ANGLIA RUSKIN UNIVERSITY

FACULTY OF SCIENCE AND ENGINEERING

# Investigation of the sheltering effect of β-lactam-resistant *K. pneumoniae* species on two susceptible *E. coli* and *S. aureus* strains

SILVIA CAPRARI

A thesis in partial fulfilment of the requirements
of Anglia Ruskin University for the degree of
Doctor of Philosophy in Biomedical Science

Submitted: February 2021

## Acknowledgements

First, I would like to thank my supervisors, Dr Helen McRobie and Dr. James Sullivan for their help and guidance throughout this PhD journey. Helen, for always being kind supportive and available when I needed her. Thanks for sharing with me the great enthusiasm for the research I carried out during my PhD. Also, a big thanks for always having a high consideration of me as a PhD student. James, I won't finish to thank you for all the excellent assistance and guidance you have always given to me. A big thank you for "saving" my PhD when everything seemed stuck at the end of my first PhD year and for converting my project in an extremely exciting and novel research. I owe you most of the knowledge, including both the theoretical and practical skills, I acquired during my academic experience at ARU. I also owe you the the ability to think critically, solve problems during my experiments and passion for the research I developed throughout my PhD. I have always appreciated the long meetings we had and all the patience you have always showed when you gave me the explanations I needed. Also, thank you for always being so swiftly available in providing help and assistance, including reading my thesis. I am deeply grateful to my former supervisor, Dr Benjamin Evans, for his well-wishing attitude and for transferring his extraordinary knowledge and skills to me. Thank you for always being so available at giving explanations and providing the support I needed even after you left ARU. I want to thank Dr Caray Walker, Dr Clett Erridge and Dr Richard Jones for giving me helpful and useful suggestions for analyses and experiments in my research. An immense "thank you" to Dr Felicity Savage for all the encouragement she gave to me, for her understanding and support, especially in the critical moments I had during my PhD. Thank you for always being so supportive and believing in me.

I am grateful to the Frances and Augustus Newman Foundation, the organization that supported my research and provided me with a studentship that allowed me to pursue my PhD at ARU. A big thanks goes to Steven Abbott for assisting me in writing and submitting my application for this scholarship. His extraordinary help has been instrumental in the success of my application.

I want to thank all the technical staff at ARU for their guidance and support. Thank you to Imogen Duncan, for giving excellent technical support and working hard for us to make sure that everything in the lab was working and available. Also, thank you to Angela Wheatley for always being so kind and helpful in providing technical assistance.

A special thanks goes to all the Dav210 PhD students. They have been extraordinary academic fellows, as well as very nice friends. Thank you all for the friendly atmosphere you created throughout all my experience. I am particular grateful to Ebi, Adam, Alice, Denise, Max, Rachel and Hilary for transforming my intense and tough studies in an extremely enjoyable moment. Thank you all for all the laughs, support, encouragement and kind words all of you had for me, especially during the hardest part of my PhD, the writing of my thesis. My PhD would definitely not have been worth the challenge without you!

Lastly, I am grateful to my family for their support during this intense part of my life. Thanks to my husband, my little one, my mum in Italy. Thank you to everyone who believed in me throughout this hard but amazing experience!

## Abstract

There is increasing evidence that microbial interactions can happen within microbial communities resulting in an increase of the pathogen resistance to antimicrobial therapies. However, the mechanisms of these interactions remain elusive at the present day. Previously, it has been shown that in a polymicrobial community, β-lactam resistant bacteria can protect other non-resistant bacteria from the action of β-lactam drugs without any gene transfer between the resistant and non-resistant bacteria. This phenomenon was named as “sheltering effect” and occurs when the resistant bacteria releases proteins that give protection to the non-resistant bacteria living in the same environment.

*Klebsiella pneumoniae* is one of the world's most dangerous multidrug resistant pathogens. Infections from this bacterium are seriously threatening the public health due to their great ability to quickly become resistant to every antibiotic available today. Even more importantly, *K. pneumoniae* is found in the blood of patients with polymicrobial infections. This doctoral project focused on the investigation of the sheltering effect in this pathogen. Studies carried out here were designed to elucidate the causes of the variability of this phenomenon observed among different clinical isolates of *K. pneumoniae*. Furthermore, the role of the Sec-dependent pathway and of the outer membrane vesicles (OMVs) in the sheltering effect was investigated. The present study also examined how the sheltering effect changes in response to different concentrations of β-lactam in the medium. Lastly the presence of sheltering effect in presence of drugs different from the β-lactams was also investigated,

The results obtained by these tests also allowed the development of methods for the direct quantification of the sheltering effect based on the features observed on an agar plate. The causes of the variability in the sheltering effect detected on plate were investigated by carrying out Random Amplified Polymorphic DNA (RAPD), Polymerase Chain Reaction (PCR) and Whole Genome Sequencing (WGS) on the genome of the isolates to find a relationship between their genetic features and their potential of sheltering effect. Minimum Inhibitory Concentration (MIC) tests were also performed in the attempt to find a relationship between their resistance profiles and their sheltering effect potential.

The results obtained suggest that the sheltering effect is a widespread and variable phenomenon in *K. pneumoniae*. The protective effect is likely due to the extracellular release of OMV-associated β-lactamases after Sec-mediated translocation of these enzymes from the cytosol to the periplasm. The results also suggest that the sheltering effect diminishes in response to increasing concentrations of β-lactam in the medium. Furthermore, the sheltering effect was not observed in presence of drugs different from the β-lactams. Lastly, no relationship was found between the sheltering effect potential of the isolates and their genetic and resistance features analysed in this project. Therefore more studies will be necessary to elucidate the causes of this variability.



**Key words**: sheltering effect, *K. pneumoniae*, β-lactamases, OMVs, variability, polymicrobial infections.

# TABLE OF CONTENTS

# List of Figures

## 1 INTRODUCTION

## 4 RESULTS II

## 5 RESULTS III

## 6 GENERAL DISCUSSION



## 8 APPENDICES

# List of Tables

## 1 INTRODUCTION



## 2 MATERIALS AND METHODS

## 4 RESULTS II



## 5 RESULTS III



## 8 APPENDICES

**DECLARATION BY CANDIDATE**

I declare that the work presented in this thesis is my own and that the thesis presented is the one upon which I expect to be examined. Any quotation or paraphrase from the published or unpublished work of another person has been duly acknowledged in the work, which I present for examination. I further state that no part of my work has been submitted for any other qualification, either at Anglia Ruskin University, or at any other institution.

Signed  Silvia Caprari

# 1 INTRODUCTION

## 1.1 Antimicrobial drugs

### 1.1.1 Historical background

Antibiotics are perceived in nature as an arsenal of the producer microorganisms that they use against other cohabiting microorganisms to eliminate competing bacteria for the purpose of "empire building" in the microbial community (Davies, 1990; Sengupta et al., 2013). Since their discovery antimicrobial drugs have conferred extraordinary benefits on human health, thus revolutionizing medicine in the 20$^{th}$ century (Davies and Davies, 2010; Adedeji, 2016; Bloom et al., 2018). Diseases such as tuberculosis in the developed world were nearly eradicated thanks to the use of antibiotics together with vaccination (Schrager et al., 2018; Voss et al., 2018). However, although people were not aware that bacteria were responsible for infections until the last century, antibiotics have been used for millennia to treat infections (Levy, 2002; Aminov, 2010; Zaman et al., 2017). Indeed, the use of plant extracts and moulds for the treatment of infections in the earliest civilisations, like the ancient Egyptians, has been well documented (Dias et al, 2012; Petrovska, 2012). Nevertheless, until the 20th century, infections caused by bacteria, like pneumonia and diarrhoea, were the major cause of human death in the developed world, although now they are currently easy to treat (Adedeji, 2016). The first observations of chemicals with antibacterial activity dated back on the late 20$^{th}$ century. In 1909 the German physician Paul Ehrlich discovered that the Arsphenamine, an organoarsenic compound, could be used for the treatment for syphilis (Bosh and Rosich, 2008). Later, in 1928, the Scottish scientist Alexander Fleming left accidently a culture plate of *Staphylococcus aureus* uncovered. He noticed that a fungus *Penicillium notatum* had contaminated the culture plate and, more importantly, created bacteria-free zones wherever it grew on the plate. After isolating and growing the mould in pure culture, Fleming found that the fungus was extremely effective in preventing the *Staphylococcus* growth (Fleming, 1929; Tan and Tatsumura, 2015). Therefore, he hypothesized that *Penicillium notatum* produced a diffusible antibacterial agent that inhibited the growth of the bacterium. This agent was named "penicillin" and this discovery shed light on the possibility for a microorganism to produce substances that inhibit the growth of other microorganisms (Saga and Yamaguchi, 2009). After this discovery Fleming performed further studies aimed to determine the activity of penicillin *in vitro* and demonstrated its non-toxicity when injected into mice and rabbits (Bennett and Chung, 2001; Lobanovska and Pilla, 2017). Nevertheless, he failed to demonstrate the essential property of this substance, that is the ability to overcome bacterial

infections (Rolinson 1998). Subsequent attempts to obtain purified penicillin were unsuccessful until 1940, when the scientists Howard Florey and Ernst Chain showed that subcutaneous injections of penicillin were highly effective against lethal streptococcal infections in mice. This proved the chemotherapeutic activity of penicillin and thus triggered the development of other antimicrobial drugs (Chain et al., 2005). Indeed, in 1952 the Russian-born biochemist and soil microbiologist Selman Abraham Waksman discovered the first antibiotic active against tuberculosis, streptomycin, that was produced from soil bacterium *Streptomyces griseus* (Waksman et al., 1948; Kingston, 2004; Woodruff, 2014). Thereafter, many new antimicrobial drugs, like chloramphenicol, macrolide, vancomycin, tetracycline were discovered and each class of antibiotics has been improved to achieve a wider antimicrobial spectrum, as well as a more effective activity (Wilson and Cockerill, 1987; Saga and Yamaguchi, 2009).

### 1.1.2 Mode of action

Antimicrobials are commonly classified according to the cellular component or system they affect (Kapoor et al., 2017). In addition, they can be classified as bactericidal or bacteriostatic drugs based on whether they respectively induce cell death or merely inhibit cell growth (Kohansky et al., 2010). Indeed different antimicrobials have different modes of action depending on their structure and target sites (Figure 1.1)(Newton, 1965; McDermott et al., 2003; Sahra, 2019).

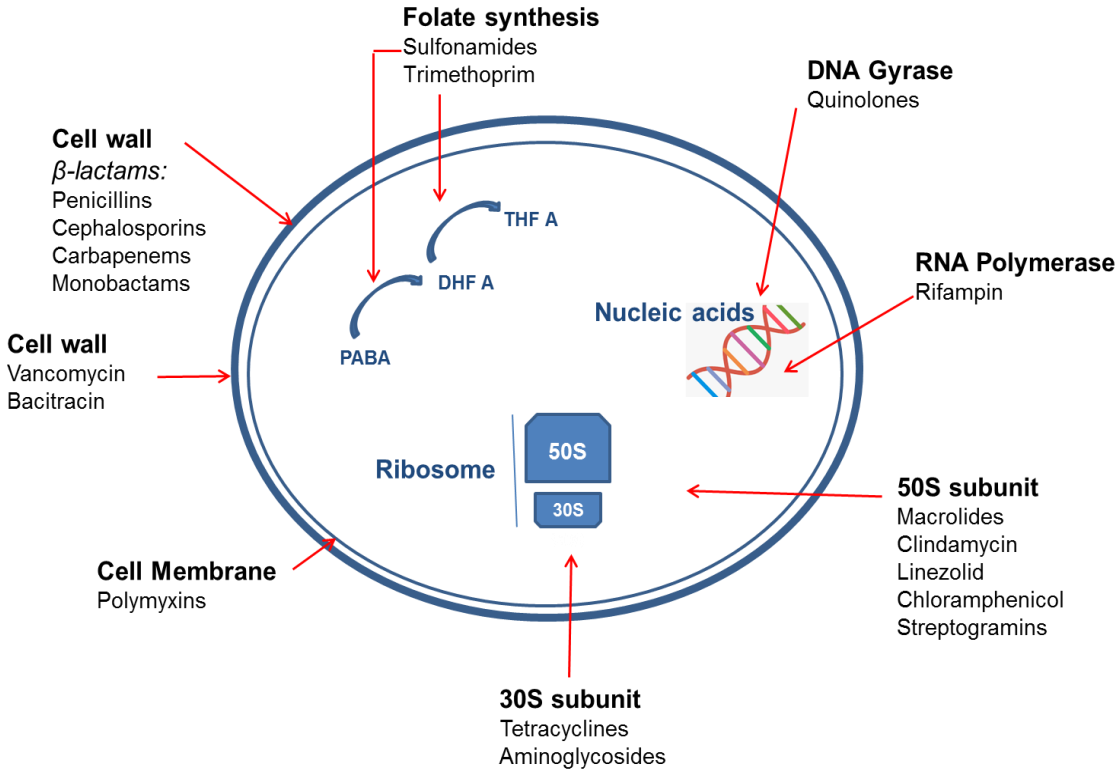



**Figure 1.1 Mechanisms of action of antimicrobials.** The classes of antimicrobials differ by their cellular targets and mechanisms of action. These can involve the inhibition of the synthesis of the cell wall (β-lactams, vancomycin, bacitracin), nucleic acids (rifampin and quinolons that target respectively the RNA polymerase and the DNA gyrase), proteins, including the drugs that target the 50S subunit of the ribosome (macrolides, clindamycin, linezolid, chloramphenicol and streptogramins) and drugs that target the 30S subunit (tetracyclines ad aminoglycosides) and folate (sulphonamide and trimethoprim). They can also act by alterating the structure of the cell membrane (polymyxins). PABA: para-aminobenzoic acid, DHF A: dihydrofolic acid, THF A: tetrahydrofolate.

One of the mechanisms of antimicrobial action is the inhibition of cell wall synthesis. The cell wall is a structural layer that surrounds the plasma membrane of bacteria (Silhavy et al., 2010). The inhibition of cell wall synthesis is the mechanism of action showed by the majority of the antibiotics (Sarkar et al., 2017). Examples of the antibiotics that target the cell wall synthesis are β-lactams (e.g. penicillins and carbapenems) and glycopeptides like vancomycin and bacitracin (Jovetic et al., 2010; Kapoor et al., 2017). These drugs selectively inhibit the bacterial growth, by blocking the synthesis of the peptidoglycan (PG). Some antimicrobials act by altering cell membranes. Cell membranes are important barriers that separate and regulate the flows of substances from the interior of all cells to the outside environment and vice versa (Yang and Hinner, 2016). A damage to the cell membrane leads to a leakage of solutes essential for the cell's survival and metabolism. Some antibiotics can injure the microbial cellular membrane, leading to leakage of cell contents and death of the microorganism (Bush, 2012; Epand et al., 2016). Examples of these drugs are

polymixin B and colistin (Gupta et al., 2009). Both polymyxin B and colistin are polycationic peptides and have both hydrophilic and lipophilic moieties. The cationic regions interact with the bacterial outer membrane (OM), by displacing magnesium and calcium bacterial counter ions in the lipopolysaccharide (LPS). Then, the hydrophobic and hydrophilic regions interact with the cytoplasmic membrane just like a detergent, solubilizing the membrane in an aqueous environment (Zavascki et al., 2007). However, the action of these antibiotics is poorly selective, thus resulting in increased levels of toxicity in the mammalian host cell. Therefore, their clinical usage is often limited to topical applications (Falagas and Kasiakou, 2006; Aggarwal and Dewan, 2018).

Some drugs are inhibitors of protein synthesis. The process of protein synthesis occurs through three main sequential phases that are initiation, elongation and termination (Schimmel, 2002). All these steps involve the ribosome and cytoplasmic accessory factors. In particular, the two ribonucleoprotein subunits of the ribosome, 50S and 30S, initiate the formation of a translational complex on the ribosome. Drugs that inhibit protein synthesis are among the broadest classes of antibiotics and can be divided into two subclasses: the 50S inhibitors and 30S inhibitors (Mehta and Champney, 2002). The 50S ribosome inhibitors work by physically blocking either initiation of protein translation or the peptidyltransferase reaction that elongates the nascent peptide chain. The 30S ribosome inhibitors block the translation by binding to the 30S subunit of ribosomes and preventing addition of amino acids to the growing peptide. 50S ribosome inhibitors include the macrolide (e.g., erythromycin), lincosamide (e.g. clindamycin), streptogramin (e.g. dalfopristin/quinupristin), amphenicol (e.g., chloramphenicol) and oxazolidinone (e.g. linezolid). 30S ribosome inhibitors include the tetracycline and aminocyclitol families of antibiotics (e.g. streptomycin, kanamycin and gentamicin) (Kohansky et al., 2010; Champney, 2020).

Another mechanism of action of antimicrobials is the Inhibition of nucleic acid synthesis. DNA and RNA are large biomolecules essential for to the replication of all living forms, including bacteria (Tan and Yiap, 2009). Some antimicrobial drugs can bind to components involved in the replication of DNA or RNA (van Eijk et al., 2017). This results in the interference of the normal cellular processes and in the inhibition of bacterial multiplication and survival. Examples of drugs that target the nucleic acid synthesis are the quinolons (Aldred et al., 2014). The quinolone class of antimicrobials target the DNA gyrase and the topoisomerase IV, enzymes involved in the maintenance of chromosomal topology (Levine et al., 1998). Metronidazole is another drug that inhibits nucleic acid synthesis by disrupting the DNA of microbial cells. However, its action is specific to anaerobic bacteria since it only occurs when it

the metronidazole is partially reduced and this reduction usually happens only in anaerobic conditons (Löfmark et al., 2010).

Some antimicrobials show antimetabolite activity. An antimetabolite acts by inhibiting a metabolite, a chemical that is part of a metabolic process (Allegra et al., 1988). Furthermore, some antibiotics can act on specific cellular processes that are necessary for the bacterial survival, for example the synthesis of the folic acid, a vitamin essential for bacteria (Fernández-Villa et al., 2019). Drugs like sulfonamides interfere with folic acid synthesis by competing for the enzyme dihydropteroate synthetase, responsible for the addition of para-aminobenzoic acid into the folic molecule. Also, trimethoprim acts by inhibiting the dihydrofolate reductase, an enzyme responsible for the synthesis of tetrahydrofolic acid, an essential precursor in the thymidine synthesis pathway involved in the DNA synthesis process (Sköld, 2010).

### 1.1.3 β-lactam antibiotics: structure and mechanisms of action

β-Lactams are one of the largest classes of antibiotics and have been amongst the most successful drugs for the treatment of bacterial infections in the last 60 years (Fisher et al., 2005; Coleman, 2011; Worthington and Melander, 2013; Bush and Bradford, 2016). They include four main classes of antibiotics: penicillins, cephalosporins, carbapenems and monobactams. All β-lactams share a core structure consisting in a beta-lactam ring which is a heteroatomic ring made of three carbon atoms and one nitrogen atom (Figure 1.2) (Rúbena et al., 2013). The chemical properties of the side chain, known as an R group, differentiates the different antibiotics within each class and affect the pharmacokinetic and antibacterial properties of β-lactam antibiotics (Chaudhry et al., 2019).

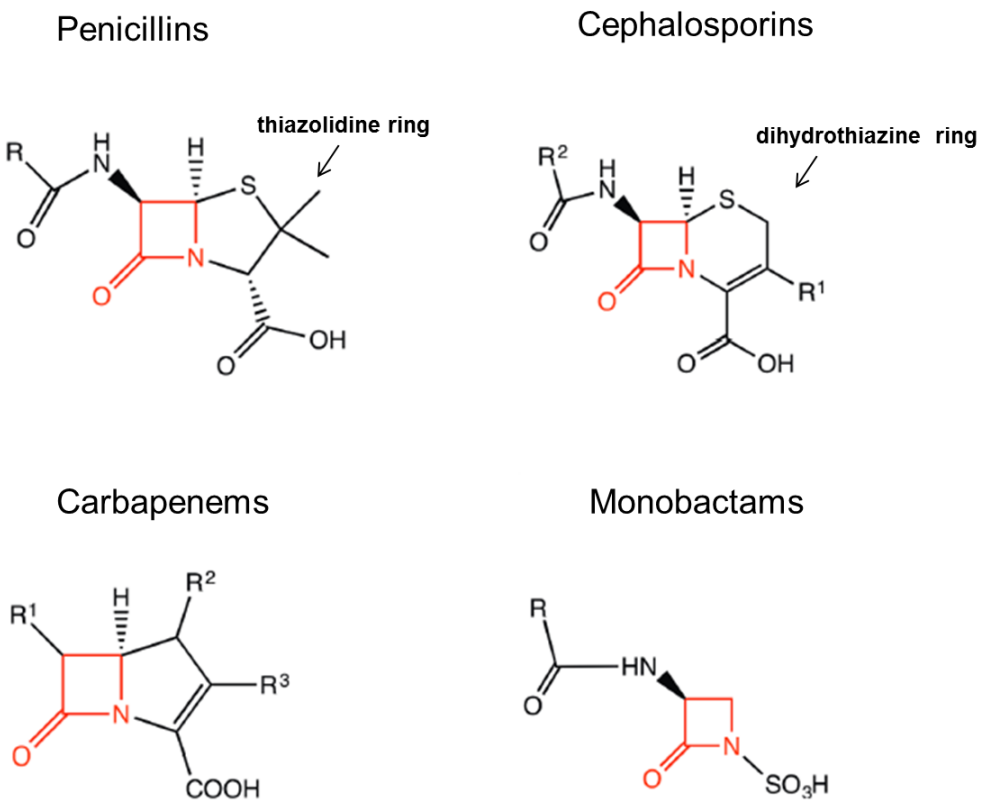



**Figure 1.2 Basic chemical structure of the β-lactam antibiotics**. The four classes of β-lactams differ in their ring structure and number of side chains (R). The β-lactam ring is highlighted in red. Adapted from Lee et al. (2016).

The β-lactam ring is fused with a thiazolidine ring in naturally occurring penicilins (for example the benzyl penicillin and the phenoxymethyl penicillin), as well as in the semisynthetic penicillins (ampicillin, carbenicillin, amozicillin, methicillin) (Miller, 2002). In cephalosporins, a dihydrothiazine ring is bound to the β-lactam ring. The cephalosporins can be further classified into generations (1st, 2nd, 3rd, 4th, and 5th generation) based on the differences in their antibacterial spectrum, structural modifications, water solubility, acid tolerability, oral bioavailability, biological half-life (Mehta and Sharma, 2016). Carbapenems have the 4:5 fused ring lactam of penicillins with a double bond between C-2 and C-3. Unlike the penicillins, in carbapenems the sulphur at C-1 position is replaced by a carbon atom. In contrast to the other β-lactams, the monobactams have the β-lactam ring is alone and not fused to another ring. Given their structural similarities, all the β-lactam antibiotics share the same mechanism of action which is the inhibition of the cell wall synthesis (Kong et al., 2011). In particular, the β-lactam antibiotics inhibit the synthesis of the peptidoglycan layer that is responsible for the cell wall structural integrity (Figure 1.3) (Bush and Bradford, 2016). The bacterial cell wall is synthetized by cross-linking of

PG units. This reaction is catalyzed by a subgroup of transpeptidases called penicillin binding proteins (PBPs), that are responsible for the formation of peptide bonds. This results in the removal of a terminal D-alanine residue from one of the PG precursors. The formation of covalent bonds between adjacent sugar molecules NAM and NAG is catalysed by the glycosyltransferases enzymes and it is responsible for conferring the cell wall rigidity and protection from osmotic forces that would lead to cell lysis. The structure of the β-lactam antibiotics resembles the natural D-Ala-D-Ala substrate for the transpeptidase. Therefore, they bind to the active site of the PBPs and form a stable β-lactam-enzyme complex that results in the inhibition of the normal transpeptidation reaction and of the cell wall synthesis. As a consequence, growing bacteria cells are highly susceptible to lysis and death (Lovering et al., 2012).

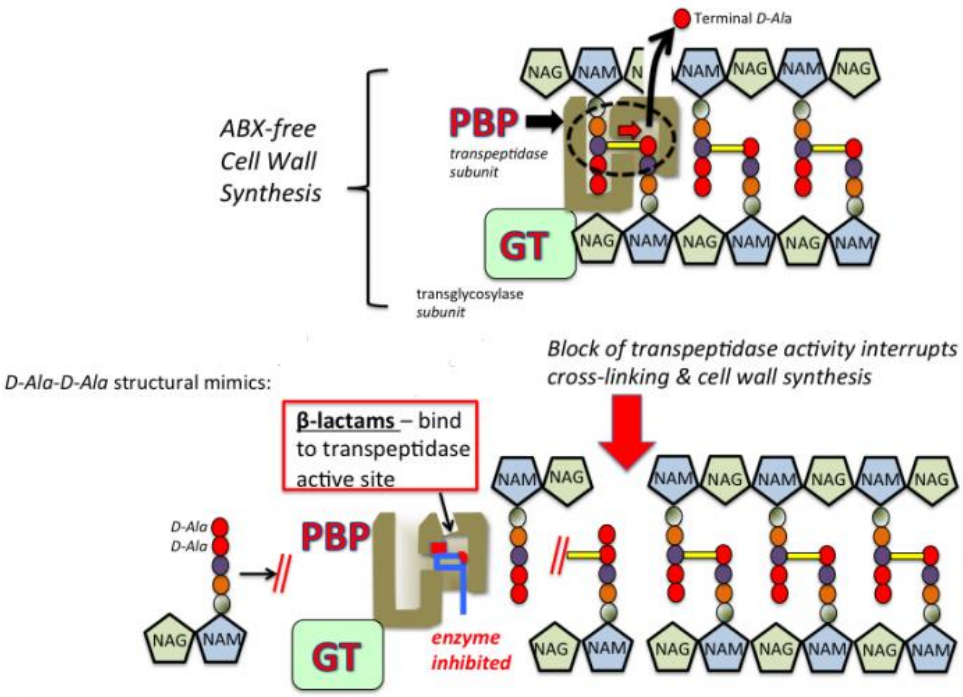



**Figure 1.3 Cell wall synthesis and mechanism of action of β-lactams.** The PG is a polymer of sugars and amino acids. The sugar component of the PG consists of alternating residues of β-(1,4) linked N-acetylglucosamine (NAG) and N-acetylmuramic acid (NAM). In the PG, the carboxyl group is replaced by a chain of three to five amino acids. ABX: Antibiotic. Adapted from Zango et al. (2019).

## 1.2 The bacterial resistance

The antimicrobial chemotherapy lead to remarkable benefits for the human health, resulting in the optimistic view that infectious diseases would be definitively defeated in the near future (Saga and Yamaguchi 2009). Since its discovery in 1928, penicillin was successful in controlling bacterial infections among World War II soldiers

(Sengupta et al., 2013; Ventola, 2015). However, shortly thereafter, penicillin resistance became an important clinical problem and by the 1950s, most of the progress of the prior decade was threatened (Spellberg and Gilbert, 2014). In response, new β-lactam antibiotics were developed and deployed (Sengupta et al., 2013; Spellberg and Gilbert, 2014). However, the first case of methicillin-resistant *Staphylococcus aureus* was identified during that same decade, in the United Kingdom in 1962 and in the United States in 1968 (Sengupta et al., 2013). Nowadays the efficacy of antibiotics is highly endangered by the rapid emergence of resistant bacteria worldwide (Ventola, 2015). Indeed bacteria have the extraordinary ability to adapt, change and develop new features that make them unsusceptible to antibiotics (Alekshun and Levy, 2007). For example some *Klebsiella pneumoniae* bacteria produce enzymes called carbapenemases, which break down carbapenem drugs and most β-lactam drugs (Psichogiou et al., 2008; Kumarasamy et al., 2010; Elshamy et al., 2020). Furthermore some *Staphylococcus aureus* bacteria can bypass the effects of trimethoprim, a drug that binds to dihydrofolate reductase and inhibits the reduction of dihydrofolic acid (DHF) to tetrahydrofolic acid (THF), an essential precursor in the thymidine synthesis pathway that is involved in the bacterial DNA synthesis (Brogden et al., 1982). This occurs by two distinct mechanisms: point mutations in the dihydrofolate reductase gene (*dfrA*), and horizontal acquisition of resistance determinants that encode drug-resistant variants of *dfrA* (Vickers et al., 2009).

Resistance to antibiotics occurs naturally over time usually through mutations in the genetic sequence. These genetic changes are spontaneous events that occur regardless of whether antibiotic is present. A bacterium carrying such a mutation is at a substantial advantage as the susceptible cells are rapidly killed by the antibiotic, leaving a resistant subpopulation (Hawkey, 1998).. In addition to these natural genetic changes, the overuse and misuse of the antibiotics remarkably accelerated the emergence and spread of this phenomenon in the last few decades, resulting in a real antibiotic resistance crisis nowadays (Aslam et al., 2018; Alam et al., 2019; Mobarki et al., 2019).Indeed global antibiotic consumption in humans increased by 65 % between 2000 and 2015, whereas consumption in animals is expected to increase by 11.5 % between 2017 and 2030. If effective interventions against antimicrobial resistance are not made, antibiotic consumption is calculated to increase worldwide by 200 % between 2015 and 2030 and cause 10 million deaths each year by 2050 (Klein et al., 2018; Sriram et al., 2021). With regards to this, the level and the complexity of the resistance mechanisms showed by bacterial pathogens rose with the increase of antimicrobial usage (Peterson and Kaur, 2018).

Antimicrobial resistance mechanisms fall into four main classes: (1) limiting uptake of a drug; (2) modifying a drug target; (3) inactivating a drug; (4) active drug efflux. Figure 1.4 illustrates these general antimicrobial resistance mechanisms.

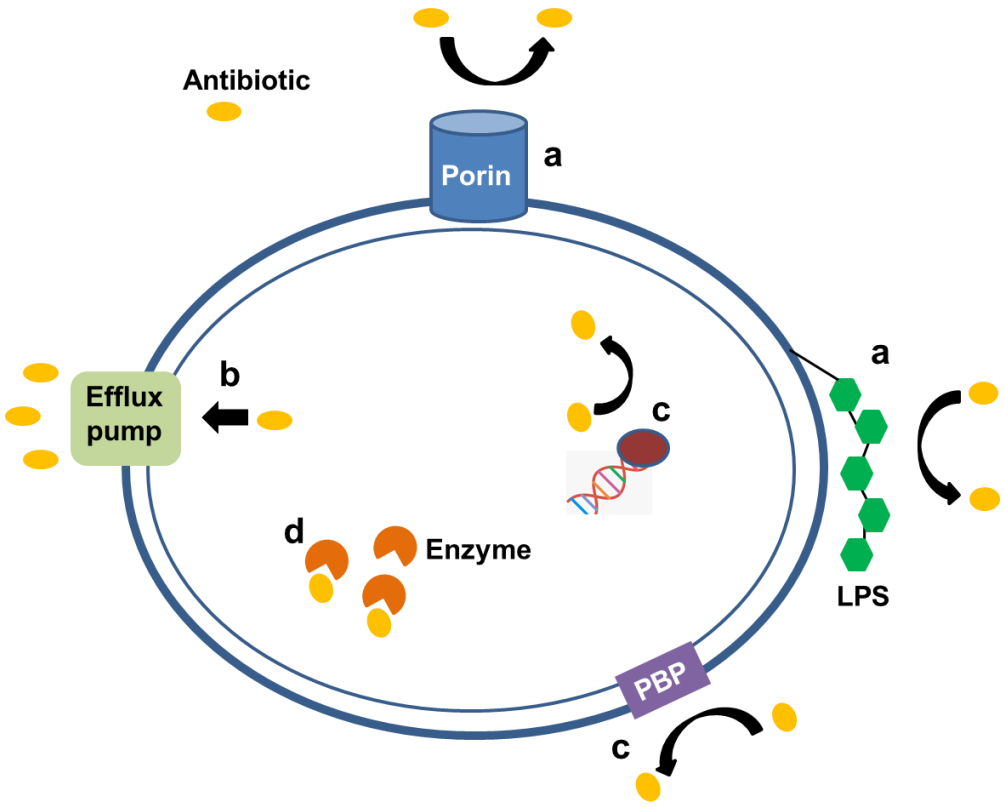



**Figure 1.4 General antimicrobial resistance mechanisms. a:** Limiting drug uptake. The structure of LPS and reduced porin expression can decrease the entry of the antibiotic in the cell. **b:** Active drug efflux. Efflux pumps can actively remove the antibiotic from the cell using energy sources like the ATP hydrolysis or proton gradients.**c:** Modifying a drug target. The target of a drug, like the Penicillin-binding proteins (PBPs) and the replication enzymes, may be modified to enable resistance to this drug. **d:** modifying a drug target. The target of a drug, like the PBPs and the replication enzymes, may be modified to enable resistance to this drug.

There are two main types of antibiotic resistance: natural and acquired. Natural resistance is the innate ability of a bacterium to resist to a drug or a class of drugs through its structural and functional features (Reygaert, 2018). This type of resistance is not related to horizontal gene transfer (HGT) and may be intrinsic if the genes for the resistance are always expressed regardless of previous antibiotic exposure or induced if these genes are only expressed or overexpressed to resistance levels after exposure to an antibiotic (Cox and Wright, 2013; Reygaert, 2018).

The LPS layer in Gram-negative bacteria provides an example of natural barrier to the uptake of certain drugs. The structure and functions of the LPS in Gram-negative bacteria provide innate resistance to certain types of molecules including large antimicrobial agents (Hayes and Wolf, 1990; Blair et al., 2014; Reygaert, 2018).

Bacteria that lack a cell wall, such as Mycoplasma and related species, are therefore intrinsically resistant to all drugs that target the cell wall including β-lactams and glycopeptides. Another example of intrinsic resistance are the efflux pumps that are expressed constitutively (Eichenberger and Thaden, 2019). The efflux pumps function primarily to rid the bacterial cell of toxic substances and many of these pumps transport a large variety of compounds (multidrug [MD] efflux pumps) (Blair et al., 2014; Eichenberger and Thaden, 2019). Most bacteria possess many different types of efflux pumps. There are five main families of efflux pumps in bacteria classified based on structure and energy source: the ATP-binding cassette (ABC) family, the multidrug and toxic compound extrusion (MATE) family, the small multidrug resistance (SMR) family, the major facilitator superfamily (MFS), and the resistance-nodulation-cell division (RND) family. This latter is found almost exclusively in Gram-negative bacteria (Figure 1.5) (Kumar and Schweizer, 2005; Piddock, 2006; Poole, 2007; Blair et al., 2014; Reygaert, 2018).

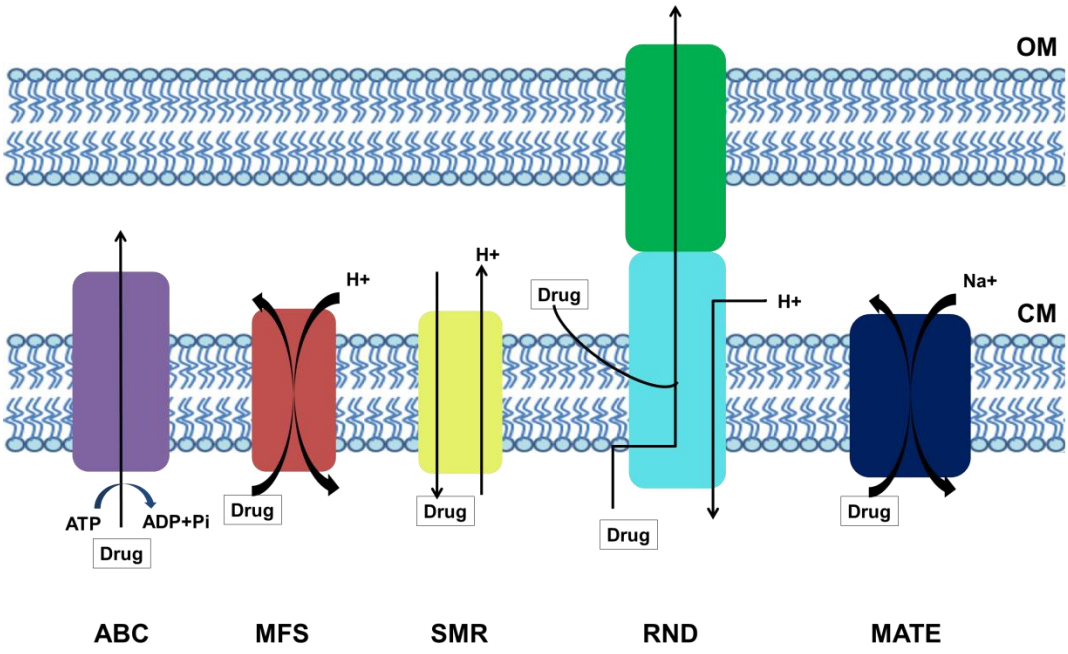



**Figure 1.5 General structure of main efflux pump families**. The ABC, MFS, SMR and MATE families are all single-component pumps which transport substrates across the cytoplasmic membrane (CM). The RND family are multi-component pumps (found almost exclusively in Gram-negative bacteria) that function in association with a periplasmic membrane fusion protein and an OM protein to efflux substrate across the entire cell envelope. The ABC pumps use the hydrolysis of ATP to ADP to transport the substrate, while the pumps of all the other families use proton gradients ($H^+$ for the pumps of the MFS, SMR and RND families and $Na^+$ for the MATE pumps).

MD efflux pumps are also a common mechanism of induced resistance. Indeed genes for multidrug resistance (MDR) can be induced at resistance levels under certain environmental stimuli or when a suitable substrate is present (Fajardo et al., 2008; Cox and Wright, 2013; Reygaert, 2018).

Bacteria can also reduce the expression of specific genes in presence of a drug. The most common example is the decrease in the number of porins present on the OM (Cornaglia et al.,1996; Reygaert, 2018). The porins are protein channels that allow the substances to enter the cell. The porin channels in Gram-negative bacteria generally allow access to hydrophilic molecules (Cornaglia et al.,1996; Gill et al., 1998). In presence of a drug, bacteria can limit drug uptake by reducing the number of porins present and sometimes stopping production entirely of certain porins (Chow and Shlaes, 1991; Cornaglia et al.,1996; Reygaert, 2018).

Resistance can also be achieved through mutations in porin-encoding genes. These mutations can have different effects, including porin loss, changes in the size or conductance of the porin channel, or a lower expression level of a porin. However, all these modifications result in a slower diffusion of the drug into the cell and, consequently, reduced bacterial killing (Fernández and Hancock, 2013). Furthermore polyamines have been found to modulate the activity of different ion channels (Baslé and Delcour, 2001; Pagès et al., 2008). For example, spermine has been reported to inhibit OmpF channel properties to protect *E. coli* from colicin action and to decrease the diffusion of norfloxacin and cefepime through OmpF (Iyer et al., 2000; Baslé and Delcour, 2001; Pagès et al., 2008).

Acquisition of genetic material that confers resistance is possible through all of the main routes by which bacteria acquire any genetic material: transformation, transposition, and conjugation (all termed HGT). The transfer involves resistance determinants borne on mobile genetic material, like plasmids, bacteriophages, transposons (Poirel et al., 2001a; Cuzon et al., 2011; Poirel et al., 2012a; Poirel et al., 2012b; Partridge et al., 2018; Peterson, 2018; Kwiecień et al., 2020). Plasmid-mediated transmission of resistance genes is the most common route for acquisition of outside genetic material (Bennett, 2008). The acquired resistance mechanisms include plasmid-encoded specific efflux pumps (such as TetK and TetL of *Staphylococcus aureus*) and enzymes that can degrade the antibiotic or modify the target of the antibiotic (Bismuth et al., 1990; Guay et al., 1993; van Hoek et al., 2011). Examples of degrading enzymes are the β-lactamases that are used by Gram-negative and Gram-positive bacteria to degrade β-lactam drugs (Tooke et al., 2019). Furthermore In Gram-negative bacteria, acquired 16S rRNA methyltransferases ArmA and NpmA confer high-level resistance to all clinically useful aminoglycosides (van Hoek et al., 2011; Lioy et al., 2014). With regards to this, the A-site of the 16S

rRNA is the target of the aminoglycoside drugs. These bactericidal antibiotics bind to the highly conserved A-site of 16S rRNA in the 30S small subunit and interfere with the decoding of mRNA. The acquired methyltransferases ArmA and NpmA act by methylating two aminoacids in the A-site of the 16S rRNA . This modification leads to decrease or loss of affinity of the antibiotic for its target site (Lioy et al., 2014).

Resistance can also be acquired by the occurrence of spontaneous mutation in genes targeted by the antibiotic (van Hoek et al., 2011). If these mutations give the bacterium an advantage in the antibiotic environment, this bacterium will grow better than its neighbors and can increase numbers thus resulting in a population of mainly resistant bacteria (Maki, 2002). There are just as many targets that may be modified by the bacteria to enable resistance to those drugs. For example, mutations that change the selectivity of certain porin channels are responsible for the occurrence of resistance to the drugs targeting those porins (Hedge and Spratt, 1985; Sanbongi et al., 2004; van Hoek et al., 2011).

## 1.3 Resistance mechanisms to β-lactam antibiotics

β-lactam are now considered one of the most important products of biotechnology. Semisynthetic penicilins and cephalosporins represent more than 60% of total antibiotic sales with estimated annual sales of $15 billion and a production volume of 37,000 tons per year (Herrera and Flores-Gallegos, 2019). Given the most widespread use of β-lactam antibiotics, the bacteria often develop resistance to these drugs (Worthington and Melander, 2013). For example, since the introduction of methicillin in the early 1960s, the continued emergence and spread of methicillin-resistant *S. aureus* (MRSA) has complicated the antimicrobial treatment of *S. aureus* (Chambers and Deleo, 2009; Chatterjee and Otto, 2013; Diekema et al., 2019). MRSA strains are now resistant to nearly all β-lactams as well as to multiple other antimicrobial classes and the prevalence of infections caused by MRSA differs around the world (Diekema et al., 1998; Hassoun et al., 2017). For example in 2017 7 of the 29 European Union countries reported 25 % or more of *S. aureus* isolates as MRSA, while in countries of South and East Asia and the Western Pacific the MRSA incidence ranged from 2.3 to 69.1% (Hassoun et al., 2017). In 2015 MRSA infections in the US occurred at a rate of 31.8% per 100,000 people, while the MRSA bacteremia rates reported in Canada ranged from 2.1 to 3.6 per 100,000 people (Laupland et al., 2013; Klevens et al., 2015; Hassoun et al., 2017).

The bacterial resistance to β-lactams can be achieved by four major strategies: altered antibiotic targets, the active extrusion of β-lactam molecules from Gram-

negative cells by efflux pumps, lack or reduced expression of OM proteins (OMPs) and production of β-lactam-hydrolyzing β-lactamase enzymes.

### 1.3.1 Altered antibiotic targets: modifications of penicillin-binding proteins (PBPs)

The penicillin-binding module contains three conserved motifs that form the active cavity. They are Ser-X-X-Lys (SXXK), the Ser-X-Asn (SXN), and the Lys-Thr/Ser-Gly (KT/SG) (Hakenbeck, 1998) There are different PBP-mediated mechanisms of β-lactam resistance. The first mechanism involves point mutations that alter aminoacid sequence of the PBPs.

Studies carried out in *Escherichia coli* showed that at least three different amino acid substitutions within the transpeptidase domain of the PBP3, conferred at least seven-fold resistance to cephalexin (Hedge and Spratt, 1985). Also, in *Streptococcus pneumoniae* five independent combinations of mutations can result in PBP 2x that have low-affinity for the cefotaxime (Laible and Hakenbeck, 1991; Sanbongi et al., 2004). In addition, mutations in the PBPs 1A and 2B play an important role in the development of resistance to ß-lactam antibiotics by *S. pneumoniae* (Sanbongi et al., 2004).

The second mechanism involves the acquisition of foreign PBP resistant to β-lactam antibiotics; for example, *S. aureus* can become resistant to methicillin and other β-lactam antibiotics through the expression of a foreign PBP, PBP2a (Hackbarth and Chambers, 1989; Stapleton and Taylor, 2002; Bæk et al., 2014).

Recombination between susceptible PBPs and those of less susceptible species is another process responsible for conferring β-lactam resistance. This hybrid protein, originated in an interspecies homologous recombination, presents lower susceptibility to β-lactams (Dougherty, 1986; Rice, 2012).

Lastly the overexpression of a PBP might result in an increase in the resistance to β-lactams. For example, when PBP5 is overexpressed, it is responsible for both natural insensitivity and acquired intrinsic resistance to penicillin in enterococci (Fontana et al. 1983; Arbeloa et al., 2004).

### 1.3.2 Efflux systems and permeability-based resistance

The presence of broad-specificity drug-efflux pumps play a key role in the antibiotic resistance by actively expelling a broad range of antibiotics, in particular in Gram-negative species (Nikaido and Pagès, 2012). The RND efflux pumps are one of the most important determinants of MDR in Gram-negative bacteria. Pumps belonging to the RND family are responsible for conferring resistance to a number of antibiotics,

including β-lactams. For example the AcrAB pump in *E. coli* is involved in the extrusion of β-lactams, chloramphenicol, ethidium bromide, fluoroquinoles, macrolides, novobiocin and rifampin (Fernando and Kumar, 2013). β-lactam resistance in Gram-negative species has also been increasingly reported as a result of the overexpression of MDR pumps (Sun et al., 2014a). One example of this is the concomitant overexpression of MexAB-OprM and MexXY occurring in clinical strains of *Pseudomonas aeruginosa* (LIanes et al., 2004). The overexpression of the two efflux systems resulted from mutations affecting multiple regulatory genes. With regards to this, *P. aeruginosa* strains with simultaneous overexpression of the two efflux systems were two- to eightfold more resistant to ticarcillin, aztreonam, and cefepime than was the wild-type reference strain PAO1. Furthermore, these strains appeared to be more resistant to cefepime than mutants overproducing MexAB-OprM or MexXY alone. (Schweizer et al., 2003; Llanes et al., 2004;; Sun et al., 2014).

The OM of Gram-negative plays a crucial role in serving as a selective permeability barrier and protecting bacteria against toxic compounds, including antibiotics. The existence of β-lactam resistance in a large number of Gram-negative bacterial species is often due to modifications in the protein composition and permeability defects in the OM (Nikaido, 2003; Delcour, 2009).The protein part of the OM is predominantly made of porins. These proteins form pores, transmembrane water-filled channels through which molecules can diffuse between cell and environment (Novikova and Solovyeva, 2009). An example of bacterial porin is LamB. This protein is a trimeric channel on the bacterial OM and is involved in the diffusion of maltodextrins across the OM (Figure 1.6) (Schirmer et al., 1995; Vinothkumar and Henderson, 2010).

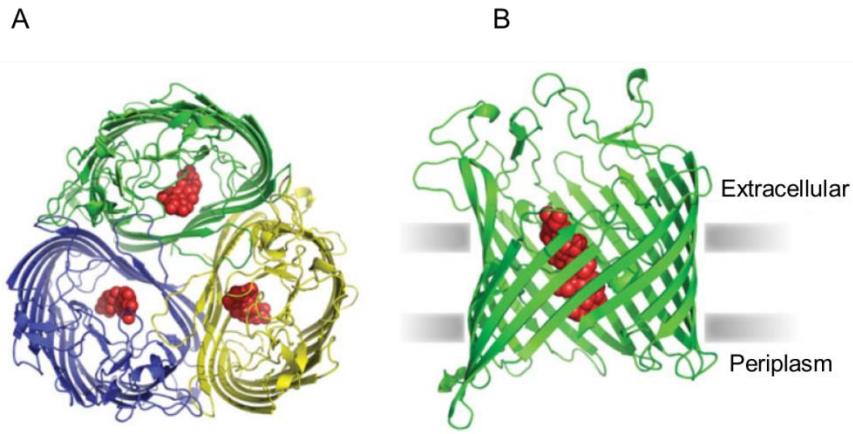



**Figure 1.6 Architecture of porins.** LamB trimer top view(A) and LamB monomer side view (B). . Each monomer is an 18-stranded β-barrel and has an individual transport pathway for the maltodextrin (red space filling model). Adapted from Vinothkumar and Henderson, (2010).

In *E. coli* and *Salmonella typhimurium* the antibiotic resistance has been reported as a result of the loss of OmpF and OmpC porins (Nikaido et al., 1983; Delcour, 2009; Ziervogel and Roux, 2013, Choi and Lee, 2019). Furthermore, mutations of key OmpF residues have been showed to be the cause of alterations in the susceptibility to antibiotics (Nikaido, 2003; Ziervogel and Roux, 2013; Igwe et al., 2016; Choi and Lee, 2019). In *Klebsiella* species OmpK36, OmpK35 are the homologues of OmpC, OmpF (Tsai et al., 2011; Sugawara et al., 2016). In addition to the major porins, minor porins have been described including OmpK37 from *K. pneumoniae*, OmpN from *E. coli* and OmpS2 from *S. typhimurium*. The percentages of identity and similarity of OmpK37 with the OmpS2 and OmpN porins are 80 and 88% and 77% and 85%, respectively (Martínez-Martínez, 2008; Doménech-Sánchez, et al., 2009).

### 1.3.3 Antibiotic-modifying enzymes: the β-lactamases

The most important mechanism of defense against β-lactam-based drugs in Gram-negative bacteria is the presence of β-lactamase enzymes (Tooke et al., 2019). These enzymes are especially important in Gram-negative bacteria as they constitute the major defence mechanism against the β-lactam drugs (Livermore 1995; Bush, 2018). In Gram-negative the β-lactamases are secreted into the periplasm or bound to the cytoplasmic membrane, while in Gram-positive they are excreted (Wilke, 2005; Fisher and Mobashery, 2016).

The antibiotic resistance is provided by breaking the antibiotics' structure. This occurs by the hydrolysis of the amide bond of the four-membered β-lactam ring (Figure 1.7)(Tooke et al., 2019).

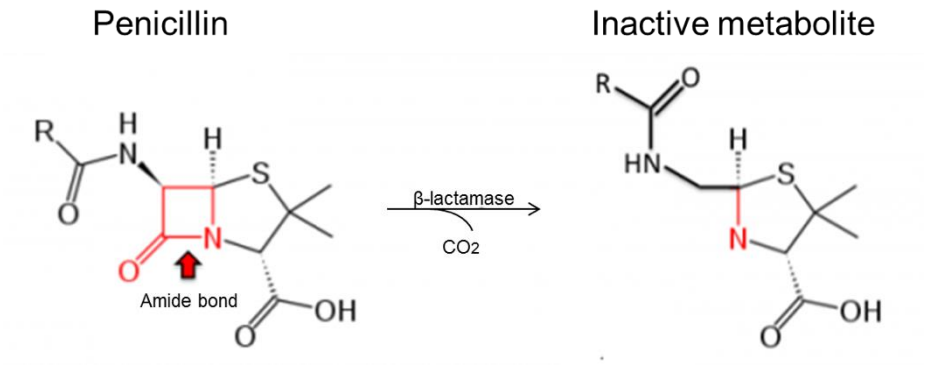



**Figure 1.7 Example of hydrolysis by β-lactamase.** The amide bond that is formed by the carboxyl group and the nitrogen atom of the β-lactam ring is cleaved by the β-lactamase. A carbon dioxide molecule is released during the process. Adapted from Tooke et al., (2019).

These enzymes provide resistance to a number of β-lactams, including penicillins, cephalosporins and monobactams (Abraham and Chain, 1940; Bush et al., 1982;

Jones et al., 1997; Bush, 2018; Palzkill, 2018). Until 20th century carbapenems have been considered unique due to their relative resistance to hydrolysis by most β-lactamases. (Papp-Wallace, 2011; Meletis, 2015). However, the rapid emergence of carbapenemases is occurring worldwide and represents an important challenge in health-care settings and a globally concern (Yan et al., 2001; Psichogiou et al., 2008; Daikos et al., 2009; Kumarasamy et al., 2010; Codjoe,and Donkor, 2018; Sheu et al., 2019; Elshamy et al., 2020). Indeed the incidence of carbapenemases is increasing globally (Hansen, 2021). A scenario that better exemplifies the concerns surrounding the spread of carbapenemases is the experience of Greece. This country shows some of the highest carbapenem-resistance rates. Prior to 2001, the prevalence of carbapenem-resistance was reported to be less than 1% (Yan et al., 2001). However in 2008 the Greek System for the Surveillance of Antimicrobial Resistance reported an increase of 30% in hospital wards and 60% in Intensive Care Units (Souli et al., 2009). By 2014, the European Centre for Disease Prevention and Control, EARS-Net reported that 62.3% of all *K. pneumoniae* isolates tested in Greece were carbapenem-resistant (Pèrez-Grajera et al., 2016, Hansen, 2021). Strategies for counteracting the β-lactamases have included the development of β-lactamase inhibitors. Although these compounds show little antibiotic activity, they can prevent bacterial degradation of β-lactams and thus extend the range of bacteria the drugs are effective against (Drawz and Bonomo, 2010).

The spread of β-lactamases has been greatly facilitated by their association with mobile genetic elements, like plasmids and transposons, that are responsible for the mobilization and transfer of genetic material between bacteria (El Salabi et al., 2013; Partridge et al., 2018). The structure of the transposons Tn*125* and Tn*4401a* is reported in Figure 1.8 as an example of genes for the β-lactamases $bla_{NDM}$ and $bla_{KPC}$ associated with mobile genetic elements (Cuzon et al., 2011; Poirel et al., 2012b).

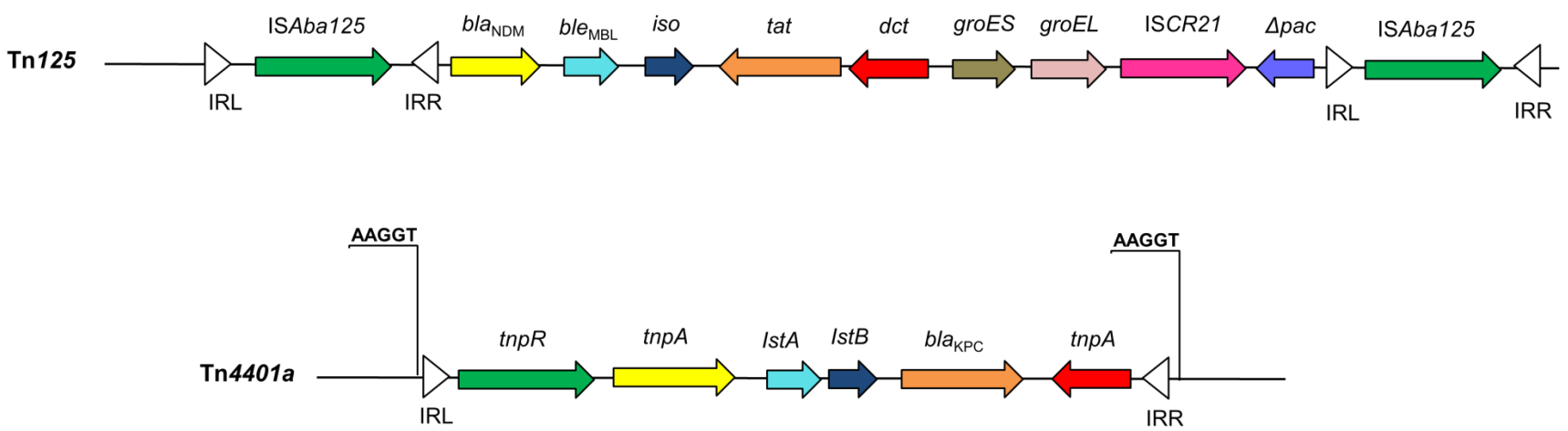



**Figure 1.8 Features of the transposons Tn*125* e Tn*4401a*, carrying respectively the *bla*$_{NDM}$ and *bla*$_{KPC}$ genes.** Genes and their transcription orientations are indicated by arrows. The variations in length of the genes are showed as differences in the length of the arrows. Gene names are abbreviated according to their corresponding proteins. For Tn*125* these genes are *ble*MBL for the bleomycin-binding protein Ble-MBL; i*so* for phosphoribosylanthranilate isomerase; *tat* for the twin-arginine translocation pathway signal sequence protein; *dct* for the divalent cation tolerance protein; Δ*pac* for truncated phospholipid acetyltransferase. *GroES* and *GroEl* are genes for two chaperonins required for the proper folding of many proteins. The mobilized element is bracketed by the two IS*Aba125* elements ended with a gene encoding the putative transposase of an ISCR-like element, termed ISCR21. In Tn*4401a* the genes *tnpA* ,*tnpR*, *IstA* and *IstB* encode proteins required for the mechanism of replication of the transposon. Both transposons are delimited by two inverted repeat sequences, IRL and IRR (white triangles)

Furthermore, these are often part of MDR cassettes located within integrons thus acquiring resistance to different antibiotic classes such as aminoglycosides and macrolides in addition to β-lactams (Bonnet, 2004; Machado et al., 2005). The structure of the integron In58 is reported in Figure 1.9 as an example of gene cassette that contains the gene for the β-lactamase VIM-2 in addition to other resistance determinants, such as the genes for the aminoglycosides and sulphonamide resistance (Poirel et al., 2001a).

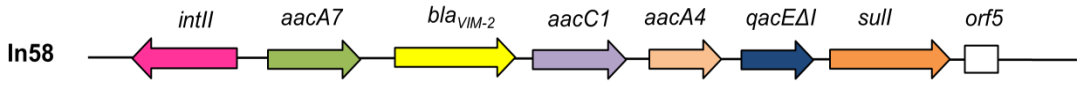



**Figure 1.9 Structure of the integron In58.** This integron contains the gene for the β-lactamase VIM-2 (*bla*$_{VIM-2}$) along with the genes for the resistance to aminoglycosides *(aacA7*and *aacC1)*, a gene for the sulfonamide resistance (*sulI*) and for the disinfectant resistance (*qacED1*). The *intI1* integrase gene encodes the integrase required for the replication of the integron. Orf5 is an open reading frame of unknown function (Poirel et al., 2001a).

### 1.3.3.1 Classification of β-lactamases

Today, over 2000 β-lactamases have been identified in naturally occurring bacterial isolates (Öztürk et al., 2015; Bush, 2018). The diversity shown by this big family of enzymes is due to the evolutionary pressure exerted by the high number of new β-lactam type antibiotics and β-lactamase inhibitors used in the treatment of infectious diseases (Gaude and Hattiholli, 2013; Bush and Bradford, 2016).

The Ambler and Bush–Jacoby systems are the two major classification schemes used to categorize the β-lactamases. Ambler classification of the β-lactamases is the most widely used classification scheme and is based on their amino acid sequences and mechanism of action. According to this classification, the β-lactamases are organized into 4 classes (A to D). The class A includes the narrow and extended spectrum of β-lactamases (ESBL) and the serine carbapenemases. The classes B and C are respectively metallo-β-lactamases and cephalosporinases. Lastly the class D includes the OXA-type enzymes (Table 1.1). (Ambler, 1997; Hall, and Barlow, 2005; Bonomo, 2017; Silveira et al., 2018).

| Type | Class | Characteristics | Examples |
|---|---|---|---|
| Narrow-spectrum β-lactamases (Livermore, 1987; Lacey and Kruczenyk, 1986;Livermore, , 1998) | A | Hydrolyze penicillin; produced primarily by *Enterobacteriaceae* | Staphylococcal penicillinase, TEM-1, TEM-2, SHV-1 |
| Extended-spectrum β-lactamases (Queenan and Bush, 2007) | A | Hydrolyze narrow and extended-spectrum β-lactam antibiotics | SHV-2, CTX-M-15, PER-1, VEB-1 |
| Serine carbapenemase (Queenan and Bush, 2007) | A | Hydrolyze carbapenems | KPC-1, IMI-1, SME-1 |
| Metallo-β-lactamases (Tamilselvi and Mugesh, 2008; Aoki et al., 2010) | B | Hydrolyze carbapenems | VIM-1, IMP-1, NDM-1 |
| Cephalosporinases (Jacoby and Munoz-Price, 2005; Jacoby, 2009;Jones, 1998) | C | Hydrolyze cephamycins and some oxyimino β-lactams; inducible; chromosomally mediated | AmpC, P99, ACT-1, CMY-2, FOX-1, MIR-1 |
| OXA-type enzymes (Ledent et al., 1993; Naas et al., 1998; Poirel et al., 2010) | D | Hydrolyze oxacillin, oxyimino β-lactams, and carbapenems; produced by *P. aeruginosa* and *A. baumannii* | OXA enzymes |

**Table 1.1 Ambler classification of β-lactamases.** For each type of β-lactamase the Ambler class, the characteristics and examples of enzymes are reported.

The classes A, C, and D are serine-β-lactamases and are all structurally similar (Bonomo, 2017) (Figure 1.10). These three classes include enzymes that hydrolyze their substrates by forming an acyl enzyme through an active site serine (Palzkill, 2018). The metallo-β-lactamases (MBLs) of class B use at least one active-site zinc ion to facilitate the hydrolysis of the β-lactam drug (Bush and Jacobi, 2010). This class is further divided into subclasses B1, B2, and B3, using sequence conservation data (Jaurin and Grundstrom, 1981; Ouellette et al., 1987; Ambler, 1997).

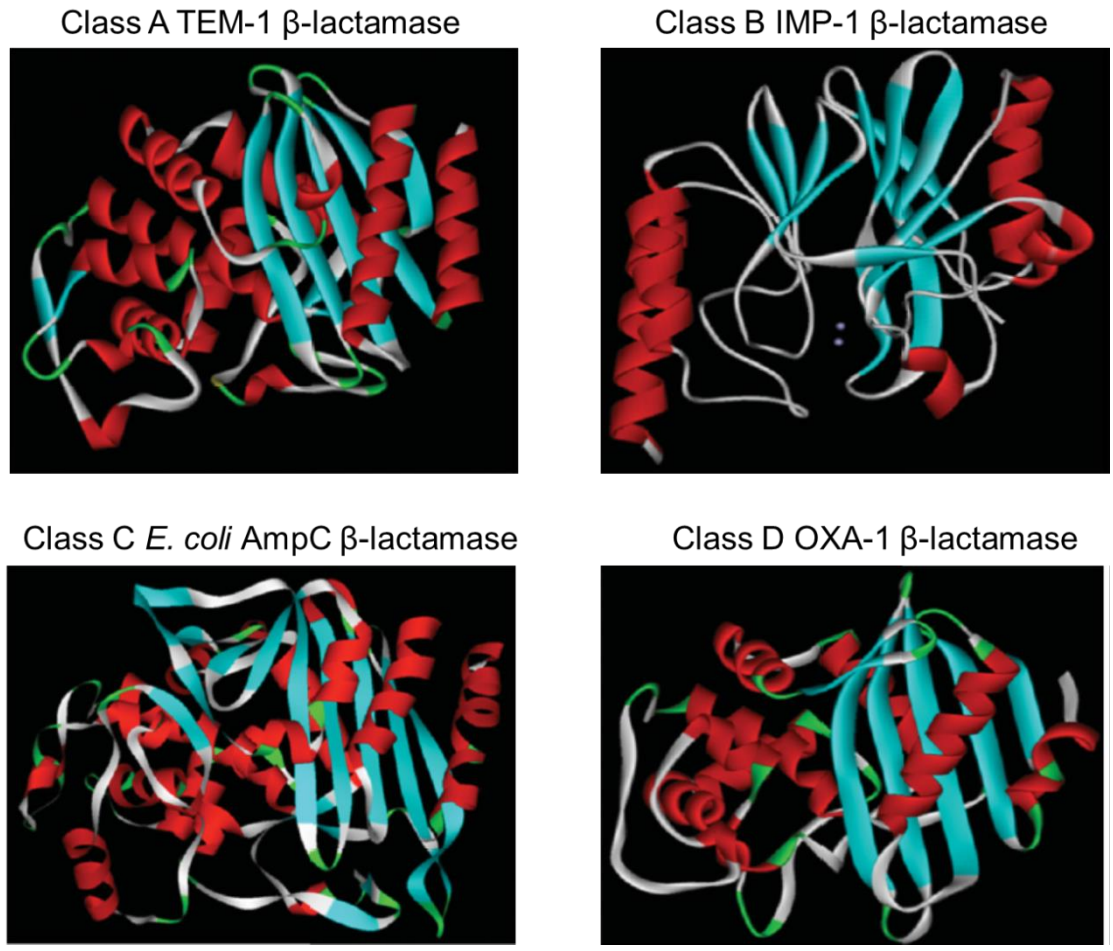



**Figure 1.10 Structure of the A, B, C and D classes of the β-Lactamases**. For each class the structure of a β-Lactamase is reported as an example. These are TEM-1 for the class A (PDB code: 1BTL), IMP-1 for the class B (PDB code: 1DD6), *E. coli* AmpC for the class C (PDB code: 6T35) and OXA-1 for the class D (PDB code: 1M6K). Each protein is coloured according to the type of secondary structure (helical residues in red and beta sheet residues in cyano) and the loop regions are showed in green. The classes A, C, and D show similarities in their protein tertiary structure that consists of an α/β/α sandwich characteristic of active-site serine β-lactamases, in which a five-stranded β-sheet is surrounded by α-helices. Class B adopts an αβ/βα sandwich fold that consists of two domains αβ with the active site located at the interface. Zinc ions are showed in purple. Adapted from Bonomo, (2017).

The Bush – Jacoby system divides the β-lactamases into 4 groups based on their substrate hydrolysis profiles (penicillin, cephalosporin, extended spectrum cephalosporin, carbapenem) and inhibitor profile (inhibition by β-lactamases inhibitors clavulanate and tazobactam) (Table 1.2).

**Table 1.2 Bush-Jacoby classification of β-lactamases**. For each Bush-Jacoby group the molecular (Ambler) class is reported

| Group | Ambler Class | Distinctive substrate(s) | Defining characteristic(s) | Representative enzymes |
|---|---|---|---|---|
| 1 | C | Cephalosporins | Greater hydrolysis of cephalosporins than benzylpenicillin; hydrolyzes cephamycins | *E. coli* AmpC, P99, ACT-1, CMY-2, FOX-1, MIR-1 |
| 1e | C | Cephalosporins | Increased hydrolysis of ceftazidime and often other oxyimino- β -lactams | GC1, CMY-37 |
| 2a | A | Penicillins | Greater hydrolysis of benzylpenicillin than cephalosporins | PC1 |
| 2b | A | Penicillins, early cephalosporins | Similar hydrolysis of benzylpenicillin and cephalosporins | TEM-1, TEM-2, SHV-1 |
| 2be | A | Extended spectrum cephalosporins, monobactams | Increased hydrolysis of oxyimino- β-lactams (cefotaxime, ceftazidime, ceftriaxone, cefepime, aztreonam) | TEM-3, SHV-2, CTX-M-15, PER-1, VEB-1 |
| 2br | A | Penicillins | Resistance to clavulanic acid, sulbactam, and tazobactam | TEM-30, SHV-10 |
| 2ber | A | Extended spectrum cephalosporins, monobactams | Increased hydrolysis of oxyimino- β-lactams combined with resistance to clavulanic acid, sulbactam, and tazobactam | TEM-50 |
| 2c | A | Carbenicillin | Increased hydrolysis of carbenicillin | PSE-1, CARB-3 |
| 2ce | A | Carbenicillin, cefepime | Increased hydrolysis of carbenicillin, cefepime, and cefpirome | RTG-4 |

| Group | Ambler Class | Distinctive substrate(s) | Defining characteristic(s) | Representative enzymes |
|---|---|---|---|---|
| 2d | D | Cloxacillin | Increased hydrolysis of cloxacillin or oxacillin | OXA-1, OXA-10 |
| 2de | D | Extended spectrum cephalosporins | Hydrolyzes cloxacillin or oxacillin and oxyimino-β-lactams | OXA-11, OXA-15 |
| 2df | D | Carbapenems | Hydrolyzes cloxacillin or oxacillin and carbapenems | OXA-23, OXA-48 |
| 2e | A | Extended spectrum cephalosporins | Hydrolyzes cephalosporins. Inhibited by clavulanic acid but not aztreonam | CepA |
| 2f | A | Carbapenems | Increased hydrolysis of carbapenems, oxyimino-β-lactams, cephamycins | KPC-2, IMI-1, SME-1 |
| 3a | B(B1) B(B3) | Carbapenems | Broad-spectrum hydrolysis including carbapenems but not monobactams | IMP-1, VIM-1, CcrA, IND-1 |
| 3b | B(B2) | Carbapenems | Preferential hydrolysis of carbapenems | CphA, Sfh-1 |

#### 1.3.3.2 Class A β-lactamases

Class A β-lactamases are generally plasmid-encoded. However, they can also be located on the bacterial chromosome (Bonomo, 2017). These enzymes are normally susceptible to inactivation by the clinically available β -lactamase inhibitor that act by one of two primary mechanisms. They may work as substrates that bind the β-lactamase with high affinity but form sterically unfavorable interactions, such as the acyl-enzyme, or may develop "suicide inhibitors," which permanently inactivate the enzyme through secondary chemical reactions in the active site. Avibactam and relebactam work by the former mechanism, while sulbactam, tazobactam, and clavulanic acid work by the latter mechanism (Drawz and Bonomo, 2010). Class A includes the β-lactamases TEM, SHV and CTX-M that are defined as extended-spectrum β-lactamases (ESBLs), a group of plasmid-mediated, rapidly evolving enzymes that represent a major therapeutic challenge today in the treatment of hospitalized and community-based patients (Rawat and Nair, 2010). ESBLs are pervasive worldwide, with over 1.5 billion people colonized with ESBL-producing Enterobacteriaceae by one estimate (Doi et al., 2017; Bezabih et al., 2021). National surveillance studies have shown the increase in prevalence of ESBL-producing isolates over time. Most European countries, including France, Italy, Spain, Belgium and Poland, exemplify these trends (Rodriguez-Villalobos et al., 2005; Livermore et al., 2006; Mugnaioli et al., 2006; Luzzaro et al., 2006; Cantón et al., 2008; Bezabih et al., 2021). In Spain, a 40-centre study performed during a 4-month period in 2000 showed ESBL prevalence rates in *K. pneumoniae* and *E. coli* of 2.7% and 0.5%, respectively. Most of the *K. pneumoniae* isolates (93%) were found in hospitalised patients, whereas 51% of *E. coli* isolates were associated with community-acquired infections (Hernández et al., 2003). Preliminary data from a new surveillance study in Spain in 2006 indicated further increases in prevalence, to 8% of *K. pneumoniae* and 6% of *E. coli* isolates (Luzzaro et al., 2006; Cantón et al., 2008). In Italy, the prevalence of ESBL producers in 1999 was 6.3 % among isolates from inpatients and 2.5 % among isolates from outpatients Luzzaro et al., 2006). However in 2003 the prevalence of ESBL producers rose to 7.4% among isolates from inpatients and 3.5% among isolates from outpatients (Mugnaioli et al., 2008).

ESBLs show increased spectrum of antimicrobial activity against β-lactams, especially against the cephalosporins with an oxyimino side chain. These cephalosporins include cefotaxime, ceftriaxone, and ceftazidime (Livermore, 2008; Rodriguez-Baño et al., 2018).

Different tests that help confirm ESBLs susceptibility are available. One test involves the use of disks containing cefotaxime and ceftazidime alone and disks that contain a

combination of these two antibiotics with clavulanic acid. These disks are placed oin Mueller-Hinton agar. A positive result is defined as a 5 mm or greater increase in the size of the zone diameter for either cefotaxime or ceftazidime tested in combination with clavulanic acid versus the zone for either antibiotic tested alone (Qureshi, 2019). A further method is the E-test screen, that evaluates third-generation cephalosporins with and without a β-lactamase inhibitor (Garrec et al.,. 2011). Finally the Vitek ESBL test, an automated broth microdilution test that uses cefotaxime and ceftazidime alone and in combination with clavulanic acid. (Garrec et al., 2011; Qureshi, 2019).

Based on comparisons of their amino acid sequences, ESBLs have been classified into nine distinct structural/evolutionary families, including TEM, SHV, CTX-M, PER, VEB, GES, TLA, BES and OXA (Gniadkowski, 2001). The largest groups are the mutants of TEM and SHV β-lactamases, with over 350 members (Bajpai et al., 2017). The mutations affecting a small number of critical amino acids in the enzyme's active site enable it to broaden the spectrum of substrates recognized. Being plasmid and transposon mediated, TEM enzymes spread worldwide and are now found in many different species of the family Enterobacteriaceae including *E. coli*, *Klebsiella* species, *Citrobacter* species, *Enterobacter* species and *Proteus* species (de Champs et al., 2001; Perilli et al., 2005; Schlesinger et al., 2005; Rawat and Nair, 2010; Jena et al., 2017; Girlich et al., 2020). Furthermore, TEM enzymes are found in *P. aeruginosa, Haemophilus influenzae*, and *Neisseria gonorrhoeae* (Castillo et al., 1998; Tristram et al., 2007; Bajpai et al., 2017). Similarly, SHV β-lactamases are usually encoded by self-transmissible plasmids, like p453, pBP60 and pZMP1, that frequently carry resistance genes to other drug classes and have become widespread throughout the world in several Enterobacteriaceae and is ubiquitous in *K. pneumoniae* (Barthélémy et al., 1988; Podbielski et al., 1991; Heritage et al., 1999; Chang et al., 2001; Liakopoulos et al., 2016).

The second largest group of ESBLs is that of CTX-M enzymes. This group of ESBLs is now the most widely distributed and globally dominant genotypes with more than 124 variants recognized (Rawat and Nair, 2010; Adamski et al., 2015; Zeynudin et al., 2018). For example, a nationwide survey of the United States during 2009–2010 revealed that 91% of ESBL-producing *E. coli* strains carried CTX-M-type genes (Doi et al., 2013; Zhang et al., 2014). Based on sequence homology this group of enzymes is divided into five subgroups: CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9 and CTX-M-25 (Mrowiec et al., 2019). Most of these subgroups have evolved from chromosomal β-lactamase genes migrated from *Kluvera* species, an enterobacterial genus of little clinical importance, to mobile DNA (Bevan et al., 2017).

KPC enzymes are an additional group of class A β-lactamases with carbapenem-hydrolyzing activity. These enzymes are carried on plasmids and are becoming an

increasingly significant problem worldwide due to their exceptional potential for dissemination (Arnold et al., 2011; Han et al., 2020). The clinical relevance of these enzymes comes from their ability to hydrolyze different groups of β-lactams, including carbapenems, cephalosporins and penicillins (Queenan and Bush, 2007). KPC enzymes are the most commonly occurring carbapenemases in the United States. For example the Consortium on resistance against carbapenems in *Klebsiella* and Enterobacteriaceae reported that the genes for the carbapenem resistance $bla_{KPC-2}$ and $bla_{KPC-3}$ were present in more than 90% of carbapenemases-producing Enterobacteriaceae circulating in the hospitals of Ohio, Pennsylvania and Michigan during the years 2013 and 2014 (Van Duin and Doi, 2017).

β-lactamases of class A include enzymes of the GES type. These enzymes confer resistance to penicillins, broad-spectrum cephalosporins and carbapenems and have been found in *P. aeruginosa*, Enterobacteriaceae and *Acinetobacter baumannii* (Poirel, et al., 2001b; Bogaerts, et al., 2010; Stewart et al., 2015, Bonnin et al., 2017). Nmc-A (non-metallo-carbapenemase-A), IMI and SME-type enzymes are other β–lactamases that confer resistance to carbapenems. However, reports of these enzymes are infrequent (Bonomo, 2017).

#### 1.3.3.3 Class B: metallo-β–lactamases (MBLs)

The MBLs show a wide substrate spectrum and can catalyze the hydrolysis of virtually all β-lactam antibiotics with the exception of monobactams. MBLs usually confer resistance to penicillins, cephalosporins, carbapenems and the clinically available β-lactamase inhibitors (Mojica et al., 2016). The genes for the MBLs are typically located on the chromosome, plasmid, and integrons (Palzkill, 2013: Mano et al., 2015). The more geographically widespread MBLs include IMP, VIM, and NDM (Mojica et al. 2015).

Among the MBLs, the NDM enzyme has experienced the fastest and widest geographical dissemination (Bahr et al., 2018). Asian continent serves as the main reservoir of NMD producers, with around 58.15% abundance of NDM-1 variant, followed by Europe and United States that show respectively 16.8% ad 10.8% abundance of NDM-1 of the total carbapenemases producers (Khan et al., 2017), NDM is the product of the $bla_{NDM-1}$ gene and is an enzyme hydrolysing a broad range of antibiotics, including the carbapenems (Khan et al., 2017). In the last few years, 17 new variants of NDM-1 evolved by changing one or two residues at different positions (Kaase et al., 2011; Khan and Nordmann, 2012; Khan and Parvez, 2014). $Bla_{NDM-1}$ is generally carried by plasmids and moves to other microorganisms via HGT, thereby increasing the probability of emergence of drug resistant strains of pathogenic

bacteria (Khan et al., 2017). Plasmids detected in these bacteria are also capable of wide rearrangement, suggesting a widespread horizontal transmission and flexibility among bacterial populations. Furthermore NDM-1 producers are not easily detected due to the lack of a routine standardized phenotypic test for MBLs with consequent probable high prevalence of unrecognized asymptomatic carriers (Khan et al., 2017). Lastly, there is a lack of available effective antibiotics for the treatment of multidrug resistant NDM-1 producers. Therefore, the emergence of bacteria carrying such genes represents a big challenge for physicians to treat infected patients (Rolain et al., 2010; Khan et al., 2017; Wang et al., 2020).

Frequently detected MBLs also include IMP-type (imipenem-resistant) and VIM-type (Verona integrin encoded MBL) with VIM-2 being the most prevalent. These MBLs are associated to different genetic structures, most commonly integrons. When these integrons are associated with transposons or plasmids, they can readily be transferred among species (Mojica et al. 2015).

#### 1.3.3.4 Class C Cephalosporinases

Class C enzymes include the AmpC β-lactamases. These are generally encoded by *bla* genes located on the bacterial chromosome, although they have been detected on plasmids as well (Mammeri et al., 2010). Organisms expressing the AmpC β-lactamase are typically resistant to penicillins, β-lactamase inhibitors (clavulanate and tazobactam) and most cephalosporins including cefoxitin, cefotetan, ceftriaxone, and cefotaxime (Bush, 2010). AmpC enzymes poorly hydrolyze cefepime, an expanded-spectrum cephalosporin, and are readily inactivated by carbapenems. Notably, AmpC cephalosporinases are very susceptible to inactivation by avibactam. (Drawz, and Bonomo, 2010; Bush and Bradford, 2016) Members of the Enterobacteriaceae family, such as *Citrobacter*, *Salmonella*, and *Shigella*, are clinically relevant producers of AmpC enzymes that resist inhibition by clavulanate and sulbactam (Bauvois and Wouters, 2007).

#### 1.3.3.5 Class D Serine Oxacillinases (OXA)

The OXA-type β-lactamases were so named because of their oxacillin-hydrolyzing abilities (Gniadkowski, 2001). These enzymes are the only ESBLs of class D and represent another relatively prevalent ESBL family (Papp-Wallace et al., 2019). These enzymes are of the utmost clinical importance due to their ability to produce resistance to carbapenems, the antibiotics of last resort for the treatment of various life-threatening infections (Antunes et al., 2014; Evans and Amyes, 2014). Among the OXA-enzymes OXA-48 is referred to as the “phantom menace” or “hidden threat” as

clinical isolates producing OXA-48 are often difficult to detect, missed by routine diagnostics. In fact, alone, OXA-48 is not a particularly efficient carbapenemase (Papp-Wallace et al., 2019). Furthermore, it possesses poor activity against broad-spectrum cephalosporins (Pfeifer et al., 2012). However, when found in combination with other resistance determinants such as loss of porins and the β-lactamase CTX-M-15, carbapenem resistance is much more pronounced (Poirel et al., 2004; Loucif et al., 2016).

OXA-48 has been detected in a variety of bacterial species, incuding *K. pneumoniae*, *E.coli*, *Enterobacter cloacae*, *Serratia marcescens*, *Citrobacter freundii*, *Providencia rettgeri*, *Klebsiella oxytoca* and *Acinetobacter baumannii*. In the UK, OXA-48 like carbapenemases are frequently reported. A study published in 2017 showed the OXA-48 like family were the most frequently reported carbapenemase in the UK, accounting for 48.5% of confirmed carbapenemase prodcuing Enterobacteriaceae (Sivaramakrishnan et al., 2020). OXA-enzymes responsible for conferring carbapenem resistance are now being reported increasingly in isolates of *A. baumanniii* (Poirel et al., 2006; Mathlouthi et al., 2018; Mentasti et al., 2020). For example, data from the United States suggest that the percentage of *A. baumannii* isolates resistant to imipenem has increased from an average of approximately 10% between 1999 and 2005 to 48% in 2008. Furthermore, meropenem resistance has increased from approximately 19% to 57.4% over the same time period (Evans and Amyes, 2014).

The global dissemination of OXA-48 has been mainly attributed to the fact that the *bla*$_{OXA-48}$ gene is localized on a conjugative IncL/M plasmid of approximately 60 kb in size. This plasmid harbors *bla*$_{OXA-48}$ within Tn*1999* transposon structures (Figure 1.11) (Poirel et al., 2012a; Carattoli et al.. 2015; Papp-Wallace et al., 2019).

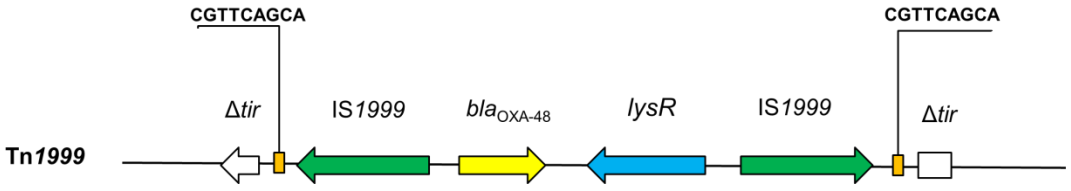



**Figure 1.11 Features of the transposon Tn*1999* carrying the *bla*$_{OXA-48}$ gene.** This transposon is inserted within a *tir* gene (Δ*tir*) and is defined by two copies of IS*1999* insertion sequences flanking bla$_{OXA-48}$. Downstream of bla$_{OXA-48}$, a *lysR* gene encode a regulatory protein of 304 amino acids. Adapted from Poirel et al., (2012a).

Together these elements facilitate the horizontal genetic exchange and therefore promote the acquisition and spread of resistance genes (Partridge et al., 2018).

Different groups of OXA-enzymes have been identified in this bacterium, namely OXA-23, OXA-24, OXA-51 and OXA-58, although OXA-51 and its derivatives may be considered to be naturally occurring β-lactamases in *A. baumannii* (Héritier et al., 2005; Graña-Miraglia et al., 2020). Among these acquired families, OXA-23 seems to be the most prevalent around the world (Mugnier et al., 2010; Graña-Miraglia et al., 2020).

### 1.3.4 Outer membrane vesicles (OMVs)

In addition to the mechanisms of resistance described, bacterial extracellular vesicles (EVs) can also confer resistance to antimicrobials (Brown et al., 2015; Qing et al., 2019). The biogenesis mechanism of Gram-positive EVs was not instantly evident as for the outer membrane vesicles (OMVs) produced by Gram-negative bacteria. Indeed, unlike the Gram-negative bacteria Gram-positives lack an outer membrane (OM) but are surrounded by thick layers of peptidoglycan (Silhavy et al., 2010). Therefore, while OMVs are generated by pinching off the OM, the generation and release of Gram-positive EVs through the thick cell wall involves the action of cell wall-degrading enzymes that weaken the peptidoglycan layer and facilitate the release of EVs (Toyofuku et al., 2017; Liu et al., 2018; Wang et al., 2018). These small, spherical nanostructures have a diameter of 20–250 nm and contain the phospholipid bilayers that are incorporated with various bacterial proteins, like lipoproteins, porins and OM proteins, LPS, and lumens carrying different cargos such as periplasmic constituents, nucleic acids (DNA, RNA), ion metabolites, PG and enzymes (Figure 1.12).

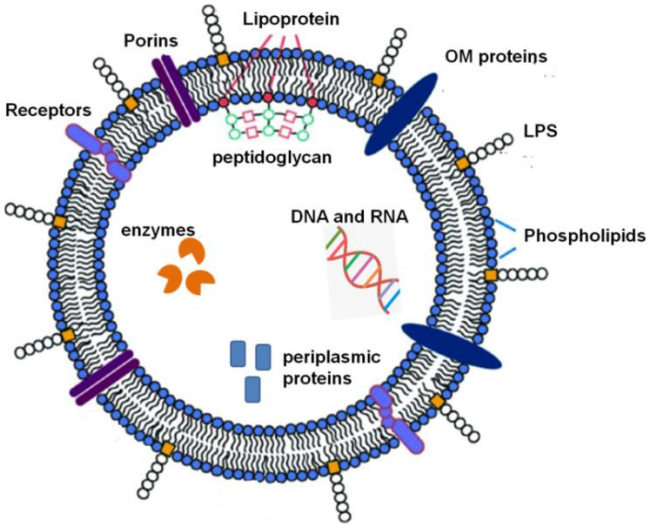



**Figure 1.12 Structure and content of Gram-negative OMVs.** OMVs are composed of a phospholipid bilayer with an outer layer of LPS, OM proteins, and receptors. Internally, OMVs possesses a thin layer of PG and contain periplasmic proteins as well as nucleic acids and enzymes. Adapted from Brown et al., (2015)

OMVs play diverse biological and pathophysiological functions including adherence to the host interface and transfer of contents to host cells, subduing effects of bacteriophages and antimicrobial peptides, responses to the stress which involves biofilm formation, inter- and intraspecies delivery of molecules, HGT, eliciting and modulating the host immune response (Kuehn, 2012; Kulkarni and Jagannadham, 2014; Jan, 2017; Qing et al., 2019). In a microbial community, there is also the potential for OMVs released by one cell to provide a benefit to other bacteria of the same or of different species (Caruana and Walper, 2020). For example, some enzymes packaged into OMVs function as “public goods” that benefit the entire bacterial community (Rakoff-Nahoum et al., 2014; Caruana and Walper, 2020)

#### 1.3.4.1 Biogenesis of OMVs

For an OMV to form, the OM needs to be liberated from the underlying PG and bulge outwards until the budding vesicle membrane undergoes fission and detaches (Schwechheimer and Kuehn, 2015; Jan et al., 2017). Previous studies suggested that reduction in the cross-linking between PG in the periplasm and Braun’s lipoprotein (Lpp) in the OM triggers the biogenesis of OMVs. Therefore OMV biogenesis often occurs in areas devoid or depleted of attachments, followed by fission without compromising envelope integrity (Figure 1.13A) (Burdett and Murray, 1974; Hoekstra et al., 1976; Schwechheimer and Kuehn, 2015).

However, the biogenesis of OMVs can also occur regardless to the total level of PG-bound Lpp. For example, studies investigated mutants that hypervesiculate as a result of a general stress response to undesirable components like misfolded proteins or high concentrations of envelope proteins, PG fragments and/or aberrant LPS. These components were found to accumulate in nanoterritories that are relatively free of bound Lpp, although the overall level of Lpp–PG crosslinks throughout the envelope as a whole remained constant. Following accumulation of this cargo in these areas, the OM could bulge outwards and bud off, effectively removing the undesirable products from the cell (Figure 1.13B). (Schwechheimer et al., 2014).

Another important factor that affects the biogenesis of OMV is the lipid composition. Indeed, specific OM regions can become enriched with particular types of LPS, phospholipids and/or specific LPS-associated molecules. These areas can act as lipid microdomains that bulge outwards as a result of their chemical features, thus leading to increased OMV production (Figure 1.13C) (Tashiro et al., 2011; Kulkarni et al., 2014).

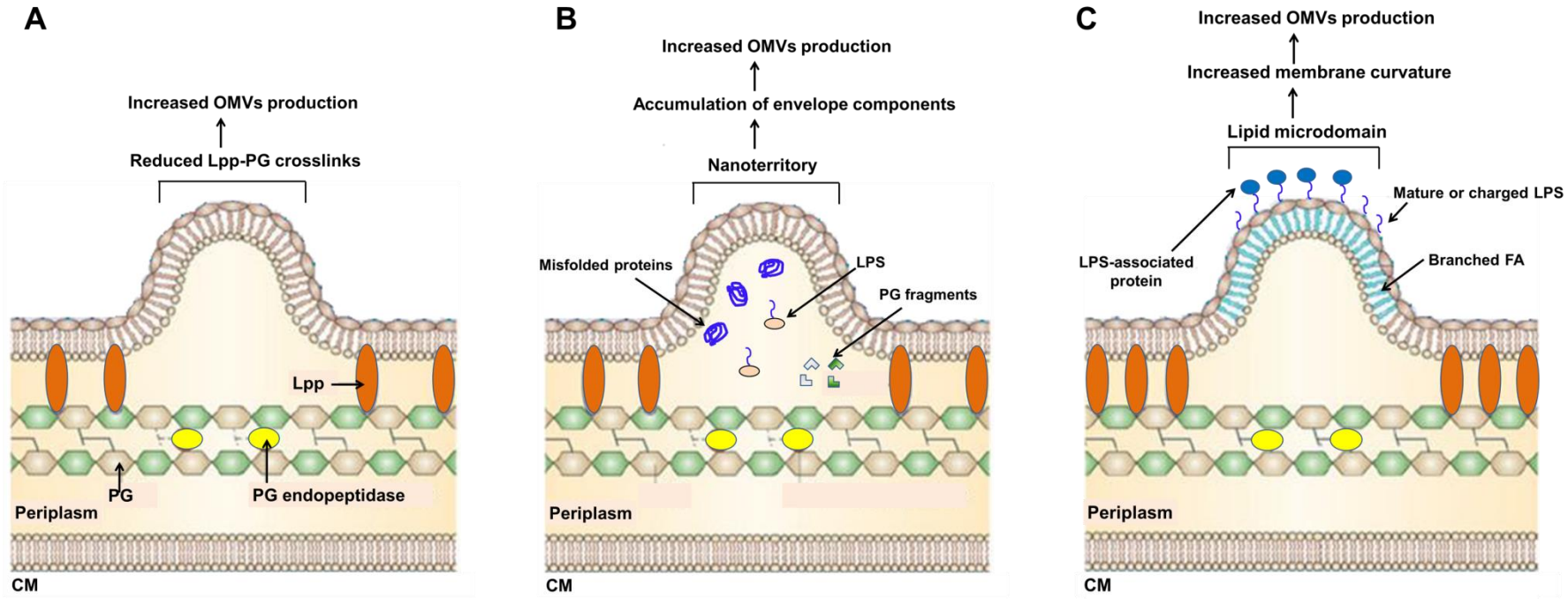



**Figure 1.13 OMVs biogenesis and cargo selection.** Different factors influence the biogenesis of OMVs. **A**. PG endopeptidases along with other enzymes regulate PG breakdown and synthesis thus governing the ability of the envelope to form crosslinks between Lpp and PG. Therefore, OMVs production is increased in areas with reduced Lpp–PG crosslinks. **B.** In regions where misfolded proteins or LPS and/or PG fragments accumulate, crosslinks are either displaced or locally depleted, promoting bulging of these OM nanoterritories and leading to increased OMVs production. **C.** Some areas of the OM can become enriched in particular types of LPS, phospholipids and/or specific LPS-associated molecules. These lipid microdomains have a propensity to bulge outwards due to their charge, their cargo or increased membrane fluidity, and this bulging leads to increased OMVs production. FA: Fatty acids. Adapted from Schwechheimer and Kuehn, (2015).

+

#### 1.3.4.2 Role of OMVs in antimicrobial resistance

The OMVs play a key role in antimicrobial resistance. Indeed, OMVs can help bacteria to battle antibiotics in ways beyond the spread of antibiotic resistance genes. For example, OMVs can act as decoys that bind or absorb antibiotics and toxins, thus providing immediate protection before the bacteria can adapt by modifying or mutating antibiotic targets (Figure 1.14A) (Manning and Kuehn, 2011). With regards to this, studies carried out in *E. coli* showed that the addition of OMVs or the use of a hypervesiculating mutant increased immediate resistance to the antimicrobials polymixin B and colistin (Manning and Kuehn, 2011). Similarly, OMVs from *Pseudomonas syringae* reduced the levels of the drugs colistin and melittin in solution by sequestering these compounds (Kulkarni et al., 2014). Phages are also a type of antimicrobial and research performed in *E. coli* showed that the viability of this bacterium cultured with the lytic T4 phage increased with the addition of OMVs as these latter irreversibly bound and inactivated the phage (Manning and Kuehn, 2011). Beyond reducing the effective antibiotic concentration in culture by adsorption, OMVs can also carry enzymes that mediate antibiotic protection, especially β-lactamases (Figure 1.14B) (Manning and Kuehn, 2011; Kulkarni et al., 2014).

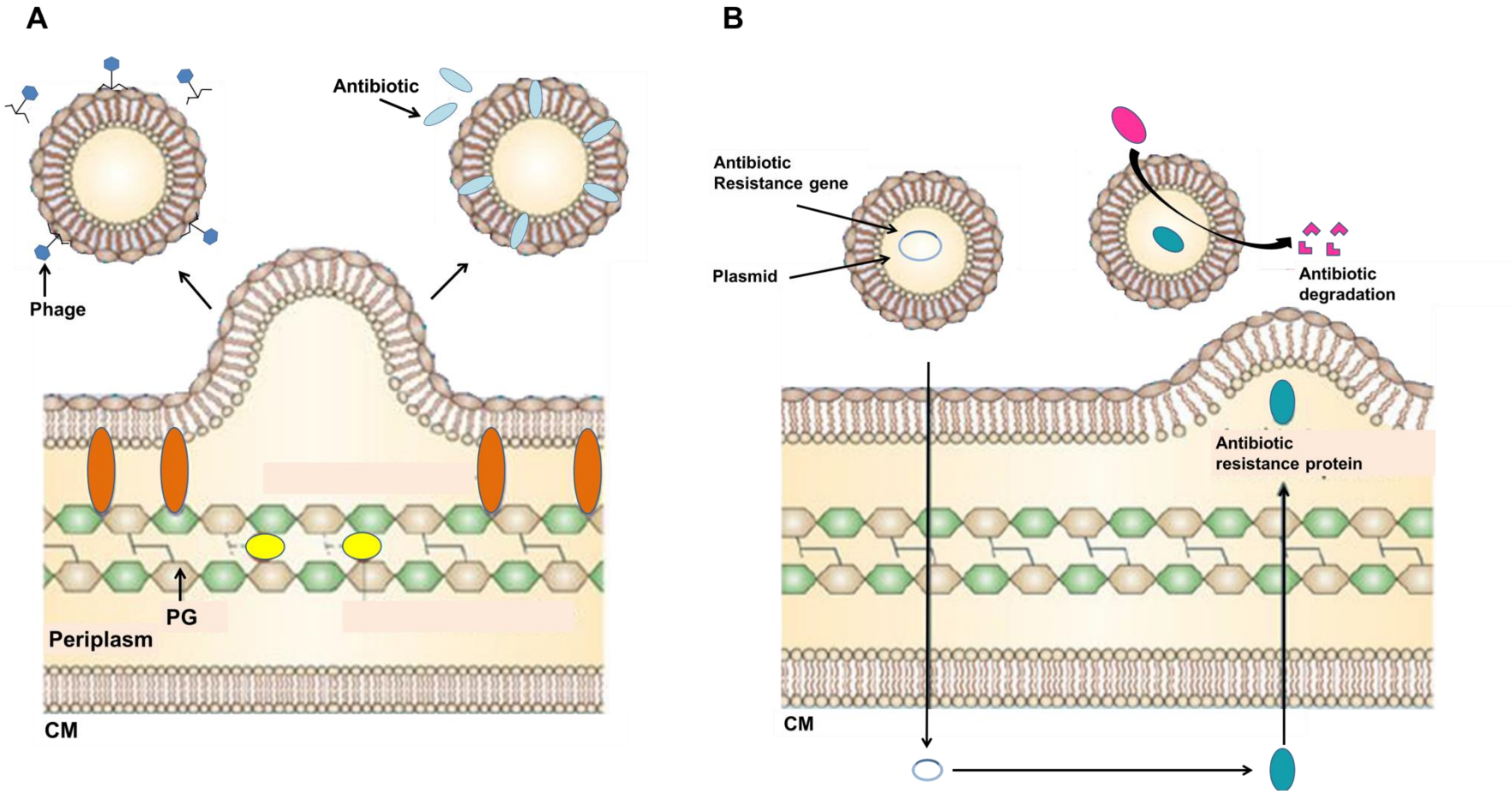



**Figure 1.14 Functions of OMVs in antimicrobial resistance. A.** OMVs can increase bacterial resistance to antibiotics and phages by acting as decoy t for for these molecules, thus protecting the bacteria cell. **B**. OMVs can also transfer DNA between cells, including antibiotic-resistance genes, and can carry enzymes that degrade antibiotics.Adapted from Schwechheimer and Kuehn, (2015).

## 1.4 Bacterial interactions

### 1.4.1 Competition and cooperation in microbial communities

Bacteria were considered to be self-contained and self-sufficient individuals until late 1800s. These organisms were thought to lack the ability of plants and animals to organize into multicellular groups. Furthermore, they were assumed to lack the ability to communicate, a key function for organizing group activities (Greenberg, 2001). However, in 1877 microbiologists including Louis Pasteur reported phenomena resulting from interactions of bacteria existing in multispecies communities (Stubbendieck and Straight, 2016). From then it became clear that a bacterial species was placed within its ecological context including the other members of its community. These communities range in scale from small, multicellular aggregates to billions or trillions of cells, such as those living in and on plants and in the gastrointestinal tract of animals (Ohno, 1997; Dong et al., 2019). In these communities microorganisms interact extensively within and between species responding to external stimuli from their environments and the fitness of a single cell depends on the interactions with other cells in the population (West et al., 2006; Stubbendieck et al., 2016). Studies over the past half-century have revealed that bacteria can communicate among themselves to carry out a broad range of complex social behaviours, including competition and cooperation (Davies, 1998; Whitehead et al., 2001).

Competition often occurs because within the microbial communities vast numbers of microbes are present. In these environments, such as a shaking liquid bacterial culture the input of new nutrients is often minimal and individuals with similar nutritional requirements, such as members of the same population, will compete for acquisition of these nutrients as they become depleted by the growing population (Hibbing et al., 2010).

Nicholson classified competition for a limiting resource into two wide groups, scramble or exploitation competition and contest or interference competition (Figure 1.15) (Nicholson, 1954; Hibbing et al., 2010; Stubbendieck and Straight, 2016). The first scenario involves rapid utilization of the limiting resource(s) without direct interaction between competitors. In particular, exploitation often occurs when one bacterial species alters its external environment through various metabolic functions and prevents other bacterial species from growing. This exploitation can arise from direct consumption of nutrients, build-up of toxic waste products or the activity of specialized bioactive metabolites (SMs), molecules produced by bacteria that are not

involved in primary metabolism but in other biological processes, like the growth and development of competing bacteria.

The latter scenario involves direct, antagonistic interactions between competitors, with the "winner" appropriating the resource(s) (Figure 1.15)(Stubbendieck and Straight, 2016).

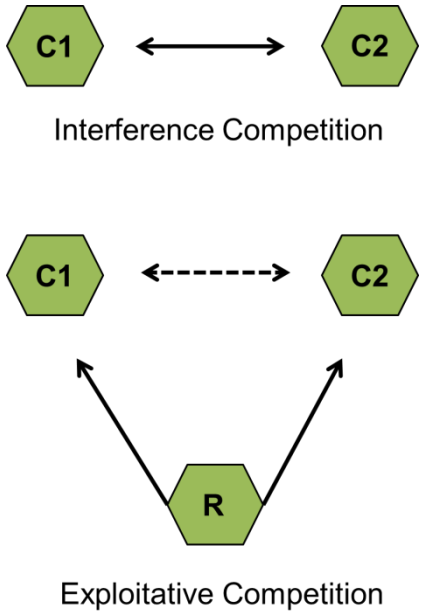



**Figure 1.15 The two major types of competitive interactions seen in microbes**. Diagrams illustrating the two major types of competitive interactions where the dashed lines indicate indirect interactions and the solid lines direct interactions. C1 = Competitor #1, C2 = Competitor #2, R = Resource. Adapted from Stubbendieck and Straight, (2016).

A typical example of interference in competitor development is to derail normal signaling processes. For example, some marine bacteria produce SMs that interfere with quorum sensing and thus disrupt subsequent downstream processes that rely on communication between competitor cells (Margaret et al., 2009).

Cooperative behaviours include complex social interactions such as division of labour and mutualism in providing shelter, foraging, reproduction, and dispersal (Wingreen and Levin, 2006). Cooperative interactions occur when a social behaviour influences positively the fitness of the recipient regardless of its fitness effect on the actor (West et al., 2002; Özkaya et a., 2017). The presence of cooperative interactions dramatically affects the evolutionary dynamics of bacterial communities, represented by the change in the frequencies of cells and species that implement different physiological strategies (such as production of public goods or not) (Cavaliere et al., 2017). The products of these microbial cooperative behaviours are generally referred

to as public goods (Smith and Schuster, 2019). In microbial cooperation, public goods are compounds produced by certain individuals that can benefit the entire population. These molecules are therefore shared among the members of the community (Figure 1.16).

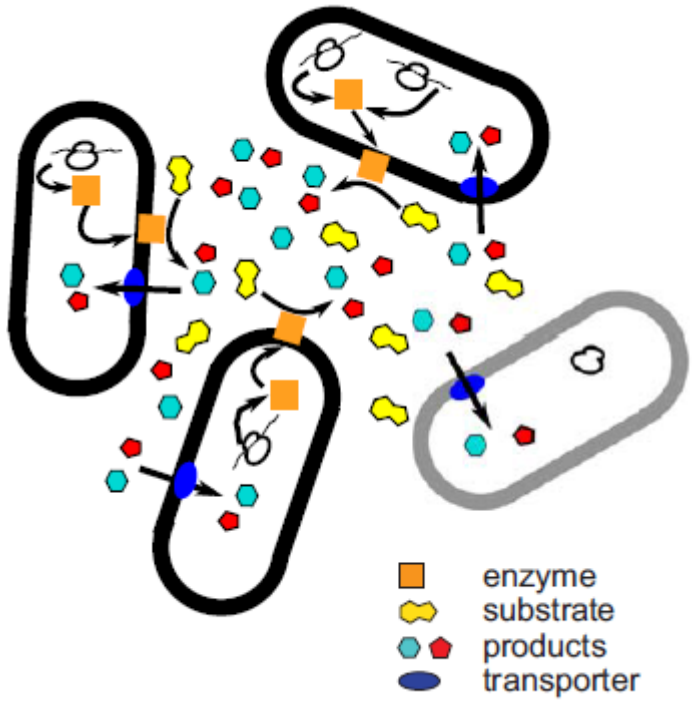



**Figure 1.16 Interactions based on shared public goods.** Cooperators (shown in black edge) produce an enzyme that splits a substrate into digestible products. Other cells (cheats, shown in grey), do not produce the enzyme but take advantage of the public goods produced by the others, by transporting the digested products inside the cell. Adapted from Cavaliere et al., (2017).

The size of the public goods ranges from large proteins to small metabolites (Cavaliere et al., 2017). These molecules may be actively secreted or may passively diffuse from the producing cell (Smith and Schuster, 2019). A broad variety of molecules can be used as public goods. They can be materials to generate protective structures like exopolysaccharides to generate biofilms, toxins to kill competitors, molecules to scavenge nutrients, enzymes for the digestion of food, biosurfactants that promote group motility over surfaces, proteins to detoxify the environment and secreted signalling molecules that coordinate the behaviour of the population in a process termed quorum sensing. (West et al., 2007; Rutherford and Bassler, 2012; Perchat et al., 2016; Side et al., 2017; Smith and Schuster, 2019; Yan and Wu, 2019). Benefit derived from public goods generally increases with population density. Antibiotics or secreted enzymes represent a good example of this aspect. Antibiotics are effective against competitors only when their concentration is above a certain threshold. Therefore they require an appropriately high density of cells involved in the antibiotic production. Similarly, as population density increases,

secreted enzymes involved in processing nutrients progressively benefit neighbouring cells rather than diffuse away (Wu, 2019).

The cooperation based on public good can be damaged by 'cheating' cells that benefit from the cooperative interactions but do not contribute to them, thus saving the cost of its production and gaining a fitness advantage over producers (Figure 1.16) (Strassmann and Queller, 2011) Thus, in mixed cultures, cheaters can increase in frequency in the population, relative to cooperators.

In microbial communities, metabolites produced by one species are used as building blocks by other species or energy sources (Cavaliere et al., 2017). The first scenario can lead to emergence of organisms fully relying on the environmental supply of certain compounds required for its growth (auxotrophic organisms). The result of the second scenario is that some cells in the community excrete metabolites that can be taken up by other cells (Figure 1.17). This mechanism is known as metabolic cross-feeding and is widespread in natural microbial communities (Smith et al., 2019).

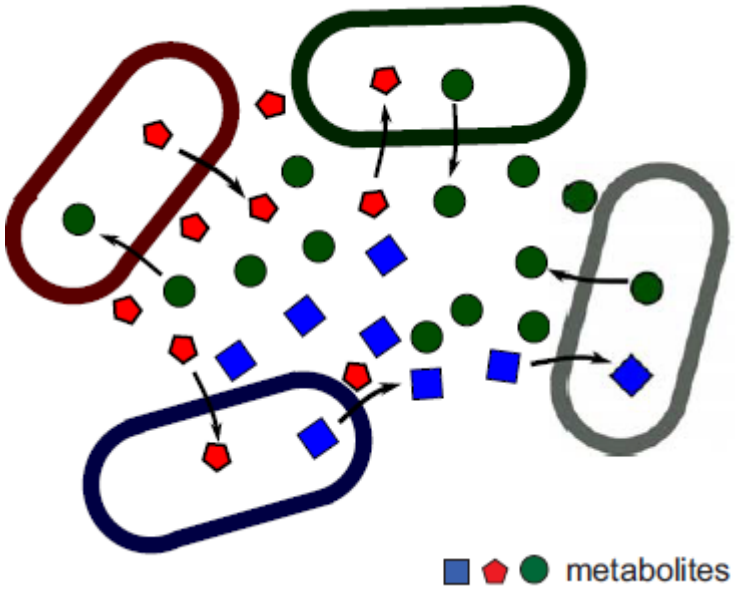



**Figure 1.17 Cross-feeding in bacteria**. The product of one strain's metabolism may be used in the nutrition of another, thus leading to a web of feeding interactions. Adapted from Cavaliere et al., (2017).

Metabolites into the environment can be released by organisms that are not able to maintain certain compounds due to leakage issues or by organisms actively secreting those compounds due to some functional benefits. With regards to this, secretion of internal metabolites can benefit both the receiving and producing organisms leading to a mutually beneficial interaction. Mutualism is the most common form of cooperative interactions seen in microbial systems (Figure 1.18A). (Freilich et al., 2011; Denton and Gokhale, 2019). The production of bacterial siderophores is a very popular example of mutually beneficial microbial interactions as these compounds

are not constricted to individual usage. Siderophores are iron-chelating compounds produced by many microbial taxa, including bacteria and fungi (Hibbing et al., 2009). These molecules facilitate the uptake and metabolism of iron in the environment, as it normally exists in an insoluble form (Neilands, 1995). In order for bacteria to access iron, cells will manufacture these compounds, and then secrete them into the environment. Once released, the siderophores will sequester the iron, and form a complex, which is recognized by bacterial cell receptors for the siderophore-Fe complexes. These complexes are then transported into the cell and reduced, making the iron metabolically accessible for the bacteria (West and Buckling, 2003; Seth and Taga, 2014).

A special type of mutualism is the syntrophy. The syntrophic partnerships can allow access to substrates that neither microbe involved in this interaction could metabolize alone (Seth and Taga, 2014). For example, the presence of a partner that actively consumes intermediates such as hydrogen allows the occurrence of an otherwise energetically unfavorable reaction, for example the fermentation of propionate to acetate to support growth (Figure 1.18B).

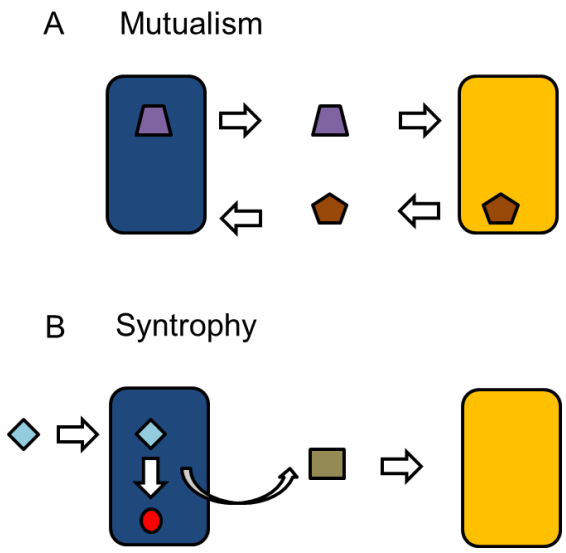



**Figure 1.18 Major cooperative Interactions between two bacterial cells (blue and yellow rectangles**. **A.** Mutualism. Cells can exchange metabolite (purple trapezium and brown pentagon that are required to support each other's growth in a mutualistic interaction. **B**. Syntrophic metabolism. The consumption of an intermediate or end product such as hydrogen(grey square) by a partner cell allows the conversion of propionate (light blue rhombus) to acetate (red circles).

### 1.4.2 Microbial cooperation in clinical settings

Interactions between different bacterial species are extremely important in clinical settings (Marjon et al., 2018). However polymicrobial diseases have not been recognized before the 20th century. Indeed several human diseases were previously characterized as being caused by just one bacterial species (monomicrobial infections) (Smith, 1982; Ingham and Sisson, 1984; Marjon et al., 2018). This was likely due to the wide use of culture dependent isolation techniques. However, with the advent of culture-independent community analysis methodologies, several diseases are becoming increasingly recognized as true polymicrobial infections because multiple microbial species are present simultaneously (Peters et al., 2012). In animals, foot rot and chronic nonprogressive pneumonia in sheep were among the first diseases found to have multiple etiologies (Jones et al., 1982a; Jones et al., 1982b; Smith et al., 1991). Respiratory disease in cattle was also shown early to have multiple etiologies (Frank, 1979; Smith, 1982). More recently, atrophic rhinitis in swine and porcine respiratory disease complex have been included (Nakai et al., 1988; Galina et al., 1994; Done and Paton, 1995; Solano et al., 1997; Thacker et al., 1999).

In humans, acute necrotizing ulcerative gingivitis and respiratory disease were recognized very early as having polymicrobial etiology (Igham and Sisson, 1984). In the 1920s, the polymicrobial etiology of respiratory disease was firmly established, and *Haemophilus influenzae* or *Streptococcus pneumoniae* were routinely found in individuals with viral respiratory disease (Hament et al., 1999). Also, later studies confirmed the polymicrobial etiology of otitis media (Nichol and Cherry, 1967; Mills, 1984; Floret, 1997; Hament et al., 1999).

Different polymicrobial diseases are commonly associated with the formation of complex structures of microbiome having different bacterial colonies or single type of cells. These microbial aggregates, known as biofilms, are immobile communities which can colonize and grow on a wide variety of surfaces including human mucosal tissues, epidermal layers and abiotic surfaces, such as intravenous and urinary catheters, cosmetic and cochlear implants,stents, artificial heart valves, and ventilator tubes (Peters et al., 2012; Hall and Mah, 2017; Sharma et al., 2019). The cells in the biofilm are embedded in extracellular polymeric substances, a matrix which is generally composed of DNA, proteins and polysaccharides (Sharma et al., 2019). The close proximity of the microorganisms enables a wide range of functions such as substrate exchange, distribution of metabolic products and removal of toxic end products so that the different species can support each other. Furthermore, the structure of biofilm communities can protect the bacteria within them from attack by

antimicrobials, shear forces and the immune system (Hall and Mah, 2017; Sharma et al., 2019).

Different polymicrobial diseases are associated with biofilms. One of the most commonly known is the dental caries (Petersen et al., 2005; Peters et al., 2012). The tooth surface becomes coated with a layer of salivary and host proteins which provides a substratum for the attachment and coaggregation of microbial species to develop into matrix-producing polymicrobial biofilm communities. These are also commonly referred to as dental plaque (Marsh, 2006). Other diseases associated with the formation of polymicrobial biofilms include otitis media, chronic infections of diabetic foot wound and Infection of the Cystic Fibrosis Lung (Segal et al., 2005; Davies et al., 2007; Farag et al., 2010; Peters et al., 2012).

The presence of a polymicrobial infection has important implications for management of a disease. Indeed interactions between microbes can modify the clinical course of the disease by increasing the severity of infections and complicate medical treatments used for the infections (Arndt and Ritts, 1961; Croxall et al., 2014; Short et al., 2014; Byrd and Segre, 2016). With regards to this, they can often impact the selection of antimicrobial therapy and the anticipated response to treatment, especially when it involves pathogens commonly exhibiting antimicrobial resistance (Marjon et al., 2018). However, despite the gravity of such infections, up to now poor research has been carried out on polymicrobial diseases. In particular it is unknown to what extent the isolates interact and whether their interactions alter bacterial growth and ecosystem resilience in the presence and absence of antibiotics (Byrd and Segre, 2016; Marjon et al., 2018).

### 1.4.3 The sheltering effect

The antibiotic tolerance has been shown to be significantly affected by the presence of more bacterial species during infections (Marjon et al., 2018). Indeed, a number of reports showed that a species normally susceptible to an antibiotic can become tolerant to this treatment in presence of a resistant species without any transfer of the resistant genes or physical contact between the two species (Schaar et al., 2011). The susceptible species remained therefore sensitive to the treatment in absence of its resistant cohort (Perlin et al., 2009; de Vos et al., 2017). This phenomenon can occur when in a polymicrobial community resistant bacteria release enzymes for the detoxification of the antibiotic into the extracellular environment thus giving protection to neighboring susceptible bacteria (Liao et al., 2015). This phenomenon is commonly regarded as sheltering effect and has been reported in a number of bacteria, especially in Gram-negative species (Brook et al., 1983; Perlin et al., 2009;

Schaar et al., 2011; Liao et al., 2014; Schaar et al., 2014). However, Gram-positive bacteria able to shelter other bacterial species have also been reported (Lee et al., 2013).

#### 1.4.3.1 Examples of sheltering effect in bacteria

All the known examples of sheltering effect in bacteria are summarised in Table 1.3.

**Table 1.3 Known examples of sheltering effect** For each sheltering bacterial species the sheltered bacteria (sheltered species) and the antibiotic treatment from which the sheltering species provides protection is reported.

| **Sheltering species** | **Sheltered Species** | **Treatment** | **Reference(s)** |
|---|---|---|---|
| *Bacteroides fragilis* | *Streptococcus pyogenes* | penicillin | Brook et al., 1983; Brook, 2004 |
| *Bacteroides melaninogenicus* | *S. pyogenes* | penicillin | Brook et al., 1983; Brook, 2004 |
| *Bacteroides oralis* | *S. pyogenes* | penicillin | Brook et al., 1983; Brook, 2004 |
| *Moraxella catarrhalis* | *S. pneumoniae; H. influenzae* | amoxicillin | Cees et al., 1994; Schaar et al., 2011 |
| *H. influenza* | *S. pyogenes* | amoxicillin | Schaar et al., 2014 |
| *E. coli* | *Salmonella enterica* | ampicillin | Perlin et al., 2009 |
| *E. coli (resistant strain RC85)* | *E. coli (sensitive strain RC85)* | ampicillin | Kim et al., 2018 |
| *A. baumannii* | *A. baumannii* | imipenem | Liao et al., 2014; Liao et al., 2015 |
| *S. aureus* | *E. coli*; *S. enterica; S. aureus*; *Streptococcus epidermidis* | ampicillin | Lee et al., 2013 |

Strains belonging to the genus Bacteroides have been among the first organisms to be reported for the presence of sheltering effect (Brook et al., 1983). With regards to this, studies carried out in the 80s demonstrated that several Bacteroides species including strains of *B. fragilis, B. melaninogenicus and B. oralis* protected susceptible streptococci by producing β-lactamase enzymes that inactivated the action of penicillin used for the treatment of tonsillitis. (Brook et al., 1981; Brook et al., 1983; Brook, 2004).

The presence of sheltering effect has been reported also in *Moraxella catarrhalis.* This Gram-negative bacterium is a common pathogen found in children with upper respiratory tract infections and in patients with chronic obstructive pulmonary disease during exacerbations (Schaar et al., 2011). Until 1975 most *M. catarrhalis* clinical strains recovered were susceptible to β-lactam antibiotics. In contrast to *M. catarrhalis*, most *S. pneumoniae* and *H. influenzae* isolates are susceptible to β-lactam antibiotics. However, *M. catarrhalis* is often found in polymicrobial infections and isolated together with *S. pneumoniae* and *H. influenzae.* In presence of these coinfections, the two susceptible species were shown to survive the amoxicillin treatment as a direct result of the protective effect exerted by *M. catarrhalis* (Cees et al., 1994; Schaar et al., 2011). However strains of β-lactam-resistant *H. influenzae* able to protect group A streptococci, Gram-positive bacteria highly sensitive to amoxicillin, have also been reported (Schaar et al., 2014).

Studies carried out in *E. coli* showed that β-lactamases-producing strains of this species were able to protect β-lactam-sensitive cohorts of other species, particularly species that could cause human disease, for example *S. enterica* (Perlin et al., 2009; Kim et al., 2018). Furthermore, the presence of sheltering effect has been reported in *A. baumannii*. Indeed, studies carried out in 2014 and 2015 reported the unexpected *in vivo* isolation of carbapenem-susceptible microorganisms from several patients with polymicrobial bacteremia during the course of carbapenem therapy. This was due to the fact that in these coinfections carbapenem-resistant *A. baumannii* protected their sensitive counterpart from the lethal action of the carbapenems (Liao et al., 2014; Liao et al., 2015).

In contrast to Gram-negative little is yet known about the sheltering effect provided by Gram-positive bacteria. However, strains of β-lactamase-producing *S. aureus* that were able to protect other ampicillin-susceptible Gram-negative and Gram-positive bacteria from the action of ampicillin have been reported (Lee et al., 2013).

### 1.4.3.2 Role of OMVs in sheltering effect

In all the resistant bacteria reported the sheltering effect is the result of the production and release of OMVs (Stentz et al., 2015). Periplasmic constituents containing β-lactam-inactivating enzymes are incorporated in the OMVs in formation when the OM forms buds, thus becoming components of the cargo once the vesicles are secreted in the extracellular environment (Liao et al., 2014; Stentz et al., 2015). The release of these vesicles triggers the degradation of the antibiotics present in the environment, where other different bacterial species live, thus providing protection from the drug to the entire bacterial community (Liao et al., 2014; Chattopadhyay and Jagannadham, 2015; Liao et al., 2015; Stentz et al., 2015).

Previous studies used gold-coupled anti-β-lactamase antibodies associated with the OMVs to track the trafficking of the chromosomally encoded β-lactamase produced and released by *P. aeruginosa.* The gold particles were found associated with the cytoplasm, the OM and emerging OMVs. This is consistent with the secretion pathway of β-lactamase which is synthesized in the cytoplasm and translocated across the plasma membrane to its periplasmic location where it is finally packaged into OMVs (Figure 1.19) (Ciofu, 2000).

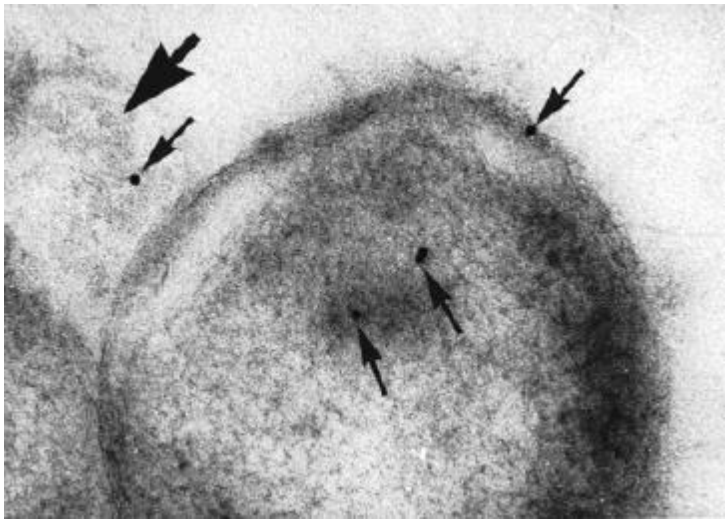


**Figure 1.19 Immunogold detection of β-lactamase on sections of a *P.aeruginosa* cell by Transmission Electron Microscopy (TEM).** The gold particles (small arrows) are associated with the cytoplasm, the OM and emerging OMVs. The large arrow points to a cluster of OMVs. Adapted from Ciofu, (2000).

More recent studies carried out in *A. baumannii* investigated more in detail the translocation of the β-lactamase OXA-58 to the periplasm (Liao et al., 2015). This research demonstrated that OXA-58 was released via OMVs after translocation to the

periplasm via the Sec system (Figure 1.20). This latter, along with the Tat system, is the major pathway for export of proteins across the cytoplasmic membrane to the bacterial periplasm in Gram-negative bacteria (Beckwith, 2013; Costa et al., 2015; Frain et al., 2019).

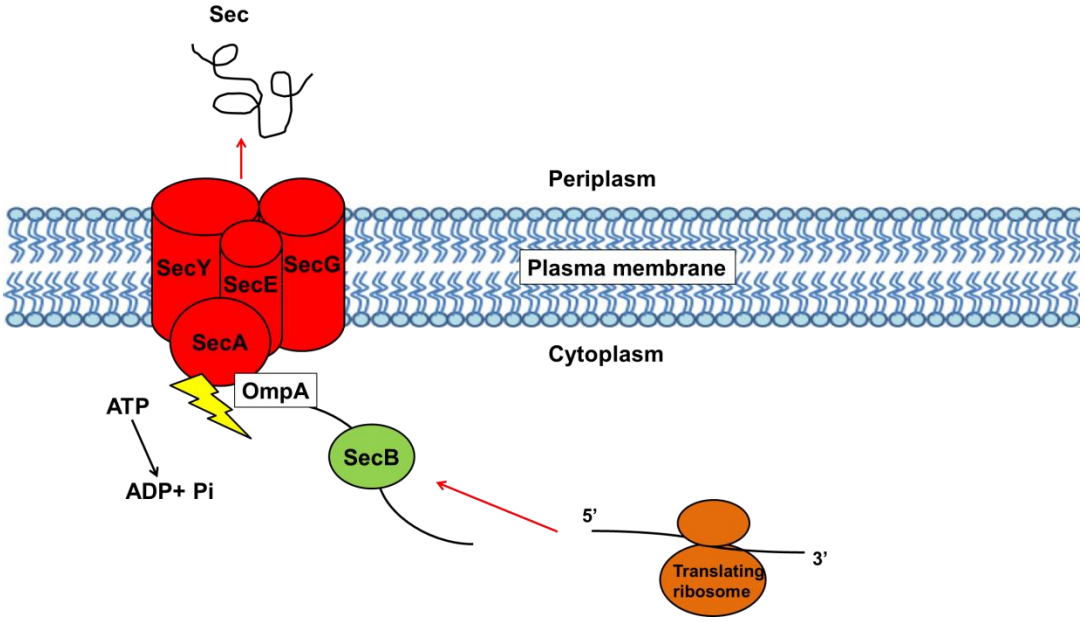



**Figure 1.20 The Sec-dependent protein transport pathway** The Sec pathway translocates cargo proteins across the plasma membrane in a loosely folded or unfolded state, here exemplified with the precursor of the OM protein A of *E. coli* (OmpA). Targeting and folding control of the cargo protein is supported by the chaperone SecB that interacts with nascent chains of the protein and prevents their folding. The Sec machinery itself is composed of the SecYEG channel and the translocation ATPase SecA, which converts chemical energy in the form of ATP into a driving force that pushes the cargo protein through the membrane. At the trans-side of the membrane, the translocated protein folds into its active and protease-resistant final conformation. Adapted from Beckwith, (2013).

.

In the SecA pathway, SecB acts as a chaperone, helping protein transport to the periplasm after complete synthesis of the peptide chains. SecA is an ATPase motor protein and moves the nascent chains to the SecYEG complex using ATP as a source of energy. SecA is inhibited in presence of Rose Bengal (Rb). This inhibitor acts as competitive inhibitor against ATP and bind to the high affinity ATP binding site of the chaperone. This prevents the binding of ATP to SecA, thus blocking its SecA ATPase and the translocation of the proteins through SecYEG. (Huang et al., 2012; Hsieh et al., 2014).

The release of OXA-58-including-OMVs from *A. baumannii* has been studied in presence and in absence of imipenem, a carbapenem antibiotic, in order to investigate whether the presence of stressors lead to an increased OMVs formation and release. These studies demonstrated that OMV-associated OXA-58 was

released in the absence of imipenem, but its release was increased upon carbapenem challenge. However, although it is not still clear whether increase in OMV-associated-β-lactamase could also be due to increase in the amount of enzyme in each OMV in addition to an increase in OMVs released, these studies showed that the antimicrobial treatment enhances OMVs formation (Figure 1.21) (Liao et al., 2015).

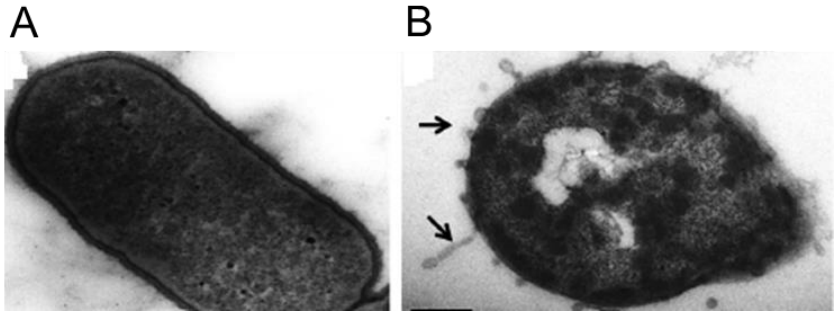



**Figure 1.21 OMVs formation increased after treatment with the imipenem.** TEM of an *A. baumannii* strain expressing wild-type OXA-58 without antibiotic treatment (**A**) and with 8 μg/ml imipenem (**B**). The OMVs are indicated by the arrows. Adapted from Liao et al., (2015).

The phenomenon of the sheltering effect due to the production and release of OMVs has not yet been identified in *K. pneumoniae.* However this bacterium represents a major threat to public health. Indeed this opportunistic pathogen causes broad spectra of diseases and shows increasingly frequent acquisition of resistance to antibiotics (Effah et al., 2020).

## 1.5 The genus *Klebsiella*

### 1.5.1 General features

The genus *Klebsiella* consists of aerobic and facultatively anaerobic, non-motile (except the species *Klebsiella aerogenes*, formerly known as *Enterobacter aerogenes*), Gram-negative rods with 0.3-1 μm in diameter and 0.6-6 μm in length arranged singly, in pairs or in short chains. (Podschun and Ullmann, 1998; Brisse and Duijkeren, 2006; Marković et al., 2013; Merla et al., 2019). Bacteria belonging to this genus show a prominent polysaccharide capsule of considerable thickness that confers the colonies their characteristic mucoid and shiny appearance on agar plates. These colonies appear large, mucoid, and red with diffusing red pigment on MacConkey agar as a result of fermentation of glucose and acid production. Figure

1.22 shows the aspect of colonies formed by *K. pneumoniae*, one of the species of *Klebsiella* genus*. Klebsiella* species are commonly found in the intestinal tract of human and animal, soil, water and botanical environment.

**Figure 1.22 Colony morphology of *K. pneumoniae* isolates on MacConkey agar plate**. In this medium, the most distinctive colony morphology characteristics of *K. pneumoniae* colonies are large, round, glistering, whitish-grey, mucoid in appearance. Adapted from Marković et al. (2013).

### 1.5.2 Pathogenesis and clinical importance

The genus *Klebsiella* is a member of the Enterobacteriaceae, a large family of Gram-negative that comprises other familiar pathogens such as *E. coli, Yersinia* species, *Salmonella* species and *Shigella* species (Podschun and Ullmann, 1998; McAdam, 2020). Because of their ability to spread rapidly in the hospital environment, members of the genus *Klebsiella* tend to cause nosocomial outbreaks (Magill et al., 2014; Guo et al., 2016; Yu et al., 2016; David et al., 2019; Li et al., 2019). *Klebsiella* species may colonize gastrointestinal tract, the skin and pharynx. They may also colonize sterile wounds, urine and may be regarded as normal flora in different parts of the colon, intestinal and biliary tract (Podschun and Ullmann 1998; Brisse et al. 2006; David et al., 2019 ).

The principal pathogenic modality of transmission of *Klebsiella* from patient to patient in hospital setting are the contaminated medical equipment, contaminated hands of medical personnel and blood products. The portals of *Klebsiella* infections are mainly surgical wounds, peritoneum, catheter entrance sites, urinary, respiratory, and biliary tracts (Goetz et al., 1995; Podschun and Ullmann 1998).

Nosocomial *Klebsiella* infections are caused mainly by *K. pneumoniae*, that is the medically most important species of the genus (Merla et al., 2019). The urinary tract

is one of the most common site of infection by *K. pneumoniae.* As with other infections, the urinary tract infections caused by *K. pneumoniae* are often associated with diabetes mellitus (Geerlings; 2008; Martin and Bachman, 2018). *K. pneumoniae* may also cause urinary tract infections associated to the use of catheters. This is probably facilitated by the ability of this pathogen to form biofilms and adhere to catheters (Schroll et al., 2010). *K. pneumoniae* is also cause of nosocomial septicaemia and is one of only a few Gram-negative rods able to cause pneumonia. With regards to this, this pathogen is a leading cause of nosocomial pneumonia. Additionally, *K. pneumoniae* is also cause of infections of wound/surgical sites (Martin and Bachman, 2018).

Currently *K. pneumoniae* represents an increasing threat to public health and is considered one of the most concerning pathogens worldwide (Effah et al., 2020). For example, according to the China Antimicrobial Surveillance Network the prevalence of carbapenem-resistant *K. pneumoniae* has increased each year in China, from 3.0% in 2005 to 25.0% in 2018, with Zhejiang Province reporting one of the highest rates of resistance (Carbapenem-resistant *K. pneumoniae* more than 50%) in China in 2018 (Hu et al., 2020). KPC- positive *K. pneumoniae* have also been increasingly reported in many European countries (Girmenia et al., 2016). In particular, in Italy, the first KPC-positive *K. pneumoniae* strains were isolated in 2008 (Giani et al., 2013). Since then several cases and outbreaks by KPC-positive *K. pneumoniae* strains were reported as a consequence of a rapid dissemination in almost all hospitals in the north, middle and south regions of the country. KPC-positive *K. pneumoniae* have spread rapidly and extensively in Italy, with a sharp increase reported by the EARS-Net surveillance system for bacteraemia isolates, from 1–2% carbapenem resistance in 2006–09 to 26.7% in 2011 and 32.9% in 2014 (Giani et al., 2013; Girmenia et al., 2016)

*K. pneumoniae* acquired high antimicrobial resistance during the last decades. Indeed *K. pneumoniae* strains are now often resistant to a broad spectrum of drugs. As a consequence of this antibiotic resistance, infections such as urinary tract infections have become recalcitrant to treatment, and more serious infections such as pneumonias and bacteremias have become increasingly life-threatening (Boucher et al., 2009; Paczosa and Mecsas, 2018). Two major types of antibiotic resistance have been commonly observed in *K. pneumoniae*. One mechanism involves the expression of ESBLs that confer *K. pneumoniae* resistance towards penicillins, first-, second- and third-generation cephalosporins as well as aztreonam (Jesus et al., 2015). The other mechanism of resistance, which is even more troubling, is the expression of carbapenemases by *K. pneumoniae*, which renders bacteria resistant to almost all available β-lactams, including the carbapenems This resistance is

resulting in an increasing worldwide problem regarding the choice of effective antibiotic treatment for hospital-acquired infections (Davies and Davies 2010; Ferreira 2019).

*K. pneumoniae* is considered as the most common pathogen in bacterial endogenous endophthalmitis associated with liver abscesses in Asia (Han 1995; Wong et al., 2000; Seale et al., 2007; Chen et al., 2010; Fujita et al., 2019). The disease also has been reported in countries of non- Asian origin (Scott et al., 2004; Lederman and Crum 2005). In addition, the *K. pneumoniae* serotype K1 is capable of causing catastrophic septic ocular or central nervous system as complications from pyogenic liver abscess especially in underlying patients (Fang et al. 2007; Wells, 2019). This serotype has been observed worldwide and is the most prevalent type in invasive infections among all the 78 *K. pneumoniae* serotypes (Lin et al., 2014).

*K. pneumoniae* has been implicated in the development of ankylosing spondylitis because of the high incidence of *Klebsiella* in the bowel flora of patients whose disease is in an active state (Ogasawara et al. 1986; Sahly and Podschun 1997; Fujita, 2019). Also, *K. pneumoniae* was associated with chronic diarrhoea in HIV-infected persons (Nguyen Thi et al. 2003).

*K. pneumoniae* subspecies *rhinoscleromatis* is associated with rhinoscleroma, a disease characterized by a chronic inflammatory process of the nasopharynx. This infection is distributed around the world and become an endemic in certain areas in Eastern Europe, Latin America, central Africa, and southern Asia (Sahly and Podschun 1997; Corelli et al., 2018).

Ozena, a chronic atrophic rhinitis caused by *K. pneumoniae* subspecies *ozaenae,* is characterized by necrosis of the mucosa and mucopurulent nasal discharge (Strampfer et al. 1987). *K. pneumoniae* subspecies *ozaenae* may cause invasive infections, especially in immunosuppressed hosts such as bacteraemia with or without meningitis, otitis, mastoiditis, urinary tract infections, wound infections, corneal ulcers, pneumonia, or brain abscess (Brisse et al. 2006; Kumar et al., 2013).

Due to a lack of available effective treatments, *K. pneumoniae* infections caused by ESBL-producing and carbapenem-resistant bacteria have significantly higher rates of morbidity and mortality than infections with nonresistant bacteria (Liu et al., 2019)

In addition to causing hospital-acquired diseases, *K. pneumoniae* is also responsible of community-acquired diseases. These are infections contracted outside of a hospital or are diagnosed within 48 hours of admission without any previous health care encounter (Martin and Bachman, 2018). Among these infections, community-acquired pneumonia is a very severe fatal illness with a rapid onset, high fever, and haemoptysis (currant jelly sputum), bulging interlobar fissure and cavitary abscesses observed by chest radiographic (Spellberg et al., 2014). The observed mortality rates

range from about 25 to 50% but can approach 100% in hospitalized, immunocompromised patient with underlying diseases such as diabetes mellitus (Waterer, 2017). The *Klebsiella* species are second to *E. coli* causing bacteraemia representing 3-8% of all nosocomial bacterial infection (Podschun and Ullmann 1998; Waterer, 2017). Community-acquired bacteraemia are usually caused by urinary tract infection, vascular catheter infection, and cholangitis (Ko, 2002).

A distinctive syndrome of community-acquired *K. pneumoniae* septicaemia with liver abscess has been reported in Taiwan and Korea since 1981 (Jun, 2018). This syndrome is characterized by high mortality (10 to 40%), and some cases have been complicated by meningitis or endophthalmitis (Fang et al., 2007; Jun, 2018). The disease has also been reported elsewhere in Asia, North America, Europe, and Australia (Chung et al., 2007; Keynan and Rubinstein 2007; Siu et al., 2012). The symptoms of the disease is characterized by fatigue, anorexia, nausea, diffuse abdominal discomfort, pleuritic chest pain, jaundice and fever (Lederman and Crum, 2005).

*K. pneumoniae* is also a common cause of community acquired bacterial meningitis in adults in Taiwan (Lee et al., 2018). Outside Taiwan, cases of *K. pneumoniae* meningitis have occurred, predominantly in other parts of Asia, Europe and North America (Yanagawa et al.,1989; Ohmori et al., 2002; Giobbia et al. 2003; Braiteh and Golden 2007; Chung, 2019). The rarity of these cases outside Asia raises the possibility of ethnicity or country of origin predisposing individuals to invasive disease (Ko et al. 2002).

The emergence of community-acquired infections has been often attributed to the recent occurrence and spread of hypervirulent strains of *K. pneumoniae* (Cheng et al., 1991; Liu et al., 2014). Indeed, hypervirulent strains are not only able to cause nosocomial infections in hospitalized patients but can also cause serious infections in a number of healthy and immunosufficient people (Liu et al., 2014). This variant differs from the classic strain by the aspect of colonies that appear hypermucoviscous on agar plate (Choby et al., 2020). The ‘string test’ was developed as a phenotypic test for hypermucoviscosity; when a colony can be stretched at least five millimetres with an inoculation loop, the isolate is considered hypermucoviscous and therefore hypervirulent (Fang et al., 2004; Sánchez-López et al., 2019; Choby et al., 2020). The hypervirulent strains show the ability to spread from the primary site of infection to other distant parts of the body, including eye, lung and central nervous system that is an unusual feature for enteric Gram-negative bacilli in immunocompromised patients (Choby et al., 2020). The genetic determinants of hypervirulence are often found on large virulence plasmids as well as chromosomal mobile genetic elements which can be used as biomarkers to distinguish hypervirulent from classic clinical isolates (Liu et

al., 2014). These distinct virulence determinants of hypervirulence include up to four siderophore systems for iron acquisition, increased capsule production, K1 and K2 capsule types and the colibactin toxin (Choby et al., 2020).

### 1.5.3 Virulence factors

*K. pneumoniae* employs many strategies to grow and protect itself from the host immune response. Importantly, many *K. pneumoniae* mutants are cleared from the lungs of mice more rapidly than wild-type *K. pneumoniae* strains, suggesting that wild-type *K. pneumoniae* employs various factors to circumvent early host responses (Cortes et al., 2002; Paczosa and Mecsas, 2018). To date, there are four major classes of virulence factors that have been well characterized in *K. pneumoniae.* These virulence factors consist of capsule, including the production of hypercapsule in hypervirulent strains; LPS; siderophores; and fimbriae, also known as pili (Paczosa and Mecsas, 2018).

#### 1.5.3.1 Capsule

The capsule is the polysaccharide matrix that coats the cell. The capsule is recognized as one of the most important virulence factors of *Klebsiella* species (Podschun and Ullmann 1998; Lawlor et al., 2006). The capsular material forms thick bundles of fibrillous structures covering the bacterial surface in extensive layers to protect the bacterium from phagocytosis by macrophage, and prevents killing of the bacteria by bactericidal serum factors (Figure 1.23) (Simoons-Smit et al. 1986; Cortes, et al. 2002; Lin et al. 2004; Brisse et al. 2006; Paczosa and Macsas, 2018).

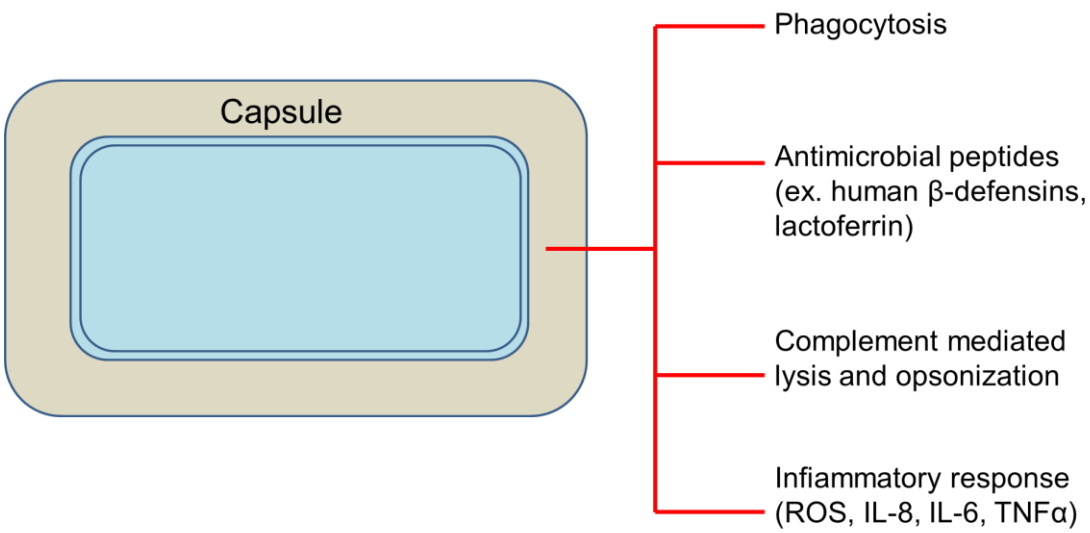



**Figure 1.23 Role of capsule in *K. pneumoniae* virulence**. A number of different functions for capsule have been characterised for *K. pneumoniae* virulence. First, capsule can prevent phagocytosis of the bacteria by immune cells. Second, it hinders the bactericidal action of antimicrobial peptides such as human beta defensins and lactoferrin by binding these molecules distal from the OM. Third, it inhibits complement components from interacting with the membrane, thus preventing complement-mediated lysis and opsonization. Finally, it averts the fulminant activation of the immune response, as measured by decreased reactive oxygen species (ROS), IL-8, IL-6, and TNFα production, by assisting in the activation of a NOD-dependent pathway and shielding LPS from recognition by immune cell receptors. Adapted from Paczosa and Macsas, (2018).

Furthermore, hypervirulent. *K. pneumoniae* strains produce a hypercapsule, responsible for the hypermucoviscous aspect of these strains. The hypercapsule consists of a mucoviscous exopolysaccharide bacterial coating that is more robust than that of the typical capsule thus contributing significantly to the pathogenicity of hypervirulent *K. pneumoniae* (Fang et al., 2004;Sánchez-López et al., 2019; Choby et al., 2020). Both classical capsule and hypercapsule are made up of strain-specific capsular polysaccharides named K antigens (i.e., K1 and K2, up through K78) (Kuo-Ming Yeh et al., 2007; Choby et al., 2020). The genes needed for the production of capsule in both classical and hypervirulent *K. pneumoniae* strains are located on a chromosomal operon, *cps*, where both the organization and the sequence of the genes are conserved compared *to E. coli* (Arakawa et al., 1991; Shu et al., 2009; Paczosa and Macsas, 2018). The *cps* gene cluster harbors a number of genes involved in capsule production (*wzi*, *wza*, *wzb*, *wzc*, *gnd*, *wca*, *cpsB*, *cpsG*, and *galF*), capsule attachment to the OM (*wzi*) in the polymerization of capsular polysaccharides (*wzy*, also known as *orf4*) and surface assembly (*wza*, *wzc*, *orf5*, and *orf6)*. *K. pneumoniae* produces a variety of capsule types (Arakawa et al., 1991; Shu et al., 2009). This diversity in capsule types arises from the glycosyltransferase activities of

the additional genes *wbaP, wbaZ, wcaN, wcaJ,* and *wcaO* (Rahn et al., 1999; Shu et al., 2009). Furthermore, comparative analysis of clinical isolates of *K. pneumoniae* has shown a large amount of diversity in the *cps* gene cluster sequence between strains and even within strain types (Shu et al., 2009; Chen et al., 2014; Chiang et al., 2015)

Based on the most frequently isolated serotypes collected from patients and results from mouse experiments, K1 and K2 strains are normally more virulent than strains of other serotypes (Lin et al., 2010; Choby et al., 2020).

During *K. pneumoniae* infection, the capsule protects against the host immune response through multiple mechanisms, including inhibiting phagocytosis by immune cells, preventing activation of the early immune response, and abrogating lysis by complement and antimicrobial peptides (Podschun and Ullmann 1998; Paczosa and Macsas, 2018). Capsule-mediated prevention of bacterial binding and internationalization by immune cells helps limit early inflammatory signals, resulting in a less robust induction of the immune response (Kabha et al., 1995; Lin et al., 2004). Furthermore, *in vitro* studies have shown that the presence of capsule inhibits the deposition of the complement component C3 onto the bacterium, and reduces adhesion and phagocytosis of the bacterium by macrophages and epithelial cells (Alvarez et al. 2000; Cortes et al. 2002 Doorduijn et al., 2016). In general, the hypercapsule phenotype enhances resistance to a variety of humoral defenses, including complement killing, and other antimicrobial peptides such as human neutrophil protein 1 and lactoferrin (Fang et al., 2004). Hypervirulent *K. pneumoniae* strains were shown to be less sensitive to complement killing than classical strains (Pomakova et al., 2012). Furthermore, the hypercapsule has been correlated with increased resistance to phagocytosis by human neutrophils and macrophages compared to a number of classical strains (Fang et al., 2004; Pomakova et al., 2012).

#### 1.5.3.2 Adhesion

Fimbriae, also known as pili, are nonflagellar, filamentous projections located on the bacterial surface. They represent another important class of *K. pneumoniae* virulence factors and are important mediators of *K. pneumoniae* adhesion to mucosal and epithelial cell surfaces. This is often the first step in the development of colonization and infection (Podschun and Sahly, 1991; Podschun and Ullmann 1998). Strains of *K. pneumoniae* commonly express three types of pili known as types 1 (common pili), 3 and 6. Pili. However, In *K. pneumoniae*, type 1 and 3 fimbriae are the major

adhesive structures that have been characterized as pathogenicity factors (Figure 1.24) (Paczosa and Mecsas, 2018).

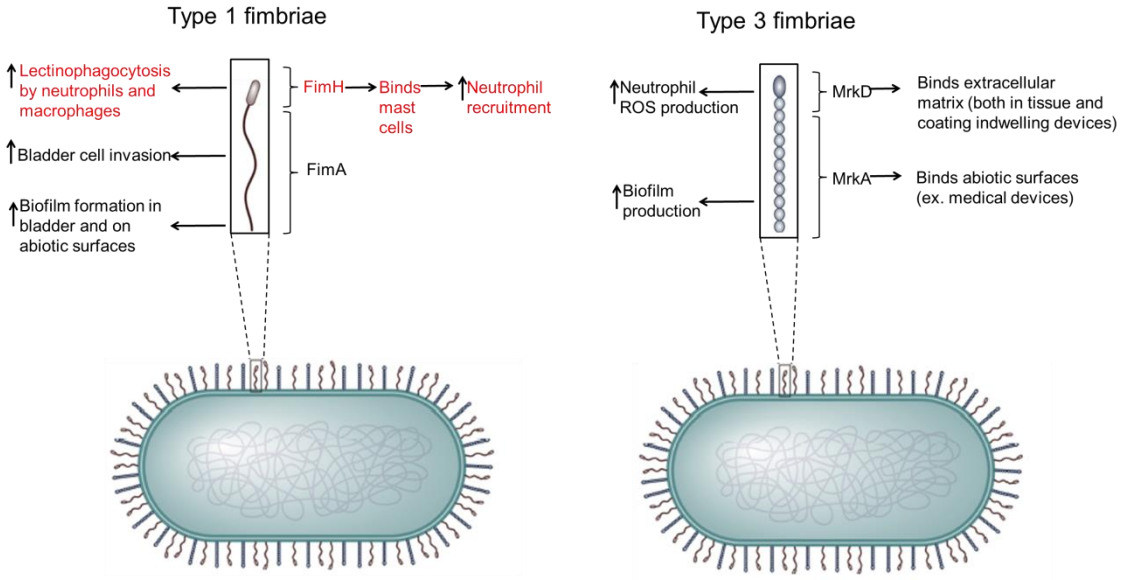



**Figure 1.24 Functions of type 1 and type 3 fimbriae during *K. pneumoniae* infection and biofilm formation.** Type 1 fimbriae are filamentous, membrane-bound, adhesive structures composed primarily of FimA subunits, with the FimH subunit on the tip. These fimbriae have a role in bladder cell invasion by *K. pneumoniae* as well as biofilm formation in the bladder and on abiotic surfaces. However, type 1 fimbriae may be a negative influence on *K. pneumoniae* virulence in vivo in a few ways. First, type 1 fimbriae amplify lectinophagocytosis of *K. pneumoniae* by macrophages and neutrophils. Second, the FimH subunit increases binding to immune cells such as mast cells, leading to increased immune cell activation and subsequent recruitment of neutrophils, which likely increases *K. pneumoniae* clearance. Functions of type 3 fimbriae during *K. pneumoniae* infection and biofilm formation. Type 3 fimbriae are helix-like, membrane-bound, adhesive structures on the surface of *K. pneumoniae*. They are composed primarily of MrkA subunits, with the MrkD subunit on the tip. Type 3 fimbriae have been found to be necessary for *K. pneumoniae* biofilm production and binding to medical devices. MrkD has specifically been found to bind extracellular matrix, such as that exposed on damaged tissues and coating indwelling devices, while MrkA binds abiotic surfaces, such as medical devices both prior to insertion into patients and after insertion when coated with host matrix. Type 3 fimbriae have been shown to have a possibly detrimental role, as their presence on *K. pneumoniae* increases reactive oxygen species (ROS) production by neutrophils. Adapted from Paczosa and Mecsas, (2018).

Type 1 fimbriae are expressed in more than 80% of clinical isolates of *K. pneumoniae* and a number of studies have shown these fimbriae are an important virulence factor in *K. pneumoniae* urinary tract infection (Podschun and Sahly 1991; Podschun and Ullmann 1998; Brisse *et al.* 2006; Struve et al. 2009). *K. pneumoniae* type 1 fimbriae bind D-mannosylated glycoproteins, and therefore, binding by type 1 fimbriae is commonly termed “mannose-sensitive” binding (Schroll et al., 2010). They mediate adhesion to mannose containing structures present on host cells or in the extracellular matrix and cause agglutination of guinea pig erythrocytes (Struve et al.,

2009). A Type 3 fimbriae are helix-like filaments and are characterized by their ability to agglutinate erythrocytes treated with tannic acid. In contrast to type 1 fimbriae, type 3 fimbriae are “mannose insensitive” and therefore do not bind mannose (Paczosa and Mecsas, 2018). *In vitro* studies have revealed that type 3 fimbriae mediate binding to different structures in human; human endothelial cells, epithelia of the respiratory tract, uroepithelial cells, and type V collagen structures (Brisse et al. 2006; Struve et al., 2009). Furthermore, type 3 fimbriae are also believed to contribute to the formation of biofilms (Schroll et al., 2010).

#### 1.5.3.3 LPS

LPS, also known as endotoxin, is a major component of the outer leaflet of the cell membrane of all Gram-negative bacteria. The LPS molecule typically comprises the lipid A, a core polysaccharide and a side chain O-antigen (O-Ag) polysaccharide (Figure 1.25). Nine O-antigen types are distinguished in *K. pneumoniae* with O1 being the most frequent. The lipid A anchors the LPS molecule into the OM and is a potentially potent activator of inflammation. *K. pneumoniae* may modify its lipid A to make it less inflammatory during infection, and lipid A may also protect against the bactericidal action of cationic antimicrobial peptides. O antigen is the outermost subunit of LPS. It has important roles in protecting against complement, including preventing C1q binding to bacteria, which inhibits subsequent activation of the complement pathway, as well as binding C3b away from the OM and, thus, abrogating bacterial lysis by the complement membrane attack complex (Paczosa and Mecsas, 2018).

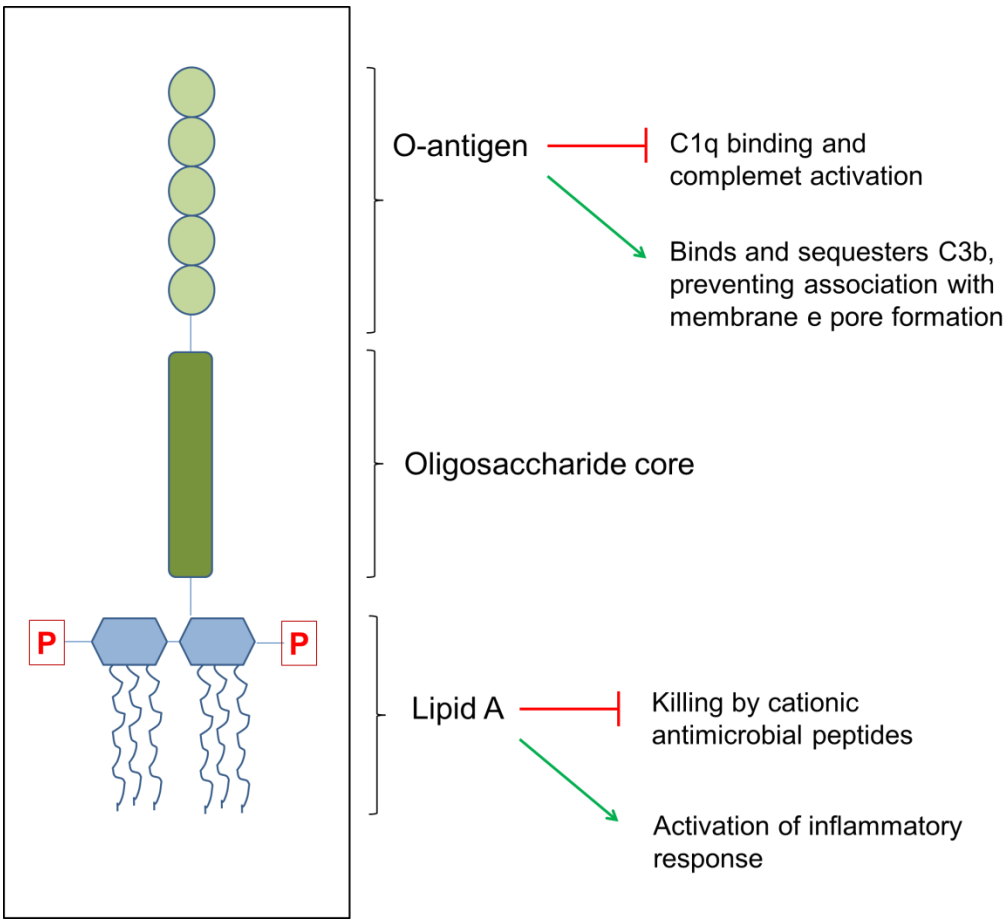



**Figure 1.25 Role of LPS in *K. pneumoniae* virulence.** LPS is composed of three major subunits: lipid A, an oligosaccharide core, and O antigen. Lipid A inserts into the bacterial membrane and is a potentially potent activator of inflammation. *K. pneumoniae* may modify its lipid A to make it less inflammatory during infection, and lipid A may also protect against the bactericidal action of cationic antimicrobial peptides. O antigen is the outermost subunit of LPS. It has important roles in protecting against complement, including preventing C1q binding to bacteria, which inhibits subsequent activation of the complement pathway, as well as binding C3b away from the outer bacterial membrane and, thus, abrogating bacterial lysis by the complement membrane attack complex. Adapted from Paczosa and Macsas, (2018).

### 1.5.3.4 Siderophores

Iron is a limited resource and must be acquired from the environment for *K. pneumoniae* to thrive during infection. This metal is not readily available in the host during infection. Indeed, the host sequesters it to restrict the growth of a number of possible pathogens as part of the nonspecific immune response (Bullen et al., 1972; Carniel, 2001). Therefore, *K. pneumoniae*, like many other bacterial pathogens, must employ tactics to acquire iron from the host in order to survive and propagate during mammalian infection (Bachman et al., 2012; Russo et al., 2015). *K. pneumoniae* strains encode several siderophores, including enterobactin, yersiniabactin,

salmochelin, and aerobactin (Figure 1.26). The production of more than one siderophore by *K. pneumoniae* is thought to be a means of optimizing successful colonization of different tissues and/or avoiding neutralization of one siderophore by the host (Brock et al., 1991; Perry et al., 1999; Paczosa and Mecsas, 2018).

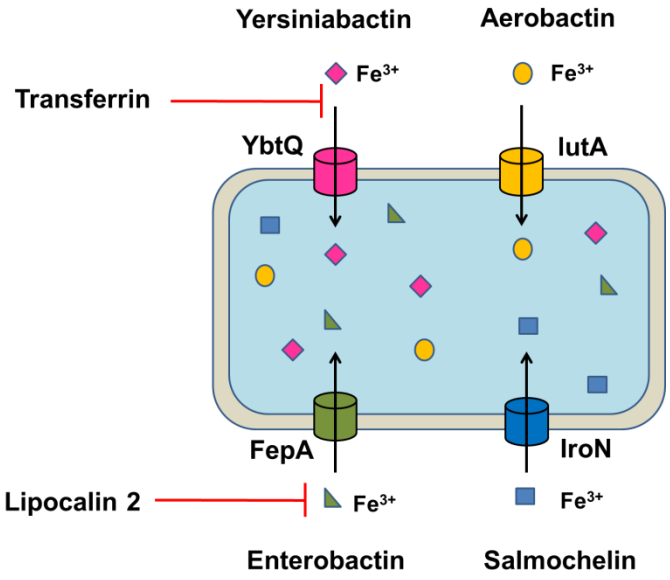



**Figure 1.26 Siderophore production and roles in virulence in *K. pneumoniae*.** The ability to acquire iron in an iron-poor environment during infection is necessary for *K. pneumoniae* pathogenesis. Therefore, bacteria secrete proteins with a high affinity for iron, called siderophores. *K. pneumoniae* strains have been found to produce one or more of the following siderophores: enterobactin, salmochelin, yersiniabactin, and aerobactin. Enterobactin is the primary siderophore used by *K. pneumoniae*, although it is inhibited by the host molecule lipocalin-2. Salmochelin is a c-glucosylated form of enterobactin that can no longer be inhibited by lipocalin-2. Yersiniabactin and aerobactin are structurally distinct from enterobactin and salmochelin. Neither siderophore can be inhibited by lipocalin-2, but yersiniabactin functionality is reduced in the presence of the host molecule transferrin. The production of a number of different siderophores may allow *K. pneumoniae* to colonize and disseminate to a number of different sites within the host, with niche-specific roles for each siderophore. FepA, IroN, YbtQ, and IutA serve as transporters specific to their corresponding siderophores of enterobactin, salmochelin, yersiniabactin, and aerobactin, respectively. Adapted from Paczosa and Macsas, (2018).

### 1.5.3.5 Other virulence factors

Several other factors were recently identified as having roles in *K. pneumoniae* virulence. These virulence factors include OMPs, porins, efflux pumps, iron transport systems, and genes involved in allantoin metabolism (Paczosa and Mecsas, 2018). OMVs have been more recently included among the *K. pneumoniae* virulence factors following studies showing that OMVs induce the innate immune response in the host (Cano et al., 2009; Lee et al., 2012). However,

these factors are not yet thoroughly characterized in *K. pneumoniae* and much work remains to be done to fully understand their mechanisms of action and clinical significance (Paczosa and Mecsas, 2018).

### 1.5.4 *K. pneumoniae* in polymicrobial infections

*K. pneumoniae* has also been isolated in polymicrobial infections in addition to monomicrobial infections. With regards to this a recent study showed that patients with complicated intra-abdominal infections were at high risk for polymicrobial bacteremia involving *K. pneumoniae* and several other microorganisms including Gram-positive and Gram-negative organisms (Arahata et al., 2018; Liu et al., 2019). Although these studies showed no substantial difference in bacteremia-related mortality in patients with monomicrobial infection by *K. pneumoniae* and those with polymicrobial infection, the outcomes for the patients with polymicrobial infection were substantially worse outcomes than those with monomicrobial infection (Liu et al., 2019). Additionally, the appropriateness of the antimicrobial therapy was substantially lower among the patients in the polymicrobial group than among those in the monomicrobial group. Another study described an aggressive case of polymicrobial bacteremia in a patient undergoing chemotherapy (Arahata et al., 2018). The blood culture isolates included *K. pneumoniae, E. coli, S. pneumoniae, M.catarrhalis.* This patient died in spite of several treatments including fluid resuscitation, mechanical ventilation and antibiotic treatments (Arahata et al., 2018). Moreover, in Southern Taiwan *K. pneumoniae* is one of the most common pathogens involved in polymicrobial peritoneal dialysis-related peritonitis that cause severe and mortality in patients affected by this disease (Lin et al., 2018).

## 1.6 Summary and aims

In conclusion *K. pneumoniae* is a pathogen with a great biomedical interest since it represents an increasing threat to public health. Although this pathogen has been identified in patients with polymicrobial antibiotic-resistant infections, the sheltering effect has not yet been investigated in *K. pneumoniae.* The present work focus on investigating this phenomenon in *K. pneumoniae*.

The specific aims of this project are:

- To Investigate whether β-lactam resistant *K. pneumoniae* species are able to shelter susceptible species from the action of β-lactam-drugs.
- To develop different methods for the quantification of the sheltering effect.

- To characterise the phenotypic variability in the sheltering effect across a panel of *K. pneumoniae* strains
- To investigate the genetic determinants of the phenotypic variability in the sheltering effect
- To investigate whether the OMVs are responsible for the sheltering effect exerted by *K. pneumoniae* similarly to the other pathogenic bacteria.
- To investigate new features of this phenomenon, including how the sheltering effect changes with time and the presence of non-β-lactam antibiotics in the medium.

# 2 MATERIALS AND METHODS

## 2.1 Growth media

The recipe of all the bacterial growth media used in this study is described in Table 2.1. All the media were sterilized by autoclaving for 15 min at 121ºC and cooled prior to bacterial inoculation.

**Table 2.1 Recipe for the preparation of the growth media used**. $DIH_2O$: deionised water.

| **Media** | **Recipe** |
|---|---|
| solid 2x Yeast Extract Tryptone (2x YT) | 3.1 % powder (OXOID)+ 1.5 % molecular agar (Fisher Scientific) dissolved in $DIH_2O$ water (g/ml) |
| liquid 2x YT | 3.1 % powder (OXOID) dissolved in $DIH_2O$ water (g/ml) |
| solid Iso-Sensitest | 3.1 % powder (OXOID) dissolved in $DIH_2O$ water (g/ml) |
| liquid Iso-Sensitest | 2.34 % powder (OXOID) dissolved in $DIH_2O$ water (g/ml) |
| solid MacConkey | 5.2 % powder (OXOID) dissolved in $DIH_2O$ water (g/ml) |
| solid Mannitol Salt Agar (MSA) | 11 % powder (OXOID) dissolved in $DIH_2O$ water (g/ml) |

## 2.2 Bacterial strains

A total of 33 clinical isolates of *K. pneumoniae* were collected as part of routine care in a Kuwait hospital in 2013-2014. These isolates were kindly provided by Dr. Benjamin Evans (personal correspondance) and were all carbapenem resistant.
The details of the bacterial strains used as reference controls for the MIC experiments (paragraph 2.4.2) as well as the bacterial strains used for the cloning experiments and confocal microscopy (paragraphs 2.15 and 2.16) are reported in Table 2.2.

**Table 2.2 MIC control strains and cloning strains used in this study**

| Name/ID | Species | Purpose | Reference or Source |
|---|---|---|---|
| NCTC 10418 | *E. coli* | Reference strain for MIC | Public Health England |
| NCTC 6571 | *S. aureus* | Reference strain for MIC | Public Health England |
| ATCC 25922 | *E. coli* | Reference strain for MIC | Public Health England |
| NCTC 10662 | *P. aeruginosa* | Reference strain for MIC | Public Health England |
| DH5α | *E. coli* | Cloning | Invitrogen |
| CIP70-10 | *A. baumannii* | Cloning | Bouvet and Grimont, 1986; Coyne et al., 2010; kindly supplied by Laurent Poirel |
| BL21 DE3 | *E. coli* | Cloning for protein expression studies | ThermoFisher Scientific |
| AbCIP70.10(pyOXA-58-GFP) | *A. baumannii* | Confocal microscopy | This study |
| AbCIP70.10(py) | *A. baumannii* | Confocal microscopy | This study |

## 2.3 Storage of isolates

*K. pneumoniae* isolates were inoculated onto 2x YT agar with 100 µg/ml ampicillin (Amp, Sigma-Aldrich). The strains *E. coli* NCTC 10418, *E. coli* ATCC 25922 and *P. aeruginosa* NCTC 10662 were inoculated onto McConkey agar. Both *A. baumannii* CIP70-10 and *E. coli* DH5α were inoculated onto plain 2x YT agar. *S. aureus* NCTC 6571 was inoculated onto MSA. The strains AbCIP70.10 (pyOXA-58-GFP) and AbCIP70.10(pyOXA-58-GFP) were grown onto 2x YT agar with 50 µg/ml kanamycin (Kan, Sigma-Aldrich). All the plates were then incubated overnight at 37°C. The freezer stocks were prepared by picking a single bacterial colony from the plate and inoculating it in 5 ml of 2x YT broth (with the appropriate antimicrobial wherever applicable). The bacteria were then grown overnight at 37°C with shaking at 220 rpm. 600 µl of each overnight culture were mixed with 400 µl of a sterile 50 % v/v glycerol solution in a cryovial. The tubes were then placed at -80°C for long-term storage.

## 2.4 Antimicrobial sensitivity testing

### 2.4.1 Antimicrobial agents

Antimicrobial agents used in this study are listed as follows: meropenem (Mer, Melford) ertapenem (Ert, Melford), ciprofloxacin (Cipr, Sigma), ceftazidime (Ceft, Fisher Scientific), cefotaxime (Cefo, Fisher Scientific), cefepime (Cefe, Fisher Scientific) and chloramphenicol (Chlor, Sigma). For each of these drugs, a stock solution was prepared by dissolving 10 mg of antimicrobial powder in the appropriate solvent as described in Table 2.3. The stock solutions were sterilized by filtration with sterile PVDF syringe filters with a 0.22 µm pore size (Millipore).

**Table 2.3 List of antimicrobials used in this study, the solvent used to dissolve each of them and the range of antimicrobial concentrations tested**. DMSO: Dimethyl sulfoxide.

| **Antimicrobial** | **Solvent** | **Antimicrobial concentrations (µg/ml)** |
|---|---|---|
| Chlor | Ethanol 100% | 0.25-128 |
| Ceft | Saturated solution $NaHCO_3$ | 0.004-128 |
| Cefo | $DIH_2O$ | 0.004-128 |
| Cefe | $DIH_2O$ | 0.004-128 |
| Cipr | $DIH_2O$ | 0.004-128 |
| Mer | DMSO | 0.015-128 |
| Ert | $DIH_2O$ | 0.015-128 |

### 2.4.2 Minimum Inhibitory Concentration (MIC)

The antimicrobial susceptibilities of the 33 *K. pneumoniae* isolates were determined by MIC, a method that identifies the lowest concentration of drug that inhibits the visible growth of a microorganism (Andrews, 2001). The tests were conducted with the agar dilution method following the BSAC guidelines (British Society for Antimicrobial Chemoterapy, 1991; Andrews, 2001).

For each antimicrobial, a specific range of 2-fold dilutions was prepared (Table 2.3) from the stock solutions. Each of these dilutions was prepared by mixing the

calculated volume of the stock solution with 20 ml of Iso-Sensitest liquid agar in a tube and pouring it into the respective plates.

The *K. pneumoniae* isolates were grown overnight in 5 ml of Iso-Sensitest broth. The bacterial cultures were diluted 1:10 with sterile distilled water and the absorbance at 600 nm ($OD_{600}$) was measured with a Biophotometer Plus (Eppendorf) after calibrating the instrument with sterile Iso-Sensitest broth. The absorbance of the bacterial culture was adjusted to be 0.08 to 0.1 that corresponds to 0.5 McFarland standard (Cockerill et al., 2012) by diluting the culture with sterile distilled water. The suspensions were further 1:10 diluted with sterile $DIH_2O$ before inoculation on Iso-Sensitest agar plates. The inoculation was carried out using a multipoint inoculator to deliver 1 µl of each bacterial suspension ($10^6$ cfu/spot) to the surface of Iso-Sensitest agar plates. Inoculated plates were incubated overnight at 37ºC.

The MIC of the isolates was the lowest concentration of drug that inhibited the visible growth of the bacteria on the plates.

The standard strains *S. aureus* NCTC 6571, *E. coli* NCTC 10418, *P. aeruginosa* NCTC 10662 and *E. coli* ATCC 25922 were used to monitor test performance since the MIC for a number of antimicrobials is known for these strains. This MIC for these control strains should be within plus or minus one two-fold dilution of the expected MIC to validate the results of the test (Andrews, 2001). The MICs of the different antimicrobials were interpreted according to the new EUCAST breakpoints (http://www.eucast.org/clinical_breakpoints/) valid from 2015. The EUCAST breakpoints are MIC values taken as a reference to determine if a strain shows sensitivity, intermediate resistance or high resistance to a given antimicrobial drug (Table 2.4).

**Table 2.4 EUCAST breakpoints values for *K. pneumoniae* (µg/ml) for Chlor, Ceft, Cefo, Cefe, Cipr, Mer and Ert.** S: sensitive; I: intermediate; R: resistant.

| Antimicrobial | S ≤ | I | R> |
|---|---|---|---|
| Chlor | 8 | - | 8 |
| Ceft | 1 | 2-4 | 4 |
| Cefo | 1 | 2 | 2 |
| Cefe | 1 | 2-4 | 4 |
| Cipr | 0.5 | 1 | 1 |
| Mer | 2 | 4-8 | 8 |
| Ert | 0.5 | 1 | 1 |

## 2.5 Genomic DNA purification

DNA extraction of the 33 *K. pneumoniae* isolates was prepared with a Cetyl trimethylammonium bromide (CTAB) genomic DNA extraction protocol (Wilson, 2001). All centrifugation steps were carried out at 13,000 rpm in a table-top microcentrifuge (Eppendorf) at room temperature. The bacterial culture of all the *K. pneumoniae* isolates was grown in 5 ml 2x YT broth with 100 µg/ml Amp and then 1.5 ml of the culture was centrifuged at 13,000 rpm for 2 min. The pellet was re-suspended in 630 µl of TE buffer (appendix 8.2). The bacterial cells were then lysed with 20 µl of 10 mg/ml chicken egg white lysozyme (Sigma-Aldrich) and the lipid structures in the cell membrane were disrupted with 40 µl of 10% sodium dodecyl sulphate (SDS) (Sigma-Aldrich). Furthermore, the samples were treated with 20 µl of 10 mg/ml ribonuclease A from bovine pancreas (Sigma-Aldrich) and 5 µl of 10 mg/ml proteinase K (PK) from *Tritirachium album* in order to degrade RNA and proteins respectively. The samples were incubated for 1 hour at 37°C and then for 1 hour at 50°C. 100 µl of 10% cetyl trimethylammonium bromide (CTAB) (Sigma-Aldrich) and 200 µl of 5 M NaCl were added and the samples were then incubated at 65°C for 10 min. The proteins and polysaccharides were separated from DNA by the addition of 500 µl of chloroform:isoamyl alcohol (24:1) (Sigma-Aldrich). Following incubation on ice for 30 mins, the samples were centrifuged for 10 min and the aqueous phase was recovered and treated with 500 µl phenol:chloroform:isoamyl alcohol (25:24:1) (Sigma-Aldrich). After centrifuging the samples for 5 min, 540 µl of isopropanol were added to precipitate the DNA. The samples were again centrifuged for 10 min, and the pellet was washed with 500 µl of 70% ethanol. Following a last step of centrifugation for 5 min, the ethanol was discarded and the DNA pellet was air-dried overnight. Lastly the pellets were resuspended in 30 µl TE buffer (appendix 8.2). After determination of DNA concentration (paragraph 2.6) the DNA samples were placed at -20°C for long-term storage.

## 2.6. Determination of DNA concentration

The quality of the DNA extracted was assessed with a nanodropTM 1000 spectrophotometer (Thermo Fisher Scientific). The instrument was cleaned with $DIH_2O$ and calibrated with TE buffer. The concentration of the DNA extracted (ng/µl) was determined by measuring the UV absorbance at 260 nm and applying the Beer-Lambert Law. The purity of the DNA was determined by calculating the ratio of absorbance at 260 and 280 nm. A ratio of circa 1.8 was considered as pure for DNA.

## 2.7 Polymerase Chain Reaction (PCR)

The PCR was used for different purposes, described as follows:

1. Identification of the β-lactamases.
   Primers for the amplification of parts of the genes known to cause resistance to β-lactams in Enterobacteriaceae were used. These genes were: *bla*$_{IMP}$, *bla*$_{VIM}$, *bla*$_{OXA-48}$, *bla*$_{KPC}$, *bla*$_{BIC}$, *bla*$_{NDM}$, the *bla*$_{CTX-M-1}$ group, the *bla*$_{CTX-M-2}$ group and the *bla*$_{CTX-M-9}$ group, *bla*$_{TEM}$, *bla*$_{SHV}$ and *bla*$_{CMY}$. Respective primers are reported in Table 2.5

2. Determination of the genetic relatedness between the isolates.
   The genetic relatedness of the 33 bacterial isolates was tested with Random Amplified Polymorphic DNA (RAPD), a type of PCR for the random amplification of segments of DNA. This technique generates a specific band pattern for a given isolate, thus allowing differentiation between genetically distinct isolates. The primers AP4, RAPD-7, Primer640 and ER1C (Table 2.5) were used in the RAPD reactions carried out on the purified genomic DNA of the isolates.

3. Confirmation of the identity of the satellite colonies in the sheltering effect experiments.
   The satellite colonies of *E. coli* NCTC 10418 and *S. aureus* NCTC 6571, as well as the colony of the *K. pneumoniae* isolate K17 were tested for the presence of *bla*$_{OXA-48}$ and 16s rDNA.

4. Amplification of *bla*$_{OXA-58}$ and GFP genes for the cloning in the pYMAb2 vector.

**Table 2.5 List of the primers used.** The target gene, the name, the sequence, the product size in bp and the reference is provided for each pair of primers listed. The product sizeddoesn't apply to "upstream GFP_R " and "T7t"" (N/A) since these primers were used for sequencing only.

| Target | Primer name | Primer sequence (5'-3') | Product size (bp) | Reference or Source |
|---|---|---|---|---|
| Random segments of DNA | ER1C | ATGTAAGCTCCTGGGGATTCAC | variable | Vogel et al., 1999 |
| Random segments of DNA | RAPD-7 | GTGGATGCGA | variable | Gniadkowski et al., 1998; Panah et al., 2012 |
| Random segments of DNA | Primer640 | CGTGGGGCCT | variable | Haryani et al., 2007; Panah et al., 2012 |
| Random segments of DNA | AP4 | TCACGATGCA | variable | Gori et al., 1996; Panah et al., 2012 |
| 16S ribosomal RNA | 27F | AGAGTTTGATCCTGGCTCAG | 1465 | Weisburg et al., 1991 |
| | 1492R | CGGTTACCTTGTTACGACTT | | |
| *bla*$_{IMP}$ | IMP-F | GGAATAGAGTGGCTTAAYTCTC | 232 | Poirel et al., 2011 |
| | IMP-R | GGTTTAAYAAAACAACCACC | | |
| *bla*$_{VIM}$ | VIM-F | GATGGTGTTTGGTCGCATA | 390 | Poirel et al., 2011 |
| | VIM-R | CGAATGCGCAGCACCAG | | |
| *bla*$_{OXA-48}$ | OXA-F | GCGTGGTTAAGGATGAACAC | 438 | Poirel et al., 2011 |
| | OXA-R | CATCAAGTTCAACCCAACCG | | |
| *bla*$_{KPC}$ | KPC-Fm | CGTCTAGTTCTGCTGTCTTG | 798 | Poirel et al., 2011 |
| | KPC-Rm | CTTGTCATCCTTGTTAGGCG | | |
| *bla*$_{BIC}$ | BIC-F | TATGCAGCTCCTTTAAGGGC | 537 | Poirel et al., 2011 |
| | BIC-R | TCATTGGCGGTGCCGTACAC | | |
| *bla*$_{NDM}$ | NDM-F | GGTTTGGCGATCTGGTTTTC | 621 | Poirel et al., 2011 |
| | NDM-R | CGGAATGGCTCATCACGATC | | |

| Target | Primer name | Primer sequence (5'-3') | Product size (bp) | Reference or Source |
|---|---|---|---|---|
| variants of *bla*$_{CTX-M}$ group 1 | MultiCTXMGp1_for | TTAGGAARTGTGCCGCTGYA | 688 | Dallenne et al., 2009 |
| | MultiCTXMGp1-2_rev | CGATATCGTTGGTGGTRCCAT | | |
| variants of *bla*$_{CTX-M}$ group 2 | MultiCTXMGp2_for | CGTTAACGGCACGATGAC | 404 | Dallenne et al., 2009 |
| | MultiCTXMGp1-2_rev | CGATATCGTTGGTGGTRCCAT | | |
| variants of *bla*$_{CTX-M}$ group 9 | MultiCTXMGp9_for | TCAAGCCTGCCGATCTGGT | 561 | Dallenne et al., 2009 |
| | MultiCTXMGp9_rev | TGATTCTCGCCGCTGAAG | | |
| variants of *bla*$_{TEM}$ | MultiTSO-T_for | CATTTCCGTGTCGCCCTTATTC | 800 | Dallenne et al., 2009 |
| | MultiTSO-T_rev | CGTTCATCCATAGTTGCCTGAC | | |
| variants of *bla*$_{SHV}$ | MultiTSO-S_for | AGCCGCTTGAGCAAATTAAAC | 713 | Dallenne et al., 2009 |
| | MultiTSO-S_rev | ATCCCGCAGATAAATCACCAC | | |
| variants of *bla*$_{CMY}$ | MultiCaseCIT_for | CGAAGAGGCAATGACCAGAC | 538 | Dallenne et al., 2009 |
| | MultiCaseCIT_rev | ACGGACAGGGTTAGGATAGY | | |
| start codon of blaOXA-58 | OXA-58 (MGext) F | AGGAGATATACATCCATGGGTATGAAATTA | 882 | this study |
| stop codon of blaOXA-58 | OXA-58 BamH1 R | GTGGTGGTGGTGGTGGTGGGATCCTAAATAATG | | |
| start codon of GFP | GFP BamH1 F | GGAGATATACATGGATCCGATAGTAAAGGAGAAGAAC | 760 | this study |
| stop codon of GFP | GFP XhoI R | CAGCCGGATCCTCGAGGTGGTGGTGG | | |
| fusion gene bla$_{OXA58}$-GFP | upstream_GFP_R | CAACAAGAATTGGGACAACTCCAGTG | N/A | this study |
| fusion gene bla$_{OXA58}$-GFP | T7t | GCTAGTTATTGCTCAGCGG | N/A | Sequencing facility, University of Cambridge |

### 2.7.1 Primer design

All the primer sequences used in this study were taken from previously published work with the exception of the primers for the cloning of *bla*$_{OXA-58}$ and *gfp*, named respectively OXA-58 (MGext)F and OXA-58 BamH1 R (Table 2.5). For these primers the sequences were manually designed without using softwares. Sites for restriction enzymes were added to the sequence of these primers. In particular, the primers for *bla*$_{OXA-58}$ were designed to add sites for respectively *Nco*I 8 base pairs upstream the start codon ATG and *Bam*H1 24 base pairs before the stop codon TGA of *bla*$_{OXA58}$. The primers for *gfp* were designed to add restriction sites for respectively *Bam*H1 and *Xho*I by replacing respectively the start codon ATG and the stop codon TGA with the sequence recognized by these two enzymes (Figure 2.1).

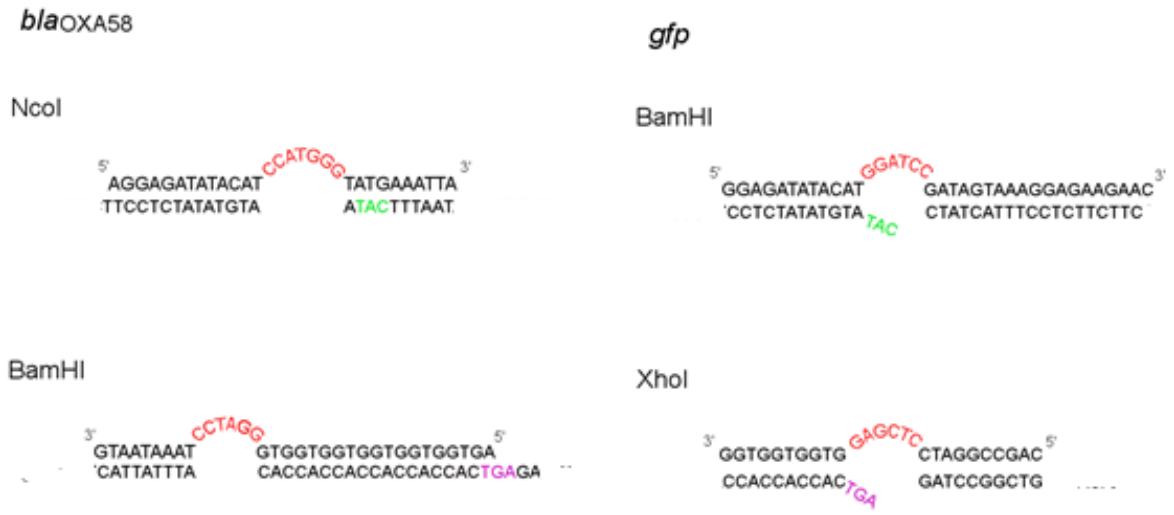



**Figure 2.1 Primer design for the cloning of *bla*$_{OXA-58}$ and *gfp*.** The sequence for the restriction sites added to the primers forward and reverse for each gene amplified is coloured in red. The start codon TAC and the stop codon TGA are reported in the sequence complementary to the primers and are coloured respectively in green and purple. The codons TAC and TGA that are replaced by the addition of the restriction sites are shown in oblique.

The primers used for the sequencing of the fusion gene *bla*$_{OXA58}$-GFP (paragraph 2.15) were respectively upstream_GFP_R and T7 terminator primer (T7t) (Table 2.5). With the exception of T7t that was provided by the Sequencing Facility of the University of Cambridge, all the other primers were synthetized by Eurofins MWG Operon, UK and re-suspended in ultra-pure water (Molecular Biology Reagent, Sigma-Aldrich) to reach a concentration of 100 µM. The primers were then 1: 10 diluted in ultra-pure water for a final working concentration of 10 µM. The stock and working primer solutions were stored at -20˚C.

### 2.7.2 PCR reaction set up

Different polymerases were used according to the purposes of the PCR reaction carried out (paragraph 2.7). These polymerases were MyTaq$^{TM}$ Red Mix (Bioline), Green Hot Start PCR Mix (Thermo Fisher Scientific) and Kod DNA Hot Start polymerase (Merck) respectively for the amplification of the β-lactam genes and confirmation of satellite colonies identity, RAPD and amplification of the genes *bla*$_{OXA58}$ and GFP for cloning (paragraph 2.15). The Green Hot Start PCR Mix was used for RAPD because the use of the Hot Start PCR allows to reduce the presence of undesired products and primer dimers due to non-specific DNA amplification at room temperatures (Birch et al., 1996). Therefore this approach is particularly suitable to RAPD applications that generate several specific PCR products (Lebedev et al., 2008). Kod DNA Hot Start polymerase is a high fidelity thermostable DNA polymerase (Kuwahara et al., 2010). Being much less subject to errors during the amplification process, KOD was used for cloning studies carried out in this project with the aim to minimize the error rate in the sequence of the genes to be cloned (Kuwahara et al., 2010). Ultra-pure water was used to complete the volume of the reaction mixture. The DNA was used at 25 ng per 25 µl reaction with MyTaq$^{TM}$ Red Mix and Green Hot Start PCR Mix and 25 ng per 50 µl reaction with Kod DNA Hot Start polymerase (Table 2.6) .

**Table 2.6 PCR mixtures used.** In each mixture the Polymerase Mixture is provided by the supplier with all the reagents, including the polymerase enzyme, deoxynucleoside triphosphates (dNTPs), Magnesium ion (Mg2+) and reaction buffer, necessary for trouble-free PCR reaction set up. Mixture A: reaction mix used for the amplification of the β-lactam genes, confirmation of satellite colonies identity and RAPD. Mixture B: reaction mix used for the amplification of the genes *bla*$_{OXA58}$ and GFP for cloning.

| **Mixture A** | **Mixture B** |
|---|---|
| 12.5 µl Polymerase Mixture | 25 µl Polymerase Mixture |
| 0.4 µmol/L Forward primer | 0.4 µmol/L Forward primer |
| 0.4 µmol/L Reverse primer | 0.4 µmol/L Reverse primer |
| 25 ng Template DNA | 25 ng Template DNA |
| Ultra-pure water to 25 µl | Ultra-pure water to 50 µl |

All amplification experiments included a negative control which contained all reagents with the exception of target DNA that was replaced by 1 µl ultra-pure water. For the amplification of the β-lactam genes and confirmation of the identity of satellite colonies PCR reactions were set-up for the amplification of the 16s rDNA as a

positive control for the presence of DNA and its amplification (primers named 27F and 1492R, Table 2.5).

### 2.7.3 Amplification reaction

The PCR reactions were carried out in a 3Prime Thermal Cycler (Techne) by setting up four different protocols according to the purpose of the PCR. These protocols are described in Table 2.7.

**Table 2.7** The four PCR protocols used in this study. For each protocol, a number (protocol No), the purpose of the PCR, the primers and the PCR reaction conditions used are reported.

| Protocol No | Purpose | Primers | Protocol description |
|---|---|---|---|
| 1 | RAPD | ER1C | 94 ˚C for 2 min;<br>35 cycles at 94 ˚C for 2 min,<br>25 ˚C for 30 s, 72 ˚C for 4 min |
| 2 | RAPD | RAPD-7, Primer640, AP4 | 94 ˚C for 2 min;<br>35 cycles at 94 ˚C for 2 min,<br>25 ˚C for 30 s, 72 ˚C for 4 min;<br>72 ˚C for 9 min |
| 3 | β-lactam gene amplification; confirmation of satellite colony identity | *bla*$_{IMP}$, *bla*$_{VIM}$, *bla*$_{OXA-48}$, *bla*$_{KPC}$, *bla*$_{BIC}$, *bla*$_{NDM}$, *bla*$_{CTX-M-1}$ group, *bla*$_{CTX-M-2}$ group *bla*$_{CTX-M-9}$ group, variants of *bla*$_{TEM}$, *bla*$_{SHV}$ and *bla*$_{CMY}$ genes<br>16S ribosomal RNA | 95 ˚C for 5 min;<br>30 cycles at 95 ˚C for 20 s,<br>52 ˚C for 20 s, 72 ˚C for 40 s;<br>72 ˚C for 1 min |
| 4 | *bla*$_{OXA-58}$ and GFP genes | OXA-58 (MGext) F<br>OXA-58 BamH1 R<br>GFP BamH1 F<br>GFP XhoI R | 95 ˚C for 2 min;<br>30 cycles at 95 ˚C for 1 min,<br>55 ˚C for 1 min, 70 ˚C for 2 min;<br>70 ˚C for 5 min |

## 2.8 Agarose gel electrophoresis

The working solution of 1x TAE buffer was made by diluting a 50x TAE stock solution prepared as described in appendix 8.2.

Agarose gels were prepared by adding agarose powder (Bioline) to 1X TAE buffer according to the percentage of agarose needed and heating the solution in microwave until complete dissolution of the agarose powder in the buffer. 10,000X GelRed (Biotium) was added to the hot solution for a final 1X concentration. The solution was then poured into an agarose gel plate and left to cool at room

temperature. 5 µl of each of the PCR product samples were applied to the gel. The gel was then run in an electrophoresis cell (BIO-RAD) at 100 V for 1 hour in 1X TAE buffer. The gel was taken out from the cell and examined with an imaging UV transilluminator (UVITEC Cambridge).

## 2.9 Purification of the PCR products

The PCR products were purified with the QIAquick PCR Purification Kit according to the manufacturer's instructions (Qiagen). All centrifugation steps were carried out at 13,000 rpm in a table-top microcentrifuge at room temperature. 5 volumes of PB Buffer were added to 1 volume of the PCR sample and mixed. This mixture was applied to a QIAquick spin column and centrifuged for 1 min. The flow-through was discarded and 750 µl PE wash were added and the column was centrifuged for 1 min. After discarding the flow-through, the column was centrifuged again for 1 min to dry the membrane. The column was placed in a clean 1.5 ml microcentrifuge tube. 30 µl of ultra-pure water were added to the column. The DNA was eluted by incubating the column at room temperature for 2 min and centrifuging it for 1 min. The concentration of the purified product was assessed using nanodrop (paragraph 2.6)

## 2.10 Screening of the sheltering effect

To confirm the presence of the sheltering effect provided by the 33 *K. pneumoniae* isolates, *E. coli* NCTC 10418, *S. aureus* NCTC 6571 and all the 33 *K. pneumoniae* isolates were grown overnight with shaking in 5 ml of 2x YT broth to allow the bacterial cultures to reach similar cell density and growth stage. The 2x YT broth included 100 µg/ml Amp for the growth of the 33 *K. pneumoniae* isolates. The $OD_{600}$ of all these overnight cultures was measured by placing 100 µl of culture in a 96 well polystyrene plate (ThermoFisher) and measuring the $OD_{600}$ with a Multimode Plate Reader (Perkin Elmer).

A sterile swab was dipped into the overnight cultures of *E. coli* NCTC 10418 or *S. aureus* NCTC 6571 (that are both susceptible to Amp) and evenly applied onto the surface of 33 2x YT agar plates containing 100 µg/ml Amp and allowed to dry. 1 µl of an overnight culture of each of the *K. pneumoniae* isolates grown overnight in 2x YT broth with 100 µg/ml Amp was then spotted on one of the 33 plates, and allowed to dry. The appearance of colonies growing around the *K. pneumoniae* inoculation area was monitored after 24 hours, 48 and 72 hours of incubation at 37˚C overnight.

As a control of the susceptibility of *E. coli* NCTC 10418 and *S. aureus* NCTC 6571 to Amp, the same procedure was carried out without applying the resistant *K. pneumoniae* on the lawn of susceptible strains.

The bacterial colonies of *E. coli* NCTC 10418 and *S. aureus* NCTC 6571 grown in the area surrounding the inoculation point of the *K. pneumoniae* isolate K17 were retrieved and streaked on a new 2x YT agar plate with 100 µg/ml Amp to confirm the susceptibility of the colonies to the antimicrobial.

The procedure described above was carried out for the preparation of the inoculum in different conditions. The isolate K17 was chosen to carry out these experiments since it showed a high potential of sheltering effect after 24 hours of incubation. Consequently reductions in the degree of this phenomenon might be easily detectable as a result of the experiments aimed to detect changes in the degree of the sheltering effect in different conditions. These latter are listed and described as follows:

1. Presence of Rose Bengal (Rb) in the 2x YT agar

The effect of Rb on the sheltering effect was studied by dissolving Rb powder (BDH) in water. This solution was used to prepare 5 2x YT agar plates at respectively 12.5, 25, 50, 100, and 200 µg/ml. All these plates also contained 100 µg/ml Amp.

As a control of the vitality of the bacteria on the media enriched with these components all the *K. pneumoniae* strains, *E. coli* NCTC 10418 and *S. aureus* NCTC 6571 were streaked on unselective 2x YT agar plates containing the Rb concentrations used.

2. Presence of Amp at different concentrations

The effect of different concentrations of Amp on the appearance of colonies of *E. coli* NCTC 10418 and *S. aureus* NCTC 6571 was assessed by repeating the same procedure for the inoculum preparation on 2x YT plates containing 25, 50 and 200 µg/ml Amp.

3. Presence of antimicrobials different from Amp

The screening of the sheltering effect was also carried out by using K17 and *E. coli* NCTC 10418 on different 2x YT agar plates containing respectively the antimicrobials Ert, Cefo, Ceft, Chlor and Cipr at respectively 1, 4, 4, 8 and 1 µg/ml.

## 2.11 Supernatant preparation for the screening of vesicles including β-lactamases

5 ml of an overnight culture of K17 grown in presence of 100 µg/ml Amp were centrifuged for 1 min at 13,000 rpm. The supernatant fraction (Sf) was retrieved and filtered twice by using sterile PVDF syringe filters with a 0.22 µm pore size. This fraction was then used for the set-up of the reaction mixtures described in Table 2.8.

**Table 2.8 Treatments of the Sf of K17 (A, B and C).** PK: Proteinase K from Tritirachium album (Sigma-Aldrich); TR: Triton X-100 (Sigma-Aldrich)

| Reaction mixtures | | |
|---|---|---|
| **A** | **B** | **C** |
| • 8 µl 10mg/ml PK<br>• K17 Sf to 800 µl | • 80 µl 5% TR<br>• K17 Sf to 800 µl | • 8 µl 10mg/ml PK<br>• 80 µl 5% TR<br>• K17 Sf to 800 µl |

These mixtures and the untreated K17 Sf were incubated at 37ºC for 1 hour and spun down for 1 min at 13,000 rpm. The supernatant was filtered once more. Then a 2x YT agar plate containing 100 µg/ml Amp and evenly spread with an overnight culture of *E. coli* NCTC 10418 was divided into four quadrants. One hole of approximately 6 mm of diameter was made in the agar of each quadrant and 100 µl of each reaction mixture A, B, C and the untreated K17 Sf were then dispensed in one of the 4 quadrants. This plate was then incubated at 37ºC for 24 hours.

## 2.12 Quantification of the sheltering effect

The two methods used for the quantification of the sheltering effect consisted of taking measurements of respectively the furthest distance from the resistant isolate at which a colony of the susceptible strain was detected (distance-based method) and the growth area occupied by the susceptible strain (area-based method). These measurements were calculated from the images of the plates captured on an imaging white light transilluminator after 24 hours incubation at 37ºC. Preliminary distance measurements were carried out to determine the furthest colony to consider for the distance-based analysis. 4 variants of this method were developed, described as follows:

- Maximum distance (variant 1). This variation consisted in measuring the linear distance between the centre of the furthest colony appearing on plate and the edge of the resistant isolate.
- Average distance of the 20 (variant 2), 10 (variant 3) or 5 (variant 4) furthest colonies. The average of the linear distance measurements between the centre of respectively the 20, 10 and 5 furthest colonies and the edge of the resistant isolate was calculated.

The area-based method consisted of measuring the growth area occupied by all the colonies and the bacterial lawn growing around the susceptible strain by using the software ImageJ (Schneider, 2012). This was computed by subtracting the area occupied only by the *K. pneumoniae* isolate (sheltering area) from the total area of the resistant isolate and the sheltered strain (sheltered area).

## 2.13 Crude DNA extraction

The DNA of K17 and of the colonies of *E. coli* NCTC 10418 and *S. aureus* NCTC 6571 was extracted by retrieving some of the bacterial culture of K17 and three colonies of *E. coli* NCTC 10418 and *S. aureus* NCTC 6571 with a loop and suspending it in 50 µl of ultra-pure water. The samples were then placed into a heating block at 100˚C for 10 min and spun for 10 s at 13,000 rpm. 1 µl of the supernatant fraction was then used for the PCR according to the instructions reported in paragraph 2.7.2.

## 2.14 Determination of growth curves

The 33 *K. pneumoniae* isolates were grown overnight in 2x YT broth containing 100 µg/ml Amp. The bacterial cultures were then 1:100 diluted in 5 ml of 2x YT broth with 100 µg/ml Amp. Immediately after the dilutions, 100 µl of each of these bacterial mixtures were dispensed in a 96 well polystyrene plate to measure their $OD_{600}$ with the Multimode Plate Reader. Then the tubes were incubated at 37ºC with shaking and the $OD_{600}$ was measured every 30 min for 4 hours, then once after 5 hours and lastly after 7 hours from the initial measurement.
At the same time intervals the $OD_{600}$ was also taken for 100 µl of a 5 ml of 2x YT broth including 100 µg/ml Amp. This was also incubated at 37ºC with shaking like all the other tubes. The broth came from the same stock used to dilute all the bacterial cultures of the *K. pneumoniae* isolates. For each time point, the normalized $OD_{600}$

values of the bacterial cultures were obtained by subtracting the $OD_{600}$ values from the $OD_{600}$ values of the bacterial cultures. The growth doubling times were determined with Excel by isolating the part of the growth curve corresponding to the logarithmic phase. The equation of this part of the curve was in the form "$A = e^{Bx}$" and the doubling time was calculated by dividing the value of ln2 (0.0693) by the value of B. This experiment was run in triplicate.

## 2.15 Cloning experiments

### 2.15.1 Plasmids

The shuttle vector pYMAb2, kindly provided as a gift by Dr Te-Li Chen, National Defense Medical Center, Taiwan (Chen et al., 2010; Kuo et al., 2013; Liao et al., 2014; Kuo et al., 2015), is derived from *E.coli* plasmid pET-28a (Novagen, Madison, WI). This plasmid contains a Kan-resistant gene and a replicon of a plasmid from *A. baumannii* reference strain ATCC 19606 (Public Health England) (Figure 2.2).

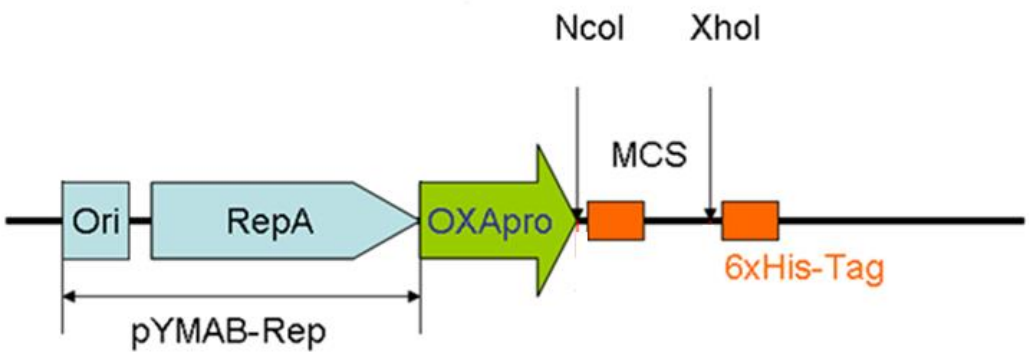



**Figure 2.2 Partial sequence of pYMAb2**. The figure shows the replicon region (pyMAB-Rep) including the Origin of replication (Ori) and the gene for the replication protein (RepA), a promoter for OXA genes (OXApro), a multiple cloning site (MCS) and a N-terminal Histidine tag (6Xhis-Tag). Diagram was provided by Dr Te-Li Chen (personal correspondence).

### 2.15.2 Small-scale plasmid preparation

Small-scale plasmid DNA extraction ("Minipreps") was carried out with the QIAprep Spin Miniprep Kit (Qiagen) according to manufacturer's protocol. All centrifugation steps were carried out at 13,000 rpm in a table-top microcentrifuge at room temperature. Bacteria containing the positive plasmid were cultured overnight in 5 ml

of 2x YT broth with 50 µg/ml Kan. 2 ml of this overnight culture were then centrifuged for 3 min. The pellet was re-suspended in 250 µl of P1 buffer and transferred to a 1.5 ml microcentrifuge tube. After the addition of the 250 µl of P2 buffer, the solution was mixed by inverting the tube 4–6 times and incubated at room temperature for 5 min. 350 µl of N3 buffer were then added to neutralize the lysate and digest any RNA present. After centrifuging the tube for 10 min, the supernatant was applied to the QIAprep Spin Miniprep column and centrifuged for 1 min. The column was then washed by adding 750 µl PE wash buffer and centrifuging it for 1 min. After discarding the flow-through, the residual PE buffer was removed by centrifuging again for 1 min. The column was placed in a clean 1.5 ml microcentrifuge tube. 50 µl of ultra-pure water were added to the column. The DNA was eluted by incubating the column at room temperature for 2 min and centrifuging it for 1 min.

### 2.15.3 Restriction digestion

All the restriction enzymes used were a fast line of enzymes offering a fast and complete digestion of DNA in a single universal buffer (FastDigest enzymes) and were supplied by ThermoFisher Scientific. The PCR products coming from the amplification for *bla*$_{OXA-58}$ and GFP gene from pET-6XHis-(-30)GFP were purified according to the protocol described in paragraph 2.9. The PCR product of the *A. baumannii bla*$_{OXA-58}$ was digested following manufacturer's protocol with the enzymes *Nco*I and *Bam*HI, while the PCR product of the GFP gene was digested with the enzymes *Bam*HI and *Xho*I. The plasmid pYMAb2 was digested with *Nco*I and *Xho*I. The restriction digestion reaction included 1 µg plasmid or PCR product, 4 µl of 10X FastDigest buffer, 1 µl (10 units) of each of the two restriction enzymes (*Nco*I and *Bam*HI for *bla*$_{OXA-58}$ gene; *Bam*HI and *Xho*I for GFP; *Nco*I and *Xho*I for pYMAb2) and ultra-pure water up to 40 µl. The mixtures were then incubated at 37ºC for 4 hours and the restriction products were then run on agarose gel (paragraph 2.8).

### 2.15.4 Purification of the DNA bands from agarose gel

The DNA bands corresponding to the digested fragments of pYMAb2 and the PCR products of *bla*$_{OXA-58}$ and GFP gene were excised from the gel using a scalpel blade on a UV transilluminator. The DNA was purified using a commercial gel extraction kit (QIAquick Gel Extraction Kit - QIAGEN) according to manufacturer's instructions. Briefly, the gel was dissolved with 500 µl QG buffer by heating to 55°C on a heating block and vortexing the sample every 2 min until complete dissolution. The melted gel solution was then placed into a binding column with 100 µl of isopropanol. The

column was centrifuged for 30 s at 13,000 rpm and the spin was repeated by placing the flow-through back to the top of the column. After discarding the flow-through, 750 µl PE buffer were added to the column and centrifuged for 1 min. This centrifugation step was repeated to completely dry the column. Finally, the DNA was eluted by incubation for 2 min at 65°C with 50 µl ultra-pure water, followed by centrifugation at 14,000 rpm for 1 min. Concentration of the eluate was then measured using NanoDrop (paragraph 2.6) and DNA quality assessed by running 5 µl of the eluted DNA solution on an agarose gel (paragraph 2.8).

### 2.15.5 DNA ligation

The enzyme T4 DNA ligase and the supplied T4 ligase buffer (ThermoFisher Scientific) was used in the ligations reactions carried out in this study. The mixture included 30 ng of each insert (purified digested fragments of $bla_{OXA-58}$ and GFP), 10 ng of purified digested pYMAb2, 2 µl T4 ligase buffer, 2 µl T4 DNA ligase enzyme and ultra-pure water to 20 µl (the ratio insert:vector was 3:1). A mixture containing all the components minus the inserts was also prepared to assess the self-ligation of pYMAb2. The mixtures were then incubated overnight at 16˚C in a 3Prime Thermal Cycler.

### 2.15.6 Making chemically competent cells of *E. coli* DH5α

Frozen *E. coli* DH5α cells (Invitrogen) were streaked on a plain 2x YT agar plate. After overnight incubation at 37ºC, a single colony was inoculated into 5 ml 2x YT media and grown at 37°C overnight with shaking. Then, 1 mL of DH5α overnight culture was inoculated in 100 ml of 2x YT broth. The cells were grown for 2 hours, chilled on ice for 15 min and centrifuged in 2 x 50 ml sterile tubes for 5 min at 4000 rpm at 4ºC with a benchtop centrifuge (Fisher Scientific AccuSpin 1). The supernatant was removed and the pellet of one tube was re-suspended in 50 ml ice-cold Transformation buffer 1 (TfbI) (appendix 8.2) and combined with the pellet from the second tube. After incubation of the cells on ice for 15 min and centrifugation for 5 min at 4000 rpm, the pellet was gently re-suspended in 4 ml of ice-cold Transformation buffer 2 (TfbII) (appendix 8.2). 100 µl of cells were then aliquoted into ice-cold 1.5 mL Eppendorf tubes, flash frozen in liquid nitrogen and stored at -80ºC.

### 2.15.7 Bacterial Transformation with heat-shock

10 µl of the ligation reaction were added to 100 µl competent DH5α *E. coli* and mixed by gently swirling. The mixture was incubated on ice for 30 min. The heat-shock procedure was carried out by placing the mixture in a heating block set up at 42ºC for 45 s and then quickly cooling it on ice for 2 min. After the addition of 900 µl of 2x YT broth, the mixture was incubated for 1 hour at 37ºC with shaking. 10 µl and 100 µl of the bacterial culture were spread onto a 2x YT agar plate with 50 µg/ml Kan. The rest of the bacterial culture was centrifuged at 13,000 rpm for 1 min. The pellet was then re-suspended in approximately 100 µl supernatant and spread to the selective agar plate. The plates were incubated overnight at 37°C.

### 2.15.8 Positive clone selection

After overnight incubation, 6 colonies were picked up from the plate with a loop and grown in 5 ml 2x YT broth with 50 µg/ml Kan overnight at 37°C with shaking. The plasmids were extracted as described in 2.15.2. The plasmids were then subjected to DNA digestion as described in 2.15.3 and DNA electrophoresis (paragraph 2.8) with two bands expected, one as the backbone pYMAb2 vector (approximately 5.4 Mb), another one as the insert fragment that was the fusion product between $bla_{OXA-58}$ and the *gfp* gene ($bla_{OXA-58}$-*gfp*, approximately 1.6 Mb). The concentration of the plasmid preparation was assessed using nanodrop (paragraph 2.6).

### 2.15.9 Making electrocompetent cells of *A. baumannii*

Frozen *A. baumannii* CIP70-10 was streaked on plain 2x YT agar plate. After overnight incubation, 1 colony was inoculated in 5 ml 2X YT broth and grown overnight at 37ºC with shaking. 2 ml of this overnight bacterial culture were inoculated in 100 ml of 2x YT broth and grown for 2 hours with shaking at 37˚C. The resultant culture was split into 2 falcon tubes of 50 ml each and centrifuged for 10 min at 4000 rpm at 4ºC. The supernatant was discarded from both tubes. The pellet from one tube was then re-suspended in 50 ml of chilled 10% glycerol and combined with the pellet in of the second tube. The tube was centrifuged for 10 min at 4000 rpm at 4ºC. This procedure, including the re-suspension of the pellet in 10% glycerol and centrifugation was repeated three times. The final wash step was performed by re-suspending the pellet in 2 ml of 10% glycerol. 100 µl of cells were then aliquoted into pre-chilled 1.5 ml microcentrifuge tubes that were flash frozen in liquid nitrogen and stored at -80ºC.

### 2.15.10 Electroporation

1 µl of pYMAb2 containing the insert OXA-58-GFP was mixed with 100 µl electrocompetent cells of *A. baumannii* CIP70.10. The same procedure was carried out by using pYMAb2 including no insert. The mixtures were then transferred to 2 mm gap polycarbonate electroporation cuvettes (Thermo Fisher Scientific) chilled on ice. The mixtures were electroporated at 2.5 kV in an electroporator chamber (Bio-Rad). 1 ml of 2x YT broth was immediately added to the electroporated cells that were grown for 1 hour at 37ºC with shaking. The resultant bacterial culture was then spread on 2x YT agar plates with 50 µg/ml Kan following the same procedure described in 2.15.7. The plates were incubated overnight at 37ºC. One transformant colony was then grown in 2x YT broth with 50 µg/ml Kan overnight at 37ºC with shaking. A freezer stock of the resultant bacterial culture was prepared as described in 2.3.

## 2.16 Confocal microscopy

*A. baumannii* CIP70-10 producing pyMAb2 with the insert $bla_{OXA-58}$-*gfp* (AbCIP70.10(pyOXA-58-GFP)) was grown in 4 tubes with 2x YT broth including 50 µg/ml Kan. Three of these tubes additionally contained respectively 0.5, 1 and 2 µg/ml meropenem (Mer) to stimulate the production and release of OMVs from the cells. All these tubes were incubated at 37ºC overnight with shaking. *A. baumannii* CIP70-10 producing pYMAb2 with no insert (AbCIP70.10(py)) was also grown in 2x YT broth with 50 µg/ml Kan at 37ºC overnight with shaking. As a positive control for the detection of GFP, 100 µl of chemically competent cells of BL21 *E. coli* were transformed with 5 µl of a His6-tagged GFP expression vector by following the procedure described in 2.15.7 (Sullivan et al., 2007). The selection of the transformants was carried out on 2x YT plates including 50 µg/ml Kan and 50 µg/ml Chlor. After overnight incubation at 37˚C, a colony was picked up and grown in 2x YT broth including 50 µg/ml Kan, 50 µg/ml Chlor, and 100 µM IPTG (ThermoScientific) overnight at 37˚C with shaking.

Multispot slides (Thermo Scientific) were prepared by loading 5 µl of 1 mg/ml Concanavalin A from Canavalia ensiformis (ConA) (Sigma Aldrich) onto the slide wells to allow the bacterial cells to adhere to the slide. These were then let them dry out for 20 min. 50 µl of all the bacterial cultures were then loaded onto the wells with ConA and let to adhere for 30 min. The excess of bacterial culture was then removed with a pipette. A coverslip was placed on the slide and sealed with nail varnish.

The slides were inspected at x100 magnification with an inverted LSM510 laser scanning microscopy (Zeiss) by using an Argon laser at 488 nm and the emission

filter BP 505-530. A drop of microscope oil was placed on the objective before inspecting.

## 2.17 Protein extraction

AbCIP70.10(pyOXA-58-GFP) and AbCIP70.10(py) were cultured overnight in 2x YT broth with 50 µg/ml Kan at 37ºC overnight with shaking. 1.5 ml of overnight culture were centrifuged at 13,000 rpm for 30 s and the supernatant removed. After discarding the supernatant, the pellet was re-suspended in 500 µl of lysis buffer (appendix 8.2) and the sample placed on ice for 10 min. The proteins were precipitated by adding 100 µl of 30% w/v thrichloroacetic acid (TCA, Sigma-Aldrich). The samples were vortexed and then placed on ice for 10 min. Following a centrifugation step at 13,000 rpm for 5 min, the supernatant was removed, and the pellet was neutralized by re-suspension in 20 µl of 1M Tris base and solubilized in 80 µl of SDS sample buffer (Sigma-Aldrich).

## 2.18 Making polyacrylamide gels

The mixtures for the resolving and stacking gels were prepared in 2X 50 ml Falcon tubes and the polymerizing agents Ammonium Persulfate (APS, Sigma Aldrich) and TEMED (ThermoFisher Scientific) were added as last components. The resolving gel contained 3.75 ml (25%) of 40% Acrylamide (ThermoFisher Scientific), 7.5 ml (50%) buffer A (appendix 8.2), 3.75 ml (25%) sterile $DIH_2O$, 150 µl (1%) APS and 15 µl (0.1%) TEMED. The mixture was then gently poured in an empty Bolt gel cassette (ThermoFisher Scientific). $DIH_2O$ was poured on the surface of the resolving gel to get even surface and the gel was left to polymerise for approximately 15 min. The stacking gel contained 625 µl (12%) of 40% 37:1 acrylamide:bis-acrylamide (ThermoFisher Scientific), 2.5 ml (49%) buffer B (appendix 8.2), 1.87 ml (37%) sterile $DIH_2O$, 50 µl (0.9%) APS and 5 µl (0.09% )TEMED. The water layer was discarded from the gel cassette and the stacking gel was poured into it followed by the insertion of 12-well combs. The stacking gel was left to polymerise for 15 min at room temperature. Once set, the combs were removed, and the wells were rinsed with $DIH_2O$. The gels were placed in a plastic bag with Buffer C (appendix 8.2) and stored at 4˚C until use.

## 2.19 Polyacrylamide gel electrophoresis

The protein samples were heated at 95ºC for 5 min before loading onto the gel. 3 µl of prestained protein molecular marker (ThermoScientific) were added to the first well. 20 µl volumes of each sample were loaded into the other wells. The electrophoresis was run in buffer C at 120 V for approximately 60 min or until the dye had reached the end of the gel.

## 2.20 Western Blot

A sheet of polyvinylidene fluoride (PVDF) transfer membrane (Immobilon-P) was cut 8.5 x 7 mm, pre-wet in methanol then soaked in Western transfer buffer (appendix 8.2) for 10 min. 6 pieces of 3 mm chromatography blotting paper (Whatman) were cut 10 x 9 mm and pre-wet in transfer buffer. The gel was retrieved from the electrophoresis apparatus. The stacking gel was removed with a scalpel blade and the resolving gel was wet in transfer buffer for 5 min. The transfer sandwich was then assembled on the lower electrode of the Western blotting apparatus (Thermo Fisher Scientific) as follows: 3 sheets of blotting paper, PVDF membrane, gel and 3 sheets of blotting paper. The top electrode was inserted and secured. The transfer blot was carried out at 125 mA and 100 V for 3 hours. Once the transfer was complete, the PVDF membrane was blocked in 15 ml blocking solution (5% w/v milk powder in Tris-buffered saline (TBS buffer, Appendix 8.2) and incubated for 1 hour with slow shaking at room temperature. After this time, the membrane was placed in a solution containing a 1:1000 dilution of Anti-GFP monoclonal antibody from mouse (Abcam) in incubation solution (blocking solution 1:3 diluted in TBS buffer) and then incubated with slow shaking at room temperature overnight. After discarding the incubation solution, the membrane was washed four times for 1 min with 50 ml TBS buffer. Finally, the membrane was incubated with shaking for 3 hours in TBS buffer that contained a 1:5000 dilution of a goat polyclonal anti-mouse secondary antibody (Sigma Aldrich). This antibody was conjugated to the green fluorescent dye CF488A. The membrane was then washed five times for 5 min with 50 ml of TBS buffer. The membrane was visualized with a G-box imager (Syngene) with a blue module lighting source and an emission filter for the wavelengths between 525-510 (Filt525).

## 2.21 Sanger sequencing

100 ng/µl of plasmid preparations was sent off for Sanger sequencing at the DNA sequencing facility of the University of Cambridge (Sanger et al., 1977), using the primers for the sequencing of the fusion gene *bla*$_{OXA58}$–GFP (Table 2.5). The samples were sequenced on the Applied Biosystems 3730XL Genetic Analyzer with a 96 capillary 50 cm array (ThermoFisher Scientific). The DNA samples were prepared with the BigDye™ Terminator v3.1 reagents (ThermoFisher Scientific). Unincorporated BigDye® terminators and salts were removed with BigDye XTerminator™ Purification Kit (ThermoFisher Scientific) prior to sequencing.

## 2.22 Illumina sequencing

### 2.22.1 Library preparation and sequencing

A single colony of the strains K3 and K9 was mixed with 1XPBS (appendix 8.2). All the mixture was then streaked on 2x YT agar plate and incubated overnight at 37°C. All the bacterial culture was then retrieved with a loop and mixed into a barcoded bead tube supplied by MicrobesNG. The sequencing was performed with the Illumina MiSeq platform provided by MicrobesNG (https://microbesng.uk/). The tube was vortexed and the liquid removed. The tubes were sent off to MicrobesNG for whole genome sequencing. Genomic DNA libraries were prepared using Nextera XT Library Prep kit (Illumina, San Diego, USA) and paired-end sequenced with 250 bp read lengths.

The sequencing of the DNA samples of all the other *K. pneumoniae* isolates was carried out at the Norwich Medical School of the University of East Anglia (UEA). Purified DNA samples were diluted to a concentration of 0.2 ng/µl in ultra-pure water. The DNA libraries were constructed by fragmenting the DNA and barcoding them with Nextera XT index kits (Illumina). Each library was adjusted to a concentration of 4 nM before being pooled together for sequencing. Paired end Sequencing was performed on an Illumina NextSeq using a NextSeq 500/550 High Output v2 kit sequencing kit (Illumina).

### 2.22.2 Assembly of reads and annotation

The assembly of reads, annotation and quality check were carried out directly by MicrobesNG by using respectively Spades, Prokka and Quast (Bankevich et al., 2012; Seeman, 2014; Gurevich et al., 2013). For all the other isolates whose genome

was sequenced at UEA, a virtual machine provided by the Cloud Infrastructure for Microbial Bioinformatics (CLIMB) (https://www.climb.ac.uk/) was used to run Spades with single and paired-end reads and the contigs were annotated with Prokka. The virtual machine was accessed by the terminal emulator Putty (https://www.chiark.greenend.org.uk/~sgtatham/putty/).

## 2.23 Softwares

### 2.23.1 Analysis of RAPD patterns

The RAPD patterns which resulted from the gel electrophoresis were analysed with the software PyElph (Pavel and Vasile, 2012). This program uses the information extracted from the analyzed gel image to compare DNA patterns and generates phylogenetic trees thus providing information about the similarity between samples of DNA sequences.

### 2.23.2 Analyses of DNA sequences

The software BioEdit (Hall, 1999) was used to visualize the sequences produced by the Sanger methodology. The software Artemis (Carver et al., 2012) was used to visualize the sequences produced by the Illumina sequencing.

DNA sequences were compared to sequences in the National Center for Biotechnology Information (NCBI) database using the basic local alignment search tool (BLAST). BLAST was used on both the sequences coming from the Sanger and the Illumina sequencing to search for the homologies with the known sequences already deposited in the NCBI database (Altschul et al., 1997).The translation of the nucleotide sequences to amino acid sequences was carried out with the tool Translator (Gasteiger et al., 2003). In particular, this program was used in cases where a homology lower than 100% at the DNA level with known genes was found.

The software ResFinder (Zankari et al., 2012) was used to detect antibiotic resistance genes in the sequence of the entire genome of the isolates sequenced. The assembled genome was uploaded on the ResFinder webpage by using a 90% identity threshold and at least 60% of the total gene length matched to the assembled sequence to identify the resistance genes in the database.

### 2.23.3 Analysis of the agar plates

The images captured from the plates were analysed with the software ImageJ (Schneider, 2012). The spatial calibration of each single image was carried out by setting a known distance value in mm on the ruler placed next to the plate while capturing the image. The surface areas values were computed by using the "Analyze particles" function of ImageJ.

### 2.23.4 Statistical analyses

The statistical analyses were performed using the statistical packages SPSS (version 24, IBM Corp.), R and R studio (R Core Team, 2013) and GraphPad Prism 7.00 for Windows (GraphPad, USA). P-values less than 0.05 were considered statistically significant. The P-values are denoted symbolically as follows: 0.01 to 0.05 (*) = significant; 0.001 to 0.01 (**) = highly significant; 0.0001-0.001(***) = very highly significant and < 0.0001 (****) = extremely significant. Analyses of differences in the sheltering provided by the *K. pneumonaie* isolates, changes of sheltering depending on different conditions, and growth curves were carried out with one-way Analysis of Variance (ANOVA) with Dunett or Tukey's multiple comparison test in GraphPad Prism. The R-squared ($R^2$) values were used to evaluate the closeness of the data to the fitted regression line in the scatter plots (Moore et al., 2013). The $R^2$ values were interpreted as follows: between 0 and 0.3 = weak positive or negative linear relationship; between 0.3 and 0.7 = moderate positive or negative linear relationship; between 0.7 and 1.0 = strong positive or negative linear relationship.

# 3 RESULTS I: METHODS FOR THE DETECTION AND QUANTIFICATION OF THE SHELTERING EFFECT IN *K. PNEUMONIAE*

## 3.1 Introduction

Different bacterial species can form small ecosystems in polymicrobial infections. Interactions between microbes in these ecosystems can result in an increase of the severity of infections (Arndt et al., 1967; Byrd and Segre, 2016; Marjon et al., 2018) and complications in treatments (Brodge et al., 2005; Peters et al., 2012; Short et al., 2014). However, the nature of these interactions in polymicrobial infections remain largely obscure since infective bacteria are commonly investigated in isolation (Marjon et al., 2018).

Bacteria can affect each other's proliferation or survival in several ways including excretion of metabolites (Fredrickson and Stephanopoulos, 1981), competition for the same nutrient (Silver and Wintermute 2010), or release in the extracellular environment of molecules that can harm or help other species living in the bacterial community (Riley and Wertz, 2002; Abrudan et al., 2015; Andersen et al., 2015).

Resistance to β-lactam drugs can arise as a result of cooperative behavior between resistant and sensitive bacteria (Dugatkin et al., 2005; Yurtsev et al., 2013). This phenomenon has been investigated in different pathogenic bacteria by using different methods. In a study carried out on *Bacteroides* species, a single infection of penicillin-susceptible streptococci, and a mixed infection of a β-lactamase-producing strain of either *Bacteroides melaninogenicus* or *Bacteroides fragilis* and penicillin-susceptible streptococci, were induced respectively in two groups of mice. The penicillin treatment prevented the formation of abscesses in animals inoculated with streptococci alone, but not in those inoculated with streptococci and *Bacteroides* species, thus suggesting that a mixed infection was responsible for the resistance to the penicillin therapy (Brook et al., 1983; Stentz et al., 2015).

This protective effect has also been reported in β-lactam-resistant non-typeable *H. influenzae*, *M. catarrhalis,* and *A. baumannii* by examining the release of β-lactamase-containing OMVs that protect β-lactam-susceptible bacterial strains present in the same extracellular environment as the resistant strains (Ciofu et al., 2000; Shaar et al., 2011; Shaar et al., 2014; Chattopadhyay and Jaganandham, 2015; Liao et al., 2015). Furthermore, studies carried out on *A. baumannii* and *E. coli* confirmed the presence of this "sheltering effect" by co-culturing the β-lactam-resistant and susceptible strains in broth containing β-lactams and then differentiating

the co-cultured bacteria on appropriate agar plates after overnight culture (Perlin et al., 2009; Liao et al., 2014). However, the use of this method is subject to the availability in research laboratories of resistant and susceptible species that can be discriminated by the colony characteristics of each species on the plates. Also, the analysis of β-lactamase-containing OMVs might be time-consuming and costly due to the number of methods involved, such as isolation of OMVs, Western Blot analyses, microscopy and quantification of activity of the β-lactamases enclosed in OMVs. Lastly, the investigation of the sheltering effect by inducing mixed infections of β-lactam resistant and sensitive strains requires *in vivo* studies on animals.

*K. pneumoniae* accounts for a number of hospital-acquired infections including septicemia, pneumonia, urinary tract infection, meningitis, and purulent abscesses at different sites (Lin et al., 2013; Kamal et al., 2017). Although this pathogen has been identified in the blood of patients with polymicrobial infections, the sheltering effect has not yet been investigated in *K. pneumoniae* (Rahim et al., 2018). In this project, the sheltering effect provided by *K. pneumoniae* has been investigated by using K17, a β-lactam-resistant *K. pneumoniae* isolated from a Kuwait hospital in 2014.

The aims of this study were:

1. To determine whether the sheltering effect is present in *K. pneumoniae*
2. To develop methods for the quantification of the sheltering effect
3. Compare the methods developed and determine the most robust for future quantification analyses.

## 3.2 Results

### 3.2.1 The sheltering effect of *K. pneumoniae* K17 on β-lactam susceptible strains

The sheltering potential of *K. pneumoniae* was investigated by carrying out point inoculations of *K. pneumoniae* strain K17 on two agar plates enriched with Amp and evenly spread with respectively *E. coli* NCTC 10418 and *S. aureus* NCTC 6571, two strains sensitive to Amp. The purpose of analyzing the sheltering effect on these two strains was to investigate if the protective effect provided by the resistant isolate was subject to differences in the species and/or the Gram group of the two susceptible strains. Colonies of these two strains were expected to grow around the inoculation area of K17 only in cases where the resistant isolate was able to shelter the susceptible strains from the antimicrobial action of Amp present in the medium. Furthermore, the appearance of colonies of *E. coli* NCTC 10418 and *S. aureus*

NCTC 6571 was assumed to be subject to the presence of both the resistant and either susceptible strain on the plate.

The results suggested the presence of the sheltering effect from K17 on both the susceptible strains, as showed by the growth of *E. coli* NCTC 10418 and *S. aureus* NCTC 6571 exclusively around K17 (Figure 3.1, A and B). For both the susceptible strains the bacterial growth in the area closest to the inoculation area of *K. pneumoniae* resulted in the formation of a continuous cover of bacteria (Figure 3.1, A and B, S+). However, the growth of *E. coli* NCTC 10418 in the form of satellite colonies was also detectable in the area around K17 (Figure 3.1, A, S+).

No growth in the area around K17 was detectable in absence of the susceptible strains (Figure 3.1 A and B, S-). Furthermore, no bacterial growth was present on plates lacking the inoculation of K17 (Figure A and B, NB). The distinction between the area occupied by the susceptible strains and the area of the resistant isolate is showed in Figure 3.1, C and D.

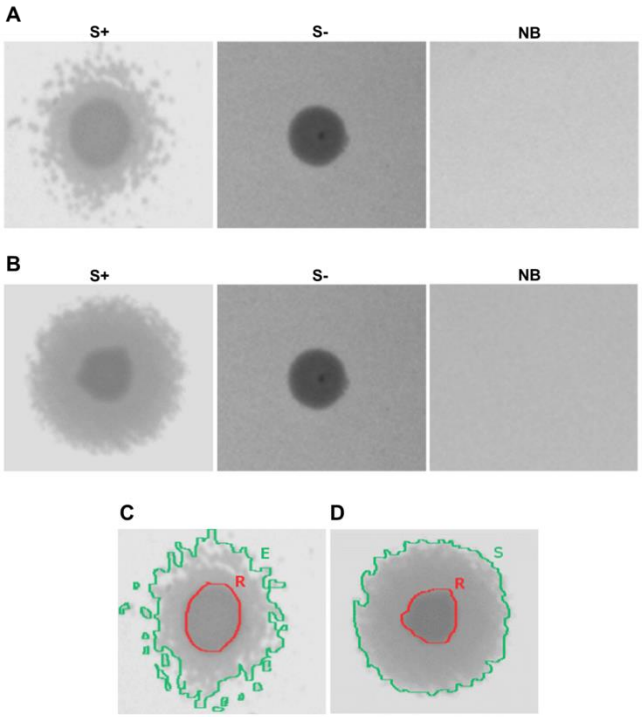



**Figure 3.1 Sheltering effect of K17 on two β-lactam susceptible strains**. The growth of *E. coli* NCTC 10418 and *S. aureus* NCTC 6571 around K17 was monitored after a point inoculation of a bacterial culture of K17 on two 100 µg/ml Amp plates evenly spread with a bacterial culture of respectively *E. coli* NCTC 10418 and *S. aureus* NCTC 6571 and incubation at 37 ˚C for 24 hours. **A and B.** The left side of the panel shows the growth of respectively *E. coli* NCTC 10418 or *S. aureus* NCTC 6571 around K17 on plates inoculated with this resistant isolate (S+). The middle picture shows the Amp plate inoculated with no susceptible strain (S-). The right side shows the appearance of two Amp plates lacking the inoculation of K17 (NB). **C and D**. The left pictures of A and B are modified to show the distinction between the area occupied by the susceptible strains and the area of the resistant isolate. The profile of the area of *E. coli* NCTC 10418 (E) and *S. aureus* NCTC 6571 (S) is coloured in green, while the outline of the area of K17 is coloured in red (R).

To confirm that the growth of the two susceptible strains was exclusively due to the sheltering effect provided by K17, a bacterial culture derived from the growth of respectively *E. coli* NCTC 10418 and *S. aureus* NCTC 6571 around K17 was streaked on 2 clean agar plates containing Amp. This test aimed to exclude the possibility that the ability of the susceptible strains to grow on a medium enriched with Amp was the result of spontaneous genetic mutations. Indeed, these mutations were expected to enable the sensitive strains to resist the action of Amp regardless of the presence of K17. The results showed that no visible bacterial growth was detected on Amp plates streaked with the bacterial culture of respectively *E. coli* NCTC 10418 and *S. aureus* NCTC 6571 growing around K17 (Figure 3.2).

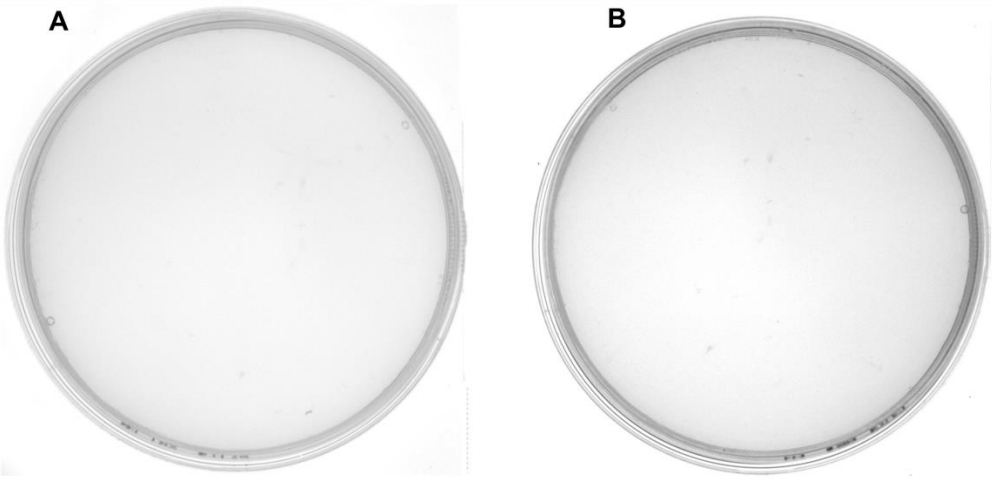



**Figure 3.2 Amp plates after 24 hours from the streaking of the bacterial culture of the susceptible strains.** Some bacterial culture of *E. coli* NCTC 10418 **(A)** and *S. aureus* NCTC 6571 **(B)** growing around K17 was retrieved with a loop and streaked on respectively two agar plates enriched with 100 µg/ml Amp. Bacterial growth was monitored on these plates after 24 hours of incubation at 37˚C.

Furthermore, to exclude that the growth of *E. coli* NCTC 10418 and *S. aureus* NCTC 6571 around K17 was the result of the transfer of plasmid-encoded β-lactamases such as the gene for OXA-48 might have been transferred, the presence of $bla_{\text{OXA-48}}$ was investigated in the cells of *E. coli* NCTC 10418 and *S. aureus* NCTC 6571 grown around K17. With regards to this, PCR reactions for $bla_{\text{OXA-48}}$ were carried out on the DNA isolated from K17 and a colony of respectively *E. coli* NCTC 10418 and *S. aureus* NCTC 6571 isolated from three different sites of their growth area. This aimed to confirm that the area occupied by the susceptible strains was not a mixture of bacteria sheltered by K17 and bacteria that acquired a β-lactamase gene from

K17. Indeed, it was expected that the whole area of susceptible strains comprised bacteria that were resistant to the action of Amp exclusively in presence of the sheltering effect provided by K17. Furthermore, a PCR for the amplification of the *16S rDNA* gene was carried out on the same DNA preparations to confirm the presence and the amplification of bacterial DNA in all samples. A PCR containing all the components needed for the amplification of the *16S rDNA* gene except for the DNA (negative control) was also prepared last to verify that no contaminating DNA was introduced into the PCR mixtures during sample processing.

The results of this analysis showed that an amplicon of the size expected for the fragment of $bla_{\text{OXA-48}}$ (743 bp) was present in the DNA samples of K17 but not in the DNA of the colonies of *E. coli* NCTC 10418 and *S. aureus* NCTC 6571 (Figure 3.3, A and B). However, the presence of an amplicon for the fragment of the *16S rDNA* gene (1465 bp) in all samples confirmed the presence and amplification of the DNA (Figure 3.3, A and B). The negative control did not produce any amplicon, thus indicating no DNA contamination in any PCR mixture prepared (Figure 3.3, B).

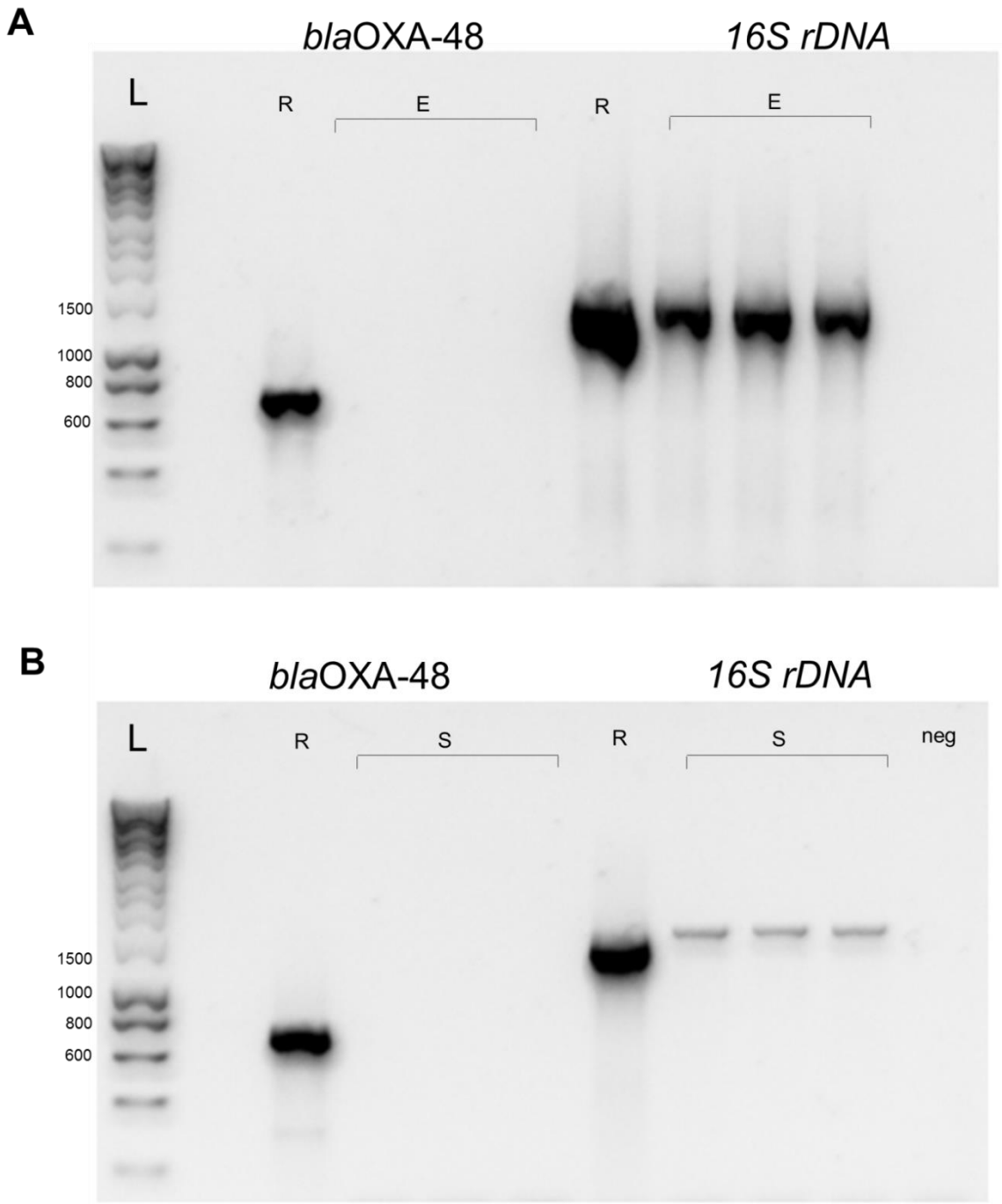



**Figure 3.3. Agarose gel showing the amplification of *bla*$_{OXA-48}$ and *16S rDNA* in the DNA of K17 and in three colonies of *E. coli* NCTC 10418 (A) and of *S. aureus* NCTC 6571 (B)**. The amplification of the fragments of *bla*$_{OXA-48}$ and *16S rDNA* is confirmed by the presence of black bands of the size expected for these fragments. The size in base pairs (bp) of the ladder (L) fragments is used as a reference to confirm the size of the amplicons. R, E and S stand for the PCR product from the DNA respectively of K17, *E. coli* NCTC 10418 and of *S. aureus* NCTC 6571. Neg: negative control. The differences in the intensity of the 16S band between *S. aureus* and K17 could be the result of a different number of DNA copies at the end of the amplification step of the PCR process.

### 3.2.2 The sheltering effect in multiple β-lactam resistant isolates of *K. pneumoniae*

To confirm that the sheltering effect detected in K17 was not a phenomenon restricted to a single isolate, 14 additional *K. pneumoniae* isolates were randomly chosen among all the 33 β-lactam-resistant isolates of this bacterium isolated form Kuwaiti clinical samples in 2014. These isolates were tested for their sheltering potential on *E. coli* NCTC 10418 and *S. aureus* 6571 and were K1, K2, K3, K7, K10, K11, K13, K18, K19, K20, K21, K23, K25 and K30. These analyses aimed to verify that the sheltering effect towards different species of susceptible bacteria was a phenomenon common within the *K. pneumoniae* species.

The screening for the sheltering effect in the 14 *K. pneumoniae* isolates was carried out with the same methods described for K17 in paragraph 3.2.1.

The results showed that all the 14 isolates were able to shelter both *E. coli* NCTC 10418 and *S. aureus* NCTC 6571, similarly to K17. No growth in the area surrounding the isolates was detected in absence of susceptible species. (Figures 3.4 and 3.5).

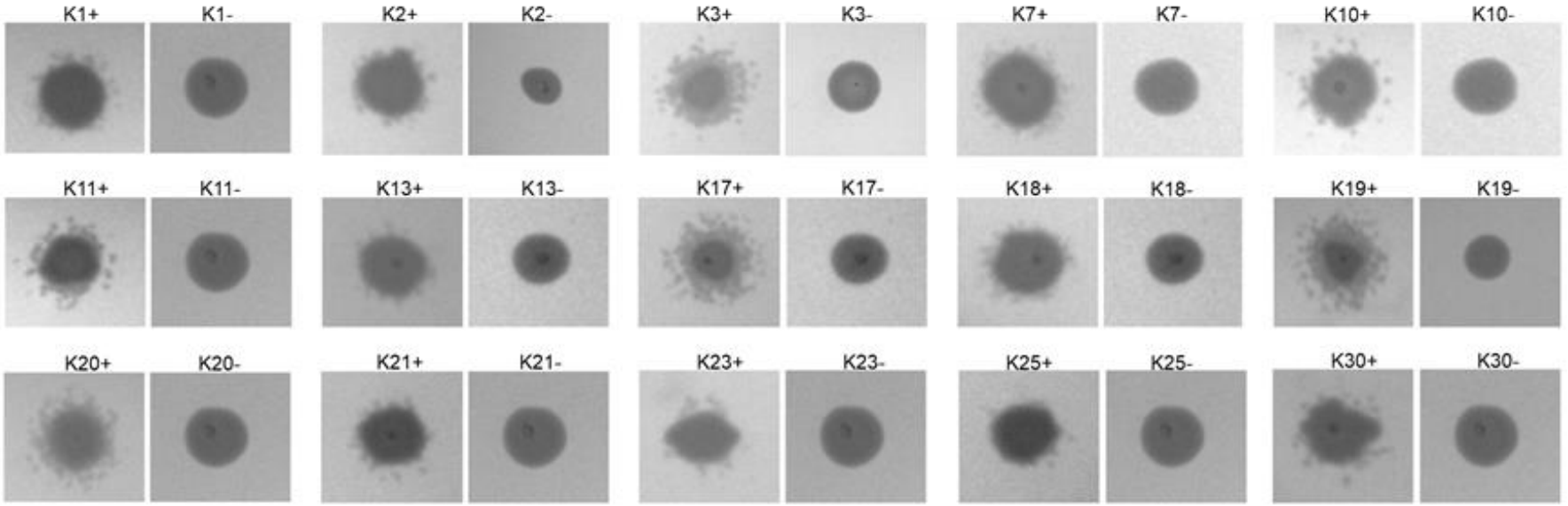



**Figure 3.4 Sheltering effect of the *K. pneumoniae* isolates on *E. coli* NCTC 10418.** The picture shows the bacterial growth on two 100 µg/ml Amp plates for each of the *K. pneumoniae* isolates tested including K17. These two plates were prepared by inoculating each *K. pneumoniae* isolate respectively in presence (+) and absence of *E. coli* NCTC 10418 (-) followed by incubation at 37°C for 24 hours. This experiment was performed in triplicate for each *K. pneumoniae* isolate (n = 3) and the image of one of the three plates used as replicates is showed in the picture as an example.

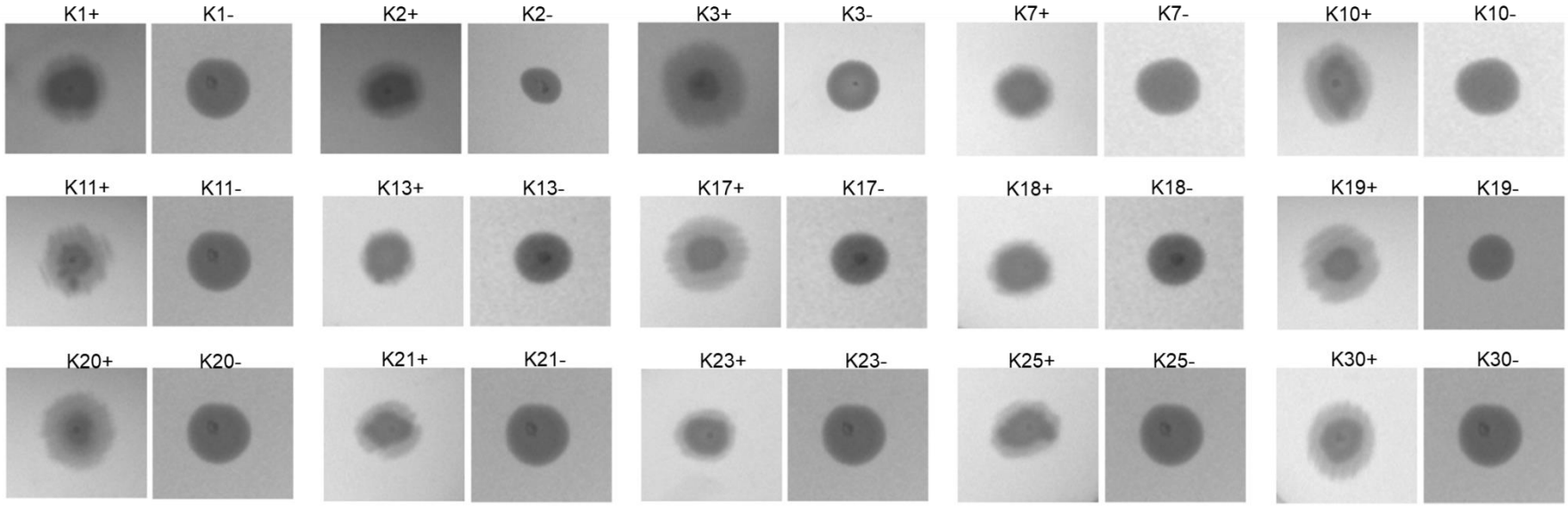



**Figure 3.5 Sheltering effect of the *K. pneumoniae* isolates on *S. aureus* NCTC 6571.** The picture shows the bacterial growth on two 100 µg/ml Amp plates for each of the *K. pneumoniae* isolates tested including K17. These two plates were prepared by inoculating each *K. pneumoniae* isolate respectively in presence (+) and absence of *S. aureus* NCTC 6571 (-) followed by incubation at 37°C for 24 hours. This experiment was performed in triplicate for each *K. pneumoniae* isolate (n = 3) and the image of one of the three plates used as replicates is showed in the picture as an example.

However, these *K. pneumoniae* isolates exhibited apparent variability in sheltering effect provided to the susceptible strains as shown by the differences in the distance of satellite colonies from the resistant isolate and in the diameter of the continuous cover of bacteria around the *K. pneumoniae* isolates (Figures 3.4 and 3.5). This observation suggested that the *K. pneumoniae* isolates might differ in the distance that the protective effect could reach from the resistant isolate, as well as by the total number of cells protected represented by the total growth area of the susceptible strain on the plate.

### 3.2.3 Comparison of two methods for the quantification of the sheltering effect

In order to investigate whether the apparent variations visually observed in the sheltering *K. pneumoniae* isolates corresponded to statistically significant differences, two methods for the quantification of the magnitude of the sheltering effect detected on plates were developed. The quantification of the sheltering effect in *K. pneumoniae* aimed to determine the degree of variability that this phenomenon shown among isolates belonging to the same bacterial species. Ultimately the differences in the sheltering strength might provide insight into the ability of *K. pneumoniae* in a mixed infection to protect other bacterial species from the action of antibiotics. Indeed, a more powerful sheltering effect might be associated with a higher degree of resistance to antimicrobial treatment during polymicrobial infections and vice versa.

The two methods were developed with the aim of quantifying the sheltering effect based on two features observed on plate. The first feature was the furthest distance from the resistant isolate at which a colony of the susceptible strain was detected (distance-based method). The second feature was the total growth area occupied by the susceptible strain (area-based method). Therefore, these two methods gave information about respectively the distance of the protective effect exerted by the resistant isolate and the proportion of susceptible cells the resistant isolate was able to shelter from the action of the β-lactam. The analyses for the quantification of the sheltering effect with these two methods were carried out on the plates where the sheltering effect of the 15 *K. pneumoniae* isolates on *E. coli* NCTC 10418 was detected.

The hypothesis behind the distance-based method was that the survival and growth of the susceptible strains inoculated on plate was affected by the protective effect exerted by the resistant bacteria growing just at the edges of the inoculation point. In addition, the more distant a colony of the susceptible strain grew from the resistant

isolate, the further the sheltering effect extended from the edge of the resistant isolate.

Therefore, in the distance-based method the linear distance between the centre of the furthest colony detected on plate and the edge of the colony of the resistant isolate was measured and the furthest distance detected was named “maximum distance”. The choice of the furthest colony to consider for the analysis was based on preliminary distance measurements aimed to determine which colony was the furthest from the resistant isolate.

This analysis was carried out on three different plates for each of the 15 resistant isolates inoculated and the three maximum distance values calculated were averaged out. The data relevant to this analysis are shown in Figure 3.6 and Table 8.1 in appendix 8.3.

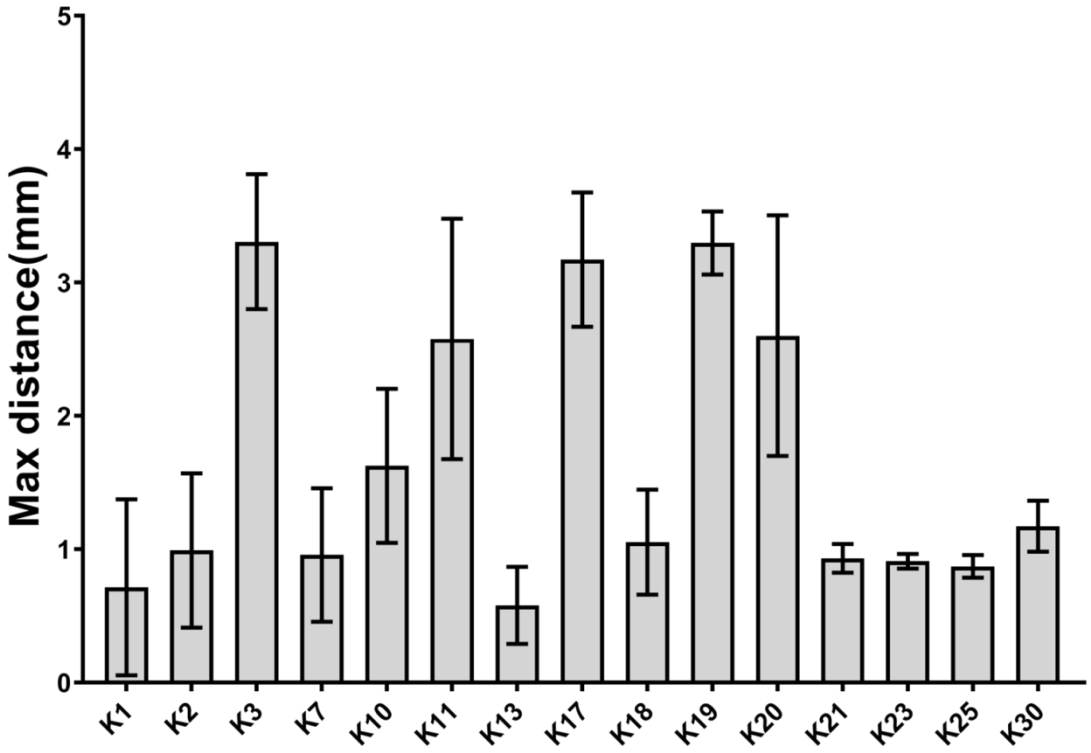



**Figure 3.6**. **Maximum distance detected on the plates inoculated with the 15 resistant isolates**. The bar chart shows the mean of the three maximum distance values of the furthest colony detected on the three different plates inoculated with the same resistant isolate. The error bars represent the standard deviation of the mean.

However taking into account only one value of maximum distance per plate might have limitations. Indeed, this procedure might be theoretically subject to the generation of skewed data due to the appearance of sporadic antimicrobial resistant mutant colonies in the sheltered species or contamination with unrelated resistant

bacteria. These random colonies might skew the analyses if they are mistaken for a sheltered colony some distance from the resistant isolate. In order to reduce the misleading effect of potential outliers on the calculation of the maximum distance value, the average of a number of maximum distance values measured on the same plate was calculated. In particular the distance values of different numbers of the furthest colonies, respectively 5, 10 and 20, on the same plate were progressively averaged out to test the effect of the increase of the sample size on the results. Also, the choice to average specific numbers of furthest colonies, respectively 20, 10 and 5, was based on a first rough visual inspection of the plates. Indeed, the number of colonies appearing on the plates was approximately in the range between 5 and 20. These observations were complicated by the observation that there was variability in the number of colonies available with some resistant isolates producing only 2 or 3 sheltered colonies while others appeared to produce around 15-25 colonies. However, the smallest sample size took into account not less than 5 colonies to calculate the average. The choice of the 20, 10 and 5 furthest colonies to consider in the analysis was based on preliminary tests aimed to determinate the colonies appearing furthest from the resistant isolate.

The average maximum distance values calculated by 20, 10 and 5 colonies calculated on the three different plates are reported respectively in Figures 3.7, 3.8 and 3.9. Furthermore, the descriptive statistics of these data is reported respectively in Tables 8.2, 8.3 and 8.4 in appendix 8.3.

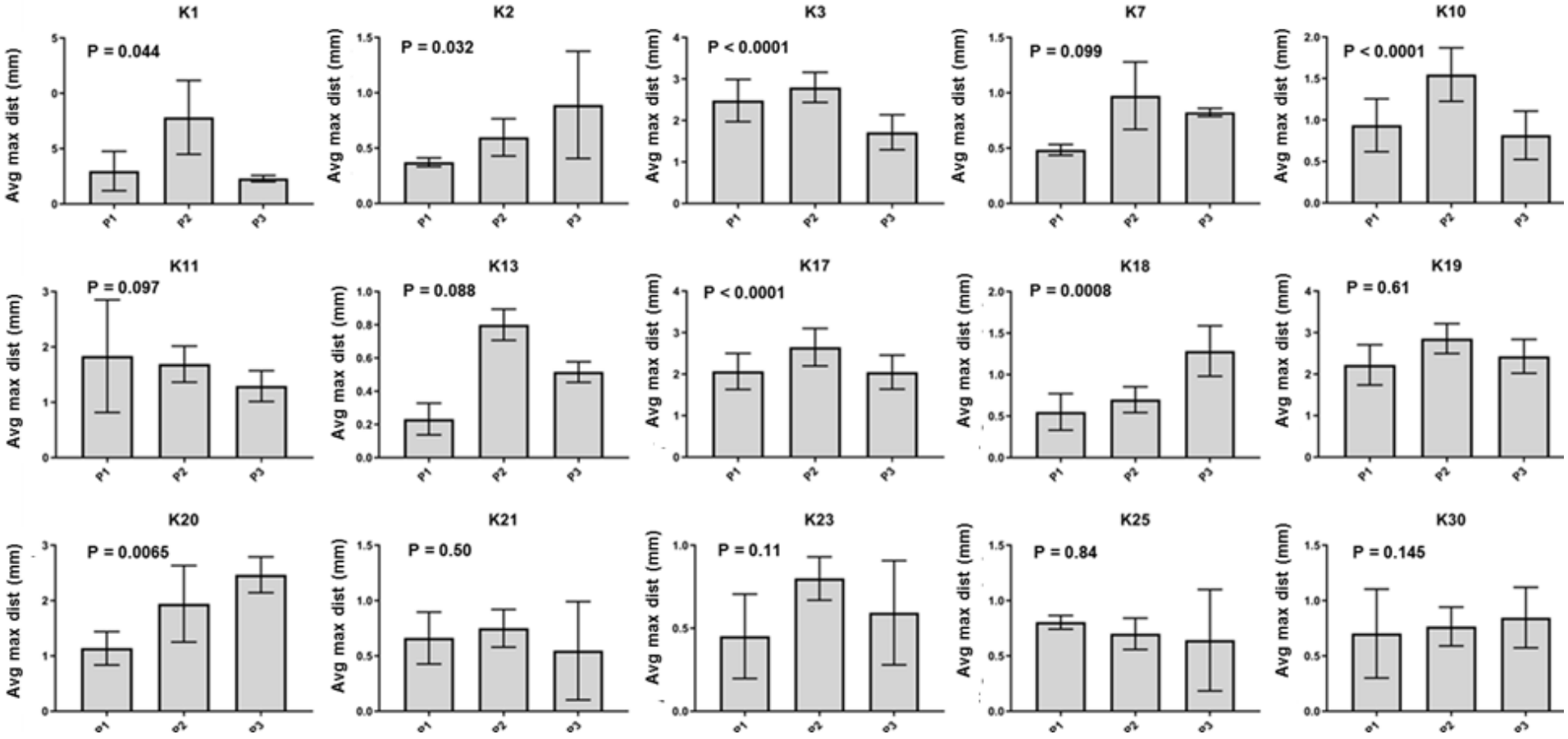



**Figure 3.7. Average maximum distance from 20 colonies detected on the plates inoculated with the resistant isolates.** The bar charts show the average of the single 20 distance values measured in the three different plates (P1, P2 and P3). The error bars represent the standard deviation of the mean for each plate. The P-value (P) obtained with one-way ANOVA for the comparison of the three plates is also reported for each resistant isolate inoculated.

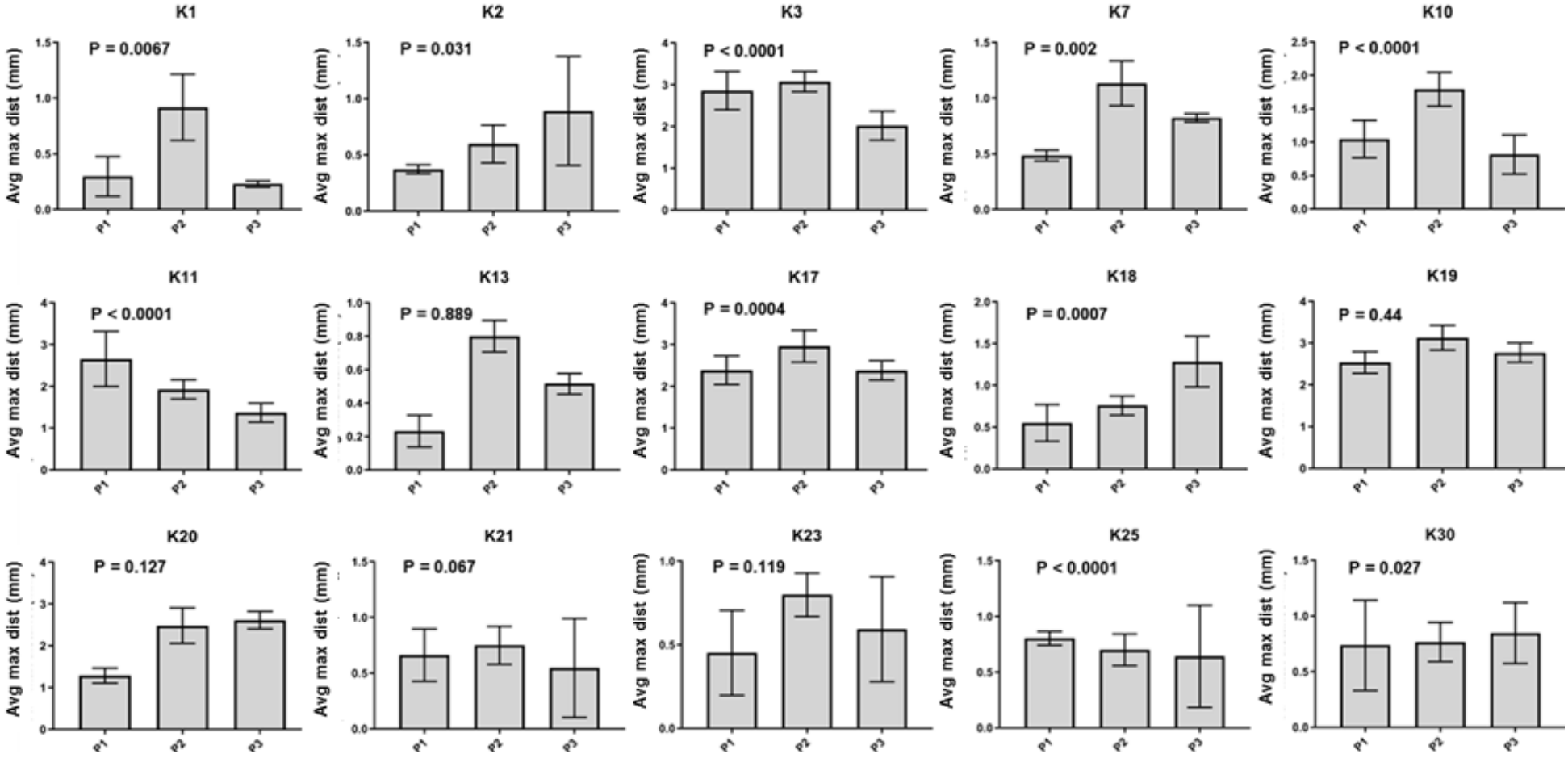



**Figure 3.8. Average maximum distance from 10 colonies detected on the plates inoculated with the resistant isolates.** The bar charts show the average of the single 10 distance values measured in the three different plates (P1, P2 and P3). The error bars represent the standard deviation of the mean for each plate. The P-value (P) obtained with one-way ANOVA for the comparison of the three plates is also reported for each resistant isolate inoculated.

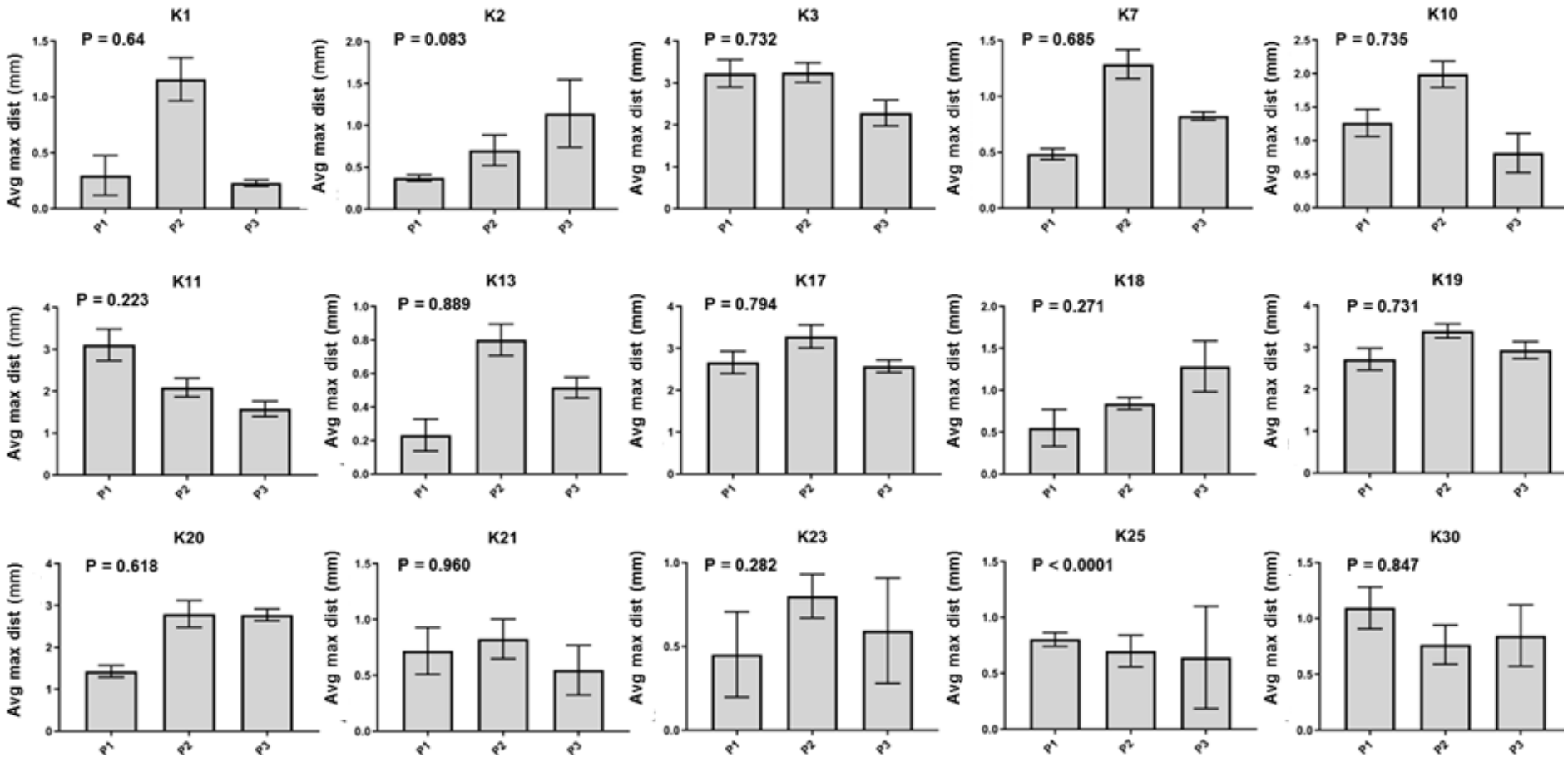



**Figure 3.9 Average maximum distance from 5 colonies detected on the plates inoculated with the resistant isolates.** The bar charts show the average of the single 5 distance values measured in the three different plates (P1, P2 and P3). The error bars represent the standard deviation of the mean for each plate reported. The P-value (P) obtained with one-way ANOVA for the comparison of the three plates is also reported for each resistant isolate inoculated.

These data showed that significant differences between the means of the 20 distance values calculated on the three plates were detectable for the isolates K1, K2, K3, K17, K18 and K20. Indeed, for these isolates the P-values calculated were less than 0.05 (Figure 3.7). Furthermore, the differences were significant for the means of the 10 distance values measured on the three plates for the isolates K1, K2, K3, K7, K10, K11, K17, K18, K25 and K30 (Figure 3.8). Lastly, no isolate showed significant differences between the mean values of the 5 distance values calculated with the exception of K25. Here the P-value was less than 0.05 (Figure 3.9).

However, these data also showed that a number of distance values were missing from the analyses, although the number of the missing values was variable depending on the number of colonies counted (Tables 8.2, 8.3 and 8.4, appendix 8.3). This was mainly due to the fact that the number of countable colonies detected on the plates inoculated with some of the isolates was less than respectively 20, 10 and 5. Furthermore, on a few plates the growth of colonies in a cover of bacteria did not allow the calculation of the distance between the centre of single colonies and the edge of the resistant isolate. In particular, with the exception of the 20 colonies growing in all the three plates inoculated with two resistant isolates (K3 and K17), the number of countable colonies was less than 20 on at least one plate inoculated with all the other 13 resistant isolates (Table 8.2 appendix 8.3). The number of the furthest colonies was also less than 10 on at least one plate inoculated with 11 resistant isolates (K1, K2, K7, K10, K13, K18, K20, K21, K23, K25, K30) (Table 8.3, appendix 8.3). Lastly, the number of countable colonies was less than 5 on at least one plate inoculated with 8 resistant isolates (K1, K2, K7, K13, K18, K21, K25 and K30). However, 5 colonies were countable on all the plates inoculated with *7* resistant isolates (K3, K10, K11, K17, K19, K20 and K23) (Table 8.4, appendix 8.3).

To determine if the calculation of the maximum distance from one furthest colony and the average maximum distance of the 20, 10 and 5 furthest colonies produced similar results the four variants of the distance-based method were compared. In particular, this aimed to investigate if the increase of the sample size affected significantly the results obtained. The data relevant to this analysis are reported in Figure 3.10.

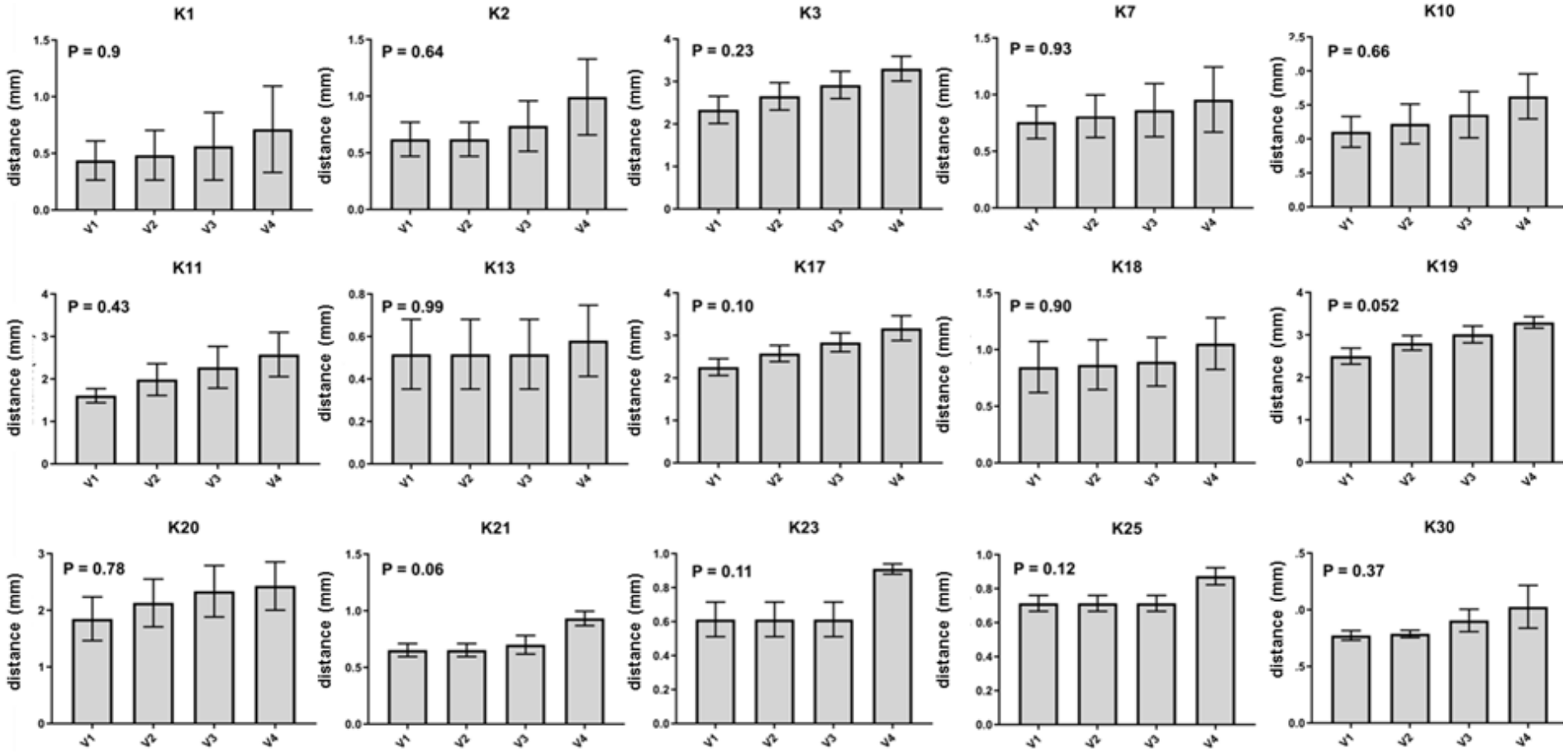



**Figure 3.10 Comparison of the results obtained with the four variants of the distance-based method.** For each resistant isolate inoculated a bar chart showing the mean distance values and the corresponding standard errors calculated with the four different distance-based variants (V1: average maximum distance of the 20 furthest colonies; V2: average maximum distance of the 10 furthest colonies; V3: average maximum distance of the 5 furthest colonies; V4: maximum distance of one furthest colony) is reported. The P-value (P) obtained with one-way ANOVA for the comparison of the four variants is also reported for each resistant isolate inoculated.

These data showed that no significant differences between the mean values of the distance values calculated with the four different variants for each sheltering isolate was detectable. Indeed all the P-values calculated were higher than 0.05 (Figure 3.10, and Table 8.5, appendix 8.3).

In the area-based method the entire area occupied by sheltered *E. coli* NCTC 10418 on a plate was determined. Unlike the distance-based procedure this method allowed to include both individual satellite colonies and the continuous cover of bacteria surrounding the resistant species in the analysis by subtracting the area occupied by the resistant isolate (sheltering area) from the total area occupied by both the resistant isolate and the sheltered strain (sheltered area). Therefore, unlike all the variants of the distance-based method, the area-based method allowed to take into account the continuous cover of bacteria around the resistant isolate and the area occupied by each single colony of the susceptible species. This method was assumed to reduce most the skewing effect of potential outliers since the major contribution to the calculation of the sheltered area was given from the area forming the susceptible lawn around the resistant isolate. The data relevant to this analysis are shown in Figure 3.11 and Table 8.6 in appendix 8.3.

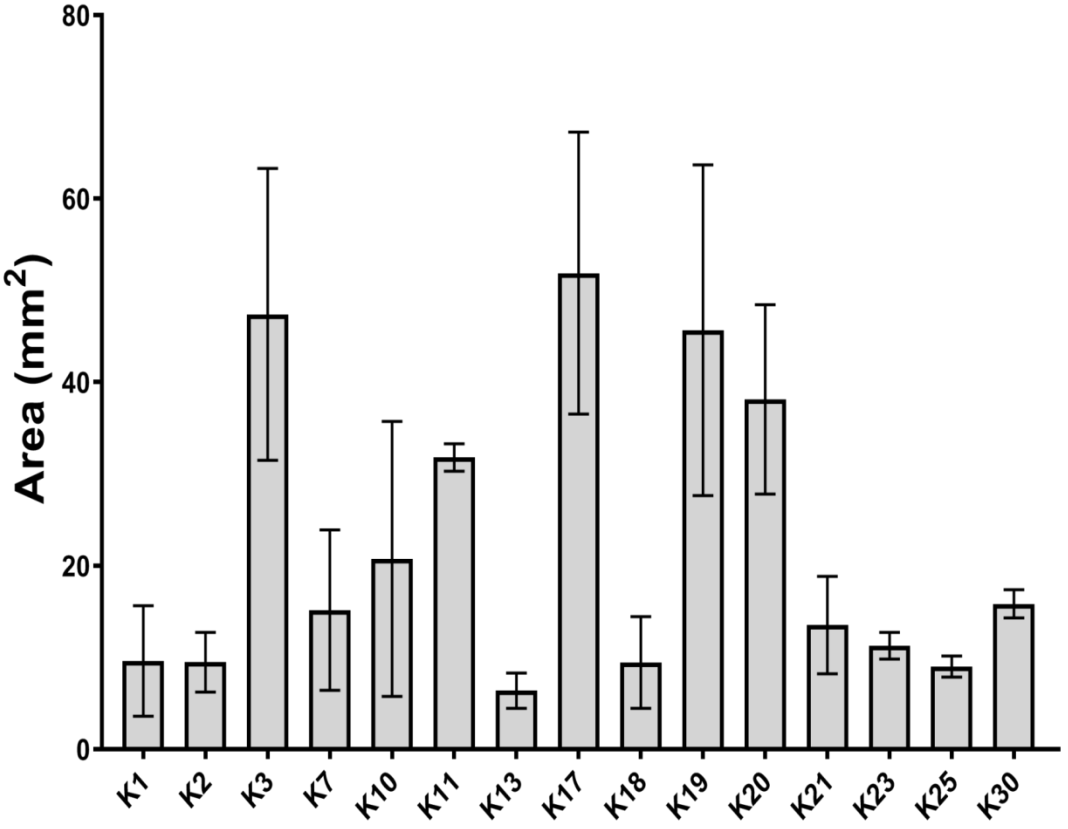



**Figure 3.11 Sheltered areas of *E. coli* NCTC 10418 calculated for each resistant isolates.** The bar chart shows the mean of the sheltered areas calculated on the three different plates inoculated with the same resistant isolate. The error bars represent the standard deviation of the mean.

The sheltered area values were plotted against the average maximum distance values of the furthest 20, 10 and 5 colonies and the values of maximum distance. A strong relationship was detected ($R^2$ > 0.8) between the results obtained with the area-based method and each of the distance-based variants when these two variables were analysed with the linear regression on a scatter plot (Figure 3.12).

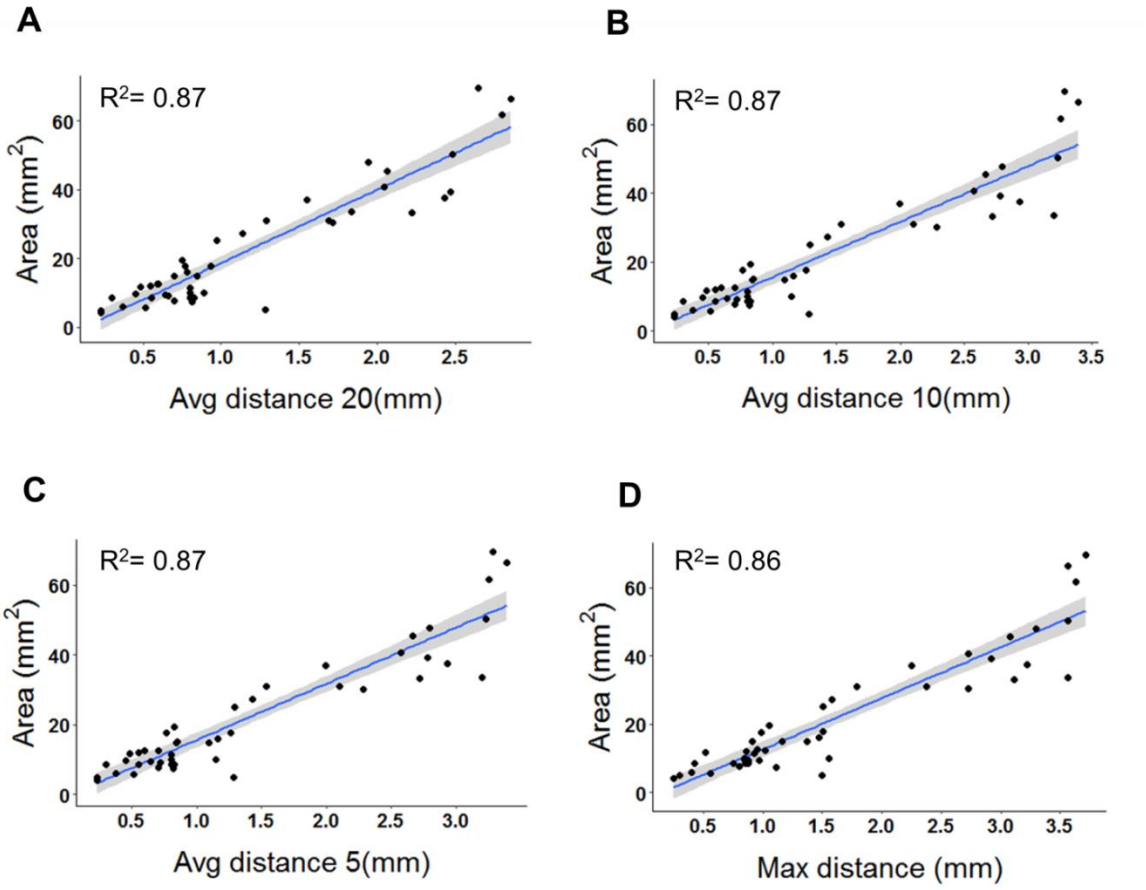



**Figure 3.12 Relationship between the sheltered areas and the distance values.** Each point in the scatter plot represents the sheltered area and the value of the average maximum distance of the furthest 20 (A), 10 (B) and 5 (C) colonies and the value of maximum distance (D) calculated on one of the three plates inoculated with the 15 resistant isolates. The line estimated by the linear regression is depicted as the solid line and the blue shaded area represents the 95% confidence interval around the line. The $R^2$ value is reported in each graph to express the closeness of the data to the fitted regression line.

The positive trend exhibited by the values plotted suggested that the distance from the resistant isolate at which a susceptible colony could grow was linearly related to the extension of the growth area of the sheltered strain. Indeed, higher distances were associated to bigger growth areas. Similarly, smaller distances also corresponded to reduce growth areas. This suggested that the distance-based method with its variants and the area-based method produced comparable results.

This suggests that any of these procedures might be used for future analyses aimed to quantify the sheltering effect on plate. However, the area-based method would be the preferred procedure in case of random mutants present on the plate since the quantification of the sheltering effect would likely be marginally affected by these colonies. In the next chapters the degree of sheltering variability between the different isolates will be investigated by taking into account a larger sample of clinical isolates of *K. pneumoniae*.

## 3.3 Discussion

The sheltering effect has been investigated in different pathogenic bacteria by using different methods, including the induction of mixed infections in animals (Brook et al., 1983) and investigation of OMVs carrying β-lactamases (Ciofu et al., 2000; Schaar et al., 2011; Schaar et al., 2014; Chattopadhyay and Jaganandham, 2015; Liao et al., 2015). However, the use of these methods requires respectively the availability of laboratory animals to carry *in vivo* bacterial infections and the use of time-consuming techniques and expensive equipment. Co-culturing resistant and susceptible species in selective liquid medium followed by the discrimination of each species by the colony characteristics on agar plates (Perlin et al., 2009; Liao et al., 2014) is a much cheaper and time-saving technique to confirm the presence of the sheltering effect. However, the use of this procedure requires the availability of resistant and sensitive strains forming colonies that can be visually differentiated on agar plates.

The presence of the sheltering effect has here been investigated in the pathogen *K. pneumoniae*. In this project, a straightforward and time-saving protocol was developed to detect the sheltering effect provided by *K. pneumoniae*. The method developed in this project involved the direct spreading of a bacterial culture of a β-lactam-susceptible strain on a selective agar plate followed by the point inoculation of a bacterial culture of a β-lactam resistant isolate. This method was tested by using *E. coli* NCTC 10418 and *S. aureus* NCTC 6571 as β-lactam-susceptible strains and K17, one of the *K. pneumoniae* β-lactam-resistant isolates available in the laboratory.

The bacterial growth of both the susceptible strains was detected only around K17 after overnight incubation and appeared in the form of single colonies and a continuous lawn of bacteria in the area immediately surrounding the inoculation area of K17. This suggested that K17 was able to protect both the susceptible strains from the action of the drug. This effect was not species-specific since the two susceptible strains belonged to different species. Furthermore, it was restricted neither to Gram-positive nor Gram-negative species since the two strains belonged to different Gram groups. The effect was detectable only if both the resistant and the susceptible

strains were inoculated on the same plate, thus confirming that the susceptible strains were able to grow only in presence of the resistant isolate (Figure 3.1).

No bacterial growth on clean selective plates was detected after the streaking of the bacterial culture of both the susceptible strains growing around K17 (Figure 3.2). This confirmed that the ability of the *E. coli* NCTC 10418 and *S. aureus* NCTC 6571 to grow on a medium enriched with Amp was unlikely the result of spontaneous genetic mutations. Indeed, these mutations were expected to enable the sensitive strains to resist the action of Amp regardless of the presence of K17. Moreover, the results of the PCR analyses confirmed that the ability of the susceptible strains to grow in presence of Amp was not the result of the transfer of the β-lactam resistance gene $bla_{\text{OXA-48}}$ from K17 to *E. coli* NCTC 10418 and *S. aureus* NCTC 6571 (Figure 3.3). However, the genome of K17 carries genes for the β-lactamases TEM, SHV, CTX-M, OXA-1 and AmpH in addition to OXA-48. Therefore, any of these genes might be transferred by bacterial conjugation thus conferring resistance to the susceptible strain. Although the presence of these additional genes in the bacterial culture retrieved from the susceptible strains was not tested by PCR, the complete lack of bacterial growth on the clean selective plates suggested no β-lactam resistance genes were transferred from K17. All these observations indicated that the bacterial growth of *E. coli* NCTC 10418 and *S. aureus* NCTC 6571 was due to the sheltering effect provided by the resistant isolate that enabled the susceptible strains to grow despite the presence of the β-lactam in the medium.

15 β-lactam resistant *K. pneumoniae* isolates, including K17, were able to shelter *E. coli* NCTC 10418 and *S. aureus* 6571 from Amp present in the agar (Figures 3.4 and 3.5). Similarly to K17, all the other 14 resistant isolates showed no specificity for the bacterial species and Gram group they sheltered. This observation suggested that the sheltering effect detected for K17 was not a phenomenon found randomly in one of the β-lactam resistant isolates. Instead, the sheltering effect could be found in multiple β-lactam resistant strains of *K. pneumoniae.*

Although all the resistant isolates were able to shelter both the susceptible strains, they showed variability in the degree of sheltering effect they provided. This variability reflected the difference among the isolates in the distance that the protective effect could reach from the resistant species, as well as by the area of growth of the susceptible strain represented by the diameter of the continuous cover of bacteria growing around the *K. pneumoniae* isolates. Therefore, quantifying the sheltering effect by measuring the distance reached by the sheltering effect and the growth area of the susceptible strain protected might represent a new method to type bacterial strains.

The MIC is a method routinely used to determine the resistance degree of a microorganism to an antimicrobial (Andrews, 2001). The use of the MIC allows the quantification of the antimicrobial resistance of a strain or isolate by determining the lowest concentration of a drug that inhibits the visible growth of the organism after overnight incubation. However, the MIC takes into account no cooperative interactions that might occur between different bacteria during polymicrobial infections. Indeed, the MIC determines only the antimicrobial resistance profile of the specific strain or isolate responsible for the resistance. The quantification of the sheltering effect might represent a method to type bacterial strains based on the antimicrobial resistance detected in the presence of both resistant and susceptible bacteria. Therefore, it might be used to predict the degree of antimicrobial resistance during polymicrobial infections of resistant and susceptible bacterial strains, thus giving information about the level of resistance to the drug therapy that a polymicrobial infection might cause.

The sheltering effect has been previously quantified by counting the bacterial colonies of the susceptible strains that could survive only in β-lactam-containing broth in the presence of different concentrations of the resistant strain (Liao et al., 2014). Furthermore, Western Blot analyses and chromogenic substrates were used to respectively determine the amount and the activity of the β-lactamases inside the OMVs that degraded the β-lactam in the medium and consequently protected the susceptible strains (Ciofu et al., 2000; Schaar et al., 2011; Liao et al., 2015).

In this project two different methods for the quantification of the sheltering effect detected on plate were developed with the aim to study the variability detected in the sheltering within the resistant isolates of *K. pneumoniae.* The purpose of these two methods was measuring respectively how far the protective effect could extend from the resistant isolate and the area of the susceptible strain that benefited from the protection from the β-lactam. The quantification of the sheltering effect with these methods was carried out by using a software-based calculation of respectively the distance and area values directly from the image of the plate.

Four variants of the distance-based method were tested. In the maximum distance variant, the distance of the furthest satellite colony from the resistant species was measured and the mean and standard deviation of the single distance values taken from three different plates was calculated (Figure 3.6). However, this method took into account only 1 distance per plate. Therefore, it might be susceptible to the presence of colonies of the susceptible strain that might grow on the plate because of genetic mutations conferring the ability to resist to the antimicrobial in the medium. These colonies would represent outliers and grow at a distance greater than those expected for the sheltered colonies that are close to the resistant species. Indeed,

they might skew the mean value calculated from the single distance values on each single plate used as a replicate for the experiments, thus producing misleading results. The skewing effect of a potential outlier might be reduced by increasing the number of plates to use as replicates. However, using a high number of plates is costly and time-consuming.

The variants based on the average of the maximum distance involved the calculation of the average distance of the furthest 20, 10 and 5 satellite colonies detected on the single plates. Unlike the maximum distance these variants took into account a larger sample size per plate, thus diminishing the need to use several single plates as replicates to increase the sample size. Indeed, the use of these variants of the distance-base method was envisaged to diminish the misleading effect of potential outliers per plate since different single distance values were averaged out. Furthermore, taking a number of distance values on the same plate was expected to be more accurate than measuring just one distance value. Indeed the increase in the sample size might better represent the behavior of the whole group of the satellite colonies. However significant variations between the replicates were detectable for a number of isolates, especially when the average distance was calculated for the furthest 20 and 10 satellite colonies. Furthermore all the variants based on the average distance produced missing values due to the lack of a sufficient number of satellite colonies that could be measured on plate (Figures 3.7, 3.8 and 3.9 and Tables 8.2, 8.3, and 8.4 in appendix 8.3). In addition, the aggregation of satellite colonies in a cover of bacteria did not allow the identification of single countable colonies. This resulted in the calculation of the mean from a sample size smaller than that expected. However the confluent growth of satellite colonies might be reduced by carrying out dilutions of the overnight culture of the susceptible species used for the experiments on plate. Indeed, this would allow to reduce the bacterial concentration and consequently increase the possibility to obtain single countable colonies on plate. These observations suggest that the use of the average distance-based variants could represent an advantage on the maximum distance variant only on plates where a selected number of single colonies is clearly visible.

However, all the four distance-based variants tested including the measurement of the maximum distance and the calculation of the average of the furthest 20, 10 and 5 colonies produced comparable results. Indeed, no significant differences were detectable when these methods were compared with one-way ANOVA (Figure 3.10 and Table 8.5 in appendix 8.3). This suggested that any of these variants of the distance-based method could be used for the quantification of the sheltering effect.

The last method tested consisted of the calculation of the whole area occupied by the sheltered strain, including the compact cover of bacteria around the resistant isolate

and the areas occupied by all the single colonies of the susceptible strain (Figure 3.11 and Table 8.6 in appendix 8.3). Linear regression analyses showed that the calculation of the sheltered area gave results comparable to those produced with the distance-based method, thus suggesting that either the distance-based and the area-based method could be used for the quantification of the sheltering effect (Figure 3.12).

However, the calculation of the susceptible area might be the preferred method to quantify the sheltering effect in case of outliers present on the plate. Indeed, the contribution of each single colony is assumed to be much smaller than the contribution given by the lawn of bacteria around the resistant isolate to the calculation of the sheltered area. Therefore, the presence of a contaminating outlier is expected to have much less misleading effect in the quantification of the sheltering with the area-based method than the distance-based one.

# 4 RESULTS II: THE VARIABILITY IN THE SHELTERING EFFECT

## 4.1 Introduction

The increasing development of tolerance or resistance to an antimicrobial is seriously compromising the successful use of this therapeutic in the treatment of the bacterial infections (Davies and Davies, 2010; Ventola, 2015). Resistance of the Enterobacteriaceae to antimicrobials, especially of the β lactam type, is mostly due to plasmids that carry β-lactamase genes (Carattoli, 2009; Iredell et al., 2016; Partridge et al., 2018; Rozwandowicz et al., 2018).

Among the β-lactamases, the ESBLs of TEM-type and SHV-type have been reported worldwide and most frequently in Enterobacteriaceae (Bradford, 2001; Paterson et al.,2005; Dallenne et al., 2010; Liakopoulos et al., 2016; McDanel et al., 2017). However, during the past decade, the rate of dissemination of the ESBLs of CTX-M-type belonging to phylogenetic group 1 and 9 has increased dramatically worldwide (Bonnet et al., 2004; Dallenne et al., 2010; Cai et al., 2017, Shen et al., 2017; Bush, 2018). Other less prevalent ESBLs have been described as minor enzymes, including the GES, PER and VEB types (Poirel et al., 2000; Dallenne et al., 2010; Esragul and Haluk, 2010; Bontron et al., 2015; Bush, 2018). Carbapenemases represent another group of β-lactamases of special interest. Their ability to catabolise almost all the hydrolyzable β-lactams including penicillins, cephalosporins, monobactams, and carbapenems renders the bacteria that carry them highly resilient against treatment with nearly all commercially viable β-lactam antimicrobials (Queenan and Bush, 2007). Among the carbapenemases the metallo-β-lactamases IMP and VIM are particularly relevant because they have been identified in several geographical areas among many different enterobacterial species (Poirel et al., 2011). Also, the β-lactamases of NDM type have become the most prevalent metallo-β-lactamases circulating worldwide (Patel and Bonomo, 2013; Lagan and Bonomo, 2016). In addition, the emergence of the KPC carbapenemase has been witnessed mostly in *K. pneumoniae* (Nordmann et al., 2009; Poirel et al., 2010; Bathoorn et al., 2016). The carbapenemases of type OXA-48 are currently receiving particular interest. Since their first discovery in 2001, these carbapenemases have been increasingly reported worldwide. They now represent a global threat to public health due to their ability to hydrolyse carbapenems (Poirel et al., 2004; Nordmann et al., 2011; Poirel et al., 2012a;; Mathers et al., 2013; Albiger et al., 2015; Findlay et al., 2017).

One of the paths responsible for the development and evolution of resistance in bacteria is the occurrence of genetic mutations in the resistance genes that enhance

and/or change the antibiotic tolerance (Andersson and Hughes, 2010; Tobrak et al., 2012; Levin-Reisman et al., 2017). The effect of such mutations is measured by the MIC, the lowest concentration of an antimicrobial which prevents the visible growth of the bacterium (Andrews, 2001; Knopp and Andersson, 2018). MIC values of clinical isolates belonging to the same bacterial species might significantly differ due to key mutations occurring in the genes responsible for the antimicrobial resistance. Indeed, these mutations might be responsible for changes in the biochemical profile of the antimicrobial resistance enzymes thus leading to increased resistance to a specific compound or catalytic activity against new compounds (Walkiewicz et al., 2012; Suzuki et al., 2014; Kambli et al., 2015; Li et al., 2018). TEM-type beta-lactamases represent a good example of this process. Indeed, it therefore appears that mutations in these enzymes contribute to resistance to new-generation cephalosporins (Sirot et al., 1987; Sougakoff et al., 1988; Paterson and Bonomo, 2005; Munita and Arias, 2016). Genetic mutations can now be easily identified by sequencing the DNA of interest (Paareck et al., 2011; Hawkins, 2017) and potential changes in the protein function can be quickly found by using bioinformatics methods for predicting the amino acid sequence starting from the corresponding DNA sequence (Gasteiger et al., 2003).

As reported in the Introduction and Chapter 3, the sheltering effect has been reported in a number of bacterial species (Brook et al., 1983; Ciofu et al., 2000; Perlin et al., 2009; Shaar et al., 2011; Shaar et al., 2014; Chattopadhyay and Jaganandham, 2015; Liao et al., 2014; Stentz et al., 2015). These studies investigated the mechanisms of this protective effect in one specific resistant strain that was used for the experimental analyses. However, the study of the sheltering effect in a number of strains or clinical isolates might highlight differences in this phenomenon among several strains belonging to the same bacterial species. As described in Chapter 3, variability in the potential of sheltering was detected among 15 clinical isolates of *K. pneumoniae.*

The purpose of this part of the project was to investigate whether the sheltering effect was a widespread phenomenon within the *K. pneumoniae* species. In addition, this study aimed to determine if the sheltering effect occurred with different degrees of strength in different isolates of *K. pneumoniae.* Therefore, a larger sample of clinical isolates of *K. pneumoniae* was taken into account for the screening of the sheltering effect. In particular all the β-lactam resistant *K. pneumoniae* isolates available in laboratory were analyzed. There were 33 isolates and these were retrieved from immunocompromised patients with β-lactam resistant infections from a Kuwait hospital in 2014.

The aims of this study were:

- To confirm the presence of sheltering effect in all the 33 β-lactam resistant isolates of *K. pneumoniae* available in the laboratory.
- To quantify their sheltering strength by using the area-based method (described in Chapter 3)
- To investigate the relation between the variability in the sheltering potential and respectively:
    1. the growth rates of the *K. pneumoniae* isolates
    2. their resistance levels to β-lactams
    3. differences in their Random Amplified Polymorphic DNA (RAPD) genetic profile
    4. the presence or absence of genes for β-lactamases
    5. the amino acid sequence of the β-lactam-resistance genes

## 4.2 Results

### 4.2.1 Variability in the sheltering effect among the *K. pneumoniae* isolates

All the 33 *K. pneumoniae* β-lactam resistant isolates available in laboratory were tested for their sheltering potential on *E. coli* NCTC 10418 and *S. aureus* 6571. In addition to the 15 *K. pneumoniae* isolates already tested and described in Chapter 3 (K1, K2, K3, K7, K10, K11, K13, K17, K18, K19, K20, K21, K23, K25 and K30) the remaining 18 *K. pneumoniae* isolates K4, K5, K8, K9, K12, K15, K16, K22, K24, K26, K27, K28, K29, K41, K47, K48, K51 and K56 were screened for the presence of sheltering effect. As described in Chapter 3, the sheltering potential of the *K. pneumoniae* isolates was investigated by carrying out point inoculations of each of the resistant isolates on two agar plates enriched with Amp and evenly spread with respectively *E. coli* NCTC 10418 and *S. aureus* NCTC 6571. This analysis was carried out in triplicate.

Although the sheltering potential of 15 isolates had already been investigated as described in Chapter 3, the screening for these isolates was repeated in a single experiment involving the remaining 18 isolates as well. All the 33 isolates were inoculated on the same sample of overnight bacterial culture of the susceptible strain as this removed potential inter-experimental variability due to differences in culture density of the susceptible strains.

The optical density of all the overnight cultures of the 33 *K.pneumoniae* isolates, *E. coli* NCTC10418 and *S. aureus* NCTC6571 used were also measured at a wavelength of 600 nm ($OD_{600}$) and compared. This aimed to determine potential

differences between these two species in the concentration of bacteria used for the spread on plate.

The results showed that out of the 33 *K.pneumoniae* isolates tested, 22 (66%) showed sheltering effect on *E. coli* NCTC 10418 (K1, K2, K3, K7, K8, K10, K11, K12, K13, K15, K16,K17, K18, K19, K20, K21, K23, K24, K25, K27, K30 and K51) since at least 1 bacterial colony appeared after 24 hours around the inoculation area of these resistant isolates. Interestingly, no growth of *E. coli* NCTC 10418 appeared around 11 (33%) resistant isolates (K4, K5, K9, K22, K26, K28, K29, K41, K47, K48 and K56)(Figure 4.1). 25 isolates (75%) showed sheltering effect on *S.aureus* NCTC 6571 (K1, K2, K3, K4, K5, K7, K8, K10, K11, K12, K13, K15, K16, K17, K18, K19, K20, K21, K23, K24, K25, K26, K27, K30 and K51) while no bacterial growth or colonies were visually detected around the 8 isolates (24%) K9, K22, K28, K29, K41, K47, K48 and K56 (Figure 4.2). No growth in the area surrounding the isolates was detected in the absence of susceptible species (Figures. 4.1. and 4.2).

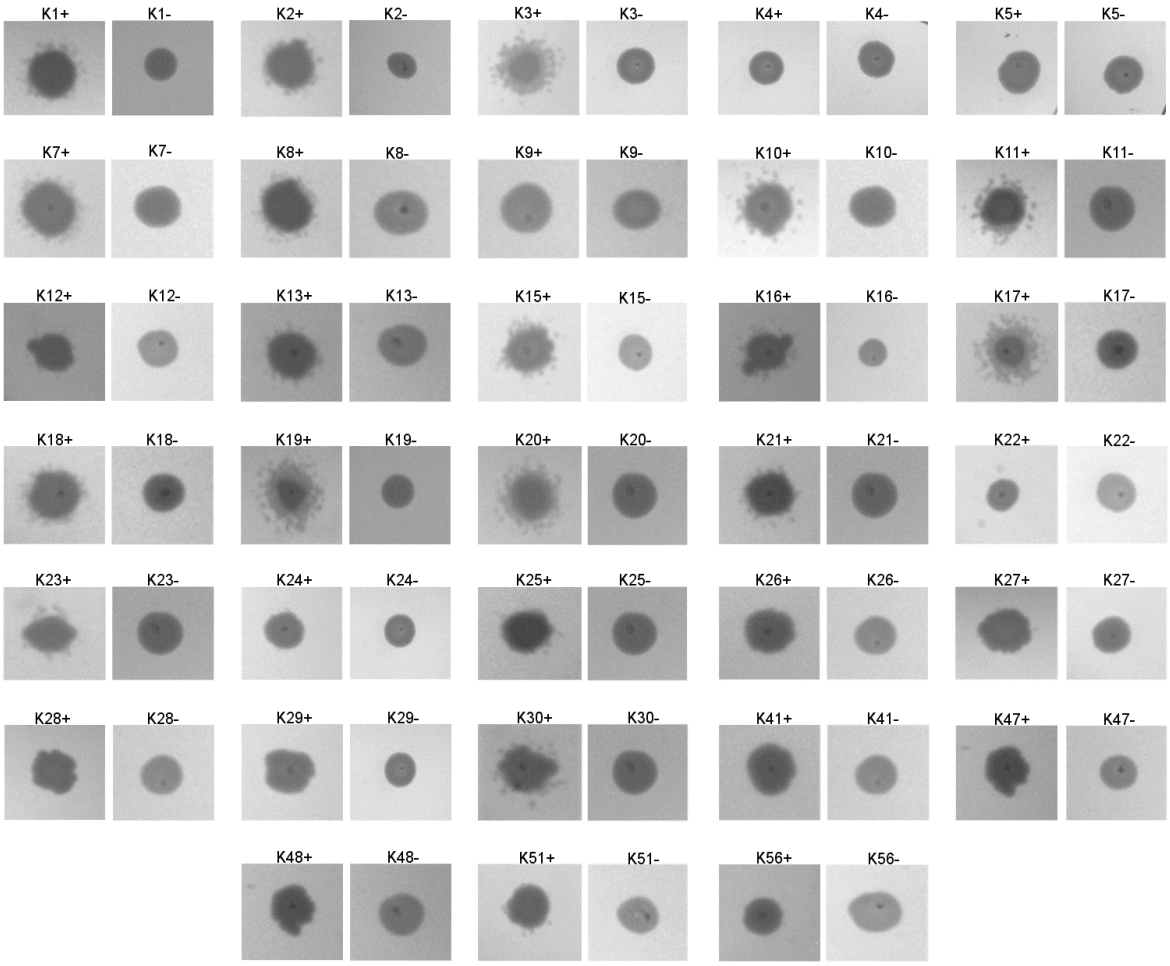



**Figure 4.1 Sheltering effect of all the *K. pneumoniae* isolates on *E. coli* NCTC 10418.** The picture shows the bacterial growth on two 100 µg/ml Amp plates for all the *K. pneumoniae* isolates tested in this project. These include the 15 *K. pneumoniae* isolates described in chapter 3 and 18 additional isolates. These two plates were prepared by inoculating each *K. pneumoniae* isolate respectively in presence (+) and absence of *E. coli* NCTC 10418 (-) followed by incubation at 37°C for 24 hours. This experiment was performed in triplicate for each *K. pneumoniae* isolate (n = 3)and the image of one of the three plates used as replicates is showed in the picture as an example.

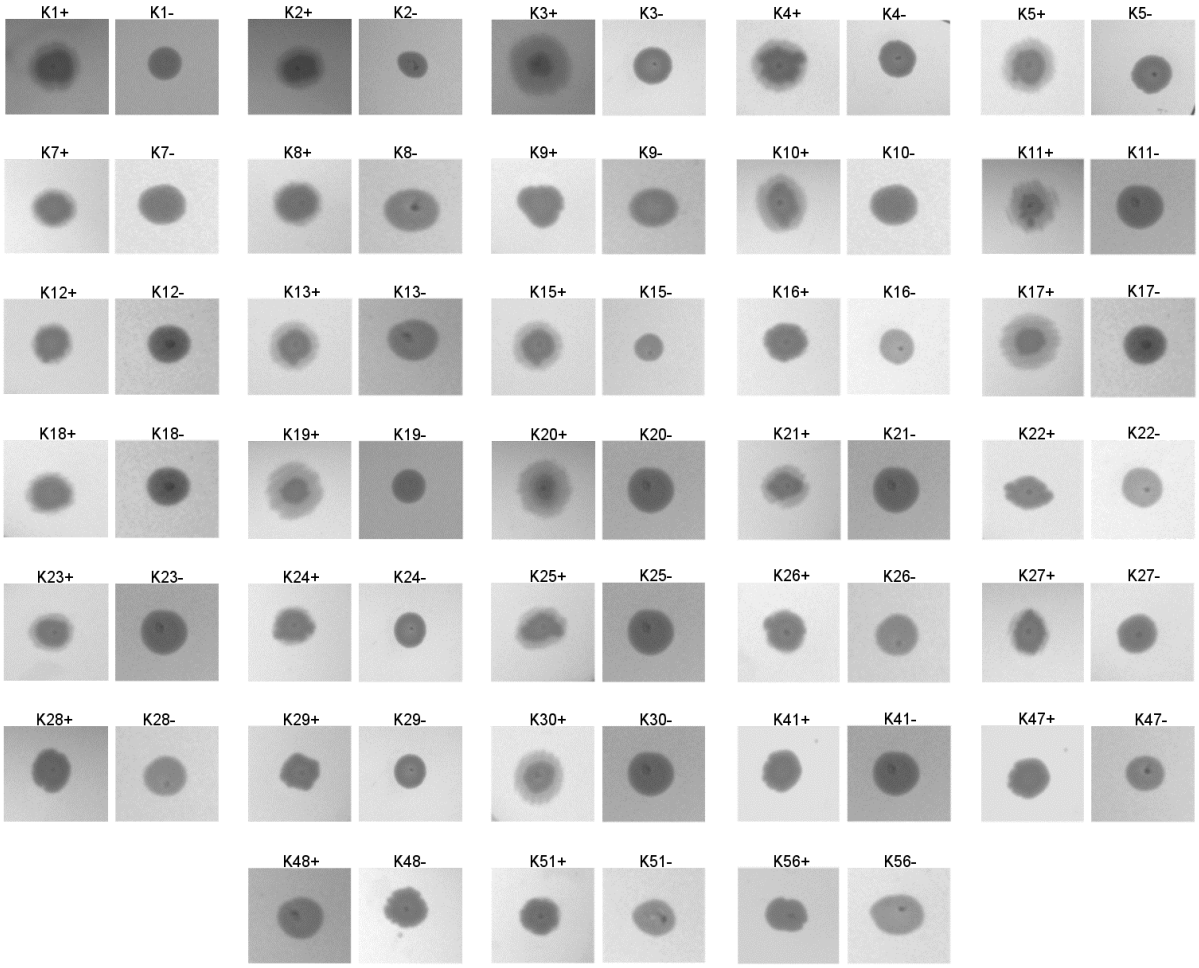



**Figure 4.2 Sheltering effect of all the *K. pneumoniae* isolates on *S. aureus* NCTC 6571.** The picture shows the bacterial growth on two 100 µg/ml Amp plates for all the 33 *K. pneumoniae* isolates tested in this project. These include the 15 *K. pneumoniae* isolates described in Chapter 3 and 18 additional isolates. These two plates were prepared by inoculating each *K. pneumoniae* isolate respectively in presence (+) and absence of *S. aureus* NCTC 6571 (-) followed by incubation at 37°C for 24 hours. This experiment was performed in triplicate for each *K. pneumoniae* isolate (n = 3) and the image of one of the three plates used as replicates is showed in the picture as an example.

The sheltering potential in the two groups of isolates producing no bacterial growth after 24 hours of incubation on plates spread with respectively *E. coli* NCTC 10418 and *S. aureus* NCTC 6571 was also monitored after 24 additional hours of incubation. This analysis aimed to confirm the presence of sheltering effect in these isolates. Indeed, these isolates might shelter the susceptible species at a slower rate of speed thus resulting in a delayed appearance of susceptible bacterial growth.

The results showed that out of 11 isolates 9 *K. pneumoniae* (81%) where no sheltering was observed after 24 hours produced at least one colony of *E.coli* NCTC 10418 after 48 hours. However, no susceptible colonies were detectable on the plates inoculated with K5 and K47 (Figure 4.3). Bacterial growth of *S. aureus* NCTC

6571 was detectable after 48 hours on the plates inoculated with all the 8 *K. pneumoniae* isolates (Figure 4.4) where no sheltering was initially observed. Again, no growth in the area surrounding the isolates was detected in absence of *E. coli* NCTC 10418 or *S. aureus* NCTC 6571 after 48 hours (Figures.4.3 and 4.4).

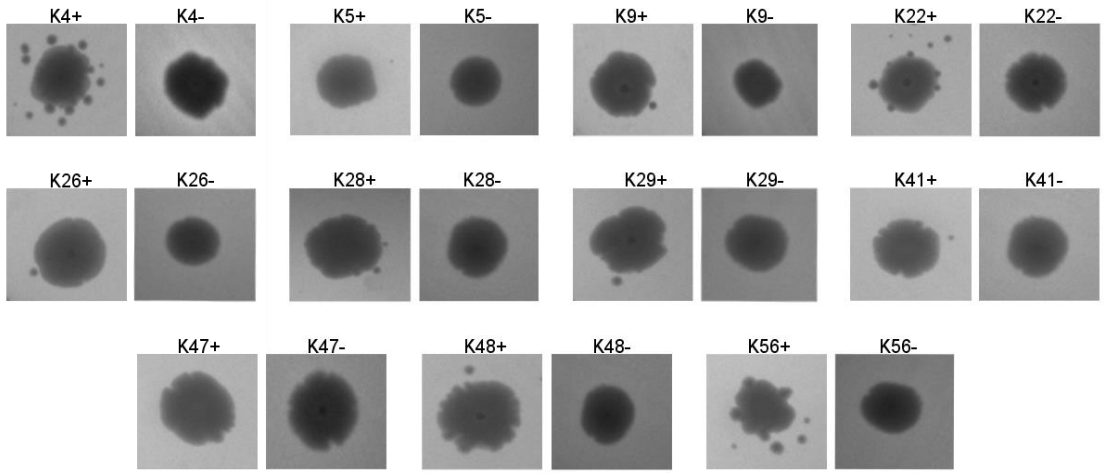



**Figure 4.3 Sheltering effect of K4, K5, K9, K22, K26, K28, K29, K41, K47, K48 and K56 on *E. coli* NCTC 10418 after 48 total hours of incubation.** The picture shows the aspect of the two 100 µg/ml Amp plates inoculated with the 11 isolates respectively in presence (+) and absence of *E. coli* NCTC 10418 (-) after 24 additional hours of incubation at 37°C. This experiment was performed in triplicate for each *K. pneumoniae* isolate (n = 3) and the image of one of the three plates used as replicates is showed in the picture as an example.

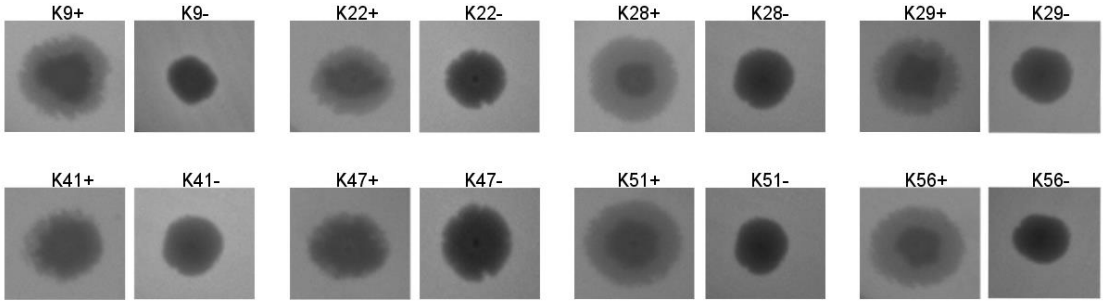



**Figure 4.4 Sheltering effect of K9, K22, K28, K29, K41, K47, K48 and K56 on *S. aureus* NCTC 6571 after 48 totall hours of incubation.** The picture shows the aspect of the two 100 µg/ml Amp plates inoculated with the 8 isolates respectively in presence (+) and absence of *S. aureus* NCTC 6571 (- ) after 24 additional hours of incubation at 37°C. This experiment was performed in triplicate for each *K. pneumoniae* isolate (n = 3) and the image of one of the three plates used as replicates is showed in the picture as an example.

Overall, these results showed that all the *K. pneumoniae* isolates had sheltering potential although the time of appearance of the sheltering effect was different depending on the susceptible strain and the isolate used.

It is interesting to note the differences in sheltering pattern morphology between the different sheltered strains. For example, with *E. coli* NCTC 10418 there was a mixture of confluent sheltered colonies adjacent to the resistant colony and distinct individual colonies at some distance from the inoculation point. In contrast, with *S. aureus* NCTC 6571 only confluent colonies directly adjacent to the resistant colony were observed. However, the area occupied by the confluent colonies of *S. aureus* NCTC 6571 appeared visually larger than occupied by the confluent colonies of *E. coli* NCTC 10418 (Figures. 4.1 and 4.2). One possibility to explain this observation was differences in the cellular density in the bacterial culture samples of *E. coli* NCTC 10418 and *S. aureus* NCTC 6571 used. To test this hypothesis, the $OD_{600}$ of the liquid overnight culture of *E. coli* NCTC 10418 and *S. aureus* NCTC6571 were measured. These results showed that the $OD_{600}$ values of the overnight bacterial culture of *S. aureus* NCTC 6571 were higher than those of *E. coli* NCTC10418 in all the three samples used as replicates (Table 4.1). This observation might suggest that the bacterial concentration of *S. aureus* NCTC 6571 was higher than that of *E. coli* NCTC 10418 in the samples used directly for the spread on plate. This might account for a major cellular density of susceptible colonies of *S. aureus* NCTC 6571 and consequently a larger area of confluent colonies directly adjacent to the resistant colony.

**Table 4.1 $OD_{600}$ values of the three samples of overnight bacterial culture of *E. coli* NCTC 10418 and *S. aureus* NCTC 6571.**

| Sample N° | *E. coli* NCTC 10418 | *S. aureus* NCTC 6571 |
|---|---|---|
| 1 | 0.416 | 0.58 |
| 2 | 0.122 | 0.47 |
| 3 | 0.375 | 0.51 |

Variability in the sheltering effect was detectable among the *K. pneumoniae* isolates inoculated on the same susceptible strain as shown in Figures 4.1 and 4.2. The variability in the sheltering effect provided by different isolates inoculated on the same susceptible strain was quantified by measuring the area occupied by the sheltered strain after the first 24 hours of incubation. The choice of a 24 hour time point was based on a clearer visual distinction between the sheltered strain and the resistant isolate. Indeed, after 48 hours of incubation the area of the continuous cover of

bacteria surrounding the resistant isolates incubated on *E. coli* NCTC 10418 was not visible on the plates (Figure 4.3).

Significant variability in the extent of the sheltering effect on *E. coli* NCTC 10418 and *S. aureus* NCTC 6571 was present among respectively the 22 and 25 *K. pneumoniae* isolates sheltering within 24 hours after incubation at 37ºC. Indeed, the sheltered areas of both *E. coli* NCTC 10418 and *S. aureus* NCTC 6571 were significantly different (P value < 0.001) when one-way ANOVA was carried out to compare the sheltered areas of all the isolates (Figures 4.5 A and B). This variability ranged from approximately 5 to 57 $mm^2$ for the areas of *E. coli* NCTC 10416 (Figure. 4.5, A) and from 1 to 95 $mm^2$ for the areas of *S. aureus* NCTC 6571 (Figure. 4.5, B). This was the result of a post hoc test for the pairwise comparison of the sheltered areas obtained for each *K. pneumoniae* isolate used (Tables 8.7 and 8.8, appendix 8.3).

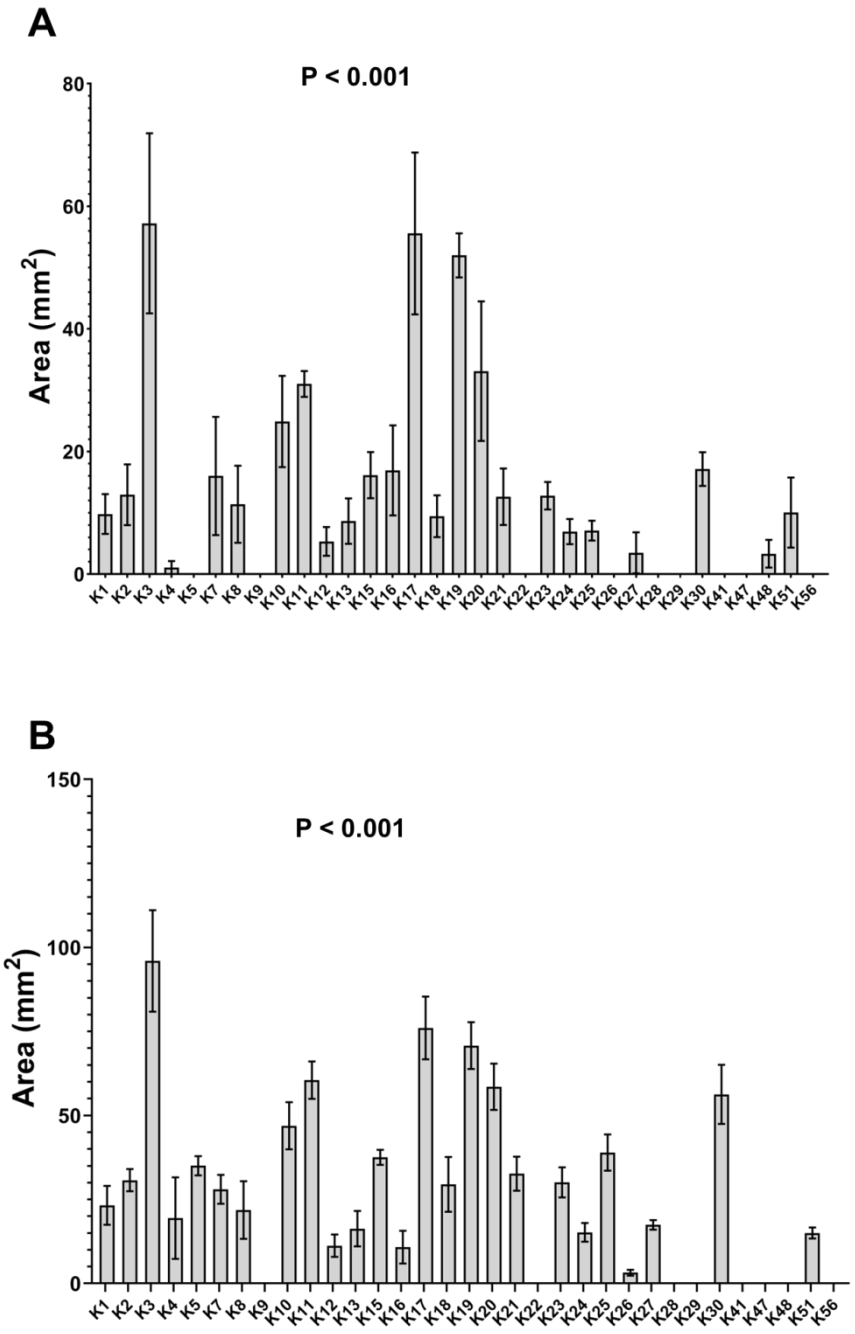



**Figure 4.5 Variability of the sheltering effect provided by the *K. pneumoniae* isolates.** The bar plots show the sheltered areas of *E. coli* NCTC10418 (A) and *S. aureus* NCTC6571 (B) appearing around each *K.pneumoniae* isolate after 24 hours of incubation at 37ºC. The error bars represent the standard error of the mean. The P-value (P) obtained with one-way ANOVA for the comparison of all the areas is also reported in each plot.

A moderate positive linear association ($R^2$= 0.51) was detected when the sheltered areas of *E. coli* NCTC 10418 and *S. aureus* NCTC 6571 growing around each *K. pneumoniae* isolate were analysed on a scatter plot, thus suggesting that the level of variability detected on *E. coli* NCTC 10418 followed the same trend as that with *S. aureus* NCTC 6571 (Figure 4.6).

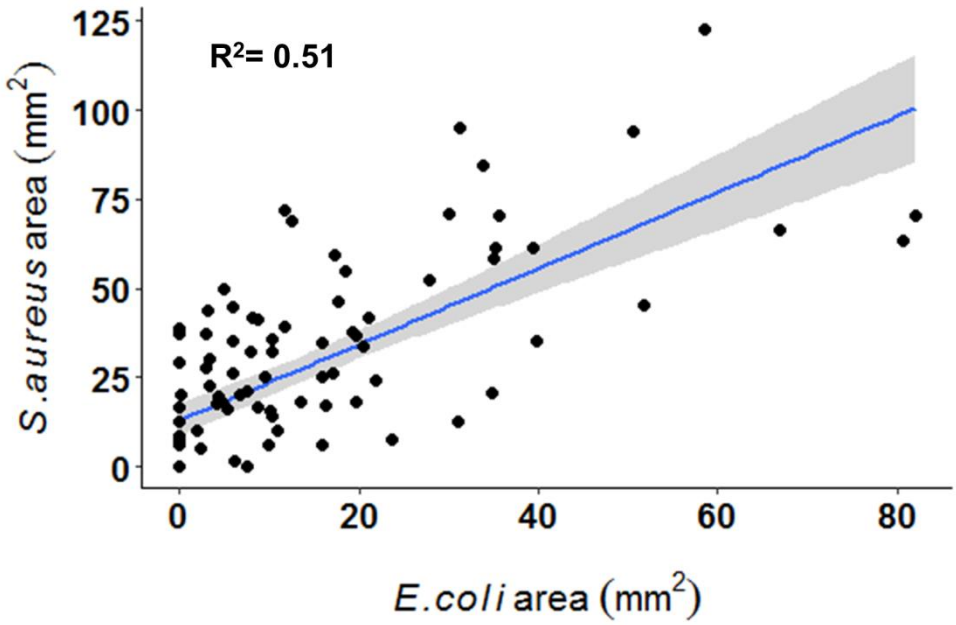



**Figure 4.6. Relationship between the sheltered areas of *E. coli* NCTC 10418 and *S. aureus* NCTC 6571.** The data points (represented as closed circles) are the values of the sheltered areas of *E. coli* NCTC 10418 and *S. aureus* NCTC 6571 for each single *K. pneumoniae* isolate. The line estimated by the linear regression is depicted as the solid line and the grey shaded area represents the 95% confidence interval around the line. The $R^2$ value is reported in each graph to express the closeness of the data to the fitted regression line.

### 4.2.2 Relationship between the variability and the growth rates of the *K. pneumoniae* isolates

One possibility to explain the variability in the sheltering effect showed by the different *K. pneumoniae* isolates could be different bacterial density in the initial *K. pneumoniae* inocula used for the experiments. In this case, higher densities of resistant bacteria could protect more susceptible cells, thus resulting in the appearance of larger sheltered areas. Therefore, in order to test whether the variability was due to a different bacterial density of the liquid overnight bacterial cultures used for the experiments, the $OD_{600}$ of these cultures was measured and compared with one-way ANOVA. However, this analysis showed no significant difference among the bacterial densities of the isolates (P value > 0.05) (Figure 4.7) suggesting that the sheltering differences observed were not simply down to differences in cell density.

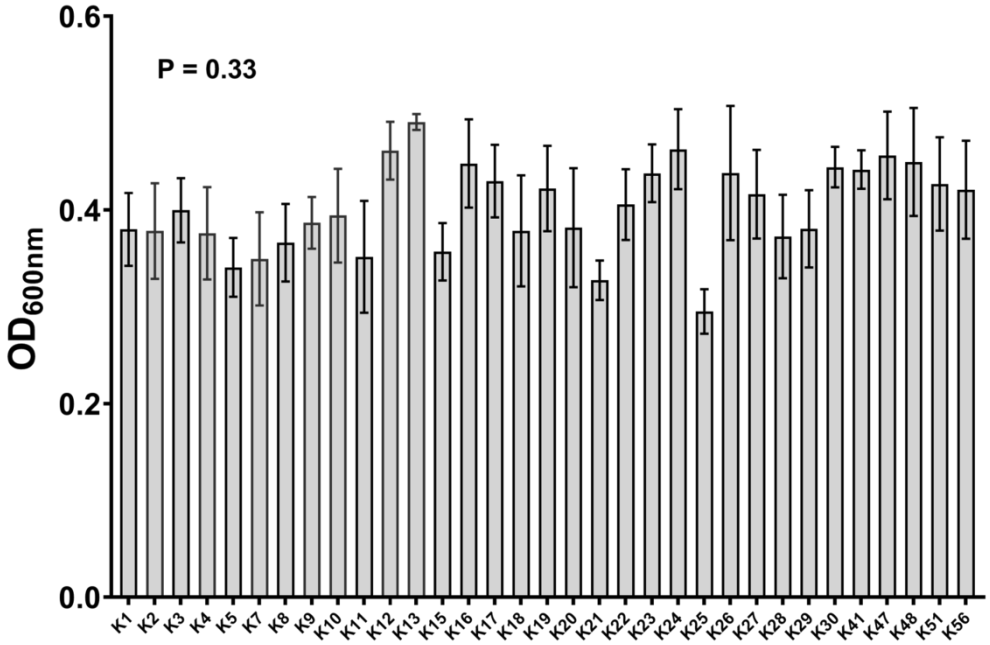



**Figure 4.7 Comparison of the $OD_{600}$ values of the bacterial cultures of all the *K. pneumoniae* isolates used.** The error bars represent the standard error of the mean for each isolate. The P value calculated with the one-way ANOVA analysis is also reported in the graph**.**

Another further consideration is that the differences detected in the sheltering effect might be due to different growth rates of the *K. pneumoniae* isolates. In this model, isolates that grew faster could provide protection to the susceptible strain in a shorter time than the isolates growing slower. This might result in the different time of appearance of the bacterial growth around different *K. pneumoniae* isolates. Therefore, the growth curves of these isolates were determined by measuring the $OD_{600}$ of the respective bacterial cultures just after dilution of the overnight culture in fresh broth. Then the measurements were taken every 30 minutes for 4 hours from the incubation at 37 ºC with shaking, then after 1 hour and after 2 hours from the last measurement. The last measurement was taken after overnight incubation (Figure 4.8). The determination of the growth curves for all the isolates was carried out in triplicate.

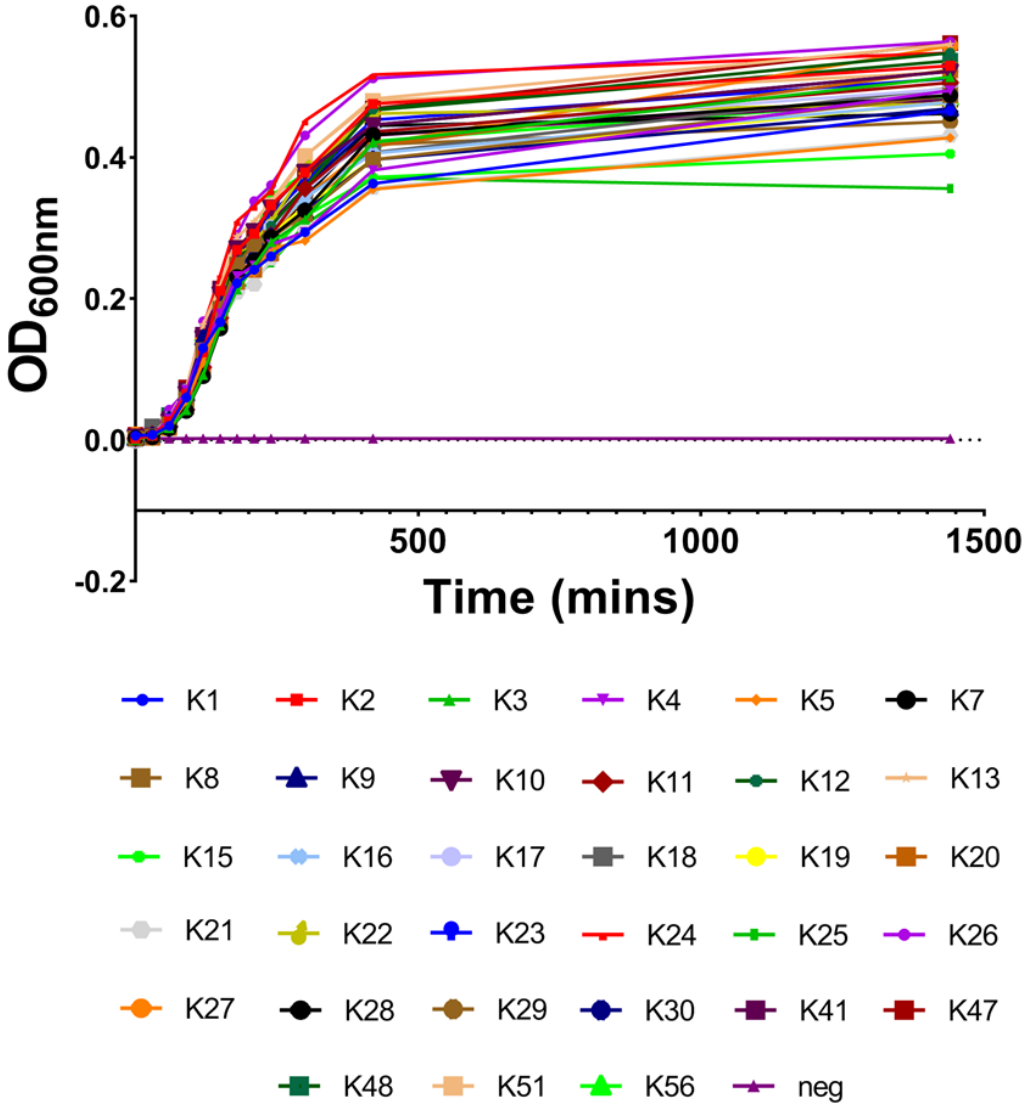



**Figure 4.8 Semi-log plot of $OD_{600}$ vs. time (in min) for the determination of the growth of the 33 *K. pneumoniae* isolates.**. The figure shows the growth curves of the 33 isolates for 1 of the 3 replicates run in this experiment. The $OD_{600}$ of all the 33 bacterial cultures was measured just after dilution of the overnight culture in fresh broth containing Amp (0 minutes), and then at 30, 60, 90, 120, 150, 180, 210, 240, 300, 360, 460 and 1440 min from the incubation at 37ºC with shaking. These time intervals were also used for the measurements of the $OD_{600}$ of liquid broth containing Amp (blank, "neg"). For each time point, the normalized $OD_{600}$ values of the bacterial cultures were obtained by subtracting the $OD_{600}$ values of the blank from the $OD_{600}$ values of the bacterial cultures. All the standard deviations calculated for the 3 replicates for each timepoint were within 5% of the mean value.

In order to determine whether the growth rates of all the isolates showed significant differences, the doubling times obtained for the respective growth curves of all isolates were compared. However, this analysis showed that the growth rates were not significantly different since the P value for this comparison was > 0.05. (Figure 4.9).

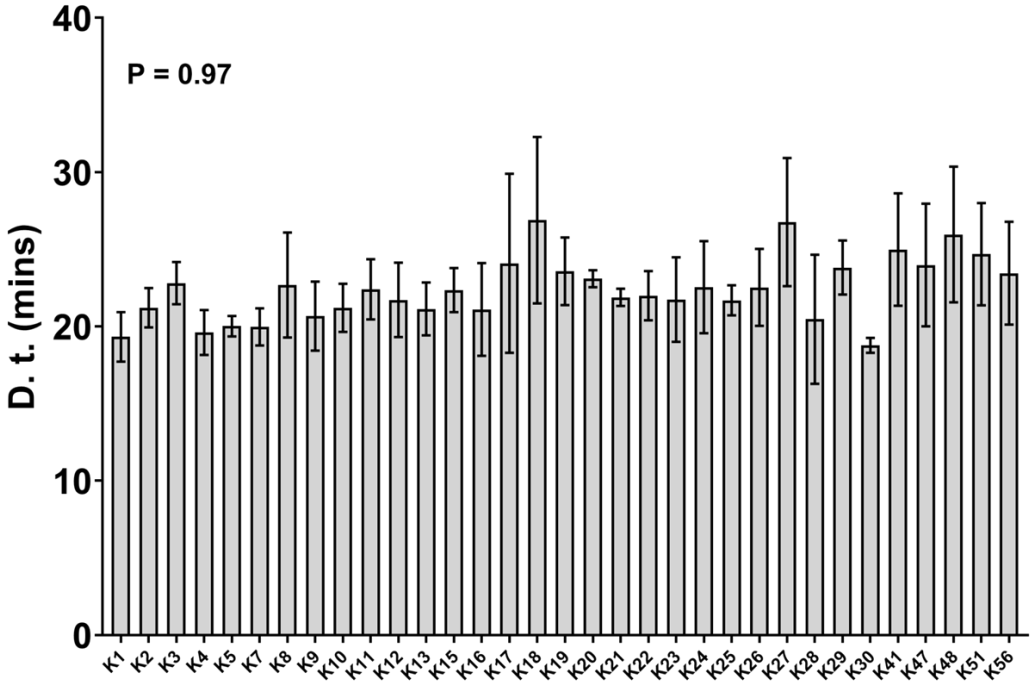



**Figure 4.9 Comparison of the doubling times (D. t.) in min obtained from the growth curves of all the *K. pneumoniae* isolates used.** The doubling time was determined by calculating the equation of the logarithmic phase of each growth curve. This equation was in the form "$A = e^{Bx}$". Then the doubling time was calculated by dividing the value of ln2 (0.0693) by the value of B. The mean of the doubling times obtained for the three growth curves used as replicates for the same isolate was calculated and the means for all the isolates were compared with one-way ANOVA. The error bars represent the standard error of the mean for each isolate. The P-value calculated for the comparison is reported.

This analysis suggested that the variability in the sheltering effect observed among the different *K. pneumoniae* isolates was due to neither differences in initial inoculum density nor to differences in the growth rates of the strains.

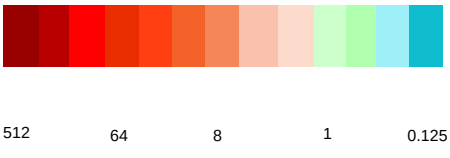
### 4.2.3 Relationship between the variability in the sheltering effect and the β-lactam resistance profiles of the *K. pneumoniae* isolates.

The variability in the extent of the sheltering effect might be related to different degrees of resistance to β-lactams. In this case, a higher degree of resistance to the β-lactams might be responsible for a stronger sheltering effect and vice versa. In order to investigate the relation between the variability and the β-lactam resistance profiles of the isolates, the MIC profiles for the β-lactam antibiotics, including the carbapenems Mer and Ert and the cephalosporins Cefo, Ceft and Cefe, were determined for all the *K. pneumoniae* isolates. The resistance to Amp was tested as well but the MIC was not determinable since all the isolates were steadily resistant to

concentrations by far above the recommended range for MIC determination for Enterobacteriaceae (0.25-128 µg/ml) (Andrews, 2001). With regards to this the highest Amp concentration tested was 2,048 µg/ml. This high resistance to Amp shown by all the isolates might be due to the fact that *K. pneumoniae* is naturally resistant to Amp and Amoxicillin, usually by the production of the SHV-1 β-lactamase encoded on the chromosome or a transferable plasmid (Nugent et al., 1979; Simpson et al., 1980; Bush et al., 1995; Arakawa et al., 1996; French et al., 1996; Chaves et al., 2001; Hæggman et al., 2004). Since all the isolates were constantly resistant to Amp, differences based on their resistance level to Amp would not have been detected. This experiment was carried out in triplicate and the mode of the three MIC values for each isolate was calculated. These results are reported in Table 4.2.

**Table 4.2 Heat-map for the MIC values (µg/ml) showed by each isolate to the β-lactam antimicrobials tested in this study.** The values reported represent the mode calculated from the three MIC values obtained for each antimicrobial tested for each isolate. Ceft, Cefo, Cefe, Mer, Ert. The colour gradient key displays a scale of the MIC values reported in the table.

| Isolates | Ceft | Cefo | Cefe | Mer | Ert |
|---|---|---|---|---|---|
| K51 | 128 | 128 | 128 | 512 | 128 |
| K7 | 128 | 128 | 128 | 512 | 128 |
| K22 | 128 | 128 | 128 | 512 | 128 |
| K9 | 128 | 128 | 128 | 256 | 128 |
| K48 | 128 | 128 | 64 | 512 | 128 |
| K41 | 128 | 128 | 64 | 512 | 64 |
| K16 | 128 | 128 | 32 | 512 | 64 |
| K10 | 128 | 128 | 64 | 256 | 64 |
| K47 | 128 | 128 | 32 | 512 | 32 |
| K56 | 128 | 128 | 32 | 512 | 32 |
| K27 | 128 | 128 | 32 | 512 | 32 |
| K30 | 64 | 128 | 128 | 256 | 128 |
| K23 | 64 | 128 | 128 | 128 | 128 |
| K25 | 32 | 128 | 64 | 128 | 128 |
| K21 | 32 | 128 | 128 | 64 | 128 |
| K17 | 32 | 128 | 128 | 64 | 128 |
| K19 | 32 | 128 | 128 | 64 | 128 |
| K15 | 32 | 128 | 128 | 64 | 64 |
| K3 | 32 | 128 | 128 | 16 | 128 |
| K20 | 16 | 128 | 128 | 64 | 128 |
| K11 | 8 | 128 | 128 | 64 | 128 |
| K12 | 32 | 128 | 16 | 0.5 | 1 |
| K13 | 32 | 128 | 16 | 2 | 2 |
| K1 | 32 | 64 | 8 | 2 | 4 |
| K2 | 16 | 64 | 64 | 1 | 4 |
| K24 | 32 | 128 | 16 | 2 | 2 |
| K8 | 16 | 128 | 8 | 4 | 8 |
| K18 | 32 | 64 | 8 | 4 | 4 |
| K5 | 64 | 16 | 0.5 | 8 | 8 |
| K4 | 1 | 4 | 2 | 8 | 64 |
| K26 | 0.25 | 1 | 0.25 | 16 | 4 |
| K28 | 0.25 | 1 | 1 | 128 | 16 |
| K29 | 0.125 | 1 | 1 | 128 | 16 |



To test the potential relationship between MICs and sheltering the sheltered areas for *E.coli* NCTC10418 were plotted against the MIC values of each of these antimicrobials. In all cases, no linear trend was detected between these two variables. Indeed, the observed data did not appear to fit a linear model, as showed in all the scatter plots in Figures 4.10, 4.11, 4.12, 4.13 and 4.14. Also the $R^2$ value was calculated in order to estimate how close the data were to the fitted regression line. However, this value was close to zero in all the scatter plots, thus suggesting lack of linear relationship between the MIC profiles and the sheltered areas.

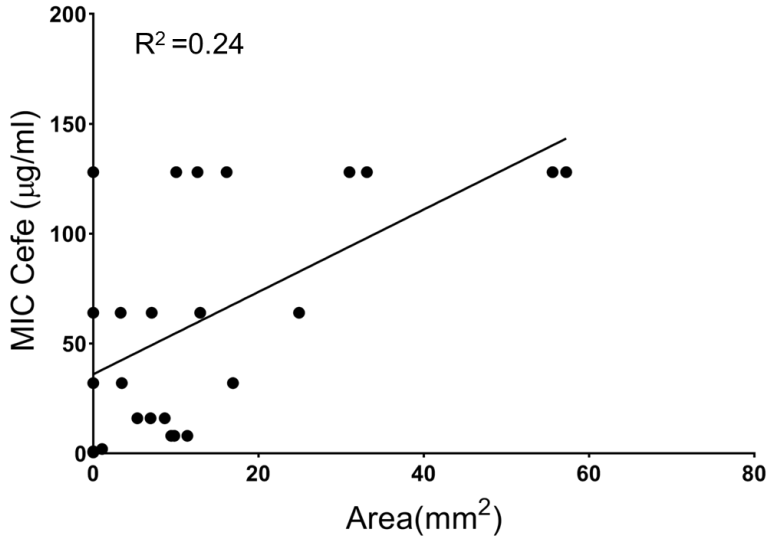



**Figure 4.10 Relationship between sheltering effect and the MIC profiles for Cefe of all the isolates.** The areas of the sheltered *E. coli* NCTC10418 growing around each *K. pneumoniae* isolate is plotted against the MIC value of each isolate for Cefe. The line estimated by the linear regression is depicted as the solid line. However, the data points are randomly scattered on the coordinate plane and appear not to concentrate near the straight line predicted for the linear relationship. The calculated $R^2$ value is also reported.

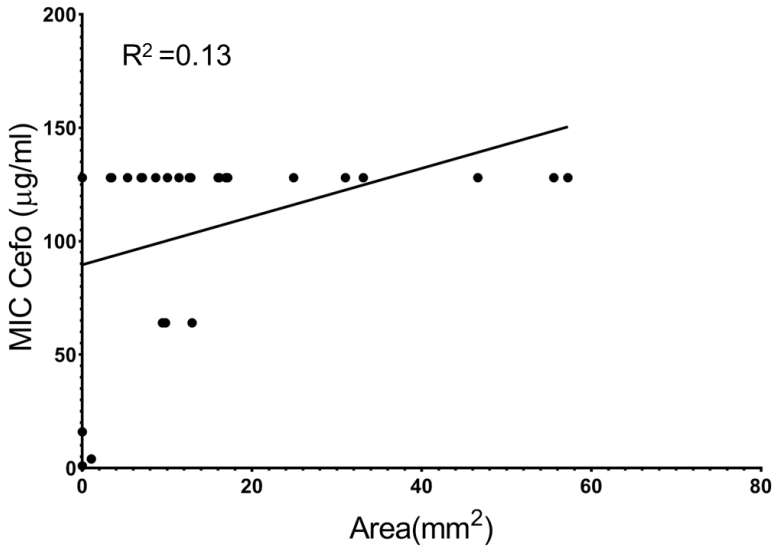



**Figure 4.11 Relationship between sheltering effect and the MIC profiles for Cefo of all the isolates.** The areas of the sheltered *E. coli* NCTC10418 growing around each *K. pneumoniae* isolate is plotted against the MIC value of each isolate for Cefo. The line estimated by the linear regression is depicted as the solid line. However, the data points are randomly scattered on the coordinate plane and appear not to concentrate near the straight line predicted for the linear relationship. The calculated $R^2$ value is also reported.

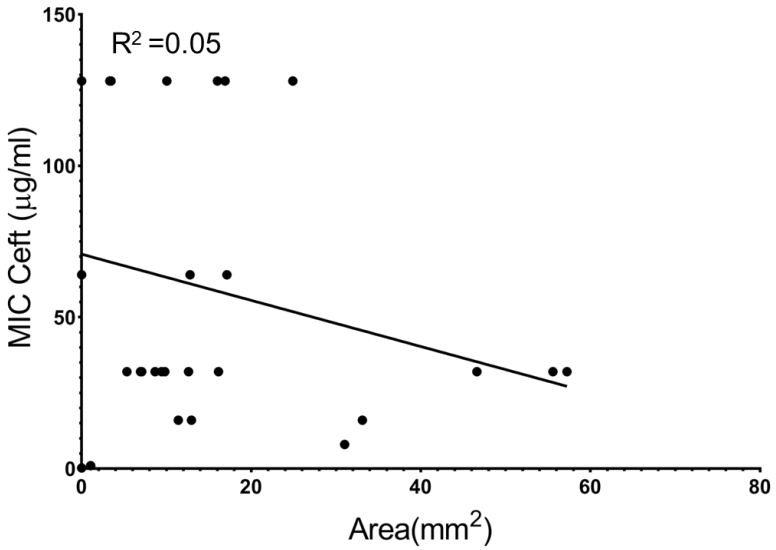



**Figure 4.12 Relationship between sheltering effect and the MIC profiles for Ceft of all the isolates.** The areas of the sheltered *E. coli* NCTC10418 growing around each *K. pneumoniae* isolate is plotted against the MIC value of each isolate for Ceft. The line estimated by the linear regression is depicted as the solid line. However, the data points are randomly scattered on the coordinate plane and appear not to concentrate near the straight line predicted for the linear relationship. The calculated $R^2$ value is also reported.

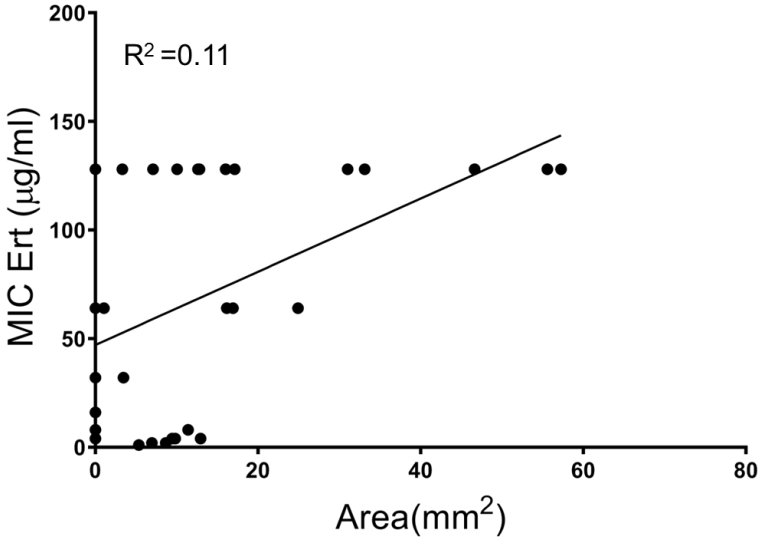



**Figure 4.13 Relationship between sheltering effect and the MIC profiles for Ert of all the isolates.** The areas of the sheltered *E. coli* NCTC10418 growing around each *K. pneumoniae* isolate is plotted against the MIC value of each isolate for Ertapenem. The line estimated by the linear regression is depicted as the solid line. However, the data points are randomly scattered on the coordinate plane and appear not to concentrate near the straight line predicted for the linear relationship. The calculated $R^2$ value is also reported.

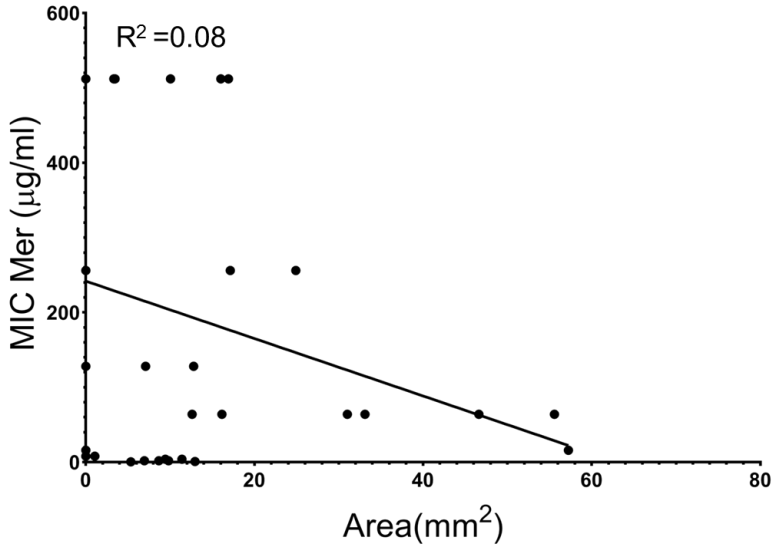



**Figure 4.14 Relationship between sheltering effect and the MIC profiles for Mer of all the isolates.** The areas of the sheltered *E. coli* NCTC10418 growing around each *K. pneumoniae* isolate is plotted against the MIC value of each isolate for Mer. The line estimated by the linear regression is depicted as the solid line. However, the data points are randomly scattered on the coordinate plane and appear not to concentrate near the straight line predicted for the linear relationship. The calculated $R^2$ value is also reported.

### 4.2.4 Relationship between the variability and the genetic features of the *K. pneumoniae* isolates.

Although no relationship between the sheltering potential and the level of resistance to the β-lactams was found, the differences in the sheltering potential might be the result of differences in the genetic features among the *K. pneumoniae* isolates. Therefore, a set of genotyping analyses were carried out in order to investigate this potential relationship.

#### 4.2.4.1 Relationship between sheltering variability and RAPD profiles of the isolates

A first genotypic analysis involved the investigation of the whole genomic DNA for the determination of the genetic profiles of the *K. pneumoniae* isolates with RAPD analysis. This is an inexpensive and quick method to differentiate between genetically distinct individuals based on the pattern of amplified products of different molecular weight that is characteristic of the isolates (SaadatianFarivaret al., 2018). The RAPD reactions were performed with different primers. This aimed to test their discriminatory capacity based on the number of bands in the pattern produced by each isolate. However, among all these primers, ER1C generated patterns with the highest number of bands on an agarose gel (Figure 4.15). Furthermore for most isolates a pattern was not visually detectable with the RAPD carried out with the primers ER1C (Voget et al., 1999) AP4 (Gori et al., 1996; Panah et al., 2012), RAPD-7 (Gniadkowski et al., 1998; Panah et al., 2012), Primer640 (Haryani et al., 2007; Panah et al., 2012)(Figures 8.1, 8.2, 8.3, appendix 8.4).

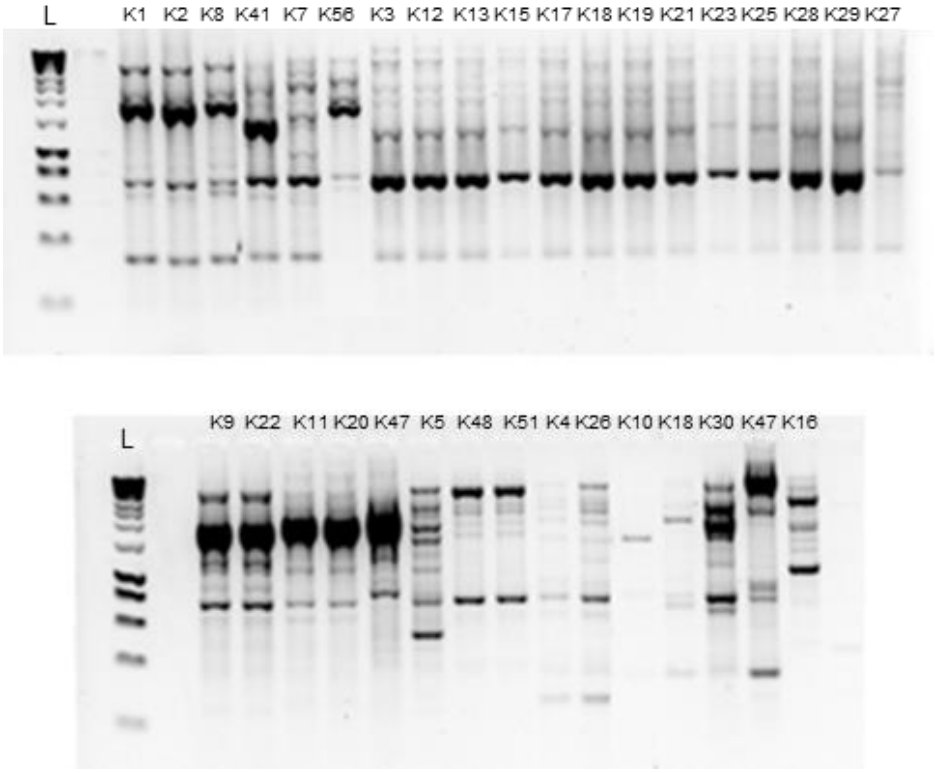



**Figure 4.15 RAPD results obtained with ER1C primer on the DNA of the isolates.** The amplification of the DNA with RAPD produced a pattern of bands of different sizes on agarose gel. This pattern was specific for each isolate. L: ladder

PyElph software was used to analyse and compare the DNA patterns of the different *K. pneumoniae* isolates generated with ER1C primer. The similarity level between two DNA patterns is expressed by using the Dice coefficient (Dice, 1945; Pavel and Vasile, 2012) and is calculated as follows:

$$Dice\ (Si,Sj) = \frac{2*number\ of\ common\ bands\ in\ lanes\ i\ and\ j}{number\ bands\ in\ lane\ i + number\ bands\ in\ lane\ j}\ [\%]$$

Then, the distance matrix is computed as 100 times matrix of ones minus the similarity matrix (Pavel and Vasile, 2012). This matrix is used for generating phylogenetic trees by five clustering methods including Neighbor Joining, Single linkage, Complete linkage,Unweighted Pair Group Method with Arithmetic Mean (UPGMA) and Weighted Pair Group Method with Arithmetic Mean (WPGMA). All the dendrograms produced comparable results. Indeed, they showed that strong genetic relatedness was found among the isolates K23, K21, K19, K17, K15, K13, K12, K3, K29, K28, K25, K24. These were grouped in one large cluster at the distance value of "0" in all the trees. Additionally, the following clusters of multiple isolates at distance

of “0” were detected: K1, K2 and K8; K20 and K11; K22 and K9; K26 and K4; K51 and K48. All the other clusters were made of single isolates (Figure 4.16).

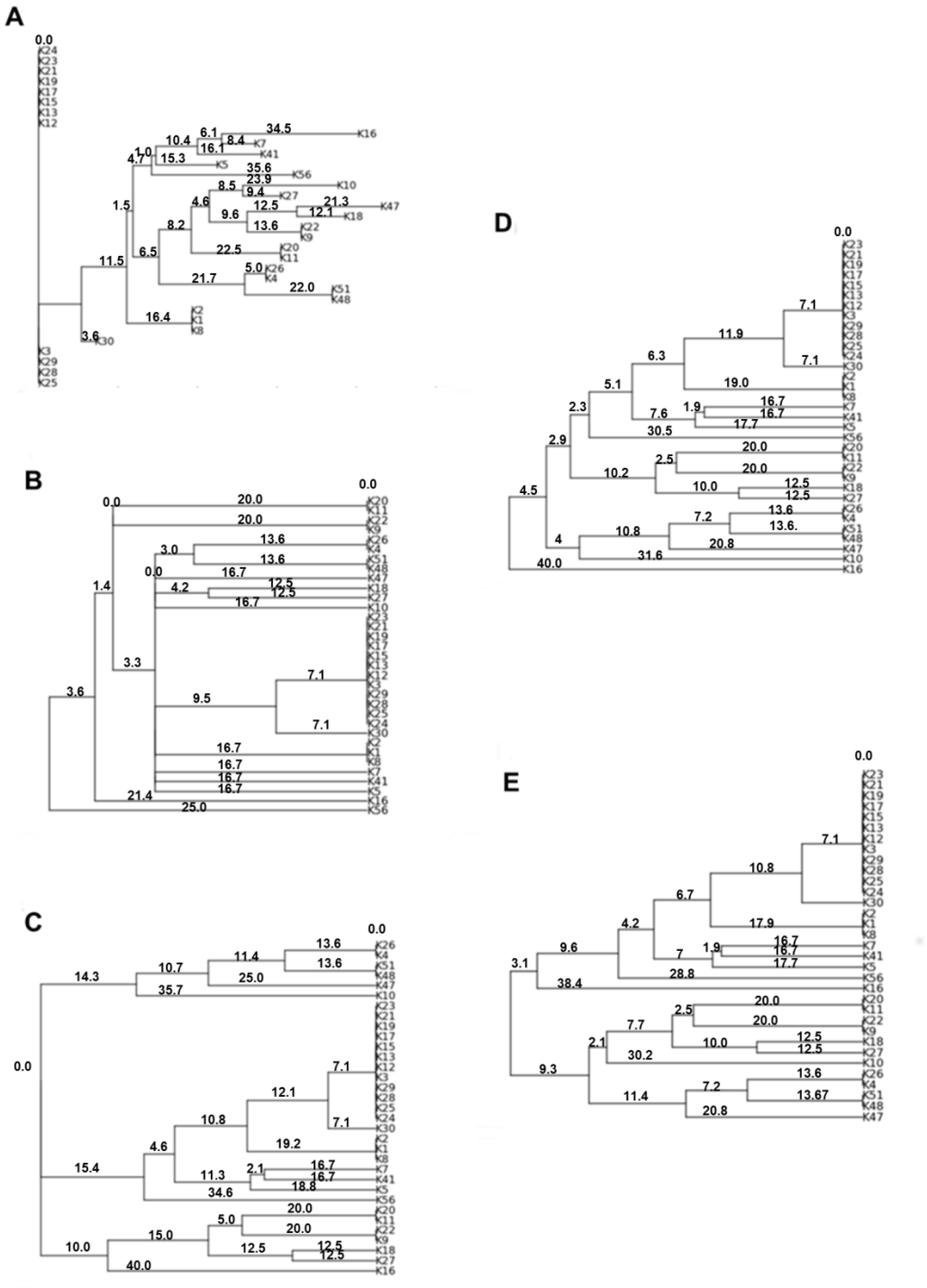



**Figure 4.16 Phylogenetic trees generated from the analysis of the pattern of bands produced by each isolate.** The dendrograms were generated respectively with A) Neighbor Joining, B) Single linkage, C) Complete linkage, D) UPGMA and E) WPGMA.The genetic distances are shown above the branches. The isolates that are clustered in the same group (represented as a vertical line) at the value of “0” have no genetic distance. The *K. pneumoniae* isolates are indicated with their isolate code in the trees.

RAPD types were therefore assigned based on the analysis of the clusters generated with UPGMA. This method was randomly chosen among the algorithms used to produce the dendrograms since all of them produced the same grouping of clusters (Figure 4.17). Isolates with the same RAPD profile were therefore expected to be genetically similar.

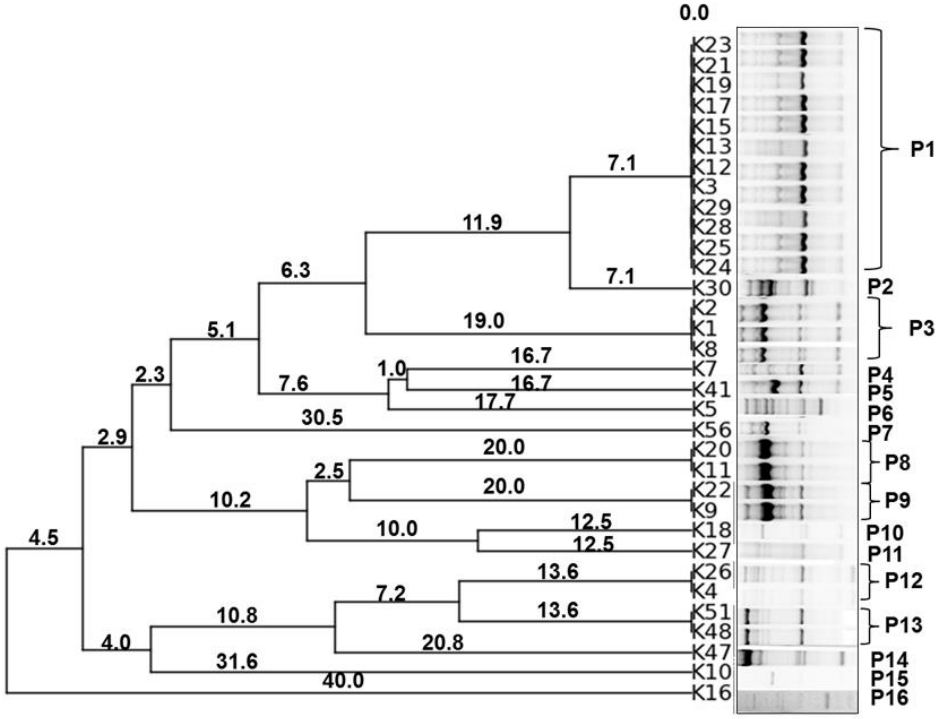



**Figure 4.17 RAPD types of *K. pneumoniae* isolates.** The UPGMA dendrogram is shown with the RAPD profile for each isolate.The analysis of the RAPD results revealed 16 different profiles (designed P1 to P16) for the *K. pneumoniae* isolates. Among these, some isolates shared the same RAPD profile and they were assigned the same RAPD number (P1, P3, P8, P9, P12 and P13).

In order to determine whether isolates with similar genetic features showed similarities in the potential of sheltering effect as well, the *K. pneumoniae* isolates were divided into 9 sheltering groups and 1 non-sheltering group (Group 1) based on the interval of values measured for the sheltered areas of *E. coli* NCTC 10418 described in paragraph 4.2.1. This interval ranged from 3.40 $mm^2$, which was the minimum value at which the sheltering effect was visually detectable, to a maximum value of 57.20 $mm^2$. The non-sheltering group included all the isolates with no sheltering effect detectable after 24 hours of incubation (K4, K5, K9, K22, K26, K41, K47 and K56). To classify the sheltering isolates the interval calculated between the maximum and minimum value (53.8 $mm^2$) was divided into 9 sheltering groups (Groups 2-10). Each of these groups covered a range unit of 5.97 $mm^2$. The choice to

group the isolates by ranges of sheltered area values was based on the possibility of finding a correspondence between the grouping of the isolates with similar genetic features and the grouping of isolates with similar sheltering potential. The non-sheltering group and the sheltering groups are listed in Table 4.3.

**Table 4.3 Ranges of *E. coli* NCTC 10418 sheltered areas**. The groups of *K. pneumoniae* isolates producing sheltered areas having values within each of the ranges listed are reported.

| Groups | Sheltered areas (mm2) | Isolates |
|---|---|---|
| 1 | 0-3.39 | K4, K5, K9, K22, K26, K28, K29, K41, K47, K56 |
| 2 | 3.40-9.37 | K12, K13, K24, K25, K27, K48 |
| 3 | 9.38-15.35 | K1, K2, K8, K18, K23, K51 |
| 4 | 15.36-21.33 | K7, K15, K16, K30 |
| 5 | 21.34-27.31 | K10 |
| 6 | 27.32-33.27 | K11, K20 |
| 7 | 33.28-39.25 | |
| 8 | 39.26-45.23 | |
| 9 | 45.24-51.21 | |
| 10 | 51.22-57.20 | K3, K17, K19 |

However, no correspondence between these groups of isolates and the RAPD profiles was identified. Indeed, isolates with the same RAPD profile were classified in different sheltering groups. As an example the isolates K28, K29, K3, K17, K19, K15, K12, K13, K24 and K25 having RAPD profile P1 were split in different sheltering groups including group 1, 10, 4 and 2. Vice versa, sheltering groups included isolates having different RAPD profiles. As an example, the non-sheltering group 1 included the isolates K4, K5, K9, K22, K26, K28, K29, K41, K47 and K56 having RAPD profiles respectively of P12, P6, P9, P9, P12, P1, P1, P5, P14 and P7. The only exception was for the isolates K11 and K20. Indeed, they had the same profile (P8) and belonged to the same sheltering group (Group 6).

#### 4.2.4.2 Relationship between sheltering variability and β-lactamases of the isolates

Variations in the content of specific genes might not be detectable with the RAPD technique since the amplification of segments of DNA is completely random. In particular, the isolates could have variations in the content of the major genes for the β-lactamases that are responsible for providing the sheltering effect to susceptible species by degrading the β-lactams and therefore providing the protective effect towards susceptible species (Liao et al., 2014; Liao et al., 2015). Therefore, in an attempt to find a relationship between the genetic features of the isolates and their sheltering potential the presence or absence of the major genes for the β-lactamases in the isolates was investigated. This analysis aimed to determine whether isolates with the same sheltering potential shared the same β-lactam resistance genotype despite the overall genetic features in their genome. Therefore, a PCR-based screening of the most commonly found ß-lactamase families (*bla*$_{TEM}$, *bla*$_{SHV,}$ *bla*$_{CTX-M}$, group 1, 2 and 9, *bla*$_{VEB}$, *bla*$_{PER}$), including carbapenemases genes (*bla*$_{KPC}$, *bla*$_{OXA-48,}$ *bla*$_{IMP,}$ *bla*$_{VIM,}$ *bla*$_{NDM}$ and *bla*$_{GES}$) was carried out. The results of this analysis are reported in Table 4.4 and in the Figures 8.4, 8.5, 8.6, 8.7, 8.8 and 8.9 (appendix 8.4).

**Table 4.4 Analysis of the presence of the ß-lactamase genes in the *K. pneumoniae* isolates. +: presence of the gene; -: absence of the gene**

| | **bla$_{OXA-48}$** | ***bla*$_{TEM}$** | ***bla*$_{SHV}$** | ***bla*$_{IMP}$** | ***bla*$_{VIM}$** | ***bla*$_{BIC-1}$** | ***bla*$_{KPC-2}$** | ***bla*$_{NDM-1}$** | ***bla*$_{CTX-M}$ group 1** | ***bla*$_{CTX-M}$ group 2** | ***bla*$_{CTX-M}$ group 9** | ***bla*$_{CMY}$** | ***bla*$_{GES}$** | ***bla*$_{PER}$** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| K1 | + | + | + | - | - | - | - | - | + | - | - | - | - | - |
| K2 | + | + | + | - | - | - | - | - | + | - | - | - | - | - |
| K3 | + | + | + | - | - | - | - | - | + | - | - | - | - | - |
| K4 | + | - | + | - | - | - | - | - | - | - | - | - | - | - |
| K5 | + | + | + | - | - | - | - | - | - | - | - | + | - | - |
| K7 | - | + | + | - | - | - | - | + | + | - | - | - | - | - |
| K8 | + | + | + | - | - | - | - | - | + | - | - | - | - | - |
| K9 | - | + | + | - | - | - | - | + | - | - | - | - | - | - |
| K10 | - | + | + | - | - | - | - | + | + | - | - | - | - | - |
| K11 | + | + | + | - | - | - | - | - | - | + | + | - | - | - |
| K12 | + | + | + | - | - | - | - | - | + | - | - | - | - | - |
| K13 | + | + | + | - | - | - | - | - | + | - | - | - | - | - |
| K15 | + | + | + | - | - | - | - | - | + | - | - | - | - | - |
| K16 | - | + | + | - | - | - | - | + | + | - | - | - | - | - |
| K17 | + | + | + | - | - | - | - | - | + | - | - | - | - | - |
| K18 | + | + | + | - | - | - | - | - | + | - | - | - | - | - |
| K19 | + | + | + | - | - | - | - | - | + | - | - | - | - | - |
| K20 | + | + | + | - | - | - | - | - | - | + | + | - | - | - |
| K21 | + | + | + | - | - | - | - | - | + | - | - | - | - | - |
| K22 | - | + | + | - | - | - | - | + | - | - | - | - | - | - |
| K23 | + | + | + | - | - | - | - | - | + | - | - | - | - | - |
| K24 | + | + | + | - | - | - | - | - | + | - | - | - | - | - |
| K25 | + | + | + | - | - | - | - | - | + | - | - | - | - | - |
| K26 | + | - | + | - | - | - | - | - | - | - | - | - | - | - |
| K27 | - | + | + | - | - | - | - | + | + | - | - | - | - | - |
| K28 | + | - | + | - | - | - | - | - | - | - | - | - | - | - |
| K29 | + | - | + | - | - | - | - | - | - | - | - | - | - | - |
| K30 | + | + | + | - | - | - | - | - | + | - | - | - | - | - |
| K41 | - | + | + | - | - | - | - | + | + | - | - | - | - | - |
| K47 | - | + | + | - | - | - | - | + | + | - | - | - | - | - |
| K48 | - | + | + | - | - | - | - | + | + | - | - | - | - | - |
| K51 | - | + | + | - | - | - | - | + | + | - | - | - | - | - |
| K56 | - | + | + | - | - | - | - | + | + | - | - | - | - | - |

According to this analysis, the *K. pneumoniae* isolates could be divided in 7 groups based on the presence of specific genes (Table 4.5).

**Table 4.5 Groups of isolates classified by their content of β-lactamase genes**

| Group | Set of resistance genes | Isolates |
|---|---|---|
| 1 | $bla_{\text{OXA-48}}$, $bla_{\text{TEM}}$, $bla_{\text{SHV}}$, $bla_{\text{CTX-M group 1}}$ | K1, K2, K3, K8, K12, K13, K15, K17, K18, K19 K21, K23, K24, K25, K30 |
| 2 | $bla_{\text{OXA-48}}$, $bla_{\text{SHV}}$ | K4, K26, K29 |
| 3 | $bla_{\text{OXA-48}}$, $bla_{\text{TEM}}$, $bla_{\text{SHV}}$, $bla_{\text{CYM}}$ | K5 |
| 4 | $bla_{\text{TEM}}$, $bla_{\text{SHV}}$, $bla_{\text{NDM}}$, $bla_{\text{CTX-M group 1}}$ | K7, K10, K16, K27, K41, K47,K48, K51, K56 |
| 5 | $bla_{\text{TEM}}$, $bla_{\text{SHV}}$, $bla_{\text{NDM}}$ | K9, K22 |
| 6 | $bla_{\text{OXA-48}}$, $bla_{\text{TEM}}$, $bla_{\text{SHV}}$, $bla_{\text{CTX-M group 9}}$ | K11, K20 |

These results showed that most isolates with the same set of resistance genes shared the same RAPD profile. This was the case of the isolates K3, K12, K13, K15, K17, K19, K21, K23, K24, K25 having both the same resistance genotype and RAPD profile. Also, the 3 groups of isolates K4 and K26, K48 and K51, K9 and K22, K11 and K20 having respectively the RAPD profiles P12, P13, P9 and P8 shared the same groups of resistance genes that were respectively 2, 5 and 6. Additionally K5 was the only isolate with RAPD profile P6 and the set of genes including $bla_{\text{OXA-48}}$, $bla_{\text{TEM}}$, $bla_{\text{SHV}}$, $bla_{\text{CYM}}$ (group 3). However, some isolates with the same set of genes did not share the same RAPD profile. Indeed, although the isolates K1, K2, K8 and K30 showed the resistance genotype of the group 1, did not have the same RAPD profile as the other isolates included in this group. Also, despite having the same set of resistance genes, the isolates K7, K10, K16, K27, K41, K47, K56 showed different RAPD profiles. This suggested that despite the differences in the chromosome highlighted by the RAPD analysis, some isolates carried plasmids with the same resistance genes. However, no correspondence between the non-sheltering and sheltering groups and the set of resistance genes was found. Indeed, similarly to the RAPD profiles, isolates with the same set of genes, showed different degrees of sheltering potential. As an example, the isolates K7, K10, K16, K27, K41, K47, K48, K51, K56 having the set of genes of group 4 were split in several sheltering groups, including 1, 2, 4 and 5. Similarly to the RAPD analysis, K11 and K20 were the only isolates that shared the same set of resistance genes (Group 6) and sheltering group (Group 6).

#### 4.2.4.3 Relationship between sheltering variability and the amino acid sequence of the β-lactam-resistance genes

The variability in the sheltering strength shown by the isolates might be the result of point mutations within the sequence of the β-lactamase genes. Indeed, the presence of genetic mutations might lead to amino acid changes in the final protein product that could affect the enzymatic activity of the β-lactamases and ultimately be responsible for the variability in the sheltering potential. Therefore, the DNA sequence of the β-lactamase genes of the isolates was translated in protein sequence and compared with the aim to find amino acid changes. In order to identify the genes $bla_{\text{OXA-48}}$, $bla_{\text{TEM}}$, $bla_{\text{SHV}}$, $bla_{\text{CTX-M group 1}}$ and $bla_{\text{CTX-M group 9}}$, the DNA of all the resistant isolates was wholly sequenced. However, the Illumina Sequencing Technology failed in the sequencing of the genome and plasmid DNA of the isolates K13, K23, K27. This might be due to a possible contamination in the DNA samples of these isolates or to an inadequate DNA template concentration used in the DNA sequencer. The DNA sequences of all the other isolates were submitted to ResFinder (Zankari et al., 2012) to identify the genes for the β-lactamases and their position in the genomic sequence of the isolates and calculate the percentage of identity and the query coverage with known genes deposited in the non-redundant database. The specific variant of each of these genes was also identified by ResFinder. The results of this analysis are reported in Table 8.9, appendix 8.3. However, ResFinder failed to identity the β-lactamase genes expected according to the PCR analyses. In particular no $bla_{\text{SHV}}$ gene was found in K1, K5 and K30 and none of the genes expected were found in K5, K7, K10 and K28. This might be due to a fragmentation of the gene sequence in different contigs as a result of a failure during assembly process. Since the genes of interest could not be identified by the sequencing data, the next analyses were carried out by leaving out these genes belonging to the isolates mentioned. The sequences of these genes were then retrieved from the isolates and translated in amino acid sequences by using the tool Expasy Translate (Gasteiger et al., 2003). Lastly for each gene the resultant amino acid sequences were then aligned and compared with ClustalOmega (Sievers et al., 2014).

The multiple sequence alignment of the amino acid sequences of the OXA-48-type carbapenemases revealed that with the exception of K30 all the isolates shared the same sequence. K30 differed by some amino acid substitutions. In particular the threonine (T) at residue 104, the asparagine (N) at residue 110, the glutamic acid (E) at residue 168, the serine (S) at residue 171 in all the isolates, were respectively substituted with alanine (A), aspartic acid (D), glutamine (Q) and alanine in K30 (Figure 4.18).


**Figure 4.18 Multiple alignment of the OXA-48 amino acid sequences of the *K. pneumoniae* isolates.**
For clarity only the aligned regions including the different amino acids are reported. The different amino acids are distinguished by different colours. The length of the sequence (from the first to the last amino acid) is reported for each isolate in the alignment.

The amino acid sequence of the OXA-48-type carbapenemase belonging respectively to K30 and to one of all the other isolates was then used to run a protein BLAST search against the entire database of known amino acid sequences and therefore identify the specific variant of the OXA-48-type carbapenemases present in K30 and in all the other isolates. This analysis revealed that the amino acid sequence present in K30 shared 100 % identity with OXA-181, while the sequence belonging to all the other isolates shared 100 % identity with OXA-48. Both these enzymes were variants of the OXA-48-like carbapenemases (Queslati et al., 2015; Dortet and Naas, 2017; Findlay et al., 2017). The multiple sequence alignment of the TEM amino acid sequences revealed that K5 and K1 differed from the other isolates. In particular, K5 showed a deletion in position 221 and showed 100 % identity with the variant TEM-10, belonging to the TEM family (Baraniak et al., 2015). K1 showed a serine at residue 91 instead of an arginine (R) and showed 100 % identity with the variant TEM-13 (Figure 4.19). A 100% identity with TEM-1 was found when one of the sequences of all the other isolates was submitted to Protein BLAST.

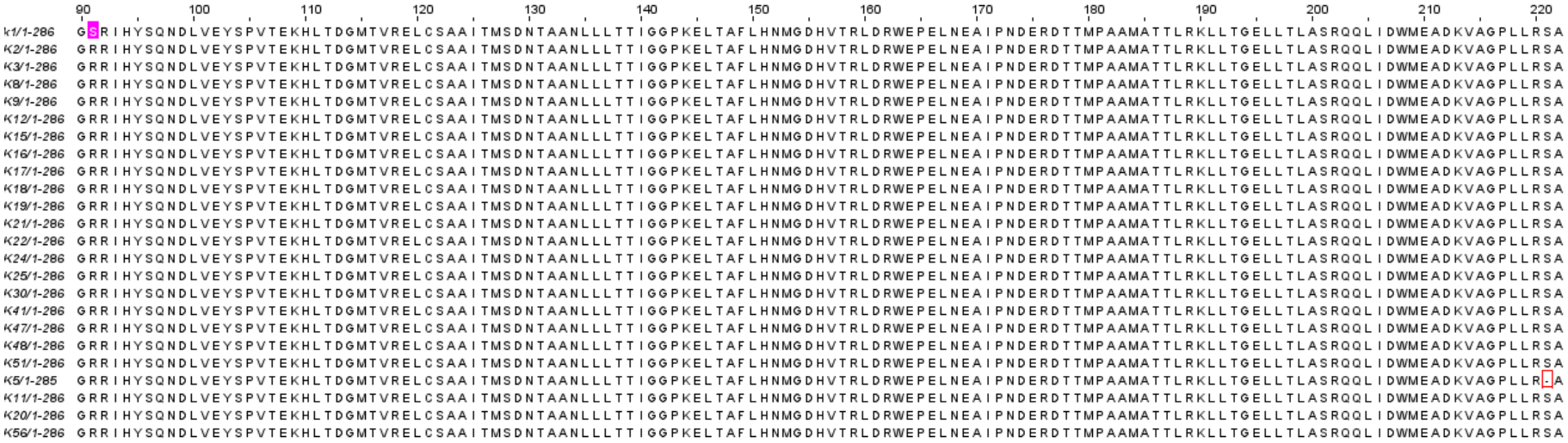



**Figure 4.19 Multiple alignment of the TEM amino acid sequences of the *K. pneumoniae* isolates.**

For clarity only the aligned regions including the different amino acids are reported. The different amino acids are distinguished by different colours.The deletion is showed as a horizontal line in the red rectangle. The length of the sequence (from the first to the last amino acid) is reported for each isolate in the alignment.

The alignment of the sequences for CTX-M showed that K11 and K20 differed from the other isolates by several amino acid substitutions reported in Figure 4.20. . Furthermore, the R at residue 167 was changed in S in K1. The sequence of both K11 and K20 shared 100% identity with CTXM-14, while the sequence of K1 had 100% identity with CTX-M-27. Additionally, the sequence belonging to all the other isolates share 100% identity with the enzyme CTX-M-15.

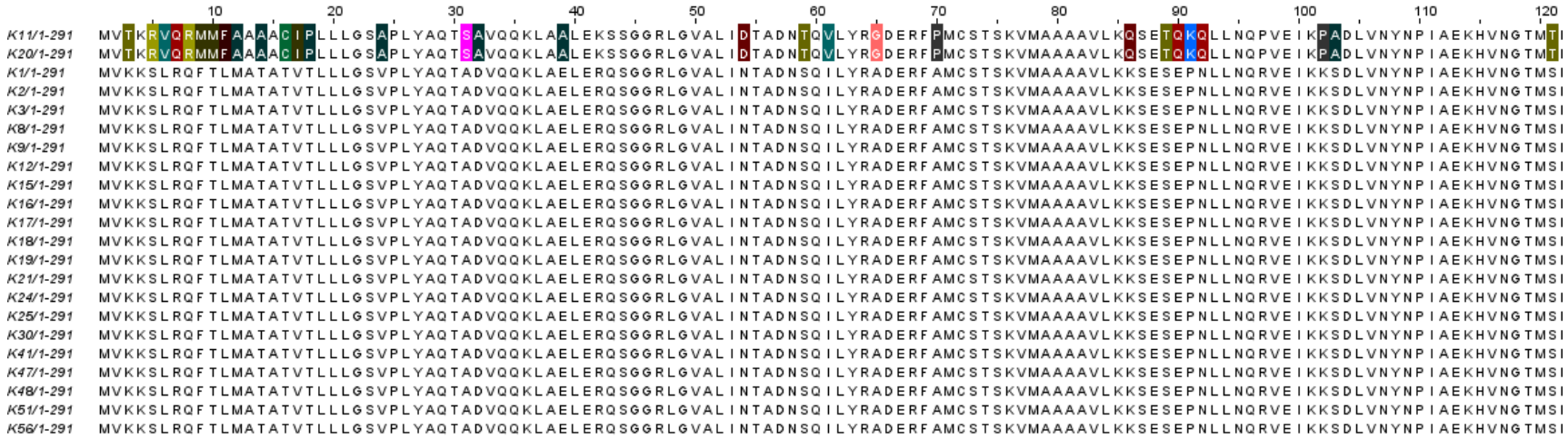



**Figure 4.20 Multiple alignment of the CTXM amino acid sequences of the *K. pneumoniae* isolates (first part).**

For clarity only the aligned regions including the different amino acids are reported. The different amino acids are distinguished by different colours.. The length of the sequence (from the first to the last amino acid) is reported for each isolate in the alignment.

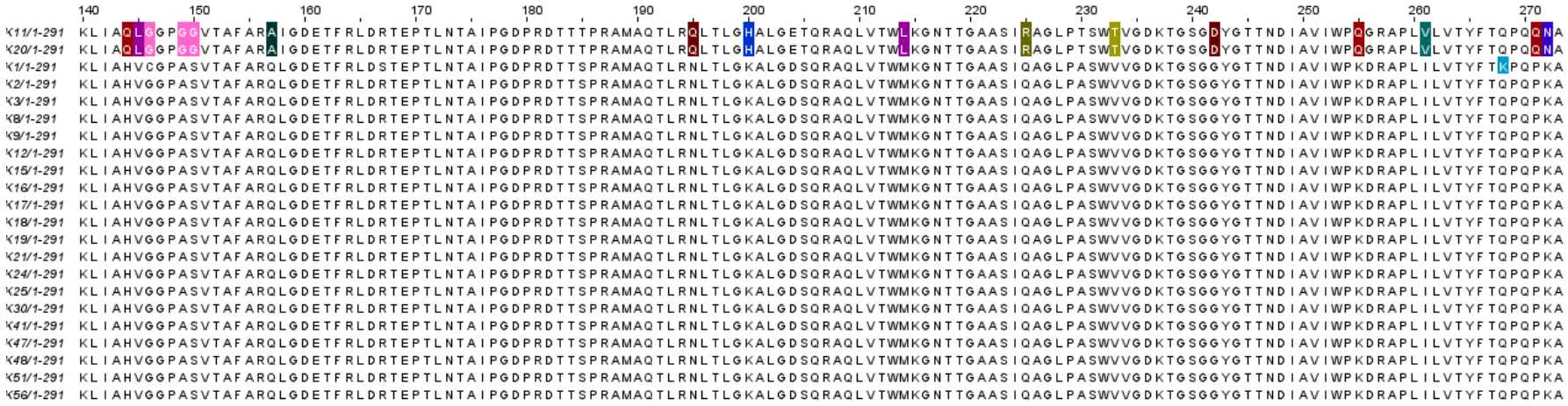



**Figure 4.20 Multiple alignment of the CTXM amino acid sequences of the *K. pneumoniae* isolates (second part).**

For clarity only the aligned regions including the different amino acids are reported. The different amino acids are distinguished by different colours..

.

With regards to the alignment of the NDM sequences, the valine (V) at residue 88 and the methionine (M) at residue 154 in the isolates were both replaced by leucine (L) in K48 and K51. The sequence of both K48 and K51 shared 100% identity with NDM-5. The sequence of all the other isolates showed 100% identity with NDM-1 (Fgure 4.21).


**Figure 4.21 Multiple alignment of the NDM amino acid sequences of the *K. pneumoniae* isolates**

For clarity only the aligned regions including the different amino acids are reported. The different amino acids are distinguished by different colours. The length of the sequence (from the first to the last amino acid) is reported for each isolate in the alignment.

.

Lastly the alignment of the sequences for SHV revealed that in K8, K2, K18, K11, K20, K48, K51, K26 and K4, an L at residue 31 replaced a Q present in all the other isolates. The sequence of K11, K20, K48, K51, K26 and K4 showed 99.65 % identity with the enzyme SHV-27. In addition, A valine (V) replaced the glycine at position 143 in all the other isolates. In the isolates K2, K8 and K18 a D replaced the G at residue 152. These isolates shared 100% identity with SHV-27. Lastly in K16, a S and a Lysine (K) in position 234 and 235 respectively replaced G and E (Figure 4.22). This sequence showed 100 % identity with SHV-12.

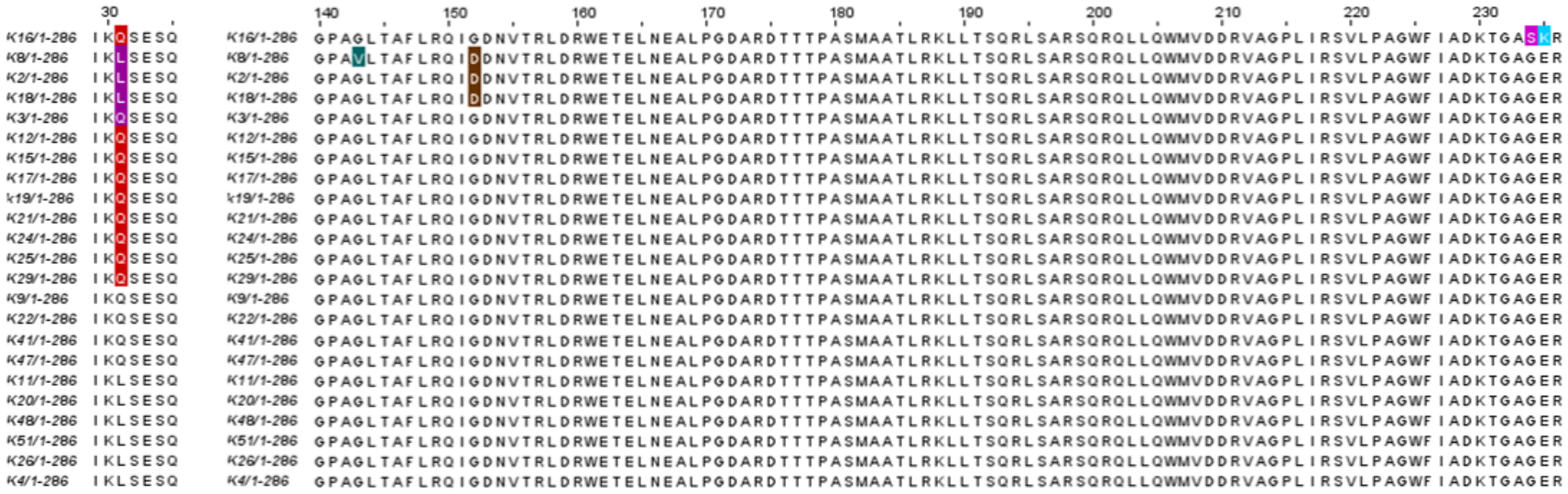



**Figure 4.22 Multiple alignment of the SHV amino acid sequences of the *K. pneumoniae* isolates.**

For clarity only the aligned regions including the different amino acids are reported. The different amino acids are distinguished by different colours. The length of the sequence (from the first to the last amino acid) is reported for each isolate in the alignment.

.

However, the variations observed in the amino acid sequence of each enzyme among the isolates did not reflect the variability level in the sheltering effect observed for the *K. pneumoniae* isolates. Indeed, the number of sheltering and non-sheltering groups was much higher than the number of different amino acid sequences found for each enzyme. With regards to this, 2 different amino acid sequences were found for the OXA-48 group, 3 for TEM, 3 for CTX-M, 4 for SHV and 2 for NDM. On the other side, the isolates were grouped in 9 sheltering groups and 1 non-sheltering group based on their sheltering potential features. Therefore, no apparent relationship between the grouping of the isolates in the sheltering groups and in the non-sheltering group and the different variants of each β-lactamase was observed.

## 4.3 Discussion

The diversity among bacterial isolates can be assessed using different methods, including phenotyping and genotyping. Different bacterial phenotypes can be identified by the morphology of colonies produced on different culture media, biochemical or serology tests, pathogenicity, and antimicrobial susceptibility (Jean and MacFaddin, 2000; Rafiq et al., 2000; Andrews, 2001; Narisara et al., 2008; Li et al., 2009; Huang et al., 2016; Knopp and Andersson, 2018). However, differences among bacterial isolates can also be determined by genotyping-based techniques. Genotyping has now become largely used for bacterial strain typing. Indeed, due to its high-resolution power, genotyping allows the discrimination of bacterial strains based on their genetic content (Li et al., 2009). Current methods for bacterial genotyping include DNA banding pattern-, DNA sequencing-, and DNA hybridization-based methods (Schwartz and Cantor, 1984; Blakesley, 1987; Thibodeau, 1987; Todd, 2001; Franca et al., 2002; Lo et al., 2006; Herschleb et al.2007; Zhang et al., 2007; Ibarz Pavón and Maiden, 2009). In this chapter the sheltering effect was investigated in all the *K. pneumoniae* β-lactam resistant clinical isolates available in our laboratory, including the 15 isolates already described in Chapter 3. The increase in the number of clinical isolates tested aimed to confirm that the sheltering effect was a phenomenon occurring throughout the *K. pneumoniae* species.

Furthermore, this analysis aimed to investigate whether different isolates significantly differed by the potential of the sheltering effect they provided to the susceptible strain. Although the *K. pneumoniae* isolates were able to shelter susceptible bacterial strains the results obtained showed that the time of detection of the sheltering effect was different depending on the resistant isolate and the susceptible strain used. Indeed,

the lawn of susceptible bacteria growing around the resistant isolate was overall more extensive for *S. aureus* NCTC 6571 than *E. coli* NCTC 10418 (Figures 4.1 and 4.2). The latter also grew as single separate colonies in the area furthest from the resistant isolate. With regards to this, the higher $OD_{600}$ detected for *S. aureus* NCTC 6571 might suggest a higher number of cells suspended in the sample used for the experiments on plate. This would account for a higher number of protected susceptible cells of *S. aureus* NCTC 6571 than *E.coli* NCTC 10418 and consequently a greater and faster sheltering effect on plates streaked with *S. aureus* NCTC 6571 than on *E. coli* NCTC 10418.

However, the potential of the sheltering effect was significantly different among the isolates inoculated on the same susceptible strain after the first 24 hours of incubation and the trend in the level of variability showed by the resistant isolates was the largely same in both the susceptible strains (Figures 4.5 and 4.6).

The determination of the growth rates of clinical isolates has previously been used to characterise and discriminate between clinical isolates from the same species (Anes et al., 2017). However, the *K. pneumoniae* isolates did not show significant differences either in the cellular density of the samples used directly for the experiments on plate or in their growth curves. Indeed, isolates producing an extensive sheltered area grew at a rate similar to those producing smaller areas (Figures. 4.8 and 4.9). Therefore, the variations in the sheltering potential among the isolates were not apparently due to differences in their growth rates.

The determination of the MIC is a method routinely used to discriminate between bacterial isolates from the same species based on their antimicrobial resistance profiles (Harada et al., 2016; Saveedra et al., 2017). Previous studies showed that surviving patients infected with microorganisms for which MICs were elevated had a significantly longer duration of hospitalisation than patients infected with microorganisms with lower MICs for the same drugs (DeFife et al., 2009). In this Chapter the relationship between the MIC profiles for selected β-lactams, including carbapenems and cephalosporins, and the strength of the sheltering effect of each *K. pneumoniae* isolate was investigated. Indeed, a stronger protective effect showed by some isolates might be the result of their higher resistance levels to β-lactams and vice versa. However, there was lack of a linear relationship between the sheltered areas produced by the isolates and their corresponding MIC values for each β-lactam tested (Figures 4.10, 4.11, 4.12, 4.13 and 4.14). Indeed, the assumption that guided the search for a relationship between the MIC and sheltering potential was that the sheltering potential was a direct result of the amount of OMVs released from each resistance isolate. Therefore, according to this assumption, a higher sheltering potential is the result of a higher number of OMVs released. Nevertheless, the lack of

correlation between the MIC and the sheltering potential might be due to the fact that the MIC is often the result of multiple resistance mechanisms. Indeed, the profile of resistance to a specific drug might vary if various resistance processes, including impermeability of the OM and increased efflux, act independently or conjointly (Aubert et al., 2001). For example, in addition to the β-lactamases packed in OMVs, the resistance profile of a bacterial strain or isolate can be determined by the activity of efflux pumps. With regards to this, *K. pneumoniae* is a good producer of MDR pumps. The efflux of the drugs in combination with the activity of β-lactamases contribute to increase the resistance to antibiotics in this bacterium (Padilla et al., 2009; Srinivasan et al., 2014; Szabo et al., 2018; Maurya et al., 2019; Ni et al., 2020). The presence of different combinations of resistance mechanisms could also explain the different patterns in the scatter plots of the different antibiotics showed in Figures 4.10, 4.11, 4.12, 4.13 and 4.14. Indeed, different β-lactams might activate different combinations of resistance mechanisms. For example, high resistance to carbapenems can sometimes be the result of the combination of efflux pump, reduced permeability, altered transpeptidases, and inactivation by β-lactamases (Sawa et al., 2020). Moreover, the ß-lactamase production alone or in conjunction with the overexpression of efflux pumps can be responsible for the difference in the resistance profiles to cephalosporins (Peña et al., 2009). Additionally, the upregulation of different porins in *K. pneumoniae* strains was found to impact the sensitivity to different drugs (Rocker et al., 2020).

In the attempt to gain insight on the source of this variability, the genotypic features among the *K. pneumoniae* isolates were determined. With regards to this, characterisation with genotype-based methods, such as RAPD and PCR, represents one of the most useful epidemiological methods for discriminating different bacterial isolates within same species, which in turn provide data to manage therapeutic treatment and the control of rapid expansion of clinically important bacteria (Li et al., 2009; Halaby et al., 2017; Al-Obaidi et al., 2018; Lomonaco et al., 2018). However, among the genotype-based methods for the bacterial discrimination, whole-genome sequencing (WGS) has become an increasingly accessible means for tracking disease outbreaks. This powerful tool can theoretically distinguish strains that differ at only a single nucleotide, thus providing a discrimination power greater than the other genotyping methods (Snitkin et al., 2012; Walker et al., 2013; Salipante et al., 2015). Indeed, WGS can theoretically distinguish strains that differ at only a single nucleotide (Pendleton et al., 2013; Pimlapas et al., 2014). With this in mind, differences in the genetic features among the *K. pneumoniae* isolates might be responsible for the differences in the sheltering potential detected among the isolates. Therefore, in this work a set of genetic analyses were carried out in the attempt to

find the source of the sheltering variability. The first test to be carried out was based on the analysis of total genomic DNA by RAPD to rapidly determine the genetic profiles of the isolates. The pattern of amplified products of different molecular weight was characteristic of the isolates and allowed to differentiate between genetically distinct individuals. Some isolates were grouped in the same cluster of the dendrogram generated based on the RAPD profiles, thus highlighting that these isolates were genetically related. The remaining isolates formed separate clusters since they did not show any genetic similarity to any other isolate (Figure 4.17). However, the groups of the isolates sharing the same genetic similarities did not correspond to the groups of the isolates sharing similar sheltering potential (Table 4.3). Therefore, the analysis of the variations found in the entire genome by RAPD did not account for the differences in the sheltering potential. With this in mind, the aim of the second analysis was to focus on the collection of resistance genes to β-lactams of the isolates, regardless of the rest of the genome. Indeed, the segments of DNA amplified with RAPD were random and therefore variations in the content of specific genes might not be detected. However, differences in the set of β-lactams resistance genes among the isolates might be responsible for variations in the sheltering since the protective effect is known to be triggered by β-lactamases released in the extracellular environment of resistant species (Liao et al., 2014; Liao et al., 2015). Nevertheless, the results obtained suggest that no relationship between the content of the β-lactams resistance genes and the sheltering groups was present. Consequently, the aim of the last analysis was to investigate the DNA sequence of the β-lactams resistance genes in the isolates. This analysis was expected to produce the most accurate results since variations in the β-lactams resistance genes were investigated at nucleotide level. Potential changes at DNA level in these genes among the isolates might alter the amino acid sequence of the corresponding proteins and possibly their enzymatic activity. This might ultimately be responsible for variations in the activity of the β-lactamases of the isolates, thus leading to differences in the sheltering effect. Again, the variations in the amino acid sequence of each β-lactamase among the isolates did not reflect the variability in the sheltering potential observed.

In conclusion, these data suggest that the variability of the sheltering effect was not the result of the different resistance profiles and the genetic features tested so far. Therefore, further analyses will be necessary to elucidate the causes of the variability observed among the *K.pneumoniae* isolates. For example, the extent of sheltering effect might be determined by differences in the expression of genes involved in their extracellular release of OMVs. Indeed, it is known that variations in these genes can alter the OMV properties, including their stability, size and cargo selection

(Nevermann et al., 2019). In the next Chapter, the differences in the genes involved in the OMVs biogenesis were investigated in the attempt to gain insight on the variability of the *K. pneumoniae* isolates.

# RESULTS III: THE ROLE OF OMVs IN THE SHELTERING EFFECT OF *K. PNEUMONIAE*

## 5.1 Introduction

OMVs are released by many Gram-negative bacteria and play important roles including the long-distance dissemination of bacterial products into the environment and the interaction with other cells, thus eliminating the need for physical contact (Roier et al., 2016). In particular, the release of OMVs is involved in the maintenance of the biofilm structure, HGT, predation, inter-kingdom communication and nutrient acquisition (Kulp and Kuehn, 2010; Perez-Cruz et al., 2015; Kim et al., 2018). The OMV's content includes LPS, cell wall components, PG, phospholipids, OM proteins (OMPs), proteins (periplasmic, cytoplasmic, and membrane-bound), ion metabolites, signaling molecules and nucleic acids (both DNA and RNA) (Lindmark et al., 2009; Bai et al., 2014; Koeppen et al., 2016; Vanaja et al., 2016; Jan, 2017**;** Malabirade et al., 2018).

OMVs can protect bacteria against a number of antibiotics by exerting effects that go beyond the degradation of antibiotics or the transfer of antibiotic resistance genes. Indeed, OMVs can act as decoys by binding or absorbing antibiotics (Schwechheimer and Kuehn, 2015; Kim et al., 2018). For example, *E. coli* was found to release OMVs involved in the binding of the antibiotic colistin and in the degradation of the antimicrobial peptide, melittin (Kulkarni et al., 2015). Also, active OMVs derived from *M. catarrhalis* and *S. aureus* were found to improve the survival of β-lactam-sensitive bacteria in the presence of β-lactam antibiotics (Shaar et al., 2011; Lee et al., 2013). Furthermore, studies carried out in *A. baumannii* showed that OMVs released from carbapenem-resistant strains could transfer a carbapenem resistance-related gene to carbapenem-susceptible *A. baumannii* (Rumbo et al., 2011).

The biogenesis of the OMVs relies on three major mechanisms. These include the dissociation of the OM in specific regions, the presence of misfolded proteins in zones devoid of crosslinks between PG and other components and changes in LPS that might generate a differential fluidity, curvature, and charge in the OM (Yeh et al., 2010; Park et al., 2012; Schwechheimer and Kuehn, 2015; Elhenawy et al., 2016; Nevermann et al., 2019). Therefore, the identification of genes involved in these

mechanisms has been increasingly gaining attention (Liechti and Goldberg, 2012; Konovalova and Kahne, 2017; Nevermann et al., 2019).

Studies carried out in *S. enterica* showed that 9 genes are involved in OMV biogenesis and encode functions related to envelope stability, accumulation of misfolded proteins, or to LPS composition (Nevermann et al., 2019). Variations of these genes are responsible for modulating OMV properties, such as size distribution and protein cargo selection. In *S. enterica* serovar Typhi these genes correspond to *ompA, nlpI*, *tolR*, *rfaE*, *waaC*, *yipP* (also known as *envC*), *mrcB*, *degS* and *hns* (Nevermann et al., 2019).

Bacterial OMVs are routinely observed by transmission electron microscopy (TEM) (Perez-Cruz et al., 2015; Bitto et al., 2017; Buzás et al., 2018; Gill et al., 2019). Nevertheless, the analysis of OMVs by TEM is time and cost consuming due to the number of methods involved and the cost of the materials used (Winey et al., 2014). Therefore, it is desirable to find a cheaper and time saving methodology to observe bacterial OMVs. An alternative to TEM might be the use of laser-scanning confocal fluorescence microscopy for determining the localization of fluorescently tagged proteins within and outside bacterial cells (Mullineaux, 2007).

Previous studies carried out in *A. baumannii* showed that the majority of the extracellular release of the carbapenem-hydrolyzing beta-lactamase OXA-58 was released via OMVs in the absence of a carbapenem. However, in the presence of carbapenems OMV formation and the OMV-associated extracellular release of OXA-58 increased (Liao et al., 2015). The release of OMVs was associated with Sec-dependent periplasmic translocation (Liao et al., 2015; Weber et al., 2017), a type II secretion system for export of proteins across the cytoplasmic membrane to the bacterial periplasm and OM (Beckwith, 2013). With regards to this, the addition of Rb, a SecA inhibitor, reduced the extracellular release of OXA-58 (Liao et al., 2015).

The purpose of this project was to investigate whether the features of the sheltering effect exerted by *K. pneumoniae* were similar to those observed for the bacterium *A. baumannii.* These features included the release of OMVs associated with Sec-dependent periplasmic translocation and the increase of the protective effect with the concentration of the drug in the medium. However, this study also aimed to investigate new features of this phenomenon, including the variations of the sheltering effect with time and the presence of non-β-lactam antibiotics in the medium. Ultimately, the focus of the present project was to test whether differences in the sequence of the proteins responsible for the OMVs biogenesis could account for the variability in the sheltering effect described in Chapter 4. Furthermore, differences in the sequence of the promoters of the genes coding for these proteins were also analysed to investigate if changes in gene expression might explain the

differences in sheltering effect observed. Indeed, genetic variations in these regions might be responsible for different expression levels by altering the copy number of these proteins. This aspect might additionally account for the differences in the sheltering effect detected among the isolates.

The aims of this study were:

- to determine whether the sheltering effect in *K. pneumoniae* involves i) OMVs and ii) the Sec-dependent pathway.
- To determine how variations in Amp, time and non-β-lactam antibiotic treatments affect the sheltering effect.
- To identify homologs of he OMVs biogenesis genes and their promoters and determine whether variation in their sequences could influence sheltering.

## 5.2 Results

### 5.2.1 Investigation into the presence of β-lactamases in OMVs released in the extracellular environment of *K. pneumoniae*

The purpose of this experiment was to confirm that the sheltering effect was due to β-lactamases included in OMVs released in the extracellular environment. With this in mind, the supernatant fraction (Sf) was retrieved after centrifugation of an overnight culture of K17 and filtration. This fraction was then used for the preparation of three different reaction mixtures. In the first mixture Sf was treated with Proteinase K (PK) with the aim of degrading any naked β-lactamases not protected in OMVs (McCaig et al., 2013). In the second reaction mixture the protease treatment with PK was combined with the use of Triton X (TR), a detergent which should solubilize vesicular proteins and make them susceptible to PK. In the third mixture Sf was treated with TR alone. After incubation, centrifugation and filtering each reaction mixture, along with the untreated Sf, was dispensed in a hole made on the agar of a plate containing 100 μg/ml Amp and evenly spread with an overnight culture of *E. coli* NCTC 10418. The growth of the susceptible strain around each hole was measured with the area-based method. If the sheltering is largely due to β-lactamases within OMVs then detergent-treatment would be expected to reduce significantly the sheltering effect. The results shown in Figure 5.1 support the hypothesis that β-lactamases are released within OMVs. Furthermore the sheltering effect was not affected by the addition of TR alone. However, there was a significant reduction (approximately 50%) in sheltering following protease treatment suggesting a role for naked enzyme but an even greater reduction was observed when detergent and

protease treatments were combined. This result suggests that approximately half of the sheltering effect observed was due to naked β-lactamases and half to the β-lactamases included in OMVs.

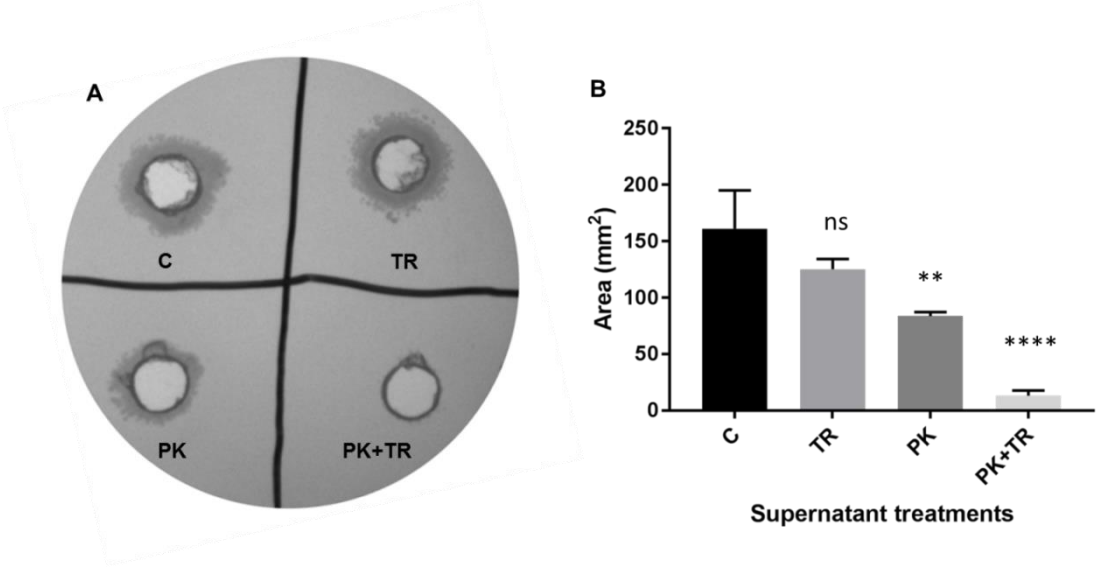



**Figure 5.1 Sheltering effect on *E coli* NCTC 10418 provided by K17 Sf untreated (C) and treated respectively with 5% TR, 10 mg/ml PK and a combination of 5% TR and 10 mg/ml PK (PK+TR).** Following the incubation at 37ºC and filtration of C, TR, PK and TR+PK , a 2x YT agar plate containing 100 µg/ml Amp was evenly spread with an overnight culture of *E.coli* NCTC 10418 and divided into four quadrants. 100 µl of respectively TR, TR+PK, PK and C were then dispensed in one of the 4 holes made in the quadrants. **A**. Appearance of the 2x YT plate after incubation of C, TR, TR+PK, PK at 37ºC overnight. **B.** Bar plot of the area occupied by the sheltered *E. coli* NCTC10418 against the different supernatant treatments incubated on the plate. The P-values were calculated by comparing the mean of each column with the mean of the C column taken as the control column. P-values ≥ than 0.05 were considered statistically not significant (ns). The P-values are denoted symbolically as follows; 0.001 to 0.01 (**) = highly significant and < 0.0001 (****) = extremely significant.

### 5.2.2 Effect of Rb on the sheltering effect on *E.coli* NCTC 10418

As described in the introduction, the sheltering effect in *A. baumanni* was inhibited by Rb, an inhibitor of the SecA-dependent periplasmic translocation (Liao et al., 2015). The aim of the analysis described here was to investigate the effect of Rb on the sheltering effect provided by *K. pneumoniae.* With regards to this, an inhibition of the phenomenon might suggest that the sheltering effect in *K. pneumoniae* occurred by a release of OMVs mediated by a Sec-dependent pathway similarly to *A. baumannii*. To confirm this hypothesis, K17 was inoculated on 6 2x YT plates enriched with 100 µg/ml Amp and evenly spread with *E. coli* NCTC 10418. These 6 plates contained Rb at the following concentrations: 0, 12.5, 25, 50, 100 and 200 µg/ml. This experiment was carried out in triplicate. As a control of the viability of the bacteria on a medium

including Rb, K17 and *E. coli* NCTC 10418 were streaked onto plates containing Rb at all the concentrations tested in this analysis.

The appearance of the satellite colonies of *E. coli* NCTC10418 around K17 was monitored after 24 hours from the incubation of the plates at 37ºC. The overnight cultures of K17 and *E. coli* NCTC 10418 grew on plates without Amp containing all the Rb concentrations used thus confirming that Rb was not lethal for these two bacterial species at any of the Rb concentrations used. Furthermore there was no observable effect on bacterial growth (Figure 5.2).

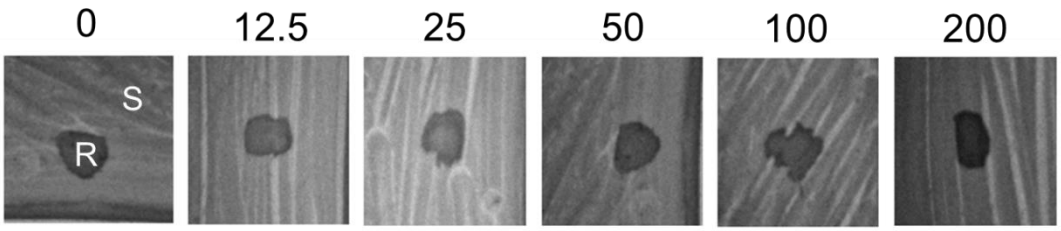



**Figure 5.2. Aspect of plates containing increasing concentrations of Rb and the inocula of K17 (R) and *E. coli* NCTC 10418 (S).** Six 2xYT plates were prepared with respectively 0,12.5, 25, 50, 100 and 200 µg/ml Rb. An overnight culture of S was evenly streaked on all the plates. 1 µl of a bacterial culture of K17 was then co-inoculated on all these plates and the growth was observed after 24 hours of incubation at 37ºC. No visible difference in the growth of S and R was detectable among all the plates.

When increasing concentrations of Rb were added to the 2x YT agar plate including 100 µg/ml Amp a large decrease in the area of the sheltered *E.coli* NCTC 10418 was detected compared to the plate with 0 µg/ml Rb (Figure 5.3, A). This reduction was not significant at 12.5 µg/ml Rb. However the increase of Rb concentration to 50, 100 and 200 µg/ml resulted in a significant decrease of the surface area (Figure 5.3, B).

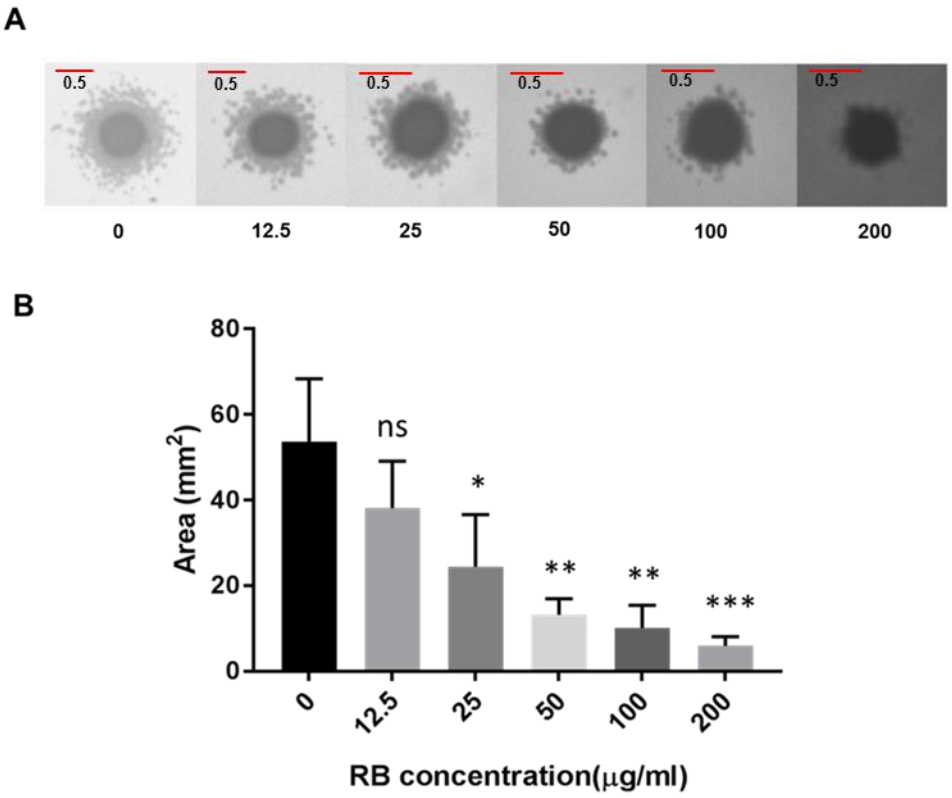



**Figure 5.3 Effect of increasing concentrations of Rb on the sheltering effect of K17 on *E. coli* NCTC 10418. A.** Appearance of satellite colonies of *E.coli* NCTC 10418 on 2xYT plates enriched with 100 µg/ml Amp and increasing concentrations of Rb (0, 12.5, 25, 50, 100 and 200 µg/ml) after 24 hours of incubation at 37ºC from the inoculation on plate. The images were taken at different magnifications. A scale bar (in cm) is reported for each image. **B.** The surface area of the sheltered *E.coli* NCTC 10418 is plotted against the different Rb concentrations. P-values were calculated by comparing the mean of each column with the mean of the 0 µg/ml column taken as the control column. P-values ≥ than 0.05 were considered statistically not significant (ns). The P-values are denoted symbolically as follows: 0.01 to 0.05 (*) = significant; 0.001 to 0.01 (**) = highly significant; 0.0001-0.001(***) = very highly significant and < 0.0001 (****) = extremely significant.

These results suggest that, as has been previously reported with other species, the Sec-dependent pathway is a mechanism by which the β-lactamases produced by *K. pneumoniae* are exported to the bacterial periplasm and OM.

### 5.2.3 Microscopic visualization of the periplasm and OMVs including the carbapenem-hydrolyzing beta-lactamase OXA-58 released by *A. baumannii.*

Previous studies showed the presence and release of OMVs that contained the carbapenem-hydrolyzing beta-lactamase OXA-58 from the bacterium *A. baumannii*. The treatment with carbapenems increased OMVs formation, resulting in an increase in the OMV-associated and OMV-independent release of extracellular OXA-58 (Liao

et al., 2015). This release occurred after translocation of this enzyme to the periplasm via the Sec system (Liao et al., 2014; Liao et al., 2015).

Since the presence of OMVs containing carbapenem-hydrolyzing β-lactamases was confirmed in *A. baumannii*, this bacterium was used to set-up an experimental protocol for the microscopic visualization of the OMVs in the extracellular environment. Furthermore, the choice to work with *A. baumannii* was due to the availability in the laboratory of the reference strain ATCC 19606 that could be used for the cloning experiments. This experimental procedure would then have been used to confirm the presence and the release of OMVs including carbapenem-hydrolyzing β-lactamases by carbapenem-resistant *K. pneumoniae.* The protocol set-up for *A. baumannii* was based on cloning experiments aimed to generate a plasmid construct including the gene for the β-lactamase OXA-58 ($bla_{OXA-58}$) fused with the gene for the Green Fluorescent Protein (*gfp*). This construct was generated by ligating the two genes to the plasmid pYMAb2 (Chen et al., 2010). This latter contains a replicon region for the replication in *A. baumannii* and a gene for the resistance to Kan for the selection of the bacteria successfully transformed (Chen et al., 2010; Kuo et al., 2013; Liao et al., 2014; Kuo et al., 2015). The fusion construct was validated by transfer of the ligation product to *E. coli* DH5α cells (Invitrogen), extraction of the plasmid from the colonies growing on 2xYT agar plates containing Kan and DNA digestion. This latter step aimed to verify that the plasmid contained the fusion gene. Furthermore the plasmid extracted was sequenced to check that the fusion gene had the desired sequence and no mutations or deletions were introduced during the PCR and cloning procedure. Following this validation process, the plasmid containing the fusion gene was transferred to electrocompetent *A. baumannii* CIP70-10 (AbCIP70.10(pyOXA-58-GFP)). The resultant fusion protein (OXA-58-GFP) was expected to be translocated to the periplasmic space, packed in OMVs and released in the medium. Cells of *A.baumannii* CIP70-10 transformed with pYMAb2 including no insert (AbCIP70.10(py)) were also visualised microscopically. The visualization of AbCIP70.10(pyOXA-58-GFP) and AbCIP70.10(py) with the confocal microscope was carried out after growth of these cells in 2x YT liquid broth. This included Kan for the maintenance of the plasmid in the cells and Mer at increasing concentrations (0, 0.25, 0.5 and 0.75 μg/ml) to stimulate the formation and the release of OMVs including OXA-58-GFP. These concentrations of Mer were not lethal for either strain Indeed, the $OD_{600}$ of both AbCIP70.10(pyOXA-58-GFP) and AbCIP70.10(py) after growth in all the different conditions described was around 0.5 and a bacterial lawn was detected when these overnight cultures were plated on 2x YT agar, thus indicating that the overnight cultures used for the microscopy were made of living cells. As a positive control for the detection of GFP, cells of *E. coli* BL21 expressing GFP were

observed at the confocal microscope as well after overnight growth in 2x YT including 50 µg/ml Kan and 50 µg/ml Chlor.5 µl.

Only sporadic green cells were detected on the slides loaded with AbCIP70.10(pyOXA-58-GFP) grown in 2x YT including Mer at all the concentrations used (Figure 5.4). The green colour might be due to the presence of OXA-58-GFP in the periplasm of AbCIP70.10(pyOXA-58-GFP) cells. In contrast, no green color was detected when cells of AbCIP70.10(py) were visualized at the confocal microscope after growth of the cells in 2x YT including all the Mer concentrations used in the experiment. However, no extracellular green spot in the extracellular environment of AbCIP70.10(pyOXA-58-GFP) was observed. Moreover, a much higher density of green cells was detected when *E. coli* BL21 expressing GFP was visualized at the confocal microscope. The low number of green AbCIP70.10(pyOXA-58-GFP) detected suggested that OXA-58-GFP was expressed at very low levels and the addition of increasing concentrations of Mer didn't result in an increase in the protein expression.

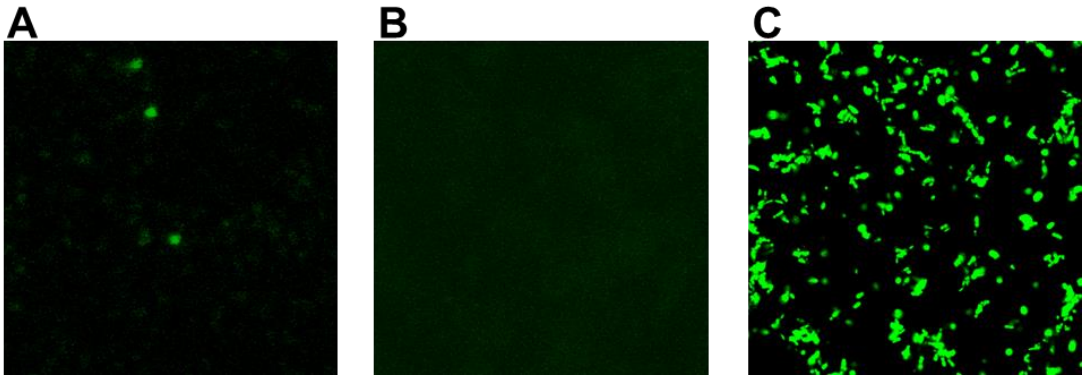



**Figure 5.4 Confocal microscopy images of AbCIP70.10(pyOXA-58-GFP) (A), AbCIP70.10(py) (B) and BL21 *E. coli* cells expressing the GFP (C).** AbCIP70.10(pyOXA-58-GFP), AbCIP70.10(py) and BL21 *E. coli* cells expressing the GFP were inspected with a LSM510 laser scanning microscopy (Zeiss) by using an Argon laser at 488 nm and the emission filter BP 505-530 and x100 magnification.

In order to confirm that AbCIP70.10(pyOXA-58-GFP) expressed the fusion protein at low levels, a Western Blot was carried out with the protein extracts of AbCIP70.10(pyOXA-58-GFP) and AbCIP70.10(py) grown overnight at the same concentrations of Mer used for the confocal microscopy experiment. The protein extract from an overnight culture of *E. coli* BL21 expressing the GFP was also tested as a positive control for the detection of the GFP with the Western Blot. A band of approximately 58 KDa was expected to appear after visualization of the membrane by transillumination as a result of the expression of OXA-58-GFP, while the GFP alone was expected to give a band of approximately 35 KDa. However, no band

corresponding to the fusion product was detected on the membrane when the protein extracts of AbCIP70.10(pyOXA-58-GFP) were run on the polyacrylamide gel and the protein bands transferred on the membrane by Western blot (Figure 5.5). Furthermore, a band of approximately 35 KDa was not detected for the protein extracts of AbCIP70.10(py). A band of approximately 80 KDa was detected on the blot for the protein extracts of both AbCIP70.10(pyOXA-58-GFP) and AbCIP70.10(py) grown at 0 (A), 0.5 (B), 1 (C) and 2 μg/ml of Mer. This might be the result of non-specific binding of the antibodies to proteins other than the fusion protein. Lastly, a band of approximately 35 KDa corresponding to the GFP was detected with the protein extract from *E. coli* BL21, thus indicating that the Western Blot procedure was carried out correctly. The absence of a band corresponding to the fusion product suggested that the fusion protein was not expressed or expressed at very low level, as already suggested by the results obtained by confocal microscopy. Therefore apparently this protocol in its current status was not applicable to the visualization of carbapenem-hydrolyzing β-lactamases-including-OMVs in *K.pneumoniae.* This indicated that the procedure set-up for the visualization of OMVs in *A. baumannii* needed to be modified and optimized to produce high levels of fusion protein. This might contribute to make the OMVs visible by confocal microscopy.

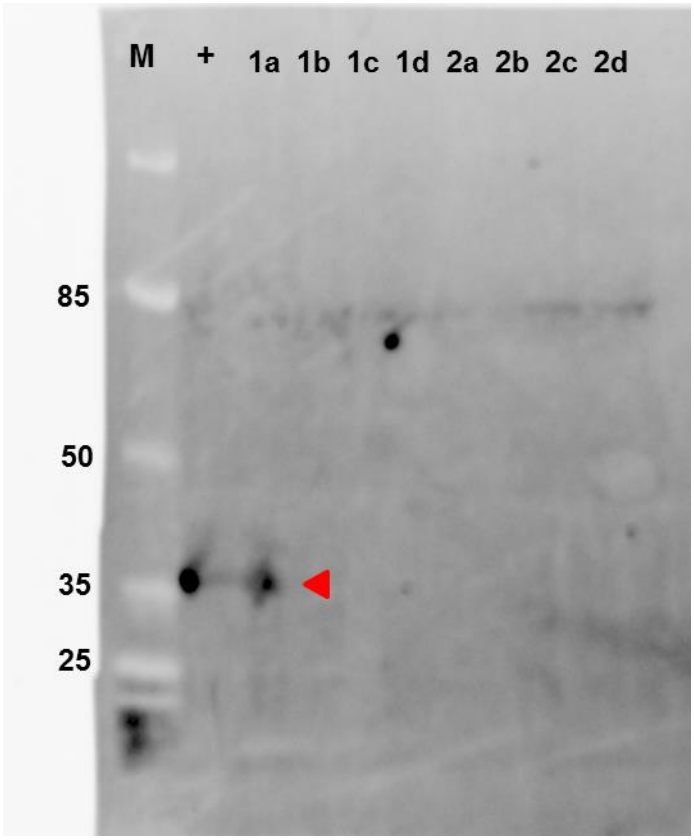



**Figure 5.5 Western blot detection of OXA-58-GFP in protein extracts from AbCIP70.10(pyOXA-58-GFP), AbCIP70.10(py) and *E. coli* BL21 expressing the GFP.** The proteins from overnight cultures of AbCIP70.10(pyOXA-58-GFP) (1) and AbCIP70.10(py) (2) grown in 2x YT with Kan and Mer at 0 (a), 0.5 (b), 1 (c) and 2 µg/ml (d) 0 were extracted and run on a polyacrylamide gel. The proteins were then transferred to a PVDF membrane by running a transfer at 125 mA and 100 V for 3 hours. This latter was incubated with Anti-GFP monoclonal antibody from mouse, washed in 1XTBS and incubated with goat polyclonal anti-mouse secondary antibody conjugated to the green fluorescent dye CF488A. After washing in 1XTBS, the membrane was visualized with a blue module lighting source and an emission filter for the wavelengths between 525-510. M:prestained protein molecular marker. The band indicated with the red arrowhead represents the GFP detected with the protein extract.

### 5.2.4 Effect of the Amp concentration on the sheltering effect on *E. coli* NCTC 10418

As described in the previous paragraph the protocol set up to visualize the OMVs failed in the detection of OMVs released in presence of increasing concentrations of β-lactams in the medium. Therefore the purpose of the present study was to investigate whether the sheltering effect exerted by *K. pneumoniae* on *E. coli* was affected by the concentration of β-lactam in the medium, by using the area-based method in alternative to the microscopy visualisation. Therefore the sheltered area of *E. coli* NCTC 10418 was measured in the presence of increasing concentrations of Amp in the medium. With this in mind, K17 was inoculated on 2x YT plates enriched

with increasing concentrations of Amp (25, 50, 100 and 200 µg/ml) and evenly spread with *E. coli* NCTC 10418. This experiment was carried out in triplicate. The appearance of satellite colonies of *E. coli* NCTC 10418 was monitored after 24 hours of incubation at 37ºC after the inoculation of *E. coli* NCTC 10418 on plates containing increasing concentrations of Amp (25, 50, 100 and 200 µg/ml). This analysis showed that the area of the sheltered *E.coli* NCTC 10418 gradually reduced with the increase of Amp from 25 to 200 µg/ml. This decrease was not statistically significant between the sheltered areas on 25 and 50 µg/ml Amp plates. However, this reduction was significant between the sheltered areas detected on 25 and 100 µg/ml Amp plates and highly significant between 25 and 200 µg/ml Amp plates (Figure 5.6).

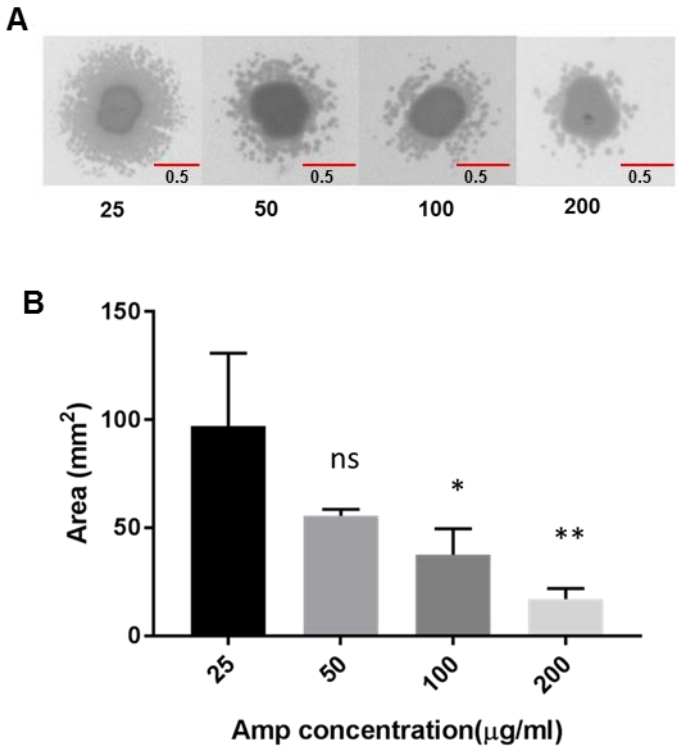



**Figure 5.6** Sheltering effect of K17 on *E.coli* NCTC 10418 on 2x YT plates enriched with 25, 50, 100 and 200 µg/ml Amp after 24 hours of incubation at 37ºC from the inoculation on plate. **A.** Appearance of sheltered *E.coli* NCTC 10418 surrounding the resistant K17 after 24 hours on plates enriched with 25, 50, 100 and 200 µg/ml Amp. The images were taken at different magnifications. A scale bar (in cm) is reported for each image. **B.** Bar plots of the surface area (in $mm^2$) occupied by the sheltered *E.coli* against the different Amp concentrations. The P-values were calculated by comparing the mean of each column with the mean of the 25 µg/ml column taken as the control column. P-values ≥ than 0.05 were considered statistically not significant (ns). The P-values are denoted symbolically as follows: 0.01 to 0.05 (*) = significant; 0.001 to 0.01 (**) = highly significant.

These results suggest that the increase of Amp in the medium slowed the growth of the susceptible species. With regards to this, increasing concentrations of Amp might have no effect or the OMV production and/or β-lactamase expression. Indeed, provided that the OMV production and/or β-lactamase expression remained constant

with the increase in Amp concentrations, the sheltering zone diminishes as the Amp concentration increases. This could be due to the fact that the area closest to the OMV-producing strain is probably the zone with the highest OMVs concentration, being this the region where the OMVs are produced and released. Instead, the OMVs are expected to diffuse in the agar medium as the distance from the resistance strain increases. Therefore, with the increase in Amp in the medium, the concentration of OMV-associated β-lactamases in the more distant areas might not be sufficient to allow appreciable growth of the *E. coli* colonies.

### 5.2.5 Effect of the time on the sheltering effect on *E.coli* NCTC 10418

The purpose of this analysis was to investigate whether the resistant strain provided a long-lasting protective effect on the susceptible strain. To confirm this hypothesis K17 was inoculated on a 2x YT plate enriched with 100 µg/ml Amp and evenly spread with *E. coli* NCTC 10418. Unlike from the experiments described in the previous paragraph, the concentration of Amp was kept constant on all the plates used in this analysis. Therefore differences in the growth of the susceptible species detected over time would not have been the result of variations in the antibiotic gradient. The appearance of the satellite colonies of *E. coli* NCTC 10418 around K17 was monitored after 24, 48 and 72 hours from the first incubation of the plates at 37ºC. The plate was not monitored after this time since at 37˚C in culture Amp was stable up to 3 days as specified in the information sheet provided by the manufacturer (Sigma Aldrich). This experiment was carried out in triplicate. This analysis showed a significant increase in the surface area occupied by the sheltered *E. coli* NCTC 10418 between 24 and 48 hours. This suggested that the resistant strain sheltered the susceptible strain in a continuous way. The surface area also increased between 48 and 72 hours although this change resulted not statistically significant (Figure 5.7).

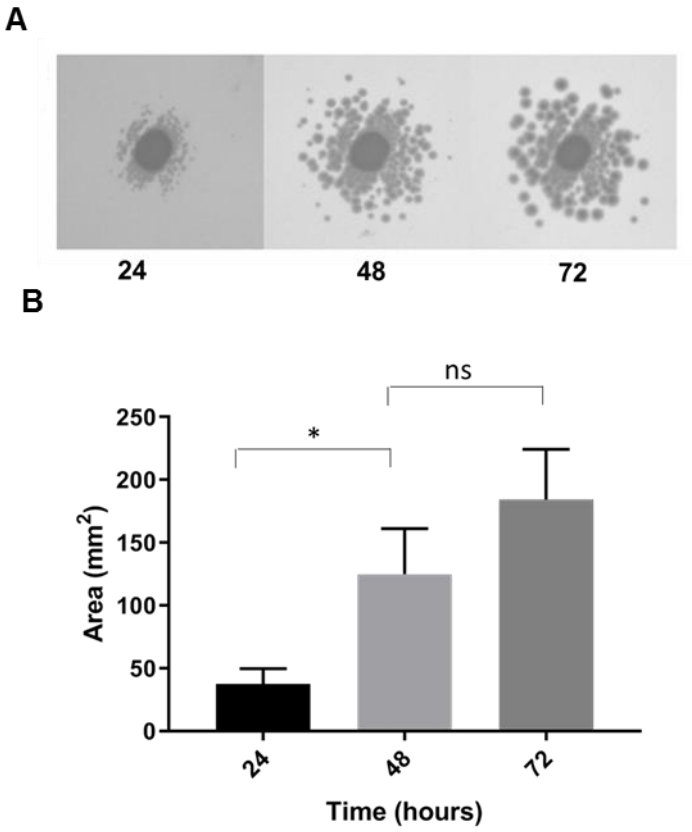



**Figure 5.7 Sheltering effect of K17 on *E.coli* NCTC 10418 monitored at 24, 48 and 72 hours of incubation at 37ºC. A.** Appearance of satellite colonies of *E.coli* NCTC 10418 around K17 at the different time points. **B.** Bar plots of the surface area (in $mm^2$) occupied by the sheltered *E.coli* NCTC 10418 against the time.The two P-values were calculated by comparing the means between respectively the 24 and 48 hours and the 48 and 72 hours. P-values ≥ than 0.05 were considered statistically not significant (ns). The P-value reported for the comparison between 24 and 48 hours is significant since it assumes a value between 0.01 to 0.05. This significance is denoted symbolically with "*" in the graph.

These results suggest that the protection to the susceptible strain was the result of a constant release of β-lactamases over time and that the appreciable growth of the colonies was dependent on the diffusion of the β-lactamases-carrying OMVs in the medium. Indeed, at 24 hours, the concentration of β-lactamases-carrying OMVs closer to the producing strain might be sufficient to allow appreciable growth of the *E. coli* colonies in this area. Further away from the producing strain, the concentration of β-lactamases-carrying OMVs might be sufficient to prevent the *E. coli* strain from being killed, but not for them to grow enough to appear as colonies visible to the naked eye. However, after more time these more distant bacteria might have been able to grow enough and form visible colonies (Figure 5.7).

### 5.2.6 Investigation of the sheltering effect in presence of non-β-lactams and β-lactams

The purpose of this experiment was to investigate whether *K. pneumoniae* was able to shelter *E.coli* from the action of drugs other than the β-lactams. Therefore, the sheltering effect on plates was investigated in the presence of different β-lactams and non β-lactams. The isolate chosen to carry out these experiments was K3 as it was one of the isolates producing the largest area of sheltered *E. coli* NCTC 10418 after 24 hours of incubation (Table 4.3, paragraph 4.2.4.1). As seen in Chapter 4, K3 was resistant to all the β-lactams tested by MIC, including Ceft, Cefe, Cefo, Mer and Ert (Table 4.2, paragraph 4.2.3) with MIC values of 32, 128, 128, 16 and 128 μg/ml, respectively. Furthermore, additional MIC tests were performed to determine the resistance profile of K3 to some non β-lactams that could be used in the experiments described in this paragraph. These non β-lactams included amikacin (Amk), gentamycin (Gent), chloramphenicol (Chlor) and ciprofloxacin (Cipr). These drugs have a mode of action different from the β-lactams, which inhibit synthesis of the bacterial cell wall. In particular, Amk, Gent and Chlor inhibit protein synthesis, while Cipr inhibits DNA synthesis (Krause et al., 2016; Kumar, 2017). The MIC tests were carried out in triplicate and the mode of the three values was calculated (Table 5.1).

**Table 5.1 MIC values (μg/ml) showed by *E. coli* NCTC 10418 and K3 to the antimicrobials tested in this study**

| Strain | Amk | Gent | Chlor | Cipr | Ert | Mer | Ceft | Cefo | Cefe |
|---|---|---|---|---|---|---|---|---|---|
| *E. coli* NCTC 10418 | 0.5 | 0.25 | 2 | 0.015 | 0.008 | 0.015 | 0.06 | 0.03 | 0.03 |
| K3 | 2 | 0.5 | 32 | 8 | 128 | 16 | 32 | 128 | 128 |

Based on the MIC values of K3 and *E. coli* NCTC 10418, Cefe, Cefo and Ert and the non β-lactams Chlor and Cipr were selected to test the sheltering effect in presence of β-lactam and non β-lactam drugs. Furthermore, the difference of several folds in the MIC value between K3 and *E. coli* NCTC 10418 for each of these drugs allowed a clear distinction to be made between sheltered and sheltering bacteria. With this in mind, the concentrations of Cefo, Cefe, Ert, Chlor and Cipr used for the sheltering effect experiments were respectively 4, 4, 1, 8 and 1 μg/ml. The volume of the drug solution corresponding to each of these concentrations was added to the liquid agar 2x YT during the preparation of the plates. The sheltering effect was also tested on 2x YT plates including 100 μg/ml of Amp. This was used as a positive control in these experiments since previous experiments described in Chapter 3 and 4 showed that K3 was able to shelter *E.coli* NCTC 10418. This experiment was carried out in

triplicate. The results showed that in all the three replicates K3 was able to shelter *E.coli* NCTC 10418 after 24 hours of incubation only in presence of β-lactams. Indeed, satellite colonies were detected on the plates containing respectively Cefo, Cefe, Ert and Amp but not on the plates containing Chlor and Cipr (Figure 5.8). To confirm that no satellite colonies on plates containing Chlor and Cipr appeared after a period of time longer than 24 hours, these plates were also monitored after 48 hours of incubation. Again, no growth in the area surrounding the isolates was detected at this time point (Figure 5.8).

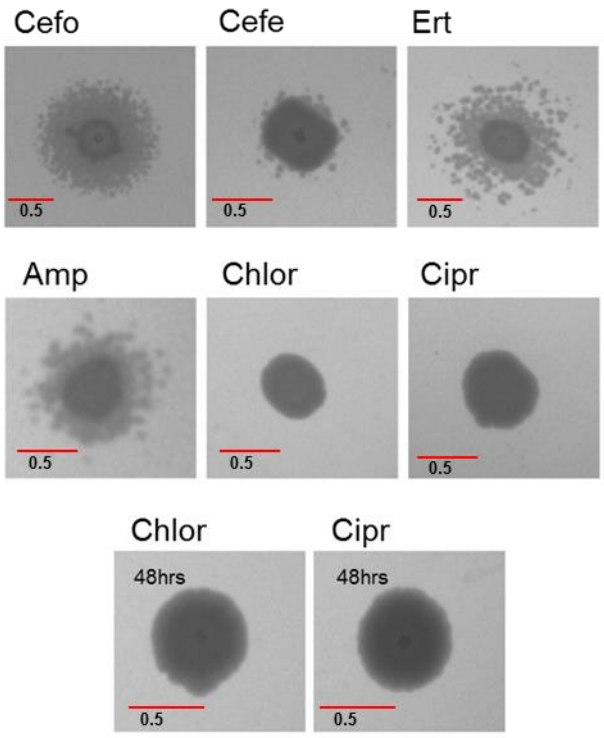



**Figure 5.8 Sheltering effect in presence of β-lactams and non β-lactams**. The picture shows the bacterial growth on 2x YT agar plates containing respectively 4 µg/ml Cefo, 4 µg/ml Cefe, 1 µg/ml Ert, 100 µg/ml Amp, 8 µg/ml Chlor and 1 µg/ml Cipr. All the plates were prepared by inoculating K3 in presence of *E. coli* NCTC 10418 followed by incubation at 37°C for 24 hours. Also the appearance of satellite colonies on the plates containing Chlor and Cipr was monitored after 48 hours of incubation at 37°C. The images were taken at different magnifications. A scale bar (in cm) is reported for each image.

These results suggested that the sheltering effect was specifically due to the action of β-lactamases produced and released in the extracellular environment by the resistant strain. These enzymes were able to degrade only β-lactam drugs in case these were present in the extracellular environment.

### 5.2.7 Studies of variability due to differences in genes involved in the OMVs biogenesis and their promoters

As already described in Chapter 4, the *K. pneumoniae* isolates were divided into 9 sheltering groups and 1 non-sheltering group. These groups are listed in Table 5.2.

**Table 5.2 Non-Sheltering and sheltering groups**. The groups of *K. pneumoniae* isolates producing sheltered areas having values within each of the ranges listed are reported.

| Sheltering groups | Isolates |
|---|---|
| 1 | K4, K5, K9, K22, K26, K28, K29, K41, K47, K56 |
| 2 | K12, K13, K24, K25, K27, K48 |
| 3 | K1, K2, K8, K18, K23, K51 |
| 4 | K7, K15, K16, K30 |
| 5 | K10 |
| 6 | K11, K20 |
| 7 | |
| 8 | |
| 9 | |
| 10 | K3, K17, K19 |

However the analyses carried out so far showed that the variations in the sheltering potential detected among the isolates were due neither to the differences in growth rates of the *K. pneumoniae* isolates nor to their genetic features including the RAPD profile and the presence of the genes for the resistance to the β-lactams. Furthermore, the variations in the amino acid sequence of the genes for the β-lactamases were not responsible for the differences in the sheltering effect among the isolates. However, the variations observed might be due to differences in genes that are not directly responsible for the resistance to the β-lactamases. Since the sheltering effect is due to the extracellular release of β-lactamases in OMVs, variations in the OMV biogenesis affecting the OMVs properties including OMVs size, stability and protein cargo might be responsible for the differences in sheltering potential observed among the isolates (Park et al., 2012; Schwechheimer and Kuehn,

2015; Elhenawy et al., 2016). The aim of the present project was to compare the amino acid sequence of the genes involved in the biogenesis of the OMVs produced by the *K. pneumoniae* isolates. Indeed, the variability in the sheltering effect might be due to differences in the sequence of these proteins that altered the properties of the OMVs.

With regards to this, the DNA sequence of the OMVs biogenesis genes identified in *S. enterica* was retrieved from the NCBI database. These genes were *ompA, nlpI*, *tolR*, *rfaE*, *waaC*, *yipP*, *mrcB*, *degS* and *hns*. With this in mind BLAST was used on each of these sequences to search for the homologies with the corresponding genes in the entire genome of the *K. pneumoniae* isolates sequenced. The DNA sequence of the corresponding genes was translated into protein sequence with the tool Translator (Gasteiger et al., 2003). Lastly the amino acid sequences were compared with the aim to find amino acid changes. However no corresponding gene was found in the genome of the isolates when the search was carried out with the sequence of the *nlpl* and *hns* genes. However these resuts don't necessarily excude the presence of these genes in *K. pneumoniae*. Indeed, the sequence of these genes in *Salmonella* might be quite different from *Klebsiella* ones that the BLAST search failed in finding significant similarity between the two species. Furthermore none of the genes analysed were found in K5, K7 and K28. Also, *tolR* was not found in K12, K21, K24, K25, K47 and K51 and *waaC* was not found in K21 and K30. Furthermore BLAST failed to identity the *degS* gene in K10 and K30. However this might be due to a fragmentation in the sequence of these genes in different contigs as a result of a failure during assembly process.

The multiple sequence alignment of the amino acid sequences of the OmpA, MrcB, RfaE, YipP and DegS proteins revealed that all the isolates shared the same primary sequence. The alignment of the sequences for WaaC showed that the amino acid in position 49 was either a glutamic acid (E) or a lysine (K) (Figure 5.9). The isolates K11, K20, K47, K1, K2 and K8 differed from the other isolates by a serine (S) instead of a threonine (T) in position 118. The isolate K20 had a leucine (L) in place of a arginine (R) in position 2. Furthermore the isolate K11 showed a histidine (H) in position 29 instead of a proline (P). Both K11 and K22 differed from the other isolates by the presence of a tryptophan (W) in position 95 instead of a glycine. The isolate K47 showed an alanine (A) in position 36 instead of a valine (V) and a T instead of an A in position 77. The position 106 was occupied by a methionine (M) in place of a L in the isolate K1.


**Figure 5.9 Multiple alignment of the WaaC amino acid sequences of the *K. pneumoniae* Isolates.** For clarity only the aligned regions including the different amino acids are reported. The different aminoacids are distinguished by different colours.

The multiple sequence alignment of the TolR amino acid sequences revealed K10 differed from all the other isolates by several amino acid substitutions reported in Figure 5.10.


**Figure 5.10 Multiple alignment of the TolR amino acid sequence of the *K. pneumoniae* isolates.**

For clarity only the aligned regions including the different amino acids are reported. The different amino acids are distinguished by different colours.

However, the variations observed in the amino acid sequence of each of these proteins among the isolates did not reflect the variability level in the sheltering effect observed for the *K. pneumoniae* isolates. With regards to this the number of sheltering and non-sheltering groups was much higher than the number of different amino acid sequences found for each protein. Indeed isolates with the same sequence were classified in different sheltering groups. As an example the isolates carrying E in the position 118 of the amino acid sequence of WaaC (K11, K20, K4, K5, K7, K9, K10, K26, K41, K48, K51, K56 and K22), were split in different sheltering groups (Table 5.2) including group 1, 2, 3, 4, 5 and 6. Vice versa, the sheltering groups included isolates having differences in their amino acid sequence. As an example the sheltering group 3 included the isolates K1, K2, K8, K18, K23 and K51 having differences in the amino acid sequence of WaaC.

However, variations in the promoter regions of the genes for the proteins involved in OMVs biogenesis might lead to differences in the expression levels of these proteins. This might ultimately affect the sheltering potential of the isolates. Since promoter regions are generally found upstream of the regulated genes and their length can vary from 100 to 1000 base pairs (Sharan, 2007), the 1000 base pairs region upstream of respectively *ompA, waaC, mrcB, rfaE, yipP, degS* and *tolR* was retrieved for each isolate. Then for each upstream region the DNA sequences all the isolates were aligned with ClustalOmega (Sievers et al., 2014). The multiple sequence alignment of the promoters of the *ompA*, *rfaE, yipP* revealed that all the isolates shared the same sequence. With regards to the promoters of the genes *waaC, mrcB*, *degS* and *TolR* a phylogenetic tree was built based on the alignment of the promoters of each gene with Jalview by using the Neighbor Joining method (Waterhouse et al., 2009). The phylogenetic trees for these promoters are reported respectively in Figures 5.11, 5.12, 5.13 and 5.14.

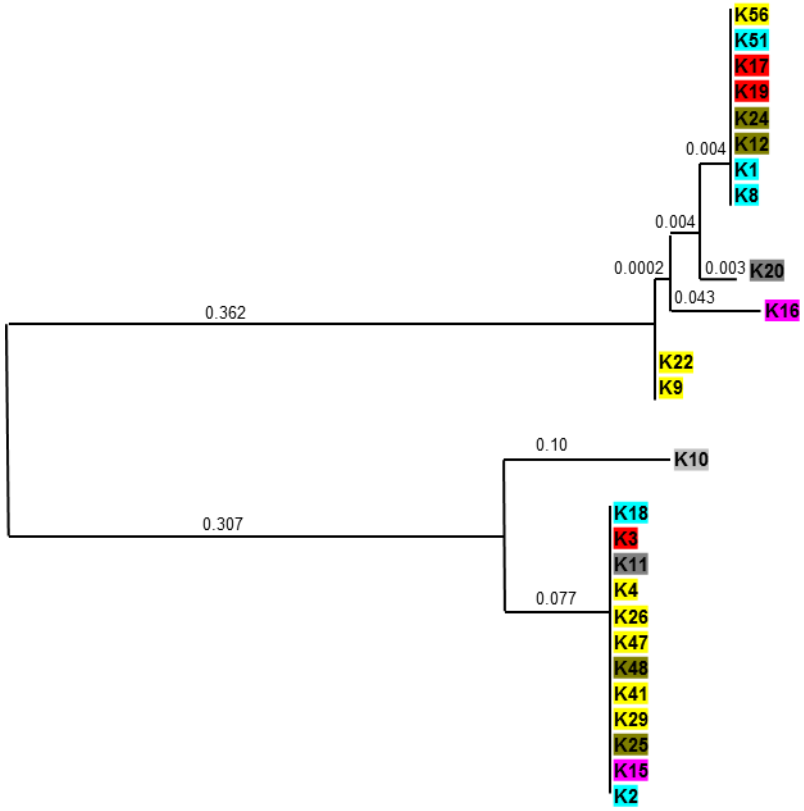



**Figure 5.11 Phylogenetic tree generated from the alignment of the promoters for *waaC*.** The genetic distances are shown above the branches. The isolates that are clustered in the same group (represented as a vertical line) have no genetic distance.

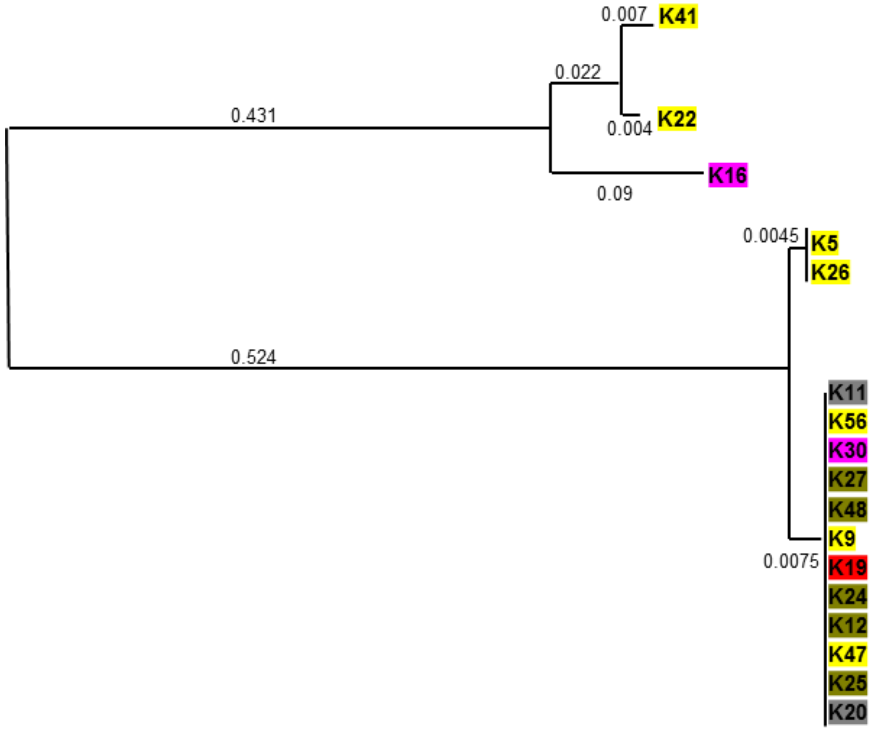



**Figure 5.12 Phylogenetic tree generated from the alignment of the promoters for *mrcB*.** The genetic distances are shown above the branches. The isolates that are clustered in the same group (represented as a vertical line) have no genetic distance.

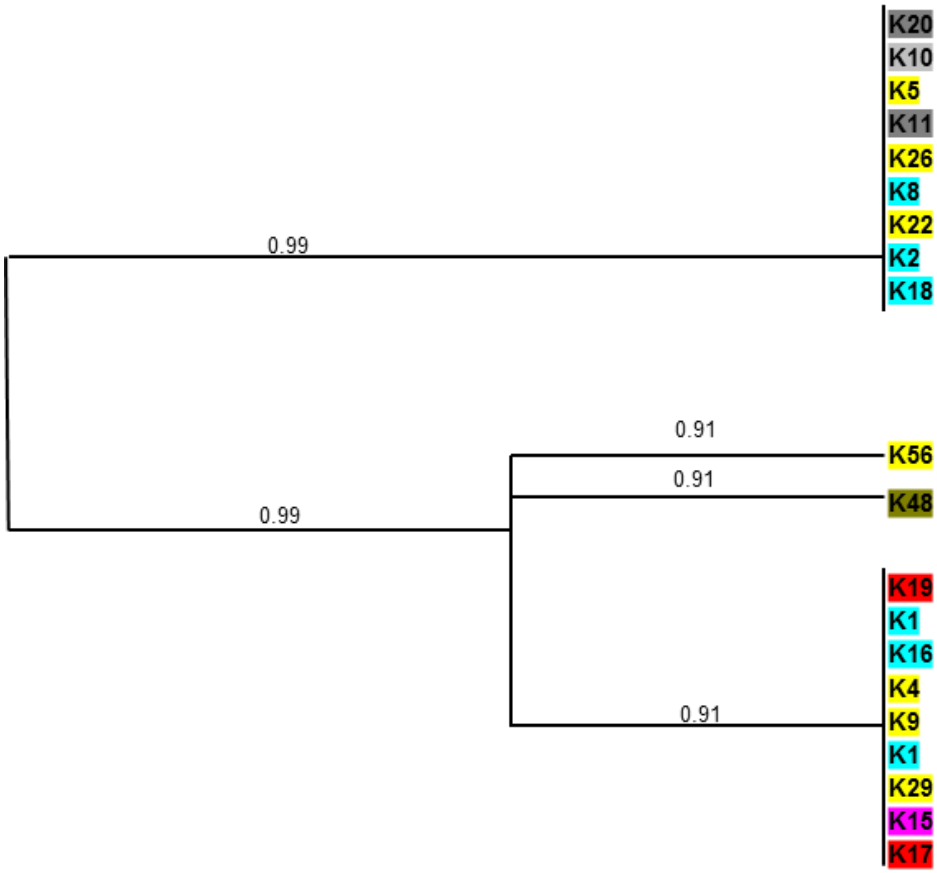



**Figure 5.13 Phylogenetic tree generated from the alignment of the promoters for *tolR*.** The genetic distances are shown above the branches. The isolates that are clustered in the same group (represented as a vertical line) have no genetic distance.

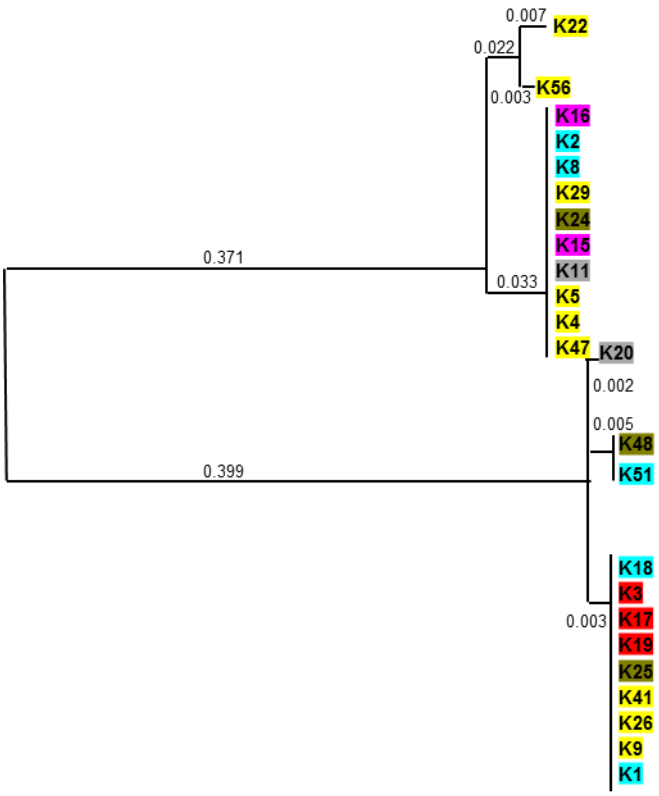



**Figure 5.14 Phylogenetic tree generated from the alignment of the promoters for *degS*.** The genetic distances are shown above the branches. The isolates that are clustered in the same group (represented as a vertical line) have no genetic distance.

However, no correspondence was identified between the different clusters of isolates for each promoter and the different sheltering groups. Indeed the results showed that in all the trees generated the clusters having no genetic distance included isolates belonging to different sheltering groups. As an example the cluster of the isolates including K18, K3, K11, K4, K26, K47, K41, K29, K15, K48, K25 and K2 shared the same upstream sequence for *waaC* but they were split in the sheltering groups 1 2 3 4 6 and 10 (Figure 5.11 and Table 5.2). Also the cluster including K19, K3, K16, K4, K9, K1, K29, K15 and K17 shared the same upstream sequence for *tolR.* Nevertheless these isolates were split in different sheltering groups that were 10, 4, 1 and 3 (Figure 5.13 and Table 5.2).

## 5.3 Discussion

The OMVs produced by Gram-negative bacteria are known to protect the producer bacterium and others thus forming a common defense for the bacterial community against specific antibiotics (Lee et al., 2012; Kulkarni et al., 2015).

Previous studies showed that *K. pneumoniae* produces OMVs that contain numerous proteins originating from OM and periplasmic space in addition to the inner

membrane and cytoplasm. Furthermore, these OMVs play a role in the pathogenesis by absorbing complement proteins, and thus inducing the innate immune response (Lee et al., 2012; Doorduijn et al., 2016). However to date, knowledge on their role in the sheltering effect has not yet been determined.

The results obtained in this project suggested that *K. pneumoniae* produced β-lactamases-containing OMVs. Indeed, the treatment of the Sf of K17 with PK, TR and PK and TR together suggested that the sheltering effect was due partly to β-lactamases included in OMVs. With regards to this both the β-lactamases included in OMVs and naked β-lactamases appeared to contribute equally to the sheltering effect in the experiments carried out here. To date, the active extracellular release of naked β-lactamases has not been reported in literature. It is therefore possible that the naked enzymes found in the extracellular environment of *K. pneumoniae* might also be β-lactamases located in the periplasmic space that were released in the medium following the rupture of the bacterial cells during the experimental procedure (Kelly and Kenneth, 2008; Shehadul Islam, et al., 2017). This observation suggested that the sheltering effect detected in *K. pneumoniae* occurred similarly to *A. baumanii.* Indeed in this bacterium the protective effect towards other susceptible species was the result of naked β-lactamases in addition to enzymes associated to OMVs (Liao et al., 2015).

The addition of increasing concentrations of Rb significantly reduced the extent of the sheltered areas of *E.coli* NCTC 10418. This suggests that the Sec-dependent pathway was responsible for the transfer of the β-lactamases produced by *K. pneumoniae* to the periplasm and OM, similarly to *A. baumannii* (Liao et al., 2015). However, a protocol for the microscopic detection of the β-lactamases responsible for the sheltering effect in *K. pneumoniae* was tested in *A. baumannii* since the production and extracellular release of carabapenemases-producing OMVs was confirmed in this bacterium (Liao et al., 2014; Liao et al., 2015). The protocol tested was based on the generation of the fusion product OXA-58-GFP, cloning in the plasmid vector pyMAb2 and transformation in electrocompetent cells of *A. baumannii* ATCC 19606. These cells were then visualised by confocal microscopy in order to detect the construct OXA-58-GFP in the periplasm and in the OMVs. However, the data obtained with confocal microscopy suggested that the transformed *A. baumannii* ATCC 19606 cells expressed the fusion product at a very low level. This result was also confirmed by the Western blot analyses. Therefore, this protocol set-up for the visualization of OMVs in *A. baumannii* needed to be modified and optimized to produce high levels of fusion protein in *A. baumannii*. A possibility could be the use of Red Fluorescent Protein (RFP) in place of GFP. Indeed, previous studies showed that the investigation of the subcellular localization of envelope proteins could be

hindered by problems with the export of GFP to the periplasm. This is due to the fact that GFP is exported to the periplasm in an unfolded conformation through the Sec system (Feilmeier et al., 2000; Dinh and Bernhardt, 2011). Therefore the variants of GFP commonly used in subcellular localization studies of periplasmic proteins do not fold properly and do not fluoresce. Instead, RFP derivatives can be effectively transported to the periplasm through the Sec system (Chen et al., 2005; Lewenza et al., 2006; Dinh and Bernhardt, 2011).

The results obtained showed that the areas formed by the susceptible strain were smaller in presence of higher antibiotic concentrations. This suggested that the increase in Amp concentrations had no effect on the OMV production and/or β-lactamase expression. However, in absence of information on the specifics of concentration gradients and diffusion rates of the OMVs. it can't be ruled out that the reduction in sheltering effect could also be explained by a reduction in OMV production or a reduction in β-lactamase expression. However, a reduction of β-lactamase expression in presence of increasing antibiotic concentrations is highly unlikely . Indeed bacteria that reduce β-lactamase expression when they need it most would not survive and be eliminated by natural selection (Shaikh et al., 2015). Furthermore, to this knowledge, no reduction in OMVs production in presence of environmental stress has been reported. Instead, the presence of stressors, including the presence of antibiotics in the medium, is known to stimulate the production of OMVs (MacDonald and Kuehn, 2013; Jan, 2017). Future research might explore whether the packaging and release of OMVs is a direct consequence of gene expression or a response to the antibiotic itself. With regards to this an inducible promoter might be used to regulate the gene expression of the β-lactamases. Then a possible study could involve the increase of antibiotic concentration while the expression of the β-lactamases is kept constant. An increased sheltering might indicate that the packaging and/or release of β-lactamases-including OMVs is directly affected by the antibiotic. A second test might involve the increase of gene expression in presence of a constant antibiotic concentration. In this case an increased OMVs packaging and release might indicate that the sheltering effect is a direct consequence of more enzyme activity rather than having more antibiotic in the extracellular environment of the bacterium.

The results obtained in this study also suggest that *K. pneumoniae* provided a constant protective effect to the susceptible strain over time. Indeed, the growth of colonies in sites more and more distant from the resistant strain at different times suggested that the OMVs were produced in a continuous way and diffused as they were released from the resistant strain.

The results obtained suggested that *K. pneumoniae* did not shelter *E.coli* from the action of 4 drugs different from the β-lactams, including Gent, Amk, Chlor and Cipr. However, a higher number of non β-lactams drugs should be tested in order to investigate whether the sheltering effect is exclusively due to the β-lactamases. Indeed, it is known that OMVs act as carriage of proteases and endopeptidases in addition to β-lactamases (Beveridge et al., 1997; Jan, 2017). The non β-lactams drugs tested might include antimicrobials with an mechanism of action different from that adopted by the antimicrobials already tested. A possibility could be the use of sulfonamides and trimethoprim that inhibit folic acid metabolism (Kapoor et al., 2017). Indeed, it is known that *K. pneumoniae* isolates can develop resistance to sulfonamides and trimethoprim as a result of the expression of dihydrofolate reductase and sulfonamide-resistant dihydropteroate synthases (Kumar et al., 2011; Ferreira et al., 2019). Therefore future research might focus on the presence of these enzymes in the *K. pneumoniae* OMVs provided that the sheltering effect is detected in presence of sulfonamides and trimethoprim.

The results obtained suggest that the variations in the amino acid sequence of the proteins involved in the biogenesis of the OMVs produced by the *K. pneumoniae* isolates did not reflect the variability in the sheltering potential observed. This suggests that extent of sheltering effect is determined by other proteins and/or by a more complex network of proteins. A possibility might be the study of the variations in the proteins involved in the Sec-dependent pathway (Beckwith, 2013). Differences among the amino acid sequence of these proteins might be responsible for changes in the amount of β-lactamases exported to the bacterial periplasm and OM. Also, although genetic differences in the promoters of *waaC, mrcB, tolR* and *degS* were found among the isolates, these variations did not appear to reflect the variability level in the sheltering effect. Furthermore variations in the promoter regions might not be sufficient to determine differences in the expression levels. Indeed, genetic elements that are at a distance from the gene might also be involved in the regulation of the expression level. With regards to this, in bacteria distal regulatory regions called ‘bacterial enhancers” have been found and DNA looping has been shown directly to be involved in promoter expression (Amit et al., 2011; Brunwasser-Meirom et al., 2016). Therefore genetic variations in these regions might lead to differences in the expression level of the genes involved in the OMVs biogenesis. With this in mind, future research might involve the study of the expression levels of the proteins involved in the OMVs biogenesis with experimental approaches. These could include the use of Western blot for verification of protein production in each isolate. Furthermore, experimental techniques might be used to investigate the amount of RNA produced for each gene. For example real-time reverse transcription PCR (real-

time RT-PCR) might be used to quantify gene expression (Wong and Medrano, 2005). Indeed, this technique is now considered a gold-standard methodology to study bacterial gene expression through relative quantitation of target RNAs (Rivera et al., 2015; Smith et al., 2018; Bai et al., 2020; Rocha et al., 2020).

# 6 GENERAL DISCUSSION

Microbial interactions happening within bacterial communities can result in an increase of evolutionary development and adaptation in each microorganism. Bacterial Interactions in polymicrobial infections have been found to promote the protection of other isolates from antibiotics, with the most sensitive strains experiencing the greatest benefit (Marjon et al., 2018). However, the nature of these interactions remain largely obscure since infective bacteria are commonly investigated in isolation (Dhamgaye et al., 2016; Marjon et al., 2018).

Studies carried out on a number of β-lactam resistant bacterial species show that in some cases bacteria do not need to transfer genes for resistance but instead release OMVs containing β-lactam-inactivating enzymes into their surrounding area (Liao et al., 2015). The release of these OMVs triggers the degradation of the antibiotics present in the environment, where other different bacterial species live. Therefore, this provides protection from the drug to the entire bacterial community, including resistant and non-resistant species (Liao et al., 2014; Liao et al., 2015).

The present work focused on investigating this phenomenon in *K. pneumoniae*. Here, future prospective based on the findings reported in this thesis will also be discussed.

## 6.1 Detection of the sheltering effect in *K. pneumoniae*

*K. pneumoniae* is regarded as one of the most important MDR pathogens affecting humans and a major source for hospital infections associated with high morbidity and mortality due to limited treatment options (Merla et al., 2019). Although this pathogen has been identified in the blood of patients with polymicrobial infections resistant the sheltering effect has not yet been investigated in this species (Rahim et al., 2018; Lin et al., 2013).

In this project the sheltering effect provided by *K. pneumonia*e was detected by observing satellite colonies and/or a continuous bacterial lawn formed by species susceptible to β-lactams in presence of resistant *K. pneumoniae* on Amp medium. To date, most studies used protocols based on the incubation of β-lactams-

susceptible bacterial strains with preparations of β-lactamase-carrying OMVs derived from resistant strains to detect the sheltering effect (Ciofu et al., 2000; Shaar et al., 2011; Khanna et al., 2013; Shaar et al., 2014; Chattopadhyay and Jaganandham, 2015; Liao et al., 2015). Compared to these protocols, the method developed here appears to be more time and equipment efficient. Therefore, it might be suitable for the implementation of a high throughput screening for large-scale studies aimed to quickly test the sheltering effect provided from hundreds or thousands of bacterial strains. High quality pictures of these plates would be then captured by using a scale bar, thus allowing to spatially calibrate each single image and consequently calculate the actual measurements on the plates. Moreover, the development of an automatic image analysis of the sheltering effect detected on plate might replace the more manual method used in this project for the quantification of the sheltering effect. The analysis of the sheltering effect detected on plate could involve the creation and use of a script that, by taking an image as an input, allows the quantification of the sheltering effect, i.e. the calculation of the distance at which the satellite colonies appear on the plates and the area formed by the susceptible species. This high-throughput screening could be used in the study of the sheltering effect in large numbers of clinical isolates that show β-lactam-resistance like *Klebsiella* species, *E. coli*, *A. baumannii*, *Enterobacter* species, *Salmonella* species, *Proteus* species, *Serratia marcescens*, *Shigella dysenteriae*, *Pseudomonas aeruginosa*, and *Burkholderia cepacia* (Podschun and Ullmann, 1998; Su and Chiu, 2007; Jones et al., 2001; Ferreira et al., 2011; Khanna et al., 2013; Liao et al., 2015; Sahl et al., 2015; Drzewiecka, 2016; Bassetti et al., 2018; Davin-Regli et al., 2019).

## 6.2. Variability in the sheltering effect

The sheltering effect was investigated in all the 33 *K. pneumonie* β-lactam resistant isolates available in laboratory. The results of this study showed that the sheltering effect was a widespread phenomenon in *K. pneumoniae*. However, the resistant isolates showed variability in the degree of sheltering effect they were able to provide to the susceptible species (Figures 3.4 and 3.5, paragraph 3.2.2). This variability was not the result of differences in the rate of diffusion of the protective entity through the agar. Indeed, all the plates were prepared by using the same medium preparation that contained a fixed concentration of agar. Furthermore, the variations observed were not due to differences in the inoculum density of either the resistant or the sensitive strain since the amounts of bacterium inoculated were the same for all the plates used. This suggested that the differences observed were the result of inherent variations in the protective entity.

### 6.2.1 Analyses for the study of the variability

In this project a number of different analyses were carried out to investigate the causes of sheltering variability, including the determination of the growth rates, MIC and RAPD profiles, the presence or absence of genes for β-lactamases and the determination of the amino acid sequence of the β-lactam-resistance genes. However, the differences among the isolates detected with each of these analyses were not related to the variations in the sheltering effect. Moreover, the variations found were not the result of differences in the genes involved in the OMVs biogenesis and their immediate 5' upstream promotors. Therefore, more studies are needed to elucidate the causes of variability of the sheltering effect in *K. pneumoniae.* With regards to this, future analyses might involve studies of mutagenesis aimed to detect the genes responsible for the sheltering effect. This analysis would require the selection of *K. pneumoniae* isolates whose sheltering potential is intermediate between the potential of high and low sheltering producers. For example, the isolates belonging to the $5^{th}$ and $6^{th}$ sheltering groups produce a range of sheltered areas halfway between the minimum and maximum value of sheltered areas detected for all the isolates (Table 4.3, paragraph 4.2.4.1). The mutagenesis of these isolates would allow to create mutants with a higher sheltering potential, mutants with a lower sheltering potential, and mutants that are no longer able to shelter. Moreover the high-throughput methodology described in section 6.1 above would allow rapid screening for mutants to identify those that have a sheltering effect different from the original strain. WGS might then be used to determine the sequence of the whole genome of these mutants. Indeed during the past two decades the WGS technologies has had a great impact in the identification and characterization of bacterial genetic determinants, granting the access to potential virulence determinants, known and new antibiotic resistance determinants, candidate drug compounds, mechanisms of pathogenicity, drug resistance and spread and their evolution in pathogens (Punina et al., 2015). Due to the obtained WGS data, over the last few years, a number of novel genetic targets have been identified and suggested for routine diagnostics of several pathogenic bacteria, for example tetM that encodes at tetracycline resistance protein and the genes for the β-lactamases OXA-51 and VIM-2 ($bla_{OXA-51}$ and $bla_{VIM-2}$) (Alex et al., 2012; Witney et al., 2014; Wright et al., 2014; Punina et al., 2015). Complementation tests should then be conducted to determine which mutations among all the genetic changes in the genome of the mutants are responsible for the altered sheltering effect phenotype. For example, changes found in genes involved in the OMVs biogenesis and/or packaging of the β-lactamases in OMVs might be responsible for differences in OMVs stability, OMVs

size and/or amount of enzyme packed in each OMV. Then these differences might account for the variability detected among the different isolates. In this project a relationship between the sheltering variability and the differences in the genes involved in OMVs biogenesis in *K. pneumoniae* was not found. However, the genes analysed were those retrieved by using the homolog genes in *S. Typhimurium*. Indeed in this bacterium the OMVs biogenesis has been well described (Bai et al., 2014). Therefore, it is possible that in *K. pneumoniae* additional genetic determinants are involved in this process and a mutagenesis approach could help in the identification of these genes.

A further approach could involve the use of differential proteomics to identify which proteins are increased or decreased relatively in two or more different *K. pneumoniae* isolates (Cordwell et al., 2001; Boysen et al., 2015; Soares et al., 2016). For example, the protein composition might be tested in two isolates, respectively with the highest and lowest sheltering potential (for example one isolate of the 1$^{st}$ sheltering group and one isolate of the 10$^{th}$ group, Table 4.3, paragraph 4.2.4.1) in order to investigate changes of proteome in the two isolates depending on the presence and absence of β-lactam drugs. This analysis might therefore help to identify target proteins and consequently genes that control the sheltering effect. The identification of potential targets could ultimately be used for the development of new compounds that inhibit these targets. Indeed once bacterial targets are identified , they can be incorporated into *in vitro* biochemical assays that permit high throughput screening of chemical libraries for the identification of inhibitory compounds and their antibacterial activity (Payne et al., 2006; Fields et al., 2017).

### 6.2.2 Methods for the quantification of the sheltering effect and their applications

Previous studies quantified the sheltering effect by measuring inhibitory growth zones of β-lactam-susceptible bacteria in presence of increasing concentrations of β-lactamase-containing OMVs (Schaar et al., 2011). In the present project two different methods for the direct quantification of the sheltering effect detected on plate were developed. Although these methods differed by the feature of the sheltering effect analysed (respectively the distance that the protective effect could reach from the resistant species and the area formed by the continuous cover of susceptible bacteria), both produced statistically similar results. This suggests that either quantification method could be in future used in studies of sheltering effect.

As frequently discussed in this thesis, a number of studies report that the sheltering effect is the result of the production and extracellular release of β-lactamases-

including-OMVs (Stentz et al., 2015). With regards to this, one of the causes of variability in the protective effect might be differences in the diffusion capacity of the OMVs released by the strains. For example, some strains might produce lots of large vesicles but these will not diffuse far resulting in the formation of a continuous cover of susceptible colonies growing close to the resistant strain. Instead, other strains might produce fewer but much smaller vesicles which would diffuse much further, thus leading to the appearance of isolated satellite colonies at a longer distance from the resistant strain. To date, different sizes for the OMVs have been observed in Gram-negative bacteria, ranging from 20 to 250 nm in diameter (Schwechheimer and Kuehn, 2015). Moreover, a heterogeneous population of OMVs can be produced by the same organisms (Kulp and Kuehn, 2010; Schwechheimer and Kuehn, 2015; Turner et al., 2018). However, the hypothesis that differences in the diffusion capacity of OMVs are responsible for the variability observed among the strains might be more supported if the difference between the distance-based and the area-based method in the analysis of the same event of sheltering effect was significant. Indeed, the analysis of sheltering effect provided by a producer of several large vesicles travelling a short distance should result in a high area value and a small distance value. Similarly, the sheltering effect provided by a producer of fewer and small vesicles travelling a longer distance would result in low area and high distance values. However, differences due to variations in the diffusion capacity of vesicles were not clearly evident from the analyses carried out in this study. Indeed, the different methods produced statistically similar results thus suggesting that either method could be used for the quantitative analyses. Nevertheless, the analyses for the comparison of the two methods were carried out on 15 strains of *K. pneumoniae.* Therefore, future studies might involve the use of both the distance-based and the area-based methods on a larger sample of isolates. Analysing the sheltering effect provided by a larger number of resistant *K. pneumoniae* and other species of sheltering bacteria, like *E. coli*, *A. baumannii*, might increase the possibility to find more variations in the protective entity. These could include producers of large areas (LA) and short-distance (SD) sheltering and producers of small areas (SA) and long-distance (LD) sheltering. Additionally, producers of LA and LD sheltering and producers of SA and SD sheltering might also be found (Figure 6.1). With regards to this, the analyses carried out in this study suggested that the 15 resistant strains analysed belonged to one of LA/LD or SA/SD categories since both the methods developed produced similar results for all the strains.

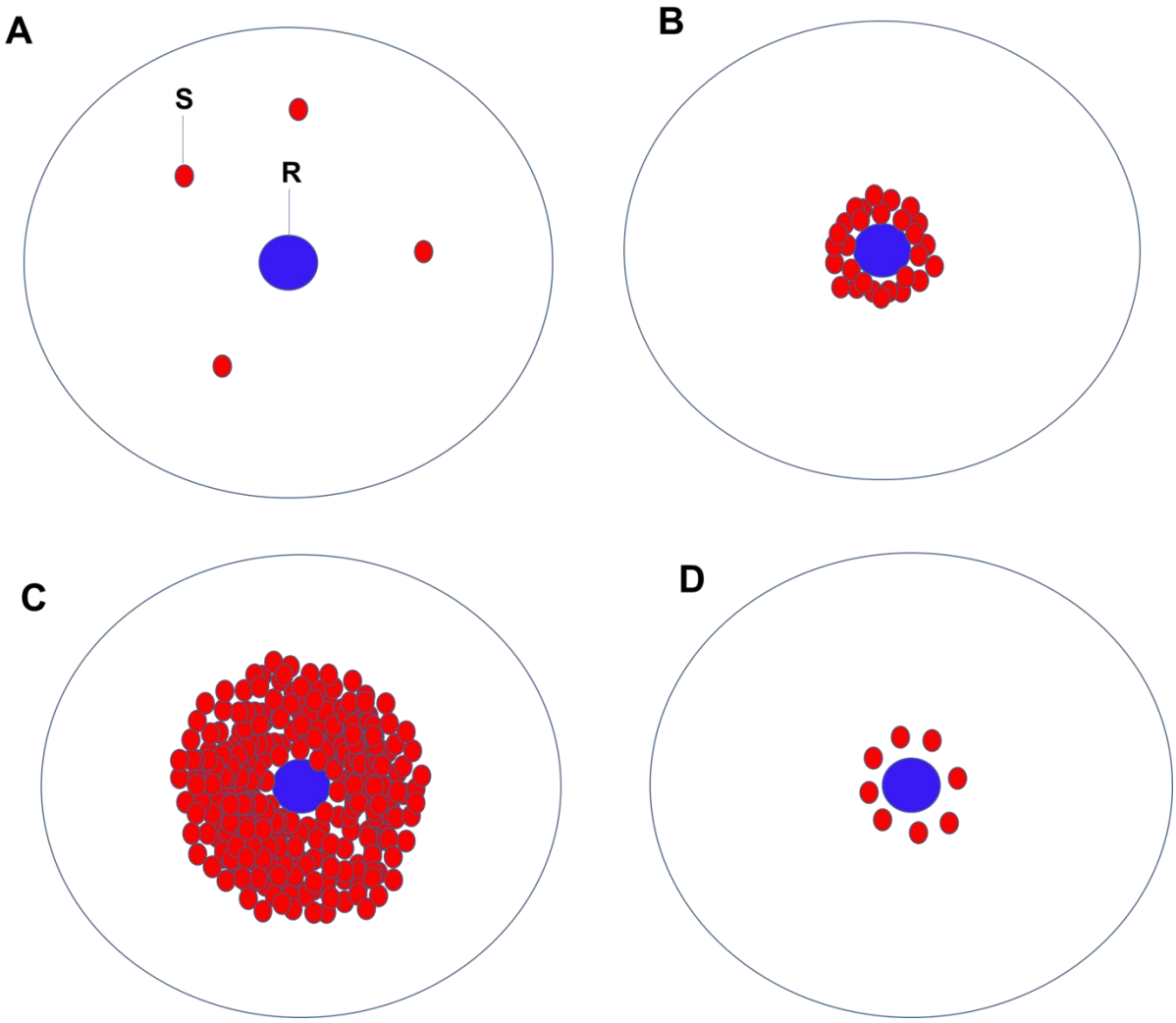



**Figure 6.1 Simplified representation of 4 possible appearances of the sheltering effect detected on plate. A.** The resistant strain (R) is responsible for a sheltering effect that produces small areas (SA) of the susceptible species (S) and at a long-distance (LD). **B.** The sheltering effect produced by R appears as a compact area of S in the immediate closeness of R. **C**. R produces a sheltering effect appearing as a large area (LA) that covers long distances (LD) from R. **D.** The sheltering effect produced by R takes the form of small areas of S growing close to R.

However, the analysis of sheltering effect on large scale might be facilitated by the use of the high throughput approach described in section 6.1. Indeed, the design of a script for the automatic calculation of the distance and area formed by the susceptible species would allow to quickly screen a large number of isolates.

## 6.3 OMVs in the sheltering effect

### 6.3.1 Protection of the susceptible species mediated by OMVs

Generally, OMVs represent a physical barrier for drugs and toxins, by acting as decoys for these molecules and therefore providing immediate protection before the bacteria can adapt by modifying or mutating antibiotic targets in the extracellular environment (Manning and Kuehn, 2011). However beyond reducing the effective antibiotic concentration in culture by adsorption, OMVs can also carry enzymes that mediate antibiotic protection, like β-lactamases. These would be responsible for an active degradation of the drugs in the extracellular environment of bacteria (Figure 1.14B, Chapter 1.3.5.2 ) (Manning and Kuehn, 2011; Kulkarni et al., 2014). Although previous studies reported the production of OMVs in *K. pneumoniae*, their role as transporters of β-lactamases to the extracellular environment has not yet been investigated (Lee et al., 2012; Doorduijn et al., 2016). The data collected here suggest that β-lactam-resistant *K. pneumoniae* produce β-lactamases-containing OMVs, as showed by the occurrence of sheltering effect in presence of protease treatment for the degradation of naked enzymes. However, the presence of β-lactamase activity in the extracellular environment was not tested in this project. Therefore, further studies might involve the detection of this activity in the agar medium where the resistant strain is inoculated with the same procedure used to test the presence of sheltering effect. This experiment would contribute to show that the sheltering effect is the result of OMVs-included β-lactamases that work actively detoxifying the extracellular environment from the drug. This study might be carried out by using a chromogenic β-lactam substrate, such as nitrocefin, that allows the rapid β-lactamase detection by changing color from yellow to red when hydrolyzed by a β-lactamase (Dai et al., 2012). The addition of nitrocefin in the agar medium used for the preparation of the plates might allow the detection of the β-lactamase activity in the extracellular environment of a *K. pneumoniae* resistant isolate inoculated on the plate. This experiment might be especially useful in the localization of the β-lactamases-including-OMVs and therefore of the distance reached from the OMVs released in the medium. However, this would require the development of an imaging system to monitor changes that are not visible to the naked eye, for example single OMVs that travel far from the resistant strain. For this purpose a magnification of at least x15,000 is desirable to visualize microscopically the OMVs (Valguarnera et al., 2018).

In addition to the detoxification of the environment from the drug, the OMVs could be responsible for the sheltering effect by entering the susceptible species and releasing

its enzymatic content. With regards to this, a number of studies reported the internalization of OMVs by host cells and release of the content inside these cells (Donoghue and Krachler, 2016; Jones et al., 2020, Cai et al., 2018). Nevertheless, to date, no study investigated the internalization of β-lactamases-including OMVs in susceptible bacterial cells in the context of the sheltering effect. Therefore, future studies might aim to test the internalization of *K. pneumoniae* β-lactamases-including OMVs in susceptible bacterial cells. A possibility might be the development of an assay based on Fluorescence Resonance Energy Microscopy (FRET), a process by which energy is transferred from an excited donor fluorophore to an acceptor when in close proximity (Mo et al., 2020). The occurrence of FRET is monitored using fluorescence microscopy by exciting the donor and measuring the emission of the acceptor and is generally used to demonstrate the close interaction of two proteins tagged with respectively two fluorescent tags, for example GFP and RFP (Mo et al., 2020). In the context of sheltering effect this assay might be used to detect the interaction between two proteins, respectively a β-lactamase tagged with GFP expressed and released in OMVs from a bacterial strain and a RFP expressed in the periplasm of a sensitive strain. The occurrence of FRET might suggest that the β-lactamase included In the OMVs are released inside the sensitive strain (Figure 6.2).

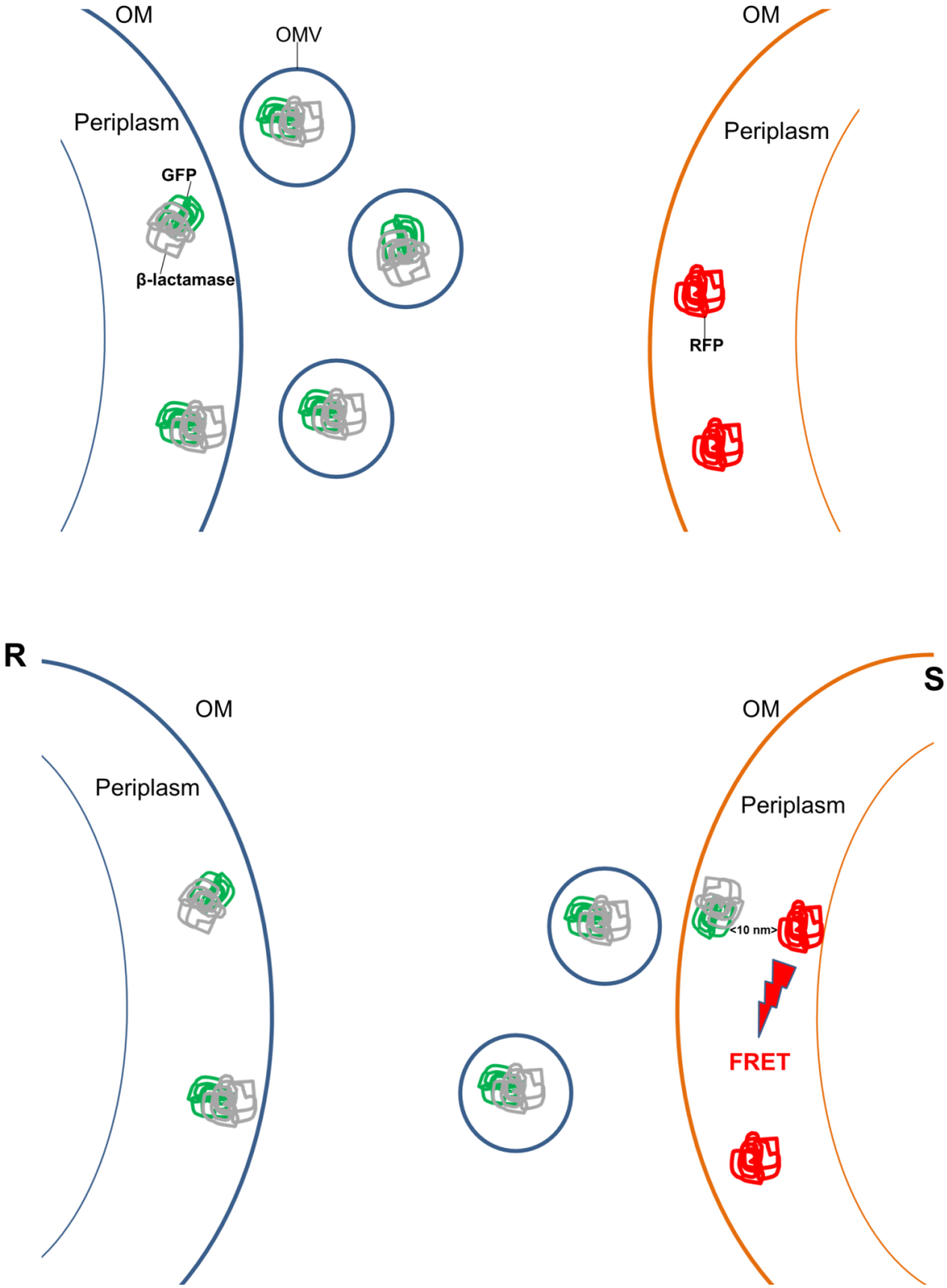



**Figure 6.2 Schematic representation of the FRET assay for the detection of the internalization of β-lactamases-including OMVs into a susceptible bacterium. Top.** OMVs are released by the OM a strain expressing a β-lactamases fused with the GFP (R) . A susceptible bacterial strain expressing the RFP in its periplasm (S) is in the surrounding of the R strain. **Bottom.** The internalization of the OMVs in S releases the β-lactamases fused with the GFP in the periplasm. The GFP transfers energy to the RFP when these two proteins are in proximity (1-10 nm) , thus allowing the RFP to emit red light (FRET).

## 6.4 The packaging of β-lactamases in OMVs as a benefit for the producer

Due to the high energy requirement for the bacterium to produce OMVs, it is reasonable to assume that the inclusion of the β-lactamases in OMVs represents a benefit for the producer. The first benefit is that the OMVs provide a mechanism to deliver the cargo in a concentrated form over longer distances. In fact, it was previously reported that by using OMVs, bacteria can deliver β-lactamases at high concentrations, thus allowing an efficient detoxification of the environment from the drug (Kulp and Kuehn, 2010; Schaar et al., 2011; Lee et al., 2013). A further benefit is the protection of these enzymes within OMVs. Indeed, a characteristic unique to OMV secretion is that the cargo can be released in a complex that provide protection from the extracellular proteases that often degrade secreted proteins (Kesty and Kuehn, 2004; Kulp and Kuehn, 2010). Future studies might involve the study of the half-life of naked β-lactamases and of the β-lactamases included in the OMVs released by *K. pneumoniae* with the aim to test the stability of these two groups of enzymes. Indeed, provided that the OMVs released by this bacterium protect the β-lactamases from degrading enzymes, the β-lactamases included in OMVs are expected to be more stable to degradation than their naked cohort.

## 6.5 Role of the Sec-dependent pathway in the sheltering effect

The results obtained showed that the sheltering effect was significantly reduced in presence of Rb, an inhibitor of the Sec-dependent pathway. Previous studies showed that the Sec-dependent pathway is responsible for the transfer of several proteins, including β-lactamases, from the cytosol to the periplasm (Liao et al., 2015). The system itself comprises two cytoplasmic proteins, respectively a chaperone protein (SecB) , an ATPase (SecA) and an integral membrane complex, also termed “holotranslocon”, that includes the SecYEG in complex with SecDFYajC and YidC. Protein domains of this transmembrane complex protrude on the periplasmic space of the cellular envelope. Previous studies that lead to the identification of the genes responsible for the OMVs biogenesis did not report involvement of the Sec genes in this process (Kulkarni and Jagannadham, 2014; Schwechheimer and Kuehn, 2015; Nevermann et al., 2019). However, to date, no study has been performed to investigate the involvement of the Sec machinery in the packaging of these proteins in OMVs. Consequently, it is not yet known whether members of the Sec-dependent

pathway play an active role in the packaging of the proteins in OMVs or whether this process occurs regardless of the machinery that transfers the proteins to the bacterial periplasm (Figure 6.3).

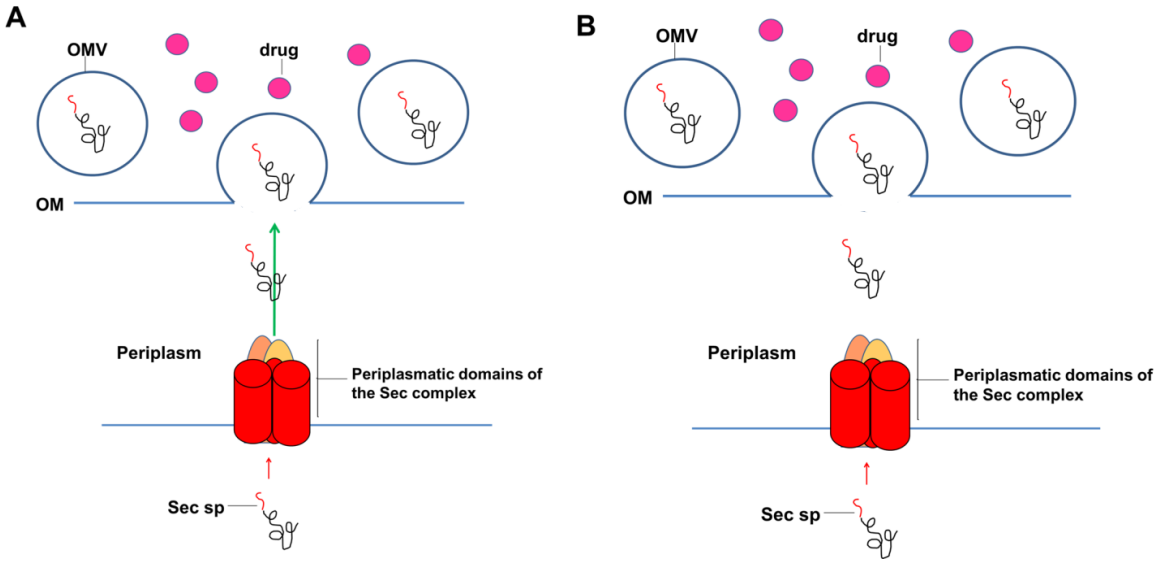



**Figure 6.3 The two hypotheses on the role of the Sec machinery in the packaging of β-lactamases in OMVs. A.** Following the translocation to the periplasm, the packaging of the β-lactamases in OMVs is assisted by periplasmic components of the Sec translocon. The involvement of these protein domains in the packaging of the β-lactamases in OMVs is represented by the green arrow. The resultant β-lactamases-including OMVs are released in the medium that contains the β-lactam drug. **B.** The Sec-dependent pathway is only required for the translocation of the enzyme in the periplasm and no Sec component has a role in the packaging. sp: signal peptide.

Therefore, more work needs to be done in order to investigate the role of this translocation pathway in the sheltering effect. In respect to this, the focus of future studies might be the use of a genetic construct carrying a β-lactamase gene fused with a twin-arginine translocation (Tat) signal peptide to investigate whether the use of a translocation system different from Sec might restore the sheltering effect in presence and inhibitor of Sec-dependent translocation such as Rose Bengal (Rb). The Tat peptide is responsible for the transfer of proteins to a membrane transporter (Tat translocon) different from the Sec complex. However, similarly to the Sec translocon, the Tat system transports proteins from the cytosol to the periplasm (Beckwith, 2013; Costa et al., 2015; Frain et al., 2019).

The involvement of the Sec-dependent pathway in the packaging of β-lactamases in OMVs might be confirmed by the inhibition of the sheltering effect produced by a resistant strain expressing a β-lactamase fused with a Tat signal peptide in presence of Rb and the β-lactam drug in the medium. Indeed, although the fusion protein is translocated to the periplasm by the Tat translocon, the lack of the periplasmic

members of the Sec machinery prevents the enzyme from being internalized in OMVs. On the contrary, the occurrence of sheltering effect might indicate that β-lactamases are packaged in OMVs regardless of the intervention of the Sec machinery in this process (Figure 6.4).

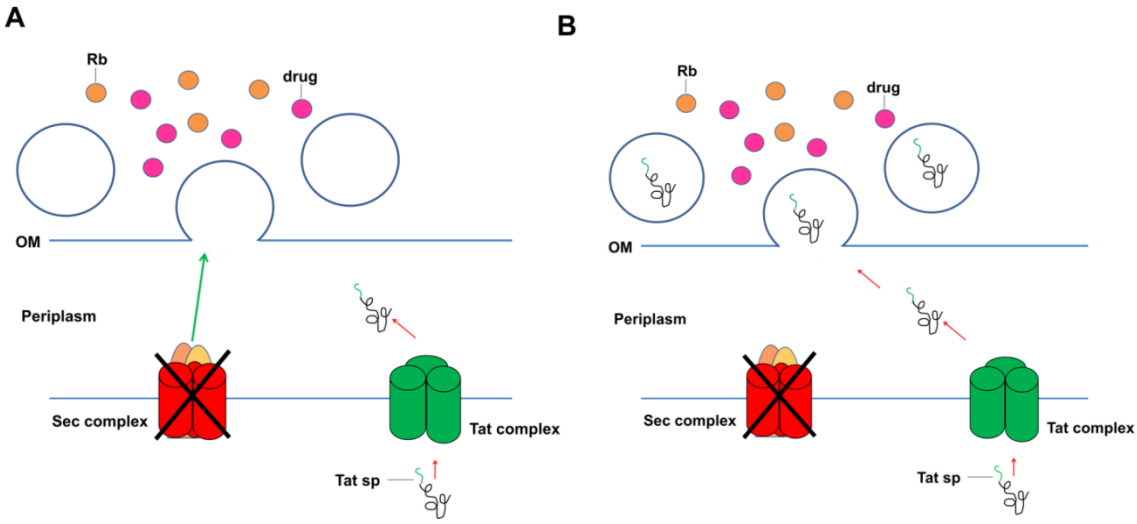



**Figure 6.4 Possible sceneries in response to the addition of Rb following the use of a β-lactamase fused with a Tat signal peptide (Tat sp). A.** The addition of Rb to the medium inhibits the Sec machinery thus blocking the translocation of β-lactamases in the periplasm and consequently the packaging of the enzymes in OMVs. A β-lactamase fused with a Tat sp is translocated to the periplasm by a Tat machinery but no packaging in OMVs occurs because of the lack of interaction with the protein members of Sec machinery involved in the transfer of the enzymes in OMVs.This transfer is represented by a green arrow. **B**. Following the translocation to the periplasm, β-lactamases are packaged in OMVs despite the lack of the Sec machinery, thus allowing the occurrence of sheltering effect in a medium containing Rb in addition to the drug.

## 6.6 Role of β-lactam concentration and time and in the sheltering effect

In this project a protocol for the confocal microscopic detection of the OMVs released by *A. baumannii* was developed with the aim to use the same procedure for the microscopic visualization of *K. pneumoniae*. The method was based on the production of a protein construct carrying the β-lactamase fused with the GFP, cloning in a plasmid vector and transformation in cells of *A. baumannii* ATCC 19606. The visualization of the construct OXA-58-GFP in the periplasm and in the OMVs was then carried out by confocal microscopy. However, this protocol needs to be modified to allow the visualization of the OMVs in the extracellular environment. A possibility might be the replacement of the GFP with the RFP that is not subject to

folding issues during the export to the periplasm (Chen et al., 2005; Lewenza et al., 2006; Dinh and Bernhardt, 2011).

Experiments testing the presence of sheltering effect on plate were used to assess variations of this phenomenon with antibiotic concentration and the time. According to these observations increasing concentrations of Amp seemed to have no effect or the OMV production and/or β-lactamase expression (Figure 5.6). However, since the production and release of β-lactamases-carrying OMVs requires an energetic cost to the bacterium, more analyses could be carried out to investigate whether the secretion of OMV-associated β-lactamases in *K. pneumoniae* is a constitutive process or whether it occurs only in presence of β-lactams. The focus of these analyses could therefore be the quantification of the OMVs released by *K. pneumoniae* in absence and in presence of Amp in the medium. A common methodology used for the quantification of OMVs released by bacteria in the medium involves the extraction of the supernatant from cultures of bacteria followed by pelleting of the OMVs by centrifugation and their resuspension in a buffer solution. The quantification of the OMVs is then generally performed by using a fluorescent lipophilic dye that binds the OMVs and allows the OMVs to emit fluorescence. The quantity of fluorescence is then used to determine the quantity of OMVs present in the sample (MacDonald and Kuehn, 2013; Donoghue and Krachler, 2016; Roier et al., 2016; Eberlein et al., 2019). Therefore, this procedure could be used with supernatants of resistant *K. pneumoniae* grown in presence and absence of Amp to determine changes in the amount of OMVs. The same method could be used to assay changes in the number of OMVs with the time of incubation. The use of the chromogenic β-lactam substrate nitrocefin could also be used to quantify the β-lactamase activity in the intracellular and extracellular fractions separated after growth of the resistant strain in presence and absence of Amp. Moreover, the extracellular fraction would be treated with protease to degrade naked β-lactamase and quantify only the enzyme present in OMVs. This would allow the comparison of the content of β-lactamases present in the bacterial cell and in the OMVs and determine changes in these amounts according to the presence of the drug.

## 6.7 Sheltering effect with non-β-lactam drugs

The main focus of the present project was the investigation of the sheltering effect in *K. pneumoniae* in presence of β-lactams, more specifically the Amp. Nevertheless, preliminary tests of the sheltering effect in presence of few non β-lactams drugs were also carried out. The results obtained suggested that the sheltering effect occurred only in presence of β-lactam antibiotics. In respect to this, several studies

investigated the sheltering effect that β-lactam-resistant strains provide to susceptible bacteria due to the β-lactamases included in OMVs (Shaar et al., 2011; Lee et al., 2013, Liao et al., 2015). In contrast, less work has been done to detect the sheltering effect as a result of non β-lactam enzymes present in OMVs. However, the sheltering effect was found to occur also in presence of non β-lactam drugs. For example, *E. coli* was found to release OMVs including proteases involved in the degradation of the antibiotic colistin and the antimicrobial peptide, melittin, thus protecting the producer bacterium and other bacterial species from the action of these drugs (Kulkarni et al., 2015). This observation suggests that more work needs to be done in order to understand whether *K. peumoniae* protects susceptible species from the action of drugs different from non-β-lactams. In respect to this, as mentioned in paragraph 5.3, Chapter 5, future studies might focus on testing the sheltering effect in presence of non-β-lactams drugs different from those used in this study. This would also require to work with *K. pneumoniae* strains that are resistant to the drugs to be tested. Provided that the sheltering effect is found with any of the drugs to be tested, studies for the identification and characterization of the protein content of the *K. pneumoniae* OMVs might be carried out to determine the enzymes responsible for this phenomenon. Previous studies characterized the protein content in OMVs produced by *K. pneumoniae* (Lee et al, 2012; You et al., 2017; Martora et al., 2019). Nevertheless, to this knowledge, no proteins for the resistance to antibiotics have been reported in OMVs produced by *K. pneumoniae.* However, the protein content and composition has been reported to vary widely between OMV preparations derived under different growth conditions (McBroom and Kuehn, 2007; Lee et al, 2012; Donoghue and Krachler, 2016). Consequently, it is reasonable to assume that enzymes for the resistance to antibiotics, including β-lactamases, might be found in the OMVs when the bacterial cells are grown under antibiotic stress. Therefore, it might be worth determining the protein content of the *K. pneumoniae* OMVs in presence of different antibiotics. A possibility might be the purification of OMVs followed by liquid chromatography-mass spectrometry (LC-MS/MS) and data analysed using softwares, for example MASCOT, that use mass spectrometry data to identify proteins from peptide sequence databases (Chen et al., 2009; Pitt, 2009).

## 6.8 The role of sheltering effect in the microbial interactions

The results obtained so far show that the sheltering effect is a widespread phenomenon in *K. pneumonia*e. Indeed β-lactam-resistant *K. pneumoniae* were able to protect β-lactam-susceptible bacterial species from the action of β-lactams. The data also suggest that this effect occurs regardless of the species of

the bacterium protected since *K. pneumoniae* was able to shelter both Gram-negative and Gram-positive species. In the context of microbial interactions the sheltering effect might be regarded as an extreme form of cooperation, i.e. altruism, as the sheltered species is apparently the only organism to receive the benefit (Refardt et al., 2013; Lewin-Epstein et al., 2017). With regards to this, forms of bacterial altruism have been reported in microbial populations under antibiotic stress. In particular, Lee et al. showed that norfloxacin-resistant *E.coli* shielded less resistant isolates from antibiotic insult by producing and sharing among the population the metabolite indole, a signaling molecule implicated in stress tolerance in *E. coli* (Hirakawa et al., 2004; Lee et al., 2010a; Lee et al., 2010b). The production of this molecule has been therefore regarded as an act of altruism as it provides protection to more vulnerable bacteria while it comes at a fitness cost to the highly resistant isolates (Lee et al., 2010a).

Nevertheless, the sheltering effect might also be simply regarded as a side effect of the extracellular release of β-lactamases from the resistant species. Indeed, the extrusion of these enzymes is a process occurring regardless of the presence of other susceptible species in the medium. With regards to this, analyses carried out in *M. catarrhalis* showed that a suspension of OMVs isolated from amoxicillin-resistant strains of this bacterium protect sensitive *S. pneumoniae* and *H. influenzae* by inactivating Amoxicillin (Schaar et al., 2011) This suggests that the resistant species produces and releases β-lactamases-included OMVs irrespective of the presence of other species in the medium.

However, the survival and growth of the susceptible species might lead to a mutually beneficial relationship between the sheltering and the sheltered species. In respect to this, the presence of benefits for the sheltering species was not investigated. Nevertheless, this was the result of the purpose of the methodology developed that was based exclusively on the detection of the benefit to the susceptible species without regard to any potential advantage to the resistant species. In the context of the microbial cooperations, the sheltered species might be involved in the production and release of public goods that can benefit the resistant species. In respect to this, cocultures made up of two or more different populations of cells were found to improve the cultivation success for certain populations (Goers et al., 2014). This has been well documented for the growth of unculturable bacteria. For example, the growth of isolates of the marine bacterial genus *Prochlorococcus* in pure culture is dependent on growth-promoting factors produced by heterotrophic bacteria (Stewart, 2012). Similarly, a number of anaerobic thermophiles in the family Clostridiaceae were found to be dependent on cell extract from *Geobacillus toebii* (Kim et al., 2008; Goers et al., 2014). Additionally growth promotion of strains of *E.coli* was also

observed when these organisms were cultured in presence of supernatants from cultures of bacteria belonging to the phyla Bacteroidetes and Firmicutes (Geesink e al., 2018). Future analyses could focus on the investigation of the existence of mutual beneficial interactions as a result of the sheltering effect. A possible experiment could involve the cultivation of resistant *K. pneumoniae* in presence of supernatants from cultures of the two susceptible strains used in this project, i.e. *E. coli* NCTC 10418 and *S. aureus* NCTC 6571. This might help to see if the supernatants of these susceptible species can exert growth-promoting effects on the resistant species, thus suggesting the presence of growth-promoting factors released in the medium by the satellite colonies of susceptible species.

An interesting study carried out in *P. aeruginosa* showed that OMVs were released after explosive bacterial cell lysis of a subpopulation of *P. aeruginosa* cells in the bacterial community. This process is thought to serve as a release of public goods in *P.aeruginosa* biofilms, thus providing benefits for the whole population (Turnbull et al., 2016). The lysis is mainly due to the upregulated expression of a prophage endolysin-encoding gene (*lys*) after exposure to exogenous stresses, like the antibiotic ciprofloxacin. Future studies might involve the investigation of this phenomenon in *K. pneumonia*e in order to test whether the explosive cell lysis of resistant β-lactam *K. pneumoniae* is a mechanism for the production of β-lactamases-including-OMVs. For example, similarly to the study carried out in *P. aeruginosa*, live-cell super-resolution microscopy might be used to detect changes in the morphology of the cells like the transition of rod-shaped cells to round cells that subsequently explode resulting in the annihilation of the cell (Turnbull et al., 2016).The formation of the OMVs might then monitored by culturing the cells in the presence of a fluorescent membrane stain that allows to visualize fluorescent particles compatible with the OMVs.

## 6.9 Clinical significance of the sheltering effect

Clinically, the role of polymicrobial infections on patient outcomes remains poorly studied. Although the seriousness of these infections has been recognized globally, poor research has been so far carried out to understand how the microorganisms interact and whether their interactions alter bacterial growth and ecosystem resilience in the presence and absence of antibiotics (Dhamgaye et al., 2016; Rupp et al., 2020).

The analysis of the sheltering effect carried out in this project might have clinical relevance. For this purpose the protocol developed here for the study of the sheltering effect might be used to quickly detect and quantify the sheltering effect in

presence of polymicrobial infections in clinical setting. The studies of variability might be used to investigate if the clinical outcome in a patient with polymicrobial infection is affected by the potential of sheltering effect the resistant strain is able to provide. For example, a strain that shows a high sheltering effect potential could be associated to severe illness and a high resistance to the antibiotic treatment. Vice versa a strain with a low potential of sheltering effect might cause milder symptoms and lower antibiotic resistance. Ultimately the identification of potential targets with the mutagenesis approach described above might allow to uncover new insights for novel therapeutic and consequently help clinic staff to treat antibiotic resistant polymicrobial infections or adopt preventative strategies.

## 6.10 Concluding remarks

In summary, the work presented here shows that *K. pneumoniae* is able to shelter susceptible species from the action of β-lactam drugs. The amount of this phenomenon varies within this bacterial species. The data obtained also suggest a role of the OMVs in the sheltering effect provided by this bacterium and additional analyses need to be carried out to elucidate the details of the mechanism of protection provided by the OMVs to the susceptible species. Furthermore, more work needs to be carried out to elucidate the causes of variability in the sheltering effect, for example differences in the number, size, and/or stability of the OMVs released or differences in the concentration of β-lactamases inside the OMVs. Lastly, future studies might investigate whether the sheltering effect benefits the producer strain in addition to the sheltered species, thus taking the form of a synergic interaction in the context of a poymicrobial community.

# 7 References


Abisado, R.G., Benomar, S., Klaus, J.R., Dandekar, A.A. and Chandler, J.R., 2018. Bacterial Quorum Sensing and Microbial Community Interactions. *mBio,* 9 (3), pp.2331-2340.

Abraham, E.P. and Chain, E., 1940. An Enzyme from Bacteria able to Destroy Penicillin. *Nature (London),*146 (3713), pp. 837-845.

Abrudan, M.I., Smakman, F., Grimbergen, A.J., Westhoff, S., Miller, E.L., van Wezel, G.P., and Rozen, D.E. 2015. Socially mediated induction and suppression of antibiosis during bacterial coexistence. *Proceedings of the National Academy of Sciences of the United States of America,* 112 (35), pp.11054-11059.

Adamski, C.J., Cardenas, A.M., Brown, N.G., Horton, L.B., Sankaran, B., Prasad, B.V.V., Gilbert, H.F. and Palzkill, T., 2015. Molecular Basis for the Catalytic Specificity of the CTX-M Extended-Spectrum β-Lactamases. *Biochemistry,* 54 (2), pp. 447-457.

Adedeji, W.A., 2016. THE TREASURE CALLED ANTIBIOTICS. *Annals of Ibadan postgraduate medicine,* 14 (2), pp. 56-57.

Aggarwal, R. and Dewan, A., 2018. Comparison of nephrotoxicity of Colistin with Polymyxin B administered in currently recommended doses: a prospective study. *Annals of clinical microbiology and antimicrobials,* 17 (1), pp.15-20.

Al-Obaidi, M.M.J., Suhaili, Z. and Desa, M.N.M., 2018. Genotyping Approaches for Identification and Characterization of Staphylococcus aureus. *IntechOpen*

Alam, M.M., Islam, M., Wahab, A. and Billah, M., 2019. Antimicrobial Resistance Crisis and Combating Approaches. *Journal of medicine*, 20 (1), pp.38-45.

Albiger, B., Glasner, C., Struelens, M.J., Grundmann, H. and Monnet, D.L., 2015. Carbapenemase-producing Enterobacteriaceae in Europe: assessment by national experts from 38 countries, May 2015. *Eurosurveillance,* 20 (45), pp.17-34.

Aldred, K.J., Kerns, R.J. and Osheroff, N., 2014. Mechanism of Quinolone Action and Resistance. *Biochemistry (Easton),* 53 (10), pp.1565-1574

Alekshun M.N. and Levy S.B., 2007. Molecular Mechanisms of Antibacterial Multidrug Resistance. *Cell,* 128 (1), pp. 1037-1050.

Alex J. McCarthy, Willem van Wamel, Stien Vandendriessche, Jesper Larsen, Olivier Denis, Cristina Garcia-Graells, Ann-Catrin Uhlemann, Franklin D. Lowy, Robert Skov and Jodi A. Lindsay, 2012. Staphylococcus aureus CC398 Clade Associated with Human-to-Human Transmission. *Applied and Environmental Microbiology,* 78 (24), pp.8845-8848

Allegra, C.J., Grem, J.L., Yeh, G.C. and Chabner, B. A., 1988. Antimetabolites, *Cancer Chemotherapy and Biological Response Modifiers,* 10 (1), pp. 1-22.

Alvarez, D., Merino, S., Tomás, J.M., Benedí, V.J. and Albertí, S., 2000. Capsular polysaccharide is a major complement resistance factor in lipopolysaccharide O side chain-deficient Klebsiella pneumoniae clinical isolates. *Infection and immunity,* 68 (2), pp. 953-955.

Ambler, R.P., 1997. The structure of β-lactamases. *Philosophical Transactions of the Royal Society of London. B, Biological Sciences,* 289 (1036), pp.321-331.

Aminov, R.I., 2010. A Brief History of the Antibiotic Era: Lessons Learned and Challenges for the Future. *Frontiers in microbiology,* 1 (10), pp. 1-7.

Amit, R., Garcia, H., Phillips, R. and Fraser, S., 2011. Building Enhancers from the Ground Up: A Synthetic Biology Approach. *Cell,* 146 (1), pp.105-118.

Andersen, S.B., Marvig, R.L., Molin, S., Johansen, H.K. and Griffin, A.S. 2015. Long-term social dynamics drive loss of function in pathogenic bacteria. *Proceedings of the National Academy of Sciences of the United States of America,* 112 (34), pp.10756-10761.

Andersson, D.I. and Hughes, D., 2010. Antibiotic resistance and its cost: is it possible to reverse resistance? *Nature Reviews Microbiology,* 8 (4), pp.260-271.

Andrews, J.M. 2001. Determination of minimum inhibitory concentrations. *The Journal of Antimicrobial Chemotherapy,* 48 (1), pp. 5-16.

Anes, J., Hurley, D., Martins, M. and Fanning, S., 2017. Exploring the Genome and Phenotype of Multi-Drug Resistant Klebsiellapneumoniae of Clinical Origin. *Frontiers in Microbiology,* 8 (1913), pp. 1-15.

Antunes, N.T., Lamoureaux, T., Toth, M., Stewart, N. K., Frase, H. and Vakulenko, S.B., 2014. Class D -Lactamases: Are They All Carbapenemases? *Antimicrobial Agents and Chemotherapy*, 58 (4), pp. 2119 –2125

Aoki, N., Ishii, Y., Tateda, K., Saga, T., Kimura, S., Kikuchi, Y., Kobayashi, T., Tanabe, Y., Tsukada, H., Gejyo F., and Yamaguchi, K., 2010. Efficacy of Calcium-EDTA as an Inhibitor for Metallo-β-Lactamase in a Mouse Model of Pseudomonas aeruginosa Pneumonia. *Antimicrobial Agents and Chemotherapy,* 54 (11), pp.4582-4588.

Arahata, K., Yamaguchi, R. and Terashima, T., 2018. A Case of Polymicrobial Bacteremia in a Patient Undergoing Chemotherapy. *Case Reports Infectious diseases*, 1 (5), pp. 156-162.

Arakawa, Y., Ohta, M., Wacharotayankun, R., Mori, M., Kido, N., Ito, H., Komatsu, T., Sugiyama, T. and Kato, N., 1991. Biosynthesis of Klebsiella K2 capsular polysaccharide in Escherichia coli HB101 requires the functions of rmpA and the chromosomal cps gene cluster of the virulent strain Klebsiella pneumoniae Chedid (O1:K2). *Infection and immunity,* 59 (6), pp.2043-2050.

Arbeloa, A., Segal, H., Hugonnet, J., Josseaume, N., Dubost, L., Brouard, J., Gutmann, L., Mengin-Lecreulx, D. and Arthur, M., 2004. Role of Class A Penicillin-

Binding Proteins in PBP5-Mediated β-Lactam Resistance in Enterococcus faecalis. *Journal of Bacteriology,* 186 (5), pp.1221-1228.

Arndt, W.F. and Ritts, R.E.,1961. Synergism between staphylococci and proteus in mixed infection. *Proceedings of the Society for Experimental Biology and Medicine*, 108 (1), pp. 166–169.

Arnold, R.S., Thom, K.A., Sharma, S., Phillips, M., Kristie Johnson, J. and Morgan, D.J., 2011. Emergence of Klebsiella pneumoniae Carbapenemase-Producing Bacteria. *Southern medical journal (Birmingham, Ala.),* 104 (1), pp.40-45.

Aslam, B., Wang, W., Arshad, M.I., Khurshid, M., Muzammil, S., Rasool, M.H., Nisar, M.A., Alvi, R.F., Aslam, M.A., Qamar, M.U., Salamat, M.K.F. and Baloch, Z., 2018. Antibiotic resistance: a rundown of a global crisis. *Infection and drug resistance*, 11, pp.1645-1658.

Aubert, D., Poirel, L., Chevalier, J., Leotard, S., Pages, J. and Nordmann, P., 2001. Oxacillinase-Mediated Resistance to Cefepime and Susceptibility to Ceftazidime in Pseudomonas aeruginosa. *Antimicrobial Agents and Chemotherapy,* 45 (6), pp.1615-1620.

Bachman, M.A., Lenio, S., Schmidt, L., Oyler, J.E. and Weiser, J.N., 2012. Interaction of Lipocalin 2, Transferrin, and Siderophores Determines the Replicative Niche of Klebsiella pneumoniae during Pneumonia. *mBio,* 3 (6), pp. 45-56.

Bæk, K.T., Gründling, A., Mogensen, R.G., Thøgersen, L., Petersen, A., Paulander, W. and Frees, D., 2014. β-Lactam Resistance in Methicillin-Resistant Staphylococcus aureus USA300 Is Increased by Inactivation of the ClpXP Protease. *Antimicrobial agents and chemotherapy,* 58 (8), pp.4593-4603.

Bahr, G., Vitor-Horen, L., Bethel, C.R., Bonomo, R.A., González, L.J. and Vila, A.J., 2018. Clinical Evolution of New Delhi Metallo-β-Lactamase (NDM) Optimizes Resistance under Zn(II) Deprivation. *Antimicrobial agents and chemotherapy,* 62 (1), pp. 450-470.

Bai, J., Kim, S.I., Ryu, S. and Yoon, H., 2014. Identification and Characterization of Outer Membrane Vesicle-Associated Proteins in Salmonella enterica Serovar Typhimurium. *Infection and immunity,* 82 (10), pp. 4001-4010.

Bai, B., Ren, J., Bai, F. and Hao, L., 2020. Selection and validation of reference genes for gene expression studies in Pseudomonas brassicacearum GS20 using real-time quantitative reverse transcription PCR. *PloS one,* [e-journal] 15 (1), pp.e0227927-e0227940..

Bajpai, T., Pandey, M., Varma, M. and Bhatambare, G., 2017. Prevalence of TEM, SHV, and CTX-M Beta-Lactamase genes in the urinary isolates of a tertiary care hospital. *Avicenna journal of medicine,* 7 (1), pp.12-16.

Bankevich, A., Nurk, S., Antipov, D., Gurevich, A.A., Dvorkin, M., Kulikov, A.S., Lesin, V.M., Nikolenko, S.I., Pham, S., Prjibelski, A.D., Pyshkin, A.V., Sirotkin, A.V., Vyahhi, N., Tesler, G., Alekseyev, M.A. and Pevzner, P.A., 2012. SPAdes: A New Genome Assembly Algorithm and Its Applications to Single-Cell Sequencing. *Journal of Computational Biology,* 19 (5), pp.455-477.

Baraniak, A., Fiett, J., Mrówka, A., Walory, J., Hryniewicz, W. and Gniadkowski, M., 2005. Evolution of TEM-Type Extended-Spectrum β-Lactamases in Clinical Enterobacteriaceae Strains in Poland. *Antimicrobial Agents and Chemotherapy,* 49 (5), pp.1872-1880.

Barker, W. H., Mullooly, J. P., 1982. Pneumonia and influenza deaths during epidemics: implications for prevention. *Archives of Internal. Medicine*,142 (4), pp. 85–89.

Barthélémy, M., Peduzzi, J. and Labia, R., 1988. Complete amino acid sequence of p453-plasmid-mediated PIT-2β-lactamase (SHV-1). *Biochemical journal,* 251 (1), pp.73-79.

Baslé, A. and Delcour, A. H., 2001. Effect of two polyamine toxins on the bacterial porin OmpF. Biochem. *Biophysics Research Community*, 285 (10), pp. 550–554.

Bassetti, M., Vena, A., Croxatto, A., Righi, E. and Guery, B., 2018. How to manage Pseudomonas aeruginosa infections. *Drugs in Context,* 7(5), pp.1-18.

Bauvois, C. and Wouters, J., 2007. Crystal Structures of Class C β-Lactamases: Mechanistic Implications and Perspectives in Drug Design. 2007. *Enzyme-Mediated Resistance to Antibiotics.* Washington, DC, USA:ASM Press. 5 (9), pp.145-161.

Beckwith, J., 2013. The Sec-dependent pathway. *Research in microbiology,* 164 (6), pp.497-504.

Bennett, J.W. and Chung, K., 2001. Alexander Fleming and the discovery of penicillin. 2001. *Advances in Applied Microbiology.* United States. *Science & Technology*, 24 (5), pp.163-184.

Bennett, P.M., 2008. Plasmid encoded antibiotic resistance: acquisition and transfer of antibiotic resistance genes in bacteria. *British journal of pharmacology,* 153 (S1), pp.S347-S357.

Bevan, E.R., Jones, A.M. and Hawkey, P.M., 2017. Global epidemiology of CTX-M β-lactamases: temporal and geographical shifts in genotype. *Journal of antimicrobial chemotherapy,* 72 (8), pp.2145-2155.

Beveridge, T. J., Makin, S. A., Kadurugamuwa, J. L. and Li, Z., 1997. Interactions between biofilms and the environment. *FEMS Microbiology*, 20 (3), pp. 291–303.

Bezabih, Y.M., Sabiiti, W., Alamneh, E., Bezabih, A., Peterson, G.M., Bezabhe, W.M. and Roujeinikova, A., 2021. The global prevalence and trend of human intestinal carriage of ESBL-producing Escherichia coli in the community. *Journal of antimicrobial chemotherapy,* 76 (1), pp.22-29.

Birch, D. E., Kolmodin, L. and Young, K.K.Y., 1996. Simplified Hot Start PCR. *Nature,* 381 (8), pp. 445-446.

Bismuth, R, Zilhao, R., Sakamoto, H., Guesdon, J. L. and Courvalin, P,, 1990. Gene heterogeneity for tetracycline resistance in Staphylococcus spp. *Antimicrobial Agents and Chemotherapy,* 34 (8), pp.1611-1614.

Bitto, N.J., Chapman, R., Pidot, S., Costin, A., Lo, C., Choi, J., D'Cruze, T., Reynolds, E.C., Dashper, S.G., Turnbull, L., Whitchurch, C.B., Stinear, T.P., Stacey, K.J. and Ferrero, R.L., 2017. Bacterial membrane vesicles transport their DNA cargo into host cells. *Scientific reports,* 7 (1), pp.7072-7086.

Blakesley, R.W., 1987. Restriction endonuclease: cleavage, ligation, and sensitivity. *Gene amplification and analysis,* 5 (6), pp.51-102.

Blair, J.M., Richmond, G.E. and Piddock, L.J., 2014. Multidrug efflux pumps in Gram-negative bacteria and their role in antibiotic resistance. *Future Microbiology,* 9 (5), pp. 1165–1177.

Bloom, D.E., Black, S., Salisbury, D. and Rappuoli, R., 2018. Antimicrobial resistance and the role of vaccines. *Proceedings of the National Academy of Sciences of the United States of America,* 115 (51), pp.12868-12871.

Bogaerts, P., Naas, T., Garch, F., Deplano, G., Delaire, T., Huang, T., Nordmann , P.and Glupczynski, Y., 2010. GES Extended-Spectrum β-Lactamases in Acinetobacter baumannii Isolates in Belgium. *Antimicrobial Agents and Chemotherapy,* 54 (11), pp.4872-4878.

Bonnet, R., 2004. Growing group of extended-spectrum beta-lactamases: the CTX-M enzymes. *Antimicrobial agents and chemotherapy,* 48 (1), pp.1-14.

Bonnin, R.A., Jousset, A.B., Urvoy, N., Gauthier, L., Tlili, L., Creton, E., Cotellon, G., Arthur, F., Dortet, L. and Naas, T., 2017. Detection of GES-5 Carbapenemase in Klebsiella pneumoniae, a Newcomer in France. *Antimicrobial agents and chemotherapy,* 61 (3), pp. 1-4.

Bonomo, R.A., 2017. β-Lactamases: A Focus on Current Challenges. Cold Spring Harbor Perspectives in Medicine, 7 (7), pp. 1-16.

Bontron, S., Poirel, L. and Nordmann, P., 2015. In vitro prediction of the evolution of GES-1 β-lactamase hydrolytic activity. *Antimicrobial agents and chemotherapy,* 59 (3), pp.1664-1670.

Bosch, F. and Rosich, L., 2008. The Contributions of Paul Ehrlich to Pharmacology: A Tribute on the Occasion of the Centenary of His Nobel Prize. *Pharmacology,* 82 (3), pp.171-179.

Boucher, H.W., Talbot, G.H., Bradley, J.S., Edwards, J.E., Gilbert, D., Rice, L.B., Scheld, M., Spellberg, B., Bartlett, J., 2009. Bad bugs, no drugs: no ESKAPE! An update from the Infectious Diseases Society of America. *Clinical Infectious Diseases*, 48 (76), pp. 1–12.

Bouvet, P.J.M. and Grimont, P.A.D., 1986. Taxonomy of the Genus Acinetobacter with the Recognition of Acinetobacter baumannii sp. nov., Acinetobacter haemolyticus sp. nov., Acinetobacter johnsonii sp. nov., and Acinetobacter junii sp. nov. and Emended Descriptions of Acinetobacter calcoaceticus and Acinetobacter lwoffii. *International journal of systematic bacteriology,* [e-journal] 36 (2), pp.228-240.

Boysen, A., Borch, J., Krogh, T.J., Hjernø, K. and Møller-Jensen, J., 2015. SILAC-based comparative analysis of pathogenic Escherichia coli secretomes. *Journal of microbiological methods,* 116, pp.66-79.

Bradford, P.A., 2001. Extended-spectrum-lactamases in the 21 century: Characterization, epidemiology, and detection of this important resistance threat. Clinical Microbiology Review, 14, (4) pp. 933–951.

Braiteh, F. and Golden, M. P., 2007. Cryptogenic invasive K. pneumoniae liver abscess syndrome. Internal Journal of Infectious Diseases, 11 (25), pp. 16-22.

Brisse, S. and Duijkeren, E.v., 2005. Identification and antimicrobial susceptibility of 100 Klebsiella animal clinical isolates. *Veterinary microbiology,* 105 (3), pp.307-312.

Brisse, S., Grimont, F. and Grimont, P., 2006. The Genus Klebsiella. *Prokaryotes*, 2 (6), pp. 159-196.

Brock, J.H., Williams, P.H., Licéaga, J. and Wooldridge, K.G., 1991. Relative availability of transferrin-bound iron and cell-derived iron to aerobactin-producing and enterochelin-producing strains of Escherichia coli and to other microorganisms. *Infection and Immunity,* 59 (9), pp.3185-3190.

Brogden, R.N., Carmine, A.A., Heel, R.C., Speight, T.M. and Avery, G.S., 1982. Trimethoprim: a review of its antibacterial activity, pharmacokinetics and therapeutic use in urinary tract infections. *Drugs (New York, N.Y.)*, 23 (6), pp.405-430.

Brook, I., Calhou, L., and Yocum, P., 1981. Beta-lactamase-producing isolates of Bacteroides species from children. *Antimicrobial Agents and Chemotherapy,* 18 (1), pp.164-166.

Brook, I., Pazzaglia, G., Coolbaugh, J.C. and Walker, R.I., 1983. In- vivo protection of group A β-haemolytic streptococci from penicillin by β-lactamase-producing Bacteroides species. *Journal of antimicrobial chemotherapy,* 12 (6), pp.599-606.

Brook, I., 2004. β-Lactamase-producing bacteria in mixed infections. *Clinical microbiology and infection,* 10 (9), pp.777-784.

Brown, L., Wolf, J.M., Prados-Rosales, R. and Casadevall, A., 2015. Through the wall: extracellular vesicles in Gram-positive bacteria, mycobacteria and fungi. *Nature reviews. Microbiology,* 13 (10), pp.620-630.

Brunwasser-Meirom, M., Pollak, Y., Goldberg, S., Levy, L., Atar, O. and Amit, R., 2016. Using synthetic bacterial enhancers to reveal a looping-based mechanism for quenching-like repression. *Nature communications,* 7 (1), pp.10407-10415.

Byrd, A.L. and Segre, J.A., 2016. Infectious disease. Adapting Koch's postulates. *Science,* 351 (1), pp. 224–226.

Bullen, J.J., Rogers, H.J. and Griffiths, E., 1972. Iron binding proteins and infection. British Journal of Haematology, 23 (89), pp. 389–392.

Burdett, I.D.J. and Murray, R.G.E., 1974. Electron Microscope Study of Septum Formation in Escherichia coli Strains B and B/r During Synchronous Growth. *Journal of Bacteriology,* 119 (3), pp.1039-1056.

Bush, K., Freudenberger, J.S. and Sykes, R.B., 1982. Interaction of azthreonam and related monobactams with beta-lactamases from Gram-negative bacteria. *Antimicrobial agents and chemotherapy,* 22 (3), pp.414-420.

Bush, K., Jacoby, G.A. and Medeiros, A.A., 1995. A functional classification scheme for beta-lactamases and its correlation with molecular structure. *Antimicrobial agents and chemotherapy,* 39 (6), pp.1211-1233.

Bush, K., 2010. Bench-to-bedside review: The role of beta-lactamases in antibiotic-resistant Gram-negative infections. *Critical care (London, England),* 14 (3), pp.1-10.

Bush, K. and Jacobi, G.A., 2010. Updated Functional Classification of Beta-Lactamases. Antimicrobial Agents Chemotherapy, 54 (3), pp. 969-976.

Bush, K., 2012. Antimicrobial agents targeting bacterial cell walls and cell membranes. *Revue scientifique et technique (International Office of Epizootics),* ] 31 (1), pp.43.

Bush, K. and Bradford, P.A., 2016. β-Lactams and β-Lactamase Inhibitors: An Overview. *Cold Spring Harbor perspectives in medicine,* 6 (8), pp.1-10.

Bush, K., 2018. Past and Present Perspectives on β-Lactamases. *Antimicrobial agents and chemotherapy,* 62 (10), pp.1-20.

Buzás, E., Tóth, E., Sódar, B. and Szabó-Taylor, K., 2018. Molecular interactions at the surface of extracellular vesicles. *Seminars in Immunopathology,* 40 (5), pp.453-464.

Cai, J., Cheng, Q., Shen, Y., Gu, D., Fang, Y., Chan, E.W. and Chen, S., 2017. Genetic and Functional Characterization of blaCTX-M-199, a Novel Tazobactam and Sulbactam Resistance-Encoding Gene Located in a Conjugative mcr-1-Bearing IncI2 Plasmid.*Antimicrobial agents and chemotherapy,*61 (7), pp. e00562-17.

Cai, W., Kesavan, D.K., Wan, J., Abdelaziz, M.H., Su, Z. and Xu, H., 2018. Bacterial outer membrane vesicles, a potential vaccine candidate in interactions with host cells based. *Diagnostic pathology,*13 (1), pp.95-99.

Cano, V., Moranta, D., Llobet-Brossa, E., Bengoechea, J. and Garmendia, J., 2009. Klebsiella pneumoniae triggers a cytotoxic effect on airway epithelial cells. *BMC microbiology,* 9 (1), pp.156-162.

Cantón, R., Novais, A., Valverde, A., Machado, E., Peixe, L., Baquero, F. and Coque, T.M., 2008. Prevalence and spread of extended-spectrum β-lactamase-producing Enterobacteriaceae in Europe. *Clinical microbiology and infection,* 14, pp.144-153.

Carattoli, A., 2009. Resistance Plasmid Families in Enterobacteriaceae. *Antimicrobial agents and chemotherapy,* 53 (6), pp.2227-2238.

Carattoli, A., Seiffert, S. N., Schwendener, S., Perreten, V., and Endimiani, A., 2015. Differentiation of IncL and IncM plasmids associated with the spread of clinically relevant antimicrobial resistance. *PLoS One,* 1 (1), pp. 1-14.

Carniel, E., 2001. The Yersinia high-pathogenicity island: an iron-uptake island. Microbes Infections, 3 (10), pp. 561–569.

Carter, C.B., Williams, D.B. and Thomas, J.M., 2016. *Transmission electron microscopy.* Cham, Switzerland: Springer.

Caruana, J.C. and Walper, S.A., 2020. Bacterial Membrane Vesicles as Mediators of Microbe - Microbe and Microbe - Host Community Interactions. *Frontiers in microbiology,* 11, pp.432-438.

Carver, T. Harris S.R., Berriman, M., Parkhill, J. and McQuillan, J.A., 2012. Artemis: an integrated platform for visualization and analysis of high-throughput sequence-based experimental data. *Bioinformatics*, 28, (4), pp. 464-469.

Castillo, M.C.d., Sesma, F., Nader, O.M.d. and Holgado, Aida P de Ruiz, 1998. Properties of ß-lactamase from Neisseria gonorrhoeae. *Memórias do Instituto Oswaldo Cruz,* 93 (2), pp.237-241.

Cate, T. R., 1998. Impact of influenza and other community-acquired viruses. *Seminars in Respiratory Infections*,13 (5), pp. 17–23.

Cavaliere, M., Feng, S., Soyer, O.S. and Jiménez, J.I., 2017. Cooperation in microbial communities and their biotechnological applications. *Environmental microbiology,* 19 (8), pp.2949-2963.

Cecil, J.D., Sirisaengtaksin, N., O'Brien-Simpson, N.M. and Krachler, A.M., 2019. Outer Membrane Vesicle-Host Cell Interactions. *Microbiology spectrum,* 7 (1), pp. 1-18.

Cees Ho, Etienne E. M. Van Dijke, Cees M. Verduin, Jan Verhoef and Hans van Dijk, 1994. Experimental Evidence for Moraxella-Induced Penicillin Neutralization in Pneumococcal Pneumonia. *The Journal of infectious diseases,* 170 (6), pp.1613-1616.

Chain, E., Florey, H.W., Gardner, A.D., Heatley, N.G., Jennings, M.A., Orr-Ewing, J., Sanders, A.G. and Peltier, L.F., 2005. THE CLASSIC: Penicillin as a Chemotherapeutic Agent. *Clinical Orthopaedics and Related Research,* 439, pp.23-26.

Chambers, H.F. and Deleo, F.R., 2009. Waves of resistance: *Staphylococcus aureus* in the antibiotic era. *Nature Reviews Microbiology*, 7 (5), pp. 629–41.

Champney, W.S., 2020. Antibiotics targeting bacterial ribosomal subunit biogenesis. *Journal of Antimicrobial Chemotherapy,* 75 (4), pp. 787-806.

Chang, F., Siu, L. K., Fung, C., Huang, M. and Monto, H.,, 2001. Diversity of SHV and TEM β-Lactamases in Klebsiella pneumoniae: Gene Evolution in Northern Taiwan and Two Novel β-Lactamases, SHV-25 and SHV-26. *Antimicrobial Agents and Chemotherapy,* 45 (9), pp.2407-2413.

Chatterjee, S.S. and Otto, M., 2013. Improved understanding of factors driving methicillin-resistant Staphylococcus aureus epidemic waves. Clinical Epidemiology, 5 (24), pp. 205–17.

Chattopadhyay, M.K. and Jaganandham, M.V., 2015. Vesicles-mediated resistance t o antibiotics in bacteria. *Frontiers in microbiology,* 6 (758), pp. 1-3.

Chaudhry, S.B., Veve, M.P. and Wagner, J.L., 2019. Cephalosporins: A Focus on Sid e Chains and β-Lactam Cross-Reactivity. *Pharmacy,* 7 (3), pp.103-115.

Chaves, J., Ldona, M.G., Segura, C., Coira, A., Reig, R. and Ampurdanes, C., 2001. SHV-1 -Lactamase Is Mainly a Chromosomally Encoded Species-Specific Enzyme in Klebsiella pneumoniae. *Antimicrobial agents and chemotherapy*, 45 (10), pp. 2856-2861.

Chen, J.C., Viollier, P.H. and Shapiro, L., 2005. A membrane metalloprotease particip ates in the sequential degradation of a Caulobacter polarity determinant. *Molecular M icrobiology,* 55 (4), pp.1085-1103.

Chen, Y., Zhang, J., Xing, G. and Zhao, Y., 2009. Mascot-derived false positive pepti de identifications revelead by manual analysis of tandem mass spectra. Journal of pr oteome research, 8 (6), pp. 3141-3147.

Chen, K-J., Hwang, Y-S., Wang, N-K. and Chao, A-N., 2010. Endogenous Klebsiella pneumonia endophthalmitis with renal abscess: Report of two cases. *International Jo urnal of Infectious Diseases,* 14 (5), pp. 429-432

Chen, T., Chang, W., Kuo, S., Lee, Y., Chen, C., Siu, L., Cho, W., and Fung, C., 2010. Contribution of a Plasmid-Borne blaOXA-58 Gene with Its Hybrid Promoter Provided by IS1006 and an ISAba3-Like Element to -Lactam Resistance in Acinetobacter Genomic Species 13TU. *Antimicrobial Agents and Chemotherapy,* 54 (8), pp.3107-3112.

Chen, L., Mathema, B., Chavda, K.D., DeLeo, F.R., Bonomo, R.A. and Kreiswirth, B. N., 2014. Carbapenemase-producing Klebsiella pneumoniae: molecular and genetic decoding. *Trends in microbiology (Regular ed.),* 22 (12), pp.686-696.

Cheng, D.L.,, Liu, Y.C., Yen, M.Y., Liu, C.Y. and, Wang, R.S.,.1991. Septicmetastaticl esions of pyogenic liver abscess. Their association withKlebsiella pneu-moniaebacter aemia in diabetic patients. *Archives of. Internal Medicine*, 151 (15), pp. 1557–1559.

Chiang, T., Yang, Y., Yeh, K., Chiu, S., Wang, N., Lin, T., Huang, L., Chang, F., Siu, L.K., Lin, J. and Chen, J., 2015. Quantification and comparison of virulence and char acteristics of different variants of carbapenemase-producing Klebsiella pneumoniae cl inical isolates from Taiwan and the United States. *Journal of Microbiology, Immunolo gy and Infection,* 49 (1), pp.83-90.

Choby, J.E., Howard-Anderson, J. and Weiss, D.S., 2020. Hypervirulent Klebsiella pn eumoniae – clinical and molecular perspectives. *Journal of internal medicine,* 287 (3), pp.283-300.

Choi, U. and Lee, C., 2019. Distinct Roles of Outer Membrane Porins in Antibiotic Resistance and Membrane Integrity in Escherichia coli. *Frontiers in microbiology,* 10, pp.953-961.

Chow, J.W. and Shlaes, D.M., 1991. Imipenem resistance associated with the loss of a 40 kDa outer membrane protein in Enterobacter aerogenes. Journal of Antimicrobia l Chemotherapy, 28 (35), pp. 499–504.

Chung, D.R., Lee, S.S., Lee, H.R., Kim, H.B., Choi, H.J., Eom, J.S., Kim, J.S., Choi, Y.H., Lee, J.S., Chung, M.H., Kim, Y.S., Lee, H., Lee, M.S. and Park, C.K., 2007. Emerging invasive liver abscess caused by K1 serotype Klebsiella pneumoniae in Korea. *The Journal of infection,* 54 (6), pp.578-583.

Chung, M., Borges, V., Gomes, J.P., de Lencastre, H. and Tomasz, A., 2019. Phenotypic signatures and genetic determinants of oxacillin tolerance in a laboratory mutant of Staphylococcus aureus. *PloS one,* 13 (7), pp. 48-65.

Ciofu, O., 2000. Chromosomal beta-lactamase is packaged into membrane vesicles and secreted from Pseudomonas aeruginosa. *Journal of antimicrobial chemotherapy,* 45 (1), pp.9-13.

Cockerill, F.R., Wikler, M.A., Alder, J., Dudley, M.N., Eliopoulos, G.M., Ferraro, M. J., Hardy, D.J., Hecht, D.W., Hindler, J.A., Patel, J.B., Powell, M., Swenson, J.M., Thomson, R.B., Traczewski, M.M., Turnidge, J.D., Weinstein, M.P., Zimmer, B.L. 2012. Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically; Approved Standard—Ninth Edition. Volume 32 Number 2.

Codjoe, F.S. and Donkor, E.S., 2018. Carbapenem Resistance: A Review. *Medical Sciences,* 6 (1), pp. 1-28.

Coleman, K., 2011. Diazabicyclooctanes (DBOs): a potent new class of non-β-lactam β-lactamase inhibitors. *Current Opinion in Microbiology,* 14 (5), pp.550-555.

Cordwell, S.J., Nouwens, A.S. and Walsh, B.J., 2001. Comparative proteomics of bacterial pathogens. *Proteomics (Weinheim),*1 (4), pp.461-472.

Corelli, B., Almeida, A.S., Sonego, F., Castiglia, V., Fevre, C., Brisse, S., Sansonetti, P.J. and Tournebize, R., 2018. Rhinoscleroma pathogenesis: The type K3 capsule of Klebsiella rhinoscleromatis is a virulence factor not involved in Mikulicz cells formation. *PLoS neglected tropical diseases,* 12 (1), pp.432-445.

Cornaglia G, Mazzariol A, Fontana R., 1996. Diffusion of carbapenems through the outer membrane of enterobacteriaceae and correlation of their activities with their periplasmic concentrations. *Microbiology Drug Resistance*, 2 (156), pp. 273–276.

Cortes, G., 2002. Molecular Analysis of the Contribution of the Capsular Polysaccharide and the Lipopolysaccharide O Side Chain to the Virulence of Klebsiella pneumoniae in a Murine Model of Pneumonia. *Infection and immunity,* 70 (5), pp.2583-2590.

Costa, T.R.D., Felisberto-Rodrigues, C., Meir, A., Prevost, M.S., Redzej, A., Trokter, M. and Waksman, G., 2015. Secretion systems in Gram-negative bacteria: structural and mechanistic insights. *Nature reviews. Microbiology,* 13 (6), pp.343-359.

Cox, G. and Wright, G.D., 2013. Intrinsic antibiotic resistance: Mechanisms, origins, challenges and solutions. *International Journal of Medical Microbiology*, 303 (2013), pp. 287-292.

Coyne, S., Guigon, G., Courvalin, P. and Périchon, B, 2010. Screening and Quantification of the Expression of Antibiotic Resistance Genes in Acinetobacter baumannii with a Microarray. *Antimicrobial Agents and Chemotherapy,* 54 (1), pp.333-340.

Croxall, G., Weston, V., Joseph, S., Manning, G., Cheetham, P. and McNally, A., 2010. Increased human pathogenic potential of Escherichia coli from polymicrobial urinary tract infections in comparison to isolates from monomicrobial culture samples. *Journal of medical microbiology,* 60 (1), pp.102-109.

Cuzon, G., Naas, T. and Nordmann, P., 2011. Functional Characterization of Tn4401, a Tn3-Based Transposon Involved in blaKPC Gene Mobilization. *Antimicrobial Agents and Chemotherapy,* 55 (11), pp.5370-5373.

DAI XiDa XIANG SiHai LI Jia GAO Qiang YANG KeQian, 2012. Development of a colorimetric assay for rapid quantitative measurement of clavulanic acid in microbial samples. *Science China. Life sciences,* 55 (2), pp.158-163

Daikos, G. L., Petrikkos, P., Psichogiou, M., Kosmidis, C., Vryonis, E., Skoutelis, A., et al. (2009). Prospective observational study of the impact of VIM-1 metallo-beta-lactamase on the outcome of patients with *Klebsiella pneumoniae* bloodstream infections. *Antimicrobial. Agents Chemotherapy.* 53, (5), pp. 1868–1873.

Dallenne, C., Da Costa, A., Decré, D., Favier, C. and Arlet, G., 2009. Development of a set of multiplex PCR assays for the detection of genes encoding important β-lactamases in Enterobacteriaceae. *Journal of antimicrobial chemotherapy,* 65 (3), pp.490-495.

Datta, N. and Kontomichalou, P.,1965. Penicillinase synthesis controlled by infectious R Factors in Enterobacteriaceae. *Nature*, 208 (5007), pp. 239–244.

David, S., Reuter, S., Harris, S.R., Glasner, C., Feltwell, T., Argimon, S., Abudahab, K., Goater, R., Giani, T., Errico, G., Aspbury, M., Sjunnebo, S., Feil, E.J., Rossolini, G.M., Aanensen, D.M. and Grundmann, H., 2019. Epidemic of carbapenem-resistant Klebsiella pneumoniae in Europe is driven by nosocomial spread. *Nature microbiology,* 4 (11), pp.1919-1929.

Davies, J., 1990. What are antibiotics? Archaic functions for modern activities. Mol. *Microbiology*, 4 (5), pp. 1227–1232

Davies, J. and Davies, J., 2010. Origins and Evolution of Antibiotic Resistance. *Microbiology and Molecular Biology Reviews,* 74 (3), pp. 417-433.

Davies, D.G., 1998. The Involvement of Cell-to-Cell Signals in the Development of a Bacterial Biofilm. *Science (American Association for the Advancement of Science),* 280 (5361), pp.295-298.

Davies, J.C., Alton, E.W. and Bush, A., 2007. Cystic fibrosis. *British Medical Journal*, 335 (1), pp. 1255–1259.

Davin-Regli, A., Lavigne, J.P. and PAGES, J., 2019. Enterobacter spp.: update on taxonomy, clinical aspect and emerging antimicrobial resistance. *Clinical microbiology reviews,* 32 (4), pp. 35-46.

De Champs, C., Monne, C., Bonnet, R., Sougakoff, W., Sirot, D., Chanal., C. and Sirot, J., 2001. New TEM variant (TEM-92) produced by Proteus mirabilis and Providencia stuartii isolates. *Antimicrobial agents and chemotherapy,* 45 (4), pp.1278-1280.

DeFife, R., Scheetz, M.H., Feinglass, J.M., Postelnick, M.J. and Scarsi, K.K., 2009. Effect of Differences in MIC Values on Clinical Outcomes in Patients with Bloodstream Infections Caused by Gram-negative Organisms Treated with Levofloxacin. *Antimicrobial Agents and Chemotherapy,* 53 (3), pp.1074-1079.

Delcour, A.H., 2009. Outer membrane permeability and antibiotic resistance. *BBA - Proteins and Proteomics,* 1794 (5), pp.808-816.

Denton, J. and Gokhale, C., 2019. Synthetic Mutualism and the Intervention Dilemma. *Life (Basel, Switzerland),* 9 (1), pp.1-17.

Dhamgaye, S., Qu, Y. and Peleg, A.Y., 2016. Polymicrobial infections involving clinically relevant Gram-negative bacteria and fungi. *Cellular Microbiology*, 18: 1716–1722.

Dias, D., Urban, S. and Roessner, U., 2012. A historical overview of natural products in drug discovery. *Metabolites*,2(2): pp. 303–336.

Dice, L.R., 1945. Measures of the Amount of Ecologic Association Between Species. *Ecology,* 26(3), pp. 297–302.

Diekema, D.J. , Pfaller, M.A. and Turnidge, J., 1998. Sentry Participants Group. Genetic relatedness of multidrug-resistant, methicillin (oxacillin)-resistant Staphylococcus aureus bloodstream isolates from SENTRY Antimicrobial Resistance Surveillance Centers worldwide. Microbiology Drug Resistance, 2000 (6), pp. 213–21.

Diekema, D., Pfaller, M., Shortridge, D., Zervos, M. and Jones, D., 2019. Twenty-Year Trends in Antimicrobial Susceptibilities Among Staphylococcus aureus From the SENTRY Antimicrobial Surveillance Program. *Open Forum Infectious diseases*, 6 (7), pp. S47-S53.

Dinh, T. and Bernhardt, T.G., 2011. Using Superfolder Green Fluorescent Protein for Periplasmic Protein Localization Studies. *Journal of Bacteriology,* 193 (18), pp. 4984-4987.

Doi Y., Park, Y.S., Rivera, J.I., Adams-Haduch, J.M., Hingwe, A., Sordillo, E.M., Lewis, J.S., Howard, W.J., Johnson, L.E., Polsky, B., Jorgensen, J.H., Richter, S.S., Shutt, K.A. and Paterson, D.L., 2013. Community-associated extended-spectrum beta-lactamase-producing Escherichia coli infection in the United States. Clinical Infectious Disease, 56 (5), pp. 641-648.

Doi, Y., Iovleva, A. and Bonomo, R.A., 2017. The ecology of extended-spectrum β-lactamases (ESBLs) in the developed world. *Journal of travel medicine,* 24 (suppl_1), pp.S44-S51.

Doménech-Sánchez, A., Hernández-Allés, S., Martínez-Martínez, L., Benedí, V.J. and Albertí, S., 1999. Identification and Characterization of a New Porin Gene of Klebsiella pneumoniae: Its Role in β-Lactam Antibiotic Resistance. *Journal of bacteriology,* 181 (9), pp.2726-2732

Done, S. H. and Paton, D. J., 1995. Porcine reproductive and respiratory syndrome: clinical disease, pathology andimmunosuppression. *Veterinary Records*, 136 (8), pp. 32–35.

Dong, C., Wang, L., Li, Q. and Shang, Q., 2019. Bacterial communities in the rhizosphere, phyllosphere and endosphere of tomato plants. *PloS one,* 14 (11), pp.1-17.

Donoghue, E.J. and Krachler, A.M., 2016. Mechanisms of outer membrane vesicle entry into host cells. Cell Microbiology, 18(11), pp. 1508-1517.

Doorduijn, D.J., Rooijakkers, S.H.M., van Schaik, W. and Bardoel, B.W., 2016. Complement resistance mechanisms of Klebsiella pneumoniae. *Immunobiology (1979),* 221 (10), pp.1102-1109.

Dortet, L. and Naas, T., 2017. Noncarbapenemase OXA-48 Variants (OXA-163 and OXA-405) Falsely Detected as Carbapenemases by the β Carba Test. *Journal of clinical microbiology,* 55 (2), pp.654-655.

Dougherty, T.J., 1986. Genetic analysis and penicillin-binding protein alterations in Neisseria gonorrhoeae with chromosomally mediated resistance. *Antimicrobial Agents and Chemotherapy,* 30 (5), pp.649-652.

Drawz, S. and Bonomo, R., 2010. Three Decades of β-Lactamase Inhibitors. *Clinical Microbiology Reviews,* 23 (1), pp.160-201.

Drzewiecka, D., 2016. Significance and Roles of Proteus spp. Bacteria in Natural Environments. *Microbial ecology,* 72 (4), pp.741-758.

Dugatkin, L.A., Perlin, M., Lucas, J.S., and Atlas, R. 2005. Group-beneficial traits, frequency-dependent selection and genotypic diversity: an antibiotic resistance paradigm. *Proceedings of the Royal Society B: Biological Sciences,* 272 (1558), pp.79-83.

Eberlein, C., Starke, S., Doncel, ÁE., Scarabotti, F. and Heipieper, H.J., 2019. Quantification of outer membrane vesicles: a potential tool to compare response in

Pseudomonas putida KT2440 to stress caused by alkanols. *Applied microbiology and biotechnology,* 103 (10), pp.4193-4201

Effah, C.Y., Sun, T., Liu, S. and Wu, Y., 2020. Klebsiella pneumoniae: an increasing threat to public health. *Annals of clinical microbiology and antimicrobials,* 19 (1), pp.1-10.

Eichenberger, E.M. and Thaden, J.T., 2019. Epidemiology and Mechanisms of Resistance of Extensively Drug Resistant Gram-negative Bacteria. *Antibiotics*, 8 (37), pp. 1-21

El Salabi, A., Walsh, T.R. and Chouchani, C., 2013. Extended spectrum β-lactamases, carbapenemases and mobile genetic elements responsible for antibiotics resistance in Gram-negative bacteria. *Critical Reviews in Microbiology,* 39 (2), pp.113-122.

Elhenawy, W., Bording-Jorgensen, M., Valguarnera, E., Haurat, M.F., Wine, E. and Feldman, M.F., 2016. LPS Remodeling Triggers Formation of Outer Membrane Vesicles in Salmonella. *mBio,* 7 (4), pp. e00940-e00955.

Elshamy, A.A. and Aboshanab, K.M., 2020. A review on bacterial resistance to carbapenems: epidemiology, detection and treatment options. *Future science OA,* 6 (3), pp.438-445.

Endimiani, A., Hujer, A.M., Perez, F., Bethel, C.R., Hujer, K. M., Kroeger, J., Oethinger, M., Paterson, D.L., Adams, M.D., Jacobs, M.R., Diekema, D.J., Hall., G., Jenkins, S.G., Rice, L.B. Tenover, F.C. and Bonomo, R.A., 2009. Characterization of blaKPC-containing *Klebsiella pneumoniae* isolates detected in different institutions in the Eastern USA. *Journal of. Antimicrobial Chemotherapy*, 63, (1), pp. 427–437.

Engebrecht, J., Nealson, K. and Silverman, M., 1983. Bacterial bioluminescence: Isolation and genetic analysis of functions from Vibrio fischeri. *Cell (Cambridge),* 32 (3), pp.773-781.

Engebrecht, J. and Silverman, M., 1984. Identification of genes and gene products necessary for bacterial bioluminescence. *Proceedings of the National Academy of Sciences – PNAS,* 81 (13), pp.4154-4158.

Epand, R.M., Walker, C., Epand, R.F. and Magarvey, N.A., 2016. Molecular mechanisms of membrane targeting antibiotics. *BBA - Biomembranes,* 1858 (5), pp. 980-987.

Esragul, A. and Haluk V., 2010. Minor extended-spectrum b-lactamases : *Expert Review of Anti-Infective Therapy*,.8 (11), pp.1251-1258.

Evans, B.A. and Amyes, S.G.B., 2014. OXA β-lactamases. *Clinical microbiology reviews,* 27 (2), pp.241-263.

Fajardo, A., Martinez-Martin, N., Mercadillo, M., 2008. The neglected intrinsic resistome of bacterial pathogens. *PLoS One* 3 (5), pp. e1619-e1630.

Falagas, M.E. and Kasiakou, S.K., 2006. Toxicity of polymyxins: a systematic review of the evidence from old and recent studies. *Critical care (London, England),* 10 (1), pp.R27.

Fang, C., Chuang, Y., Shun, C., Chang, S. and Wang, J., 2004. A Novel Virulence Gene in Klebsiella pneumoniae Strains Causing Primary Liver Abscess and Septic Metastatic Complications. *The Journal of experimental medicine,* 199 (5), pp.697-705.

Fang, C., Lai, S., Yi, W.., Hsueh, P., Liu, K. and Chang, S., 2007. Klebsiella pneumoniae Genotype K1: An Emerging Pathogen That Causes Septic Ocular or Central Nervous System Complications from Pyogenic Liver Abscess. *Clinical infectious diseases,* 45 (3), pp.284-293.

Farag, Y.M.K. and Gaballa, M.R., 2010. Diabesity: an overview of a rising epidemic. *Nephrology, dialysis, transplantation,* 26 (1), pp.28-35.

Feilmeier, B.J., Iseminger, G.D. Schroeder, Webber, H. and Phillips, G.J., 2000. Green fluorescent protein functions as a reporter for protein localization in Escherichia coli. *Journal of Bacteriology*, 182 (14), pp. 4068–4076.

Fernández, L. and Hancock, R.E.W., 2013. Adaptive and Mutational Resistance: Role of Porins and Efflux Pumps in Drug Resistance. Clinical microbiology reviews, 26 (1), pp.163-170.

Fernández-Villa, D., Aguilar, M.R. and Rojo, L., 2019. Folic Acid Antagonists: Antimicrobial and Immunomodulating Mechanisms and Applications. *International journal of molecular sciences,* 20 (20), pp.4996.

Fernando, D. and Kumar, A., 2013. Resistance-Nodulation-Division Multidrug Efflux Pumps in Gram-negative Bacteria: Role in Virulence. *Antibiotics (Basel),* 2 (1), pp.163-181.

Ferreira, C.M., Ferreira, W.A., Almeida, Nayanne Cristina Oliveira da Silva, Naveca, F.G. and Barbosa, Maria das Graças Vale, 2011. Extended-spectrum beta-lactamase-producing bacteria isolated from hematologic patients in Manaus, State of Amazonas, Brazil. *Brazilian journal of microbiology,* 42 (3), pp.1076-1084.

Ferreira, R.L., da Silva, Brenda C M, Rezende, G.S., Nakamura-Silva, R., Pitondo-Silva, A., Campanini, E.B., Brito, M.C.A., da Silva, Eulália M L, Freire, Caio César de Melo, da Cunha, A.F. and Pranchevicius, M.d.S., 2019. High Prevalence of Multidrug-Resistant Klebsiella pneumoniae Harboring Several Virulence and β-Lactamase Encoding Genes in a Brazilian Intensive Care Unit. *Frontiers in microbiology,* 9 (15), pp.3198-3221.

Fields, F.R., Lee, S.W. and McConnell, M.J., 2017. Using bacterial genomes and essential genes for the development of new antibiotics. *Biochemical pharmacology,* 134, pp.74-86.

Findlay, J., Hopkins, K.L., Loy, R., Doumith, M., Meunier, D., Hill, R., Pike, R., Mustafa, N., Livermore, D.M. and Woodford, N., 2017. OXA-48-like carbapenemases in the UK: an analysis of isolates and cases from 2007 to 2014. *The Journal of Antimicrobial Chemotherapy,* 2 (5), pp.1340-1349.

Fisher, J.F., Meroueh, S.O. and Mobashery, S., 2005. Bacterial Resistance to β-Lactam Antibiotics: Compelling Opportunism, Compelling Opportunity. *Chemical Reviews,* 105 (2), pp. 395-424.

Fisher, J.F. and Mobashery, S., 2016. β-Lactam Resistance Mechanisms: Gram-positive Bacteria and Mycobacterium tuberculosis. *Cold Spring Harbor perspectives in medicine,* 6 (5), pp. 1-19.

Fleming, A., 1929. On the antibacterial action of cultures of a penicillium, with special reference to their use in the isolation of B. influenzae. *Experimental pathology*, 10 (1), pp. 226-236.

Floret, D., 1997. Virus-bacteria co-infections. *Archives of Pediatry,* 4 (10), pp. 1119–1124.

Fontana, R., Cerini, R., Longoni, P., Grossato, A. and Canepari, P., 1983. Identification of a streptococcal penicillin-binding protein that reacts very slowly with penicillin. *Journal of bacteriology,* 155 (3), pp.1343-1350.

Frain, K.M., Robinson, C. and van Dijl, J.M., 2019. Transport of Folded Proteins by the Tat System. *Journal of protein chemistry,* 38 (4), pp.377-388.

Frank, G.H., 1979. *Pasteurella haemolytica* and respiratory disease in cattle*In Proceedings of the83rd Annual Meeting of The United States Animal Health Association*, 43 (7),pp. 153–160.

Fredrickson, A.G., Stephanopoulos, G., 1981. Microbial competition. *Science* 213, (2), pp.972–979.

Freilich, S., Zarecki, R., Eilam, O., Segal, E.S., Henry, C.S., Kupiec, M., Gophna, U., Sharan, R. and Ruppin, E., 2011. Competitive and cooperative metabolic interactions in bacterial communities. *Nature communications,* 2 (1), pp.589-596.

French, G.L., Shannon, K.P. and Simmons, N., 1996. Hospital outbreak of Klebsiellapneumoniae resistant to broad-spectrum cephalosporins and beta-lactam-beta-lactamase inhibitor combinations by hyperproduction of SHV-5 beta-lactamase. *Journal of Clinical Microbiology,* 34 (2), pp.358-363

Fujita, M., Takahashi, A., Imaizumi, H., Hayashi, M., Okai, K., Abe, K. and Ohira, H., 2019. Endogenous Endophthalmitis Associated with Pyogenic Liver Abscess Caused by Klebsiella pneumoniae. *Internal medicine (Tokyo, 1992),* 58 (17), pp.2507-2514.

Galdadas, I., Lovera, S., Pérez-Hernández, G., Barnes, M.D., Healy, J., Afsharikho, H., Woodford, N., Bonomo, R.A., Gervasio, F.L. and Haider, S., 2018. Defining the architecture of KPC-2 Carbapenemase: identifying allosteric networks to fight antibiotics resistance. *Scientific reports,* 8 (1), pp.12916-13.

Galina, L., Pijoan, C., Sitjar, M., Christianson, W. T., Rossow, K. and Collins, J. E., 1994. Interaction between Streptococcus suisserotype 2 and porcine reproductive and respiratory syndrome virus in specific pathogen-free piglets. Veterinary Records, 134 (5), pp. 60–64.

GARDNER, A., WEST, S.A. and WILD, G., 2011. The genetical theory of kin selection. *Journal of evolutionary biology,* 24 (5), pp.1020-1043.

GARREC, H., DRIEUX-ROUZET, L., GOLMARD, J., JARLIER, V. and ROBERT, J., 2011. Comparison of Nine Phenotypic Methods for Detection of Extended-Spectrum β-Lactamase Production by Enterobacteriaceae. *Journal of Clinical Microbiology,* 49 (3), pp.1048-1057.

Gasteiger, E. Gattiker, A., Hoogland, C., Ivanyi, I., Appel, R.D. and Bairoch, A., 2003. ExPASy: the proteomics server for in-depth protein knowledge and analysis. *Nucleic Acids Research,* 31, (13), pp. 3784-3788.

Gaude, G. and Hattiholli, J., 2013. Rising bacterial resistance to beta-lactam antibiotics: Can there be solutions? *Journal of Dr. NTR University of Health Sciences,* 2 (1), pp.4-9.

Geerlings, S.E., 2008. Urinary tract infections in patients with diabetes mellitus: epidemiology, pathogenesis and treatment. *International journal of antimicrobial agents,* 31 (Suppl. 1), pp.54-57.

Geesink, P., Tyc, O., Küsel, K., Taubert, M., van de Velde, C., Kumar, S. and Garbeva, P., 2018. Growth promotion and inhibition induced by interactions of groundwater bacteria. *FEMS microbiology ecology,* 94 (11), pp. 35-45.

Giani, T., D'Andrea, M.M., Pecile, P., Borgianni, L., Nicoletti, P., Tonelli, F., Bartoloni, A. and Rossolini, G., 2009. Emergence in Italy of Klebsiella pneumoniae sequence type 258 producing KPC-3 Carbapenemase. *Euro Surveillance 2013,* 18 (22), pp. 34-43.

Gibbs, K.A. and Shen, A., 2016. Editorial: Signals to sociality: how microbial communication fashions communities. *FEMS Microbiology Reviews,*40 (6), pp.795-797.

Gill, M.J., Simjee, S. and Al-Hattawi, K., 1998 Gonococcal resistance to β-lactams and tetracycline involves mutation in loop 3 of the porin encoded at the penB locus. *Antimicrobial Agents Chemotherapy*, 42 (25), pp. 2799–2803.

Gill, S., Catchpole, R. and Forterre, P., 2019. Extracellular membrane vesicles in the three domains of life and beyond. *FEMS microbiology reviews,* 43 (3), pp.273-303.

Giobbia, M., Scotton, P. G., Carniato, A., Cruciani, M., Farnia, A. and Daniotti, E., 2003. Community-acquired K. pneumoniae bacteremia with meningitis and endophthalmitis in Italy. Journal of Infectious Diseases, 7 (15), pp. 234-235.

Girlich, D., Bonnin, R.A., Dortet, L. and Naas, T., 2020. Genetics of Acquired Antibiotic Resistance Genes in Proteus spp. *Frontiers in microbiology,* 21 (11), pp.256.

Girmenia, C., Serrao, A. and Canichella, M., 2016. Epidemiology of Carbapenem Resistant Klebsiella pneumoniae Infections in Mediterranean Countries. Mediterrean Journal of Hematology and Infectious Diseases, 8 (1), pp. 1-15

Gniadkowski, M., Pałucha, A., Grzesiowski, P. and Hryniewicz, W. 1998. Outbreak of Ceftazidime-Resistant Klebsiellapneumoniae in a Pediatric Hospital in Warsaw, Poland: Clonal Spread of the TEM-47 Extended-Spectrum β-Lactamase (ESBL)-Producing Strain and Transfer of a Plasmid Carrying the SHV-5-Like ESBL-Encoding Gene. *Antimicrobial Agents and Chemotherapy,* 42 (12), pp.3079-3085.

Gniadkowski, M., 2001. Evolution and epidemiology of extended-spectrum β-lactamases (ESBLs) and ESBL-producing microorganisms. *Clinical Microbiology and Infection,* 7 (11), pp.597-608.

Goers, L., Freemont, P. and Polizzi, K.M., 2014. Co-culture systems and technologies: taking synthetic biology to the next level. *Journal of the Royal Society interface,* 11 (96), pp.20140065-20140076.

Goetz, A. M., Rihs, J.D., Chow, J.W., Singh, N. and Muder, R.R., 1995. An outbreak of infusion-related K. pneumoniae bacteremia in a liver transplantation unit. Clinical Infectious Diseases, 21 (6), pp. 1501-1503.

Gori, A., Espinasse, F., Deplano, A., Nonhoff, C., Nicolas, M.H. and Struelens, M.J. 1996. Comparison of pulsed-field gel electrophoresis and randomly amplified DNA polymorphism analysis for typing extended-spectrum-beta-lactamase-producing Klebsiella pneumoniae. *Journal of Clinical Microbiology,* 34 (10), pp. 2448-2453.

Graña-Miraglia, L., Evans, B.A., López-Jácome, L.E., Hernández-Durán, M., Colín-Castro, C.A., Volkow-Fernández, P., Cevallos, M.A., Franco-Cendejas, R. and Castillo-Ramírez, S., 2020. Origin of OXA-23 Variant OXA-239 from a Recently Emerged Lineage of Acinetobacter baumannii International Clone V. *mSphere,* [e-journal] 5 (1), pp.801-807.

Greenberg, E., 2003. Tiny teamwork. *Nature (London),* 424 (6945), pp.134-138.

Guay, G. and Rothstein, D.M.,1993. Expression of the tetK gene from Staphylococcus aureus in Escherichia coli: comparison of substrate specificities of TetA(B), TetA(C), and TetK efflux proteins. *Antimicrobial Agents and Chemotherapy,* 37 (2), pp.191-198.

Guo, L., An, J., Ma, Y., Ye, L., Luo, Y., Tao, C. and Yang, J., 2016. Nosocomial Outbreak of OXA-48-Producing Klebsiella pneumoniae in a Chinese Hospital: Clonal Transmission of ST147 and ST383. *PloS one,* 11 (8), pp. 128-135.

Gupta, S., Govil, D., Kakar, P.N., Prakash, O., Arora, D., Das, S., Govil, P. and Malhotra, A., 2009. Colistin and polymyxin B: A re-emergence. *Indian journal of critical care medicine,* 13 (2), pp.49-53.

Gurevich, A., Saveliev, V., Vyahhi, N. and Tesler, G., 2013. QUAST: quality assessment tool for genome assemblies. *Bioinformatics (Oxford, England),* 29 (8), pp.1072-1075.

Hackbarth, C.J. and Chambers, H.F., 1989. Methicillin-resistant staphylococci:genetics and mechanisms of resistance. Antimicrobial Agents Chemotherapy, 33 (4), pp. 991-994.

Hæggman, S., Löfdahl, S.,. Paauw, A, Verhoef J. and Brisse, S., 2004. Diversity and Evolution of the Class A Chromosomal Beta-Lactamase Gene in Klebsiellapneumoniae. *Antimicrobial Agents and Chemotherapy,* 48 (7), pp.2400-2408.

Hakenbeck, R.,1998. Mosaic genes and their role in penicillin-resistant *Streptococcus pneumoniae*. *Electrophoresis,* 19 (6), pp.597-601.

Hakenbeck, R., Brückner, R., Denapaite, D. and Maurer, P., 2012. Molecular mechanisms of β-lactam resistance in Streptococcus pneumoniae. *Future microbiology,* 7 (3), pp.395-410.

Halaby, T., Kucukkose, E., Janssen, A.B., Rogers, M.R.C., Doorduijn, D.J., van der Zanden, Adri G M, Al Naiemi, N., Vandenbroucke-Grauls, Christina M J E and van Schaik, W., 2016. Genomic Characterization of ColistinHeteroresistance in Klebsiellapneumoniae during a Nosocomial Outbreak. *Antimicrobial agents and chemotherapy,* 60 (11), pp.6837-6843.

Hall, T.M.,1999. Bioedit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series*, 41, pp. 95-98.

Hall, B.G. and Barlow, M., 2005. Revised Ambler classification of β-lactamases. *Journal of antimicrobial chemotherapy,* 55 (6), pp.1050-1051.

Hall, C.W. and Mah, T., 2017. Molecular mechanisms of biofilm-based antibiotic resistance and tolerance in pathogenic bacteria. *FEMS microbiology reviews,* 41 (3), pp.276-301.

Hament, J. M., Kimpen, J. L., Fleer, A. and Wolfs T. F., 1999. Respiratory viral infection predisposing for bacterial disease: aconcise review. *FEMS Immunology Medical Microbiology*, 26 (8), pp. 189–195.

Han, S. H., 1995. Review of hepatic abscess from K. pneumoniae. An association with diabetes mellitus and septic endophthalmitis. *West Journal of Medicine*, 162 (56), pp. 220-224.

Han, R., Shi, Q., Wu, S., Yin, D., Peng, M., Dong, D., Zheng, Y., Guo, Y., Zhang, R. and Hu, F., 2020. Dissemination of Carbapenemases (KPC, NDM, OXA-48, IMP, and VIM) Among Carbapenem-Resistant Enterobacteriaceae Isolated From Adult and Children Patients in China. *Frontiers in cellular and infection microbiology,* 10, pp.314-321.

Hansen, G.T., 2021. The continuous evolution: perspective on the epidemiology of carbapenemase resistance among Enterobacterales and other Gram-negative bacteria. *Infecttious Diseases and Therapy*, 10 (25), pp. 75-92.

Harada, K., Shimizu, T., Mukai, Y., Kuwajima, K., Sato, T., Usui, M., Tamura, Y., Kimura, Y., Miyamoto, T., Tsuyuki, Y., Ohki, A. and Kataoka, Y., 2016. Phenotypic and Molecular Characterization of Antimicrobial Resistance in Klebsiella spp. Isolates from Companion Animals in Japan: Clonal Dissemination of Multidrug-Resistant

Extended-Spectrum β-Lactamase-Producing Klebsiellapneumoniae. *Frontiers in microbiology, 29 (7)*, pp. 1-12.12

Haryani, Y., Noorzaleha, A.S., Fatimah, A.B., Noorjahan, B.A., Patrick, G.B., Shamsi nar, A.T., Laila, R.A.S. and Son, R., 2007. Incidence of Klebsiella pneumonia in street foods sold in Malaysia and their characterization by antibiotic resistance, plasmid prof iling, and RAPD–PCR analysis. *Food Control,* 18 (7), pp.847-853.

Hassoun, A., Linden, P.K. and Friedman, B., 2017. Incidence , prevalence and mana gement of MRSA bacteremia across patient populations-a review of recent developm ents in MRSA management and treatment. *Critical care,* 21 (211), pp. 1-15.

Hayes, J.D. and Wolf, C.R., 1990. Molecular mechanisms of drug resistance. *Biochemistry Journal*, 272 (15), pp. 281-295.

Hawkey, P.M., 1998. The origins and molecular basis of antibiotic resistance. *BMJ,* 317 (7159), pp.657-660.

Hawkins, G.A., 2017. Chapter 5 - Analysis of Human Genetic Variations Using DNA Sequencing. 2017. *Basic Science Methods for Clinical Researchers.* Elsevier Inc. , pp.77-98.

Hedge, P. J. and Spratt, B. G., 1985. Amino acid substitutions that reduce the affinity of penicillin-binding protein 3 of E. coli for cephalexin. *Journal of Biochemistry*, 151 (9), pp.111-121.

Heidi, B., Kaplan, A. and Greenberg, E. P., 1987. Overproduction and Purification of the luxR Gene Product: Transcriptional Activator of the Vibrio fischeri Luminescence System. *Proceedings of the National Academy of Sciences - PNAS,* 84 (19), pp.6639-6643.

Heritage, J., M'Zali, F.H., Gascoyne-Binzi, D. and Hawkey, P.M., 1999. Evolution and spread of SHV extended-spectrum β-lactamases in Gram-negative bacteria. *Journal of antimicrobial chemotherapy,* 44 (3), pp.309-318.

Héritier, C., Poirel, L., Fournier, P., Claverie, J., Raoult, D. and Nordmann, P., 2005. Characterization of the Naturally Occurring Oxacillinase of Acinetobacter baumannii. *Antimicrobial agents and chemotherapy,* 49 (10), pp.4174-4179.

Hernández, H., Pascual, A., Cantón, R., Martínez-Martínez, L., 2003. Escherichia coli y Klebsiella pneumoniae productores de βlactamasas de espectro extendido en hospitales españoles (Proyecto GEIH-BLEE 2000). Enferm Infecc Microbiol Clin, 21 (9) pp. 77-82

Herrera, R. and Flores-Gallegos, A., 2019. Enzymes in the Pharmaceutical Industry for β-lactam antibiotic production. *Enzymes in Food Biotechnology*, 23 (5), pp. 627-643.

Herschleb, J., Schwartz, D.C.and Ananiev, G., 2007. Pulsed-field gel electrophoresis. *Nature Protocols,* 2 (3), pp.677-684.

Hibbing, M.E., Fuqua, C., Parsek, M.R. and Peterson, S.B., 2010. Bacterial competition: surviving and thriving in the microbial jungle. *Nature reviews. Microbiology,* 8 (1), pp.15-25.

Hirakawa, H., Inazumi, Y., Masaki, T., Hirata, T. and Yamaguchi, A., 2004. Indole induces the expression of multidrug exporter genes in Escherichia coli. *Molecular microbiology,* 55 (4), pp.1113-1126.

Hoekstra, D., van der Laan, Jan Willem, de Leij, L. and Witholt, B., 1976. Release of outer membrane fragments from normally growing Escherichia coli. *Biochimica et biophysica acta. Biomembranes,* 455 (3), pp.889-899.

Hsieh, Y., Huang, Y., Jin, J., Yu, L., Yang, H., Jiang, C., Wang, B. and Tai, P.C., 2014. Mechanisms of Rose Bengal inhibition on SecA ATPase and ion channel activities. *Biochemical and biophysical research communications,* 454 (2), pp.308-312

Huang, Y., Wang, H., Gao, F., Li, M., Yang, H., Wang, B. and Tai, P.C., 2012. Fluorescein Analogues Inhibit SecA ATPase: The First Sub-micromolar Inhibitor of Bacterial Protein Translocation. *ChemMedChem,* 7 (4), pp.571-577.

Huang, M., Lin, L., Wu, Y., Honhing, H., He, P., He, Y., He, P., Li, G., Xiong, G. and Yuan, Y., 2016. Pathogenicity of Klebsiella pneumonia (KpC4) infecting maize and mice. *Journal of Integrative Agriculture,* 15 (7), pp.1510-1520.

IbarzPavón, A.B. and Maiden, M.C.J., 2009. Multilocus sequence typing. *Methods in molecular biology ,* 551 (4), pp.129-140.

Igwe, J.C., Olayinka, B.O., Ehnimidu, J.O. and Onaolapo, J.A., 2016. Impact of Outer Membrane Protein OmpC and OmpF on Antibiotics Resistance of E. coli Isolated from UTI and Diarrhoeic Patients in Zaria, Nigeria. *Clinical microbiology,* 5 (6), pp. 1-5.

Ingham, H.R. and Sisson P. R., 1984. Pathogenic synergism. *Microbiological Sciences,* 1 (5), pp. 206–208.

Iredell, J., Brown, J. and Tagg, K., 2016. Antibiotic resistance in Enterobacteriaceae: mechanisms and clinical implications. *BMJ,* 352, pp.h6420-h6427.

Jacoby, G.A. and Munoz-Price, L.S., 2005. The New β-Lactamases. *The New England journal of medicine,* 352 (4), pp.380-391.

Jacoby G., 2009. AmpC beta-lactamases. *Clinical Microbiology Review*, 22 (161), pp. 1-9.

Jan, A.T., 2017. Outer Membrane Vesicles (OMVs) of Gram-negative Bacteria: A Perspective Update. *Frontiers in microbiology,* 8, pp.1053-1058..

Jang, K., Sweredoski, M.J., Graham, R.L.J., Hess, S. and Clemons, W.M., 2014. Comprehensive proteomic profiling of outer membrane vesicles from Campylobacter jejuni. *Journal of proteomics,* 98, pp.90-98.

Jaurin, B. and Grundstrom, T., 1981. ampC cephalosporinase of Escherichia coli K-12 has a different evolutionary origin from that of beta-lactamases of the penicillinase

type. *Proceedings of the National Academy of Sciences - PNAS,* 78 (8), pp.4897-4901.

Jean F. MacFaddin, 2000. Biochemical tests for identification of medical bacteria.*Journal of Clinical Pathology,* 34 (5), pp. 572-580.

Jena, J., Sahoo, R.K., Debata, N.K. and Subudhi, E., 2017. Prevalence of TEM, SHV, and CTX-M genes of extended-spectrum β-lactamase-producing Escherichia coli strains isolated from urinary tract infections in adults. *3ournal of Biotechnology,* 7 (4), pp.1-7.

Jesus, M.B., Ehlers, M.M. and Kock, Ricardo F. Dos Santos And Marleen M, 2015. *Review - Understanding β-lactamase Producing Klebsiella pneumoniae*InTech.

Ji, G., Beavis, R.C. and Novick, R.P., 1995. Cell density control of staphylococcal virulence mediated by an octapeptide pheromone. *Proceedings of the National Academy of Sciences - PNAS,* 92 (26), pp.12055-12059.

Jones G. E., Gilmour J. S. and Rae A. G., 1982a. The effect of *Mycoplasma ovipneumoniae* and *Pasteurella haemolytica* onspecific pathogen-free lambs. *Journal of Comparative Pathology*, 92 (4), pp. 261–266.

Jones G. E., Gilmour J. S. and Rae A. G., 1982b. The effects of different strains of *Mycoplasma ovipneumoniae* on specific pathogen-free and conventionally-reared lambs. *Journal of Comparative. Pathology*, 92 (5), pp. 267–272.

Jones, R.N., Baquero, F., Privitera, G., Inoue, M. and Wiedemann, B., 1997. Inducible β-lactamase-mediated resistance to third-generation cephalosporins. *Clinical Microbiology and Infection,* 3, pp.S7-S20.

Jones R., 1998. Important and emerging beta-lactamase-mediated resistances in hospital-based pathogens: the AmpC enzymes. Diagnostic Microbiology Infectious Diseases, 31 (1), pp. 461-466.

Jones, A.M., Dodd, M.E. and Webb, A.K., 2001. Burkholderia cepacia: current clinical issues, environmental controversies and ethical dilemmas. *European Respiratory Journal,* 17 (2), pp.295-301.

Jones, E.J., Booth, C., Fonseca, S., Parker, A., Cross, K., Miquel-Clopés, A., Hautefort, I., Mayer, U., Wileman, T., Stentz, R. and Carding, S.R., 2020. The Uptake, Trafficking, and Biodistribution of Bacteroides thetaiotaomicron Generated Outer Membrane Vesicles. Frontiers in microbiology, 11, (45), pp.57-62.

Jovetic, S., Zhu, Y., Marcone, G.L., Marinelli, F. and Tramper, J., 2010. β-Lactam and glycopeptide antibiotics: first and last line of defense? *Trends in Biotechnology,* 28 (12), pp.596-604.

Jun, J., 2018. *Klebsiella pneumoniae* Liver Abscess. *Infectious Chemotherapy*, 50 (3), pp. 210-218.

Kaase, M., Nordmann, P., Wichelhaus, T.A., Gatermann, S.G., Bonnin, R.A. and Poirel, L., 2011. NDM-2 carbapenemase in Acinetobacter baumannii from Egypt. *Journal of antimicrobial chemotherapy,* 66 (6), pp.1260-1262.

Kabha, L Nissimov, A Athamna, Y Keisari, H Parolis, L A Parolis, R M Grue, J Schlepper-Schafer, A R Ezekowitz and D E Ohman, 1995. Relationships among capsular structure, phagocytosis, and mouse virulence in Klebsiella pneumoniae. *Infection and Immunity,* 63 (3), pp.847-852.

Kambli, P., Ajbani, K.,Chaitali N., Meeta, S., Anjali, S., Zarir, U., Sophia,G.B., Rodwell, T.C., Catanzaro, A., Rodrigues, C., 2015. Correlating rrs and eis promoter mutations in clinical isolates of Mycobacterium tuberculosis with phenotypic susceptibility levels to the second-line injectables. *International Journal of Mycobacteriology,* 5 (1), pp.1-6.

Kaparakis-Liaskos, M. and Ferrero, R.L., 2015. Immune modulation by bacterial outer membrane vesicles. *Nature reviews. Immunology,* 15 (6), pp.375-387.

Kapoor, G., Saigal, S. and Elongavan, A., 2017. Action and resistance mechanisms of antibiotics: A guide for clinicians. *Journal of anaesthesiology, clinical pharmacology,* 33 (3), pp.300-305.

Kelly, J.A., Dideberg, O., Charlier, P., Wery, J.P., Libert, M., Moews, P.C., Knox, J.R., Duez, C., Fraipont, C. and Joris, B., 1986. On the origin of bacterial resistance to penicillin: comparison of a beta-lactamase and a penicillin target. *Science,* 231 (4744), pp.1429-1431.

Kelly C. Rice and Kenneth W. Bayles, 2008. Molecular Control of Bacterial Death and Lysis. *Microbiology and Molecular Biology Reviews,* 72 (1), pp. 85-109.

Kenneth H. Nealson, Terry Platt and J. Woodland Hastings, 1970. Cellular Control of the Synthesis and Activity of the Bacterial Luminescent System. *Journal of Bacteriology,* 104 (1), pp.313-322.

Kester, J.C. and Fortune, S.M., 2013. Persisters and beyond: Mechanisms of phenotypic drug resistance and drug tolerance in bacteria. *Critical Reviews in Biochemistry and Molecular Biology,* 49 (2), pp.91-101.

Kesty, N.C. and Kuehn, M.J., 2004. Incorporation of Heterologous Outer Membrane and Periplasmic Proteins into Escherichia coli Outer Membrane Vesicles. *The Journal of biological chemistry*, 279 (3), pp.2069-2076

Keynan, Y. and Rubinstein, E., 2007. The changing face of Klebsiella pneumoniae infections in the community. *International Journal of Antimicrobial Agents,* 30 (5), pp.385-389.

Khan, A.U. and Nordmann, P., 2012. Spread of carbapenemase NDM-1 producers: The situation in India and what may be proposed. *Scandinavian Journal of Infectious Diseases,* 44 (7), pp.531-535.

Khan, A.U. and Parvez, S., 2014. Detection of bla(NDM-4) in Escherichia coli from hospital sewage. *Journal of medical microbiology,* 63 (10), pp.1404-1406.

Khan, A.U., Maryam, L. and Zarrilli, R., 2017. Structure, Genetics and Worldwide Spread of New Delhi Metallo-β-lactamase (NDM): a threat to public health. *BMC microbiology,* 17 (1), pp.1-12.

Khanna, A., Khanna, M. and Aggarwal, A., 2013. Serratia marcescens- a rare opportunistic nosocomial pathogen and measures to limit its spread in hospitalized patients. *Journal of clinical and diagnostic research,* 7 (2), pp.243-246.

Kim, J., Masui, R., Kuramitsu, S., Seo, J., Kim, K. and Sung, M., 2008. Characterization of growth-supporting factors produced by Geobacillus toebii for the commensal thermophile Symbiobacterium toebii. *Journal of microbiology and biotechnology,* 18 (3), pp.490-496.

Kim, S.W., Park, S.B., Im, S.P., Lee, J.S., Jung, J.W., Gong, T.W., Lazarte, J.M.S., Kim, J., Seo, J., Kim, J., Song, J., Jung, H.S., Kim, G.J., Lee, Y.J., Lim, S. and Jung, T.S., 2018. Outer membrane vesicles from β-lactam-resistant Escherichia coli enable the survival of β-lactam-susceptible E. coli in the presence of β-lactam antibiotics. *Scientific reports,* 8 (1), pp. 5402-5413.

Kingston, W. 2004. Streptomycin, Schatz v. Waksman, and the balance of credit for discovery. Journal of the History of Medicine and Allied Sciences, 71 (5), pp. 441-462.

Klein, E.Y., Van Boeckel, T.P., Martinez, E.M., Pant, S., Gandra, S., Levin, S.A., Goossens, H. and Laxminarayan, R., 2018. Global increase and geographic convergence in antibiotic consumption between 2000 and 2015. *Proceedings of the National Academy of Sciences - PNAS,* 115 (15), pp.E3463-E3470.

Klevens, R.M., Morrison, M.A., Nadle, J. Petit, S., Gersham, K. and Ray, S., 2015. Invasive methicillin.resistance S. aureus infections in the United States.*Journal of American Medical Association*, 298 (4), pp. 1763-1771.

Klöppel, G., Löhr, M., Habich, K., Oberholzer, M. and Heitz, P.U., 1985. Islet pathology and the pathogenesis of type 1 and type 2 diabetes mellitus revisited. *Survey and synthesis of pathology research,* 4 (2), pp.1-10.

Knopp, M. and Andersson, D.I., 2018. Predictable Phenotypes of Antibiotic Resistance Mutations. *MBio,* 9 (3), pp.e00770-18.

Ko, W., 2002. Community-Acquired Klebsiella pneumoniae Bacteremia: Global Differences in Clinical Patterns. *Emerging infectious diseases,* 8 (2), pp.160-166.

Koeppen, K., Hampton, T.H., Jarek, M., Scharfe, M., Gerber, S.A., Mielcarz, D.W., Demers, E.G., Dolben, E.L., Hammond, J.H., Hogan, D.A. and Stanton, B.A., 2016. A Novel Mechanism of Host-Pathogen Interaction through sRNA in Bacterial Outer Membrane Vesicles. *PLoS pathogens,* 12 (6), pp. e1005672-e1005683..

Konovalova, A., Kahne, D.E. and Silhavy, T.J., 2017. Outer Membrane Biogenesis. *Annual Review of Microbiology,* 71 (1), pp. 539-556.

Krause, K.M., Serio, A.W., Kane, T.R. and Connolly, L.E., 2016. Aminoglycosides: An Overview. *Cold Spring Harbor perspectives in medicine,* 6 (6), pp. a027029-027035.

Kuehn, M., 2012. Secreted Bacterial Vesicles as Good Samaritans. *Cell host & microbe,* 12 (4), pp.392-393.

Kumar, P., 2017. 33 - Pharmacology of Specific Drug Groups: Antibiotic Therapy. 2017. *Pharmacology and Therapeutics for Dentistry.* Elsevier Inc. , 15 (8), pp. 457-487.

Kuo, S., Yang, S., Lee, Y., Chuang, H., Chen, C., Chang, C., Chen, T., Lu, P., Hsueh, P. and Fung, C., 2013. Dissemination of imipenem-resistant Acinetobacter baumannii with new plasmid-borne blaOXA-72 in Taiwan. *BMC Infectious Diseases,* 13 (1), pp. 319.

Kuo, S., Lee, Y., Yang Lauderdale, T., Huang, W., Chuang, M., Chen, C., Su, S., Lee, K. and Chen, T., 2015. Contribution of Acinetobacter-derived cephalosporinase-30 to sulbactam resistance in Acinetobacter baumannii. *Frontiers in microbiology,* 6, (231), pp. 1-9.

Kohanski, M.A., Collins, J.J. and Dwyer, D.J., 2010. How antibiotics kill bacteria: from targets to networks. *Nature Reviews Microbiology,* 8 (6), pp.423-435.

Kong, K., Schneper, L. and Mathee, K., 2011. Beta-lactam Antibiotics: From Antibiosis to Resistance and Bacteriology. *APMIS,* 118(1), pp. 1–36.

Krishnamurthy, A., McGrath, J., Cripps, A.W. and Kyd, J.M., 2009. The incidence of Streptococcus pneumoniae otitis media is affected by the polymicrobial environment particularly Moraxella catarrhalis in a mouse nasal colonisation model. *Microbes and infection,* 11 (5), pp.545-553.

Kuehn, M.J., 2005. Bacterial outer membrane vesicles and the host-pathogen interaction. *Genes & development,* 19 (22), pp.2645-2655.

Kulkarni, H.M. and Jagannadham, M.V., 2014. Biogenesis and multifaceted roles of outer membrane vesicles from Gram-negative bacteria. *Microbiology (Society for General Microbiology),* 160 (10), pp.2109-2121.

Kulkarni, H.M., Swamy, Ch.V. and Jagannadham, M.V., 2014. Molecular characterization and functional analysis of outer membrane vesicles from the Antarctic bacterium Pseudomonas syringae suggest a possible response to environmental conditions. *Journal of Proteome Research;* 13 (50), pp. 1345–1358.

Kulkarni, H.M., Nagaraj, R. and Jagannadham, M.V., 2015. Protective role of E. coli outer membrane vesicles against antibiotics. *Microbiological Research,* 181, pp.1-7.

Kulp, A. and Kuehn, M.J., 2010. Biological Functions and Biogenesis of Secreted Bacterial Outer Membrane Vesicles. *Annual Reviews of Microbiology*, 64 (4), pp. 163–184.

KUMAR, A. and SCHWEIZER, H., 2005. Bacterial resistance to antibiotics: Active efflux and reduced uptake. *Advanced drug delivery reviews,* 57 (10), pp.1486-1513.

Kumar, S., Alfaadhel, T. and AlBugami, M.M., 2013. Klebsiella ozaenae Bacteremia in a Kidney Transplant Recipient. *Case reports in transplantation,* 20 (56), pp.493516-493532.

Kumarasamy, K.K., Toleman, M.A., Walsh, T.R., Bagaria, J., Butt, F., Balakrishnan, R., Chaudhary, U., Doumith, M., Giske, C.G., Irfan, S., Krishnan, P., Kumar, A.V., Maharjan, S., Mushtaq, S., Noorie, T., Paterson, D.L., Pearson, A., Perry, C., Pike, R., Rao, B., Ray, U., Sarma, J.B., Sharma, M., Sheridan, E., Thirunarayan, M.A., Turton, J., Upadhyay, S., Warner, M., Welfare, W., Livermore, D.M. and Woodford, N., 2010. Emergence of a new antibiotic resistance mechanism in India, Pakistan, and the UK: a molecular, biological, and epidemiological study. *The Lancet infectious diseases,* 10 (9), pp.597-602.

Kuo-Ming Yeh, A. Kurup, L. K. Siu, Y. L. Koh, Chang-Phone Fung, Jung-Chung Lin, Te-Li Chen, Feng-Yee Chang and Tse-Hsien Koh, 2007. Capsular Serotype K1 or K2, Rather than magA and rmpA, Is a Major Virulence Determinant for Klebsiella pneumoniae Liver Abscess in Singapore and Taiwan. *Journal of Clinical Microbiology,* 45 (2), pp.466-471.

Kuwahara, M, Takano, Y., Kasahara, Y., Nara, H. and Obiki, S., 2010. Study on suitability of Kod polymerase for enzymatic production of artificial nucleic acids using base/sugar modified nucleoside triphosphate. *Molecules,* 15 (11), pp. 8229-8240.

Kwiecień, E., Stefańska, I., Chrobak-Chmiel, D., Sałamaszyńska-Guz, A. and Rzewuska, M., 2020. New Determinants of Aminoglycoside Resistance and Their Association with the Class 1 Integron Gene Cassettes in Trueperella pyogenes. *International journal of molecular sciences,* 21 (12), pp.4230-4235.

Laible, G. and Hakenbeck, R., 1991. Five independent combinations of mutations can result in low-affinity penicillin-binding protein 2x of Streptococcus pneumoniae. *Journal of Bacteriology,* 173 (21), pp.6986-6990.

Lang, J. M. and Benbow, M. E., 2013. Species Interactions and Competition. *Nature Education Knowledge*, 4(4), pp. 8-10.

Laupland, K.B., Lyytikainen, O., Sogaard, M., Kennedy, K.J. and Ostergard, C., 2013. The changimg epidemiology of S. bloodstream infection: a multinational population-based surveillance study. *Clinical Microbiology Infections*, 19 (5), pp. 465-471.

Lawlor, M.S., Handley, S.A. and Miller, V.L., 2006. Comparison of the Host Responses to Wild-Type and cpsB Mutant Klebsiella pneumoniae Infections. *Infection and immunity,* 74 (9), pp. 5402-5407.

Lebedev,A., Paul,N., Yee, J., Timoshchuk, V. and Zon, G., 2008. Hot start PCR with heat-activable primers. *Nucleic Acids Research*, 36 (20), pp. 131-136.

Ledent, P., Raquet, X., Joris, B., Beeumen, J. and Frère, J.M., 1993. *A comparative study of class-D beta-lactamases*. *Biochemistry Journal*, 292 (1), pp. 555-562.

Lederman, E.R. and Crum, N.F., 2005. Pyogenic Liver Abscess with a Focus on Klebsiella pneumoniae as a Primary Pathogen: An Emerging Disease with Unique Clinical Characteristics. *The American journal of gastroenterology,* 100 (2), pp.322-331.

Lee, H.H., Molla, M.N., Cantor, C.R. and Collins, J.J., 2010a. Bacterial charity work leads to population-wide resistance. *Nature (London),*467 (7311), pp.82-85.

Lee, J. and Lee, J., 2010b. Indole as an intercellular signal in microbial communities. *FEMS microbiology reviews,* 34 (4), pp.426-444.

Lee, J.C., Lee, E.J., Lee, J.H., Jun, S.H., Choi, C.W., Kim, S.I., Kang, S.S. and Hyun, S., 2012. Klebsiella pneumoniae secretes outer membrane vesicles that induce the innate immune response. *FEMS Microbiology Letters,* 331 (1), pp.17-24.

Lee, J., Lee, E. Y., Kim, S. H., Kim, D. K., Park, K. S., Kim, K. P., Roh, T. Y and Gho, Y. S., 2013. Staphylococcus aureus extracellular vesicles carry biologically active β-lactamase. *Antimicrobial Agents Chemotherapy,* 57, (6), pp. 2589-2595.

Lee, D., Das, S., Dawson, N.L., Dobrijevic, D., Ward, J. and Orengo, C., 2016. Novel Computational Protocols for Functionally Classifying and Characterising Serine Beta-Lactamases. *PLoS computational biology,* 12 (6), pp.1-33.

Lee, B., Yeroushalmi, K., Me, H.M., Sojitra, P., Jilani, U., Iqbal, S., Ahmed, S., Verley, J. and Akella, J., 2018. Community acquired Klebsiella pneumoniae meningitis: a case report. *Germs (Bucureşti),* 8 (2), pp.92-95.

Lehmann, L. and Rousset, F., 2014. The genetical theory of social behaviour. *Philosophical transactions. Biological sciences,* 369 (1642), pp.1-5.

Levin-Reisman, 7I., Ronin, I., Gefen, O., Braniss, I., Shoresh, N., and Balaban, N.Q., 2017. Antibiotic tolerance facilitates the evolution of resistance. *Science,* 355 (6327), pp.826-830.

Levine, C., Hiasa, H. and Marians, K.J., 1998. DNA gyrase and topoisomerase IV: biochemical activities, physiological roles during chromosome replication, and drug sensitivities. *Biochimica et biophysica acta,* 1400 (1-3), pp. 29-43.

Levy, S.B., 2002. Factors impacting on the problem of antibiotic resistance. *Journal of antimicrobial chemotherapy,* 49 (1), pp.25-30.

Lewenza, S., Vidal-Ingigliardi, D. and Pugsley, A.P. 2006. Direct Visualization of Red Fluorescent Lipoproteins Indicates Conservation of the Membrane Sorting Rules in the Family Enterobacteriaceae. *Journal of Bacteriology,* 188 (10), pp. 3516-3524.

Lewin-Epstein, O., Aharonov, R. and Hadany, L., 2017. Microbes can help explain the evolution of host altruism. *Nature communications,* 8 (1), pp.1.7.

Li, W., Raoult, D. and Fournier, P., 2009. Bacterial strain typing in the genomic era. *FEMS microbiology reviews,* 33 (5), pp.892-916.

Li, B., Ye, M., Guo, Q., Zhang, Z., Yang, S., Ma, W., Yu, F. and Chu, H., 2018. Determination of MIC Distribution and Mechanisms of Decreased Susceptibility to Bedaquiline among Clinical Isolates of Mycobacterium abscessus. *Antimicrobial agents and chemotherapy,* 62 (7), pp. 11-21.

Li, X., Ma, W., Qin, Q., Liu, S., Ye, L., Yang, J. and Li, B., 2019. Nosocomial spread of OXA-232-producing Klebsiella pneumoniae ST15 in a teaching hospital, Shanghai, China. *BMC microbiology,* 19 (1), pp.235.

Liakopoulos, A., Mevius, D.J. and Ceccarelli, D., 2016. A Review of SHV Extended-Spectrum β-Lactamases: Neglected Yet Ubiquitous. *Frontiers in microbiology,* 7, (4), pp.1-27.

Liao, Y., Kuo, S., Lee, Y., Chen, C., Lin, S., Shen, L., Fung, C., Cho, W. and Chen, T., 2014. Sheltering Effect and Indirect Pathogenesis of Carbapenem-Resistant Acinetobacter baumannii in Polymicrobial Infection. *Antimicrobial agents and chemotherapy,* 58 (7), pp.3983-3990.

Liao, Y., Kuo, S., Chiang, M., Lee, Y., Sung, W., Chen, Y., Chen, T. and Fung, C., 2015. Acinetobacter baumannii Extracellular OXA-58 Is Primarily and Selectively Released via Outer Membrane Vesicles after Sec-Dependent Periplasmic Translocation. *Antimicrobial agents and chemotherapy,* 59 (12), pp.7346-7354.

Liechti, G. and Goldberg, J.B., 2012. Outer membrane biogenesis in Escherichia coli, Neisseria meningitidis, and Helicobacter pylori: paradigm deviations in H. pylori. *Front iers in cellular and infection microbiology,* 2, (29), pp. 1- 19.

Lilley, B.N. and Bassler, B.L., 2000. Regulation of quorum sensing in Vibrio harveyi by LuxO and Sigma-54. *Molecular Microbiology,*36 (4), pp.940-954.

Lin, J., Chang, F., Fung, C., Xu, J., Cheng, H., Wang, J., Huang, L. and Siu, L.K., 2004. High prevalence of phagocytic-resistant capsular serotypes of Klebsiella pneumoniae in liver abscess. *Microbes and infection,* 6 (13), pp.1191-1198.

Lin, Y., Jeng, Y., Chen, T. and Fung, C., 2010. Bacteremic community-acquired pneumonia due to Klebsiella pneumoniae: Clinical and microbiological characteristics in Taiwan, 2001-2008. *BMC infectious diseases,* 10 (1), pp.307.

Lin, J., Koh, T.H., Lee, N., Fung, C., Chang, F., Tsai, Y., Ip, M. and Siu, L.K., 2014. Genotypes and virulence in serotype K2 Klebsiella pneumoniae from liver abscess and non-infectious carriers in Hong Kong, Singapore and Taiwan. *Gut pathogens,* 6 (1), pp.21-27.

Lin, W., Tseng, C., Wu, A., Chang, Y., Kuo, T., Chao, J., Wang, M. and Wu, J., 2018. Clinical and microbiological characteristics of peritoneal dialysis-related peritonitis caused by Escherichia coli in southern Taiwan. *European Journal of Clinical Microbiology & Infectious Diseases,* 37 (9), pp.1699-1707.

Lindmark, B., Rompikuntal, P.K., Vaitkevicius, K., Song, T., Mizunoe, Y., Uhlin, B.E., Guerry, P. and Wai, S.N., 2009. Outer membrane vesicle-mediated release of cytolet hal distending toxin (CDT) from Campylobacter jejuni. *BMC microbiology,* 9 (1), pp. 1-10.

Lioy, V.S., Goussard, S., Guerineau, V., Yoon, E., Courvalin, P., Galimand, M. and Grillot-Courvalin, C., 2014. Aminoglycoside resistance 16S rRNA methyltransferases block endogenous methylation, affect translation efficiency and fitness of the host. *RNA (Cambridge),* 20 (3), pp.382-391.

Liu, Y.M., Li, B.B., Zhang, Y.Y., Zhang, W., Shen, H., Li, H. and Cao, B., 2014. Clinical and Molecular Characteristics of Emerging Hypervirulent Klebsiella

pneumoniae Bloodstream Infections in Mainland China. *Antimicrobial agents and chemotherapy,* 58 (9), pp.5379-5385.

Liu, Y., Defourny, K.A.Y., Smid, E.J. and Abee, T., 2018. Gram-positive bacterial extracellular vesicles and their impact on health and disease. *Frontiers in microbiology,* 9 (JUL), pp.1502-1510.

Liu, W, Wu, J, Wang, Z., Wu, X., Wang, G and Ren, J., 2019. Polymicrobial Bacteremia Involving Klebsiella pneumoniae in Patients with Complicated Intra-Abdominal Infections Frequency, Co-Pathogens, Risk Factors, and Clinical Outcomes. Surgical Infections, 20 (4), pp. 1-16.

Livermore, D.M., 1987a. Radiolabelling of penicillin-binding proteins (PBPs) in intact Pseudomonas aeruginosa cells: consequences of β-lactamase activity by PBP-5. *Journal of antimicrobial chemotherapy,* 19 (6), pp.733-742.

Livermore, D.M., 1987b. Clinical significance of beta-lactamase induction and stable derepression in Gram-negative rods. *European journal of clinical microbiology,* 6 (4), pp.439-445.

Livermore, D.M.,1995. β-Lactamases in laboratory and clinical resistance. *Clinical Microbiology Review*, 8 (1), pp. 557-584.

Livermore, D.M., 1998. Beta-lactamase-mediated resistance and opportunities for its control. *Journal of antimicrobial chemotherapy,* 41 Suppl D (suppl 4), pp.25-41.

Livermore, D.M., Canton, R., Gniadkowski, M., Nordmann, P., Rossolini, G.M., Arlet, G., Ayala, J., Coque, T.M., Kern-Zdanowicz, I., Luzzaro, F., Poirel, L. and Woodford, N., 2006. CTX-M: changing the face of ESBLs in Europe. *Journal of antimicrobial chemotherapy,* 59 (2), pp.165-174.

Livermore, D.M., 2008. Defining an extended-spectrum β-lactamase. *Clinical Microbiology and Infection,*14, (7) pp.3-10.

Llanes, C., Hocquet, D., Vogne, C., Benali-Baitich, D., Neuwirth, C. and Plesiat, P., 2004. Clinical Strains of Pseudomonas aeruginosa Overproducing MexAB-OprM and MexXY Efflux Pumps Simultaneously. *Antimicrobial agents and chemotherapy,* 48 (5), pp.1797-1802.

Lo, Y.M.D. and Chan, K.C.A., 2006. Introduction to the polymerase chain reaction. *Methods in molecular biology (Clifton, N.J.),* 336 (9), pp.1-10.

Lobanovska, M. and Pilla, G., 2017. Penicillin's Discovery and Antibiotic Resistance: Lessons for the Future? *The Yale journal of biology & medicine,* 90 (1), pp.135-145.

Löfmark, S., Edlund, C. and Nord,C.N., 2010. Metronidazole Is Still the Drug of Choice for Treatment of Anaerobic Infections. *Clinical Infectious Diseases,* 50 (s1), pp.S16-S23.

Logan, L.K. and Bonomo, R.A., 2016. Metallo-β-lactamase (MBL)-producing Enterobacteriaceae in U.S. Children. *Infectious Diseases* 3(2), pp 25-36.

Lomonaco, S., Crawford, M.A., Lascols, C., Timme, R.E., Anderson, K., Hodge, R.D., Fisher, D.J., Pillai, S.P., Morse, S.E., Khan, E., Hughes, M.H., Allard, M.W., and

Sharma, K., 2018. Resistome of carbapenem- and colistin-resistant Klebsiellapneumoniae clinical isolates. *PLoS One,* 13 (6), pp.e0198526.

Loucif, L., Kassah-Laouar, A., Saidi, M., Messala, A., Chelaghma, W. and Rolain, J., 2016. Outbreak of OXA-48-Producing Klebsiella pneumoniae Involving a Sequence Type 101 Clone in Batna University Hospital, Algeria. *Antimicrobial agents and chemotherapy,* 60 (12), pp.7494-7497.

Lovering, A.L., Safadi, S.S. and Strynadka, N.C.J., 2012. Structural Perspective of Peptidoglycan Biosynthesis and Assembly. *Annual review of biochemistry,* 81 (1), pp.451-478.

LUZZARO, F., MEZZATESTA, M., MUGNAIOLI, C., PERILLI, M., STEFANI, S., AMICOSANTE, G., ROSSOLINI, G.M. and TONIOLO, A., 2016. Trends in Production of Extended-Spectrum β-Lactamases among Enterobacteria of Medical Interest: Report of the Second Italian Nationwide Survey. *Journal of Clinical Microbiology,* 44 (5), pp.1659-1664.

Lyon, G.J. and Novick, R.P., 2004. Peptide signaling in Staphylococcus aureus and other Gram-positive bacteria. *Peptides (New York, N.Y. : 1980),* 25 (9), pp.1389-1403.

MacDonald, I.A. and Kuehn, M.J., 2013. Stress-Induced Outer Membrane Vesicle Production by Pseudomonas aeruginosa. *Journal of bacteriology,* 195 (13), pp.2971-2981.

Machado, E., Cantón, R., Baquero, F., Galán, J., Rollán, A., Peixe L., and Coque, T.M., 2005. Integron Content of Extended-Spectrum-β-Lactamase-Producing Escherichia coli Strains over 12 Years in a Single Hospital in Madrid, Spain. *Antimicrobial Agents and Chemotherapy,* 49 (5), pp.1823-1829.

Magill, S.S., Edwards, J.R., Bamberg, W., Beldavs, Z.G., Dumyati, G., Kainer, M.A., Lynfield, R., Maloney, M., McAllister-Hollod, L., Nadle, J., Ray, S.M., Thompson, D.L., Wilson, L.E. and Fridkin, S.K., 2014. Multistate Point-Prevalence Survey of Health Care–Associated Infections. *The New England journal of medicine,* 370 (13), pp.1198-1208.

Maki, H., 2002. Origins of spontaneous mutations: specificity and directionality of base-substituions, frameshift, and sequence-substitution mutagenesis. *Annual review of genetics*, 36 (272), pp. 279-303.

Malabirade, A., Habier, J., Heintz-Buschart, A., May, P., Godet, J., Halder, R., Etherid ge, A., Galas, D., Wilmes, P. and Fritz, J.V., 2018. The RNA Complement of Outer M embrane Vesicles From Salmonella enterica Serovar Typhimurium Under Distinct Cul ture Conditions. *Frontiers in microbiology,* 9, (2015), pp. 1-20.

Mammeri, H., Guillon, H., Eb, F. and Nordmann, P., 2010. Phenotypic and Biochemical Comparison of the Carbapenem-Hydrolyzing Activities of Five Plasmid-Borne AmpC β-Lactamases. *Antimicrobial Agents and Chemotherapy,* 54 (11), pp.4556-4560.

Manning, A.J., Kuehn, M.J., 2011. Contribution of bacterial outer membrane vesicles to innate bacterial defense. *BMC Microbiology*, 11 (3), pp. 258-265.

Mano, Y., Saga, T., Ishii, Y., Yoshizumi, A., Bonomo, R.A., Yamaguchi, K. and Tateda, K., 2015. Molecular analysis of the integrons of metallo-β-lactamase-producing Pseudomonas aeruginosa isolates collected by nationwide surveillance programs across Japan. *BMC microbiology,* 15 (1), pp.1-10.

Manoharan, A., Premalatha, K., Chatterjee, S., Mathai, D. and SARI Study Group, 2011. Correlation of TEM, SHV and CTX-M extended-spectrum beta lactamases among Enterobacteriaceae with their in vitro antimicrobial susceptibility. *Indian Journal of Medical Microbiology,* 29 (2), pp.161-164.

Margaret, E., Teasdale, J., Wallace, F., and David C. R., 2009. Secondary Metabolites Produced by the Marine Bacterium Halobacillus salinus That Inhibit Quorum Sensing-Controlled Phenotypes in Gram-negative Bacteria. *Applied and Environmental Microbiology,* 75 (3), pp.567-572.

Marjon, G.J.D.V., Zagorskib, M., McNally , A. and Bollenbach, T., 2018. Interaction networks, ecological stability, and collective antibiotic tolerance in polymicrobial infections. *Microbiology, Immunology and Infection*, 114 (1) , pp. 10666–10671.

Marković, T., Jeinić, L., Smitran, A. and Petković, M., 2013. Prevalence and antimicrobial susceptibility of extended-spectrum beta-lactamase producing urinary isolates of Escherichia coli in outpatients. *Srpski arhiv za celokupno lekarstvo,* 141 (11-12), pp.775-779.

Marsh, P.D., 2006. Dental plaque as a biofilm and a microbial community – implications for health and disease. *BMC oral health,* 6 (S1), pp.S14-S16.

Martin, R.M. and Bachman, M.A., 2018. Colonization, Infection, and the Accessory Genome of Klebsiella pneumoniae. *Frontiers in cellular and infection microbiology,* 8, pp.4-10.

Martínez-Martínez, L., 2008. Extended-spectrum β-lactamases and the permeability barrier. *Clinical microbiology and infection,* 14, pp.82-89.

Martora, F., Pinto, F., Folliero, V., Cammarota, M., Dell'Annunziata, F., Squillaci, G., Galdiero, M., Morana, A., Schiraldi, C., Giovane, A., Galdiero, M. and Franci, G., 2019. Isolation, characterization and analysis of pro-inflammatory potential of Klebsiella pneumoniae outer membrane vesicles. *Microbial pathogenesis,* 136, pp.103719-103725.

Mathers, A.J., Hazen, K.C., Carroll, J., Yeh, A.J., Cox, H.L., Bonomo, R.A. and Sifri, C.D., 2013. First Clinical Cases of OXA-48-Producing Carbapenem-Resistant Klebsiellapneumoniae in the United States: the "Menace" Arrives in the New World. *Journal of clinical microbiology,* 51 (2), pp.680-683.

Mathlouthi, N., Ben lamine, Y., Somai, R., Bouhalila-Besbes, S., Bakour, S., Rolain, J. and Chouchani, C., 2018. Incidence of OXA-23 and OXA-58 Carbapenemases Coexpressed in Clinical Isolates of Acinetobacter baumannii in Tunisia. *Microbial drug resistance (Larchmont, N.Y.),* 24 (2), pp.136-141.

Maurya, N., Jangra, M., Tambat, R. and Nandanwar, H., 2019. Alliance of Efflux Pumps with β-Lactamases in Multidrug-Resistant Klebsiella pneumoniae Isolates. *Microbial drug resistance (Larchmont, N.Y.),* 25 (8), pp.1155-1163.

McAdam, A.J., 2020. Enterobacteriaceae ? Enterobacterales ? What Should We Call Enteric Gram-negative Bacilli? A Micro-Comic Strip. *Journal of clinical microbiology,* 58 (2), pp. 1-10.

McBroom, A.J. and Kuehn, M.J., 2007. Release of outer membrane vesicles by Gram-negative bacteria is a novel envelope stress response. *Molecular microbiology,* 63 (2), pp.545-558.

McCaig, W. D., Koller, A. and Thanassi, D. G., 2013. Production of Outer Membrane Vesicles and Outer Membrane Tubes by Francisella novicida. *Journal of Bacteriology,* 195 (6), pp.1120-1132.

McDanel, J., Schweizer, M., Crabb, V., Nelson, R., Samore, M., Khader, K., Blevins, A.E., Diekema, D., Chiang, H., Nair, R. and Perencevich, E., 2017. Incidence of Extended-Spectrum β-Lactamase (ESBL)-Producing Escherichia coli and Klebsiella Infections in the United States: A Systematic Literature Review. *Infection control and hospital epidemiology,* 38 (10), pp.1209-1215.

McDermott, P.F., Walker, R.D. and White, D.G., 2003. Antimicrobials: Modes of Action and Mechanisms of Resistance. *International Journal of Toxicology,* 22 (2), pp.135-143.

Mehta, R. and Champney, W.S., 2002. 30S Ribosomal subunit assembly is a target for inhibition by aminoglycosides in E.coli. *Antmicrobial agents and chemotherapy*, 1 (46), pp. 1546-1549.

Mehta, D. and Sharma, A.K., 2016. Cephalosporins: A Review on Imperative Class of Antibiotics. *Inventi Spreading Knowledge,* 50 (1), pp. 1-7.

Meletis, G., 2015. Carbapenem resistance: overview of the problem and future perspectives. *Therapeutic advances in infectious disease,* 3 (1), pp.15-21.

Mentasti, M., Prime, K., Sands, K., Khan, S. and Wootton, M., 2020. Rapid detection of OXA-23-like, OXA-24-like, and OXA-58-like carbapenemases from Acinetobacter species by real-time PCR. *The Journal of hospital infection,* 105 (4), pp.741-746.

Merla, C., Rodrigues, C., Passet, V., Corbella, M., Thorpe, H.A., Kallonen, T.V.S. and Zong, Z., 2019. Description of Klebsiella spallanzanii sp. nov. and of Klebsiella pasteurii sp. nov. *Frontiers in microbiology,* 10 (15), pp.675-686.

Meroueh, S.O., Minasov, G., Lee, W., Shoichet, B.K. and Mobashery, S., 2003. Structural Aspects for Evolution of β-Lactamases from Penicillin-Binding Proteins. *Journal of the American Chemical Society,* 125 (32), pp.9612-9618.

Miller, E.L., 2002. The penicillins: A review and update. *Journal of Midwifery and Women's Health,* 47 (6), pp.426-434.

Mills, E.L., 1984. Viral infections predisposing to bacterial infections. Annual Reviews of Medicine, 35 (7), pp. 469–479.

Miyashiro, T. and Ruby, E.G., 2012. Shedding light on bioluminescence regulation in Vibrio fischeri. *Molecular microbiology,* 84 (5), pp.795-806.

Mo, G.C.H., Posner, C., Rodriguez, E.A., Sun, T. and Zhang, J., 2020. A rationally enhanced red fluorescent protein expands the utility of FRET biosensors. *Nature communications*,] 11 (1), pp.1848-1852.

Mobarki, N.S., Almerabi, B.A. and Hattan, A.A., 2019*. International Journal of Medicine in Developing Countries,* 3 (6), pp.561-564.

Mojica, M.F., Bonomo R.A. and Fast, W., 2016. B1-Metallo-β-Lactamases: Where Do We Stand? *Current Drug Targets,* 17 (9), pp.1029-1050.

Moore, D.S., Notz, W.I. and Flinger, M.A., 2013.The basic practice of statistics (6th ed.)

Mortensen, J., Hansen, A., Falk, M., Nielsen, I.K. and Groth, S., 1993. Reduced effect of inhaled beta 2-adrenergic agonists on lung mucociliary clearance in patients with cystic fibrosis. *Chest* 103 (1), pp. 805– 811.

Mrowiec, P., Klesiewicz, K., Małek, M., Skiba-Kurek, I., Sowa-Sierant, I., Skałkowska, M., Budak, A. and Karczewska, E., 2019. Antimicrobial susceptibility and prevalence of extended-spectrum beta-lactamases in clinical strains of Klebsiella pneumoniae isolated from pediatric and adult patients of two Polish hospitals. *The New microbiologica,* 42 (4), pp.1-8.

MUGNAIOLI, C., LUZZARO, F., DE LUCA, F., BRIGANTE, G., PERILLI, M., AMICOSANTE, G., STEFANI, S., TONIOLO, A. and ROSSOLINI, G.M., 2006. CTX-M-Type Extended-Spectrum β-Lactamases in Italy: Molecular Epidemiology of an Emerging Countrywide Problem. *Antimicrobial Agents and Chemotherapy,* 50 (8), pp.2700-2706.

Mugnier, P.D., Poirel, L., Naas, T. and Nordmann, P., 2010. Worldwide dissemination of the blaOXA-23 carbapenemase gene of Acinetobacter baumannii. *Emerging infectious diseases,* 16 (1), pp.35-40.

Mullineaux, C.W., 2007. Localization and mobility of bacterial proteins by confocal microscopy and fluorescence recovery after photobleaching. *Methods in molecular biology (Clifton, N.J.),* 390, pp. 3-15.

Munita, J.M. and Arias, C.A., 2016. Mechanisms of antibiotic resistance. *Microbiology Spectrum*, 4(2), doi: 10.1128.

Naas, T., Sougakoff, W., Casetta, A. and Nordmann, P., 1998. Molecular Characterization of OXA-20, a Novel Class D β-Lactamase, and Its Integron from Pseudomonas aeruginosa. *Antimicrobial Agents and Chemotherapy,* 42 (8), pp.2074-2083.

Nakai, T., Kume, K., Yoshikawa, H., Oyamada, T. and Yoshikawa, T., 1988. Adherence of Pasteurella multocida or Bordetellabronchiseptica to the swine nasal epithelial cell in vitro. *Infections and. Immunology*,56 (5), pp. 234–240.

Narisara, C., Vanaporn, W., Khaemaporn, B., Rachaneeporn, T., Mongkol, V., Direk, L., Wirongrong, C., Surasakdi, W., Sasithorn, P., Nicholas, J. W., Nicholas, P. J.

D.and Sharon J. P., 2007. Biological Relevance of Colony Morphology and Phenotypic Switching by Burkholderiapseudomallei. *Journal of Bacteriology,* 189 (3), pp.807-817.

Neilands, J.B., 1995. Siderophores: Structure and Function of Microbial Iron Transport Compounds. *The Journal of biological chemistry,* 270 (45), pp.26723-26726.

Nevermann, J., Silva, A., Otero, C., Oyarzún, D.P., Barrera, B., Gil, F., Calderón, I.L. and Fuentes, J.A., 2019. Identification of Genes Involved in Biogenesis of Outer Membrane Vesicles (OMVs) in Salmonella enterica Serovar Typhi. *Frontiers in microbiology,* 10, pp. 104-108.

Newton, R.A. 1965. Mechanisms of Antibiotic Action. Annual Review of Microbiology, 19: 209-240.

Nguyen Thi, P. L., Yassibanda, S., Aidara, A., Le Bouguenec, C. & Germani, Y., 2003. Enteropathogenic K. pneumoniae HIV-infected adults, Africa. *Emerging Infectious Diseases*, 9 (45), pp. 135-137.

Ni, R.T., Onishi, M., Mizusawa, M., Kitagawa, R., Kishino, T., Matsubara, F., Tsuchiya, T., Kuroda, T. and Ogawa, W., 2020. The role of RND-type efflux pumps in multidrug-resistant mutants of Klebsiella pneumoniae. *Scientific reports,* 10 (1), pp.10876-10888..

Nichol, K. P. and Cherry, J. D., 1967. Bacterial-viral interrelations in respiratory infections of children. New England Journal of Medicine, 277 (9), pp. 667–672

Nicholson, A.J. 1954. An outline of the dynamics of animal populations. *Australian Journal of Zoology,* 2 (1), pp.9-65.

Nicolas-Chanoine, M., Mayer, N., Guyot, K., Dumont, E. and Pagès, J., 2018. Interplay Between Membrane Permeability and Enzymatic Barrier Leads to Antibiotic-Dependent Resistance in Klebsiella Pneumoniae. *Frontiers in microbiology,* 9, pp.1422-1434.

Nikaido, H., Rosenberg, E.Y. and Foulds, J., 1983. Porin channels in Escherichia coli: studies with beta-lactams in intact cells. *Journal of Bacteriology,* 153 (1), pp.232-240

Nikaido, H., 2003. Molecular Basis of Bacterial Outer Membrane Permeability Revisited. *Microbiology and Molecular Biology Reviews,* 67 (4), pp.593-656.

Nikaido, H. and Pagès, J., 2012. Broad-specificity efflux pumps and their role in multidrug resistance of Gram-negative bacteria. *FEMS Microbiology Reviews,* 36 (2), pp.340-363.

Nordmann, P., Cuzon, M.D., Naas, P. 2009. The real threat of Klebsiella pneumonia ecarbapenemase-producing bacteria. *Lancet Infectious Diseases,* 9 (4), pp.228-236.

Nordmann, P., Naas, T. and Poirel, L., 2011. Global spread of Carbapenemase-producing Enterobacteriaceae. *Emerging infectious diseases,* 17 (10), pp.1791-1798.

Novikova, O.D. and Solovyeva, T.F., 2009. Nonspecific porins of the outer membrane of Gram-negative bacteria: Structure and functions. *Biochemistry (Moscow). Supplement series A, Membrane and cell biology,* 3 (1), pp.3-15.

Nugent, M.E. and Hedges, R.W., 1979. The nature of the genetic determinant for the SHV-1 beta-lactamase. *Molecular & general genetics ,* 175 (3), pp.239-247.

Ogasawara, M., Kono, D. H. & Yu, D. T. (1986). Mimicry of human histocompatibility HLA-B27 antigens by K. pneumoniae. *Infectious Immunology,* 51 (18), pp. 901-908.

Ohmori, S., Shiraki, K., Ito, K., Inoue, H., Ito, T., Sakai, T., Takase, K. and Nakano, T., 2002. Septic endophthalmitis and meningitis associated with K. pneumoniae liver abscess. *Hepatology Research,* 22 (10), pp. 307-312.

Ohno, S., 1997. The reason for as well as the consequence of the cambrian explosion in animal evolution. *Journal of Molecular Evolution,* 44 (S1), pp.S23-S27.

Ouellette, M., Bissonnette, L. and Roy, P.H., 1987. Precise insertion of antibiotic resistance determinants into Tn21-like transposons: nucleotide sequence of the OXA-1 beta-lactamase gene. *Proceedings of the National Academy of Sciences of the United States of America,* 84 (21), pp.7378-7382.

Özkaya, Ö, Xavier, K.B., Dionisio, F. and Balbontín, R., 2017. Maintenance of Microbial Cooperation Mediated by Public Goods in Single- and Multiple-Trait Scenarios. *Journal of bacteriology,* 199 (22), pp. 1-14.

Öztürk, H., Ozkirimli, E. and Özgür, A., 2015. Classification of Beta-Lactamases and Penicillin Binding Proteins Using Ligand-Centric Network Models. *PloS one,* 10 (2), pp.1-23.

Paczosa, M.K. and Mecsas, J., 2018. Klebsiella pneumoniae: Going on the Offense with a Strong Defense. *Microbiology and molecular biology reviews,* 80 (3), pp.629-661.

Padilla, E., Llobet, E., Domenech-Sanchez, A., Martinez-Martinez, L., Bengoechea, J.A. and Alberti, S., 2009. Klebsiella pneumoniae AcrAB Efflux Pump Contributes to Antimicrobial Resistance and Virulence. *Antimicrobial agents and chemotherapy,* 54 (1), pp.177-183.

Pagès, J., James, C.E. and Winterhalter, M., 2008. The porin and the permeating antibiotic: a selective diffusion barrier in Gram-negative bacteria. Nature Reviews, Microbiology, 6 (12), pp. 893-903.

Palzkill, T., 2013. Metallo-β-lactamase structure and function. *Annals of the New York Academy of Sciences,* 1277 (1), pp.91-104.

Palzkill, T., 2018. Structural and Mechanistic Basis for Extended-Spectrum Drug-Resistance Mutations in Altering the Specificity of TEM, CTX-M, and KPC β-lactamases. *Frontiers in molecular biosciences,* 5, pp.1-19.

Panah,M.A., Eftekhar, F., Ghamsari, M.M., Parvin, M., Feizabadi, M.M., 2012. Genetic profiling of Klebsiella pneumoniae: comparison of pulsed field gel

electrophoresis and random amplified polymorphic DNA. Brazilian Journal of Microbiology, 44 (3), pp. 823-828.

Papp-Wallace, K.M., Kumar, V., Zeiser, E.T., Becka, S.A. and van den Akker, F., 2019. Structural Analysis of The OXA-48 Carbapenemase Bound to A "Poor" Carbapenem Substrate, Doripenem. *Antibiotics (Basel),* 8 (3), pp.1-14.

Pareek, C., Smoczynski, R. and Tretyn, A., 2011. Sequencing technologies and genome sequencing. *Journal of Applied Genetics,* 52 (4), pp.413-435.

Park, J.S., Lee, W.C., Yeo, K.J., Ryu, K., Kumarasiri, M., Hesek, D., Lee, M., Mobashery, S., Song, J.H., Kim, S.I., Lee, J.C., Cheong, C., Jeon, Y.H. and Kim, H., 2012. Mechanism of anchoring of OmpA protein to the cell wall peptidoglycan of the Gram-negative bacterial outer membrane. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology,* 26 (1), pp. 219-228.

Partridge, S.R., Kwong, S.M., Firth, N. and Jensen, S.O., 2018. Mobile Genetic Elements Associated with Antimicrobial Resistance. *Clinical microbiology reviews,* 31 (4) pp. 1-61.

Patel, G. and Bonomo, R.A., 2013. "Stormy waters ahead": global emergence of carbapenemases. *Frontiers in microbiology,* 4 (48)pp. 1-17.

Paterson, D.L. and Bonomo R.A., 2005. Extended-spectrum beta-lactamases: a clinical update. *ClinicalMicrobioly Reviews*, 18 (4), pp.657–686.

Pavel, A.B. and Vasile, C.I., 2012. PyElph - a software tool for gel images analysis and phylogenetics. *BMC bioinformatics,* 13 (1), pp.9-14.

Payne, D.J., Gwynn, M.N., Holmes, D.J. and Pompliano, D.L., 2006. Drugs for bad bugs: confronting the challenges of antibacterial discovery. *Nature reviews. Drug discovery,* 6 (1), pp.29-40.

Peña, C., Suarez, F. Tubau, C. Juan, B. Moya, M. A. Dominguez, A. Oliver, M. Pujol and J. Ariza, 2009. Nosocomial Outbreak of a Non-Cefepime-Susceptible Ceftazidime-Susceptible Pseudomonas aeruginosa Strain Overexpressing MexXY-OprM and Producing an Integron-Borne PSE-1 ß-Lactamase. *Journal of Clinical Microbiology,* 47 (8), pp.2381-2387.

Pendleton, S., Hanning, I., Biswas, D. and Ricke, S.C., 2013. Evaluation of whole-genome sequencing as a genotyping tool for Campylobacter jejuni in comparison with pulsed-field gel electrophoresis and flaA typing. *Poultry science,* 92 (2), pp.573-580.

Perchat, S., Talagas, A., Poncet, S., Lazar, N., Li de la Sierra-Gallay, Inès, Gohar, M., Lereclus, D. and Nessler, S., 2016. How Quorum Sensing Connects Sporulation to Necrotrophism in Bacillus thuringiensis. *PLoS pathogens,*12 (8), pp.779-791.

Perez-Cruz, C., Delgado, L., Lopez-Iglesias, C. and Mercade, E., 2015. Outer-Inner Membrane Vesicles Naturally Secreted by Gram-negative Pathogenic Bacteria. *PLoS One,* 10 (1), pp. e0116896-e011699.

Perez, F., El Chakhtoura, N.G., Papp-Wallace, K.M., Wilson, B.M. and Bonomo, R.A., 2016. Treatment options for infections caused by carbapenem-resistant

Enterobacteriaceae: can we apply "precision medicine" to antimicrobial chemotherapy? *Expert Opinion on Pharmacotherapy,* 17 (6), pp.761-781.

Pèrez-Grajera, I., Lara-Fuella, N. and Fernàndez-Romero, S., 2016. Ràpido aumento de la resistencia a cefalosporinas de 3 a generacìon, imipenem y de la corresistencia en 7.140 aislados de Klebsiella pneumoniae en hemocultivos (2010-2014) segùn datose de EARS-Net Infectious Microbiology Clinics, 35 (9), pp. 480-486.

Perilli, M., Mugnaioli, C., Luzzaro, F., Fiore, M., Stefani, S., Rossolini, G.M. and Amicosante, G., 2005. Novel TEM-Type Extended-Spectrum β-Lactamase, TEM-134, in a Citrobacter koseri Clinical Isolate. *Antimicrobial Agents and Chemotherapy,* 49 (4), pp.1564-1566.

Perlin, M., Clark, D., McKenzie, C., Patel, H., Jackson, N., Kormanik, C., Powell, C., Bajorek, A., Myers, D., Dugatkin, L. and Atlas, R., 2009. Protection of Salmonella by ampicillin-resistant Escherichia coli in the presence of otherwise lethal drug concentrations. *Proceedings of the Royal Society. B, Biological sciences,* 276 (1674), pp.3759-3768.

Perry, R.D., Balbo, P.B., Jones, H.A., Fetherston, J.D. and DeMoll, E., 1999. Yersiniabactin from Yersinia pestis: biochemical characterization of the siderophore and its role in iron transport and regulation. *Microbiology,* 145 (5), pp.1181-1190.

Peters, B.M., Jabra-Rizk, M.A., O'May, G.A., Costerton, J.W. and Shirtliff, M.E., 2012. Polymicrobial Interactions: Impact on Pathogenesis and Human Disease. *Clinical microbiology reviews,* 25 (1), pp.193-213.

Petersen, P.E., Bourgeois, D., Ogawa, H., Estupinan-Day, S. and Ndiaye, C., 2005. The global burden of oral diseases and risks to oral health. *Bulletin of the World Health Organization,* 83 (9), pp.661-669.

Peterson, E. and Kaur, P., 2018. Antibiotic Resistance Mechanisms in Bacteria: Relationships Between Resistance Determinants of Antibiotic Producers, Environmental Bacteria, and Clinical Pathogens. *Frontiers in microbiology,* 9, pp.2928.

Petrovska, B.B., 2012. Historical review of medicinal plants' usage. *Pharmacognosy reviews,* 6 (11), pp. 1-5.

Pfeifer, Y., Schlatterer, K., Engelmann, E., Schiller, R.A., Frangenberg, H.R., Stiewe, D., Holfelder, M., Witte, W., Nordmann, P. and Poirel, L., 2012. Emergence of OXA-48-Type Carbapenemase-Producing Enterobacteriaceae in German Hospitals. *Antimicrobial agents and chemotherapy,* 56 (4), pp.2125-2128.

Piddock, L.J., 2006. Clinically relevant chromosomally encoded multidrug resistance efflux pumps in bacteria. *Clinical Microbiology Reviews* 19 (14), pp. 382–402.

Pimlapas, L., Eva M.N., Rolf, S.K., Ole, L. and Frank,M.A., 2014. Evaluation of Whole Genome Sequencing for Outbreak Detection of Salmonella enterica. *PLoS One,* 9 (2), pp.e87991.

Pitman, S. and Cho, K.H., 2015. The Mechanisms of Virulence Regulation by Small Noncoding RNAs in Low GC Gram-positive Pathogens. *International journal of molecular sciences,* 16 (12), pp.29797-29814.

Pitt, J.J., 2009. Principles and applcations of liquid chromatography-mass spectrometry in clinical biochemistry.The Clinical Biochemistry Review, 30 (1), pp.19-34.

Podbielski, A., Schönling, J., Melzer, B., Warnatz, K. and Leusch, H.G., 1991. Molecular characterization of a new plasmid-encoded SHV-type beta-lactamase (SHV-2 variant) conferring high-level cefotaxime resistance upon Klebsiella pneumoniae. *Journal of general microbiology,* 137 (3), pp.569-578

Podschun, R. and Sahly, H., 1991. Hemagglutinins of Klebsiella pneumoniae and K. oxytoca isolated from different sources. *Zentralblatt für Hygiene und Umweltmedizin,* 191 (1), pp.46-53.

Podschun, R. and Ullmann, U., 1998. Klebsiella spp. as Nosocomial Pathogens: Epidemiology, Taxonomy, Typing Methods, and Pathogenicity Factors. *Clinical microbiology reviews,* 11 (4), pp.589-603.

Poirel, L., Le Thomas, I., Naas, T., Karim, A. and Nordmann, P., 2000. Biochemical Sequence Analyses of GES-1, a Novel Class A Extended-Spectrum β-Lactamase, and the Class 1 Integron In52 from Klebsiellapneumoniae. *Antimicrobial Agents and Chemotherapy,* 44 (3), pp.622-632.

Poirel, L., Lambert, T., Türkoglü, S., Ronco, E., Gaillard, J. and Nordmann, P., 2001a. Characterization of Class 1 Integrons from Pseudomonas aeruginosa That Contain the blaVIM-2 Carbapenem-Hydrolyzing β-Lactamase Gene and of Two Novel Aminoglycoside Resistance Gene Cassettes : *Antimicrobial agents and chemotherapy.American Society for Microbiology.*45 (2) pp.546-552.

Poirel, L., Weldhagen, G.F., Naas, T., De Champs, C., Dove, M.G. and Nordmann, P., 2001b. GES-2, a class A beta-lactamase from Pseudomonas aeruginosa with increased hydrolysis of imipenem. *Antimicrobial agents and chemotherapy,* 45 (9), pp.2598-2603.

Poirel, L., Héritier, C., Tolün, V. and Nordmann, P., 2004. Emergence of Oxacillinase-Mediated Resistance to Imipenem in Klebsiella pneumoniae. *Antimicrobial Agents and Chemotherapy,* 48 (1), pp.15-22.

Poirel, L., Lebessi, E., Héritier, C., Patsoura, A., Foustoukou, M. and Nordmann, P., 2006. Nosocomial spread of OXA-58-positive carbapenem-resistant Acinetobacter baumannii isolates in a paediatric hospital in Greece. *Clinical Microbiology and Infection,* 12 (11), pp.1138-1141.

Poirel, L., Naas, T., and Nordmann, P., 2010. Diversity, Epidemiology, and Genetics of Class D β-Lactamases. *Antimicrobial Agents and Chemotherapy,* 54 (1), pp.24-38.

Poirel, L., Walsh, T.R., Cuvillier, V. and Nordmann, P., 2011. Multiplex PCR for detection of acquired carbapenemase genes. *Diagnostic microbiology and infectious disease,* 70 (1), pp.119-123.

Poirel, L., Bonnin, R. A., and Nordmann, P. 2012a. Genetic features of the widespread plasmid coding for the carbapenemase OXA-48. *Antimicrobial. Agents Chemotherapy*, 56, (1), pp. 559–562

Poirel, L., Bonnin, R., Boulanger, A., Schrenzel, J., Kaase, M. and Nordmann, P., 2012b. Tn125-Related Acquisition of blaNDM-Like Genes in Acinetobacter baumannii. *Antimicrobial Agents and Chemotherapy,* 56 (2), pp.1087-1089.

Poirel, L., Potron, A. and Nordmann, P., 2012c. OXA-48-like carbapenemases: the phantom menace. *Journal of antimicrobial chemotherapy,* 67 (7), pp.1597-1606.

Pomakova, D., Pomakova, D., Hsiao, C., Hsiao, C., Beanan, J., Beanan, J., Olson, R., Olson, R., MacDonald, U., MacDonald, U., Keynan, Y., Keynan, Y., Russo, T. and Russo, T., 2012. Clinical and phenotypic differences between classic and hypervirulent Klebsiella pneumonia: an emerging and under-recognized pathogenic variant. *European Journal of Clinical Microbiology & Infectious Diseases,* 31 (6), pp.981-989.

Poole, K., 2007. Efflux pumps as antimicrobial resistance mechanisms. *Annals of Medicine,* 39 (3), pp.162-176.

Psichogiou, M., Tassios, P. T., Avlamis, A., Stefanou, I., Kosmidis, C., Platsouka, E., et al. (2008). Ongoing epidemic of blaVIM-1-positive *Klebsiella pneumoniae* in Athens, Greece: a prospective survey. *Journal of Antimicrobial Chemotherapy,* 61,(1), pp. 59–63.

Punina, N.V., Makridakis, N.M., Remnev, M.A. and Topunov, A.F., 2015. Whole-genome sequencing targets drug-resistant bacterial infections. *Human genomics,* 9 (1), pp.19-24.

Qin, X., Emich, J. and Goycoolea, F.M., 2018. Assessment of the Quorum Sensing Inhibition Activity of a Non-Toxic Chitosan in an N -Acyl Homoserine Lactone (AHL)-Based Escherichia coli Biosensor. *Biomolecules (Basel, Switzerland),* 8 (3), pp. 87-95.

Qing, G., Gong, N, Chen, X, Chen, J, Zhang, H., Wang, Y., Wang, R., Zhang, S., Zhang, Z., Zhao, X., Luo, Y. and Liang, X., 2019. Natural and engineered bacterial outer membrane vesicles. *Biophysics Reports,* 5 (4), pp. 184-198.

Queenan, A.M. and Bush, K., 2007. Carbapenemases: the Versatile β-Lactamases. *Clinical Microbiology Reviews,* 20 (3), pp. 440-458.

Oueslati, S., Nordmann, P. and Poirel, L., 2015. Heterogeneous hydrolytic features for OXA-48-like β-lactamases. *Journal of antimicrobial chemotherapy,* [e-journal] 70 (4), pp.1059-1063.

Qureshi, S., 2019. Methods to screen for ESBLs. Medscape.

R Core Team. 2013. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria (URL http://www.R-project.org/).

Rafiq, M., Worthington, T., Tebbs, S.E., Treacy, R.B., Dias, R., Lambert, P.A. and Elliott, T.S., 2000. Serological detection of Gram-positive bacterial infection around prostheses. *The Journal of bone and joint surgery. British volume,* 82 (8), pp.1156-1161.

Rahim, G.R., Gupta, N. and Maheswari, P., 2018. Monomicrobial *Klebsiella pneumoniae* necrotizing fasciitis: an emerging life-threatening entity. *Clinical Microbiology and Infection*, 25 (3), pp. 316-323

Rahn, A., Drummelsmith, J. and Whitfield, C., 1999. Conserved Organization in the cps Gene Clusters for Expression of Escherichia coli Group 1 K Antigens: Relationship to the Colanic Acid Biosynthesis Locus and the cps Genes from Klebsiella pneumoniae. *Journal of bacteriology,* 181 (7), pp.2307-2313.

Rakoff-Nahoum, S., Coyne, M. and Comstock, L., 2014. An Ecological Network of Polysaccharide Utilization among Human Intestinal Symbionts. *Current biology,* 24 (1), pp.40-49.

Rawat, D. and Nair, D., 2010. Extended-spectrum β-lactamases in Gram-negative Bacteria. *Journal of global infectious diseases,* 2 (3), pp. 263-274.

Refardt, D., Bergmiller, T. and Kummerli, R., 2013. Altruism can evolve when relatedness is low: evidence from bacteria committing suicide upon phage infection. *Proceedings of the Royal Society. B, Biological sciences,* 280 (1759), pp. 1-7.

Reygaert, W.C., 2018. An overview of the antimicrobial resistance mechanisms of bacteria. *AIMS microbiology*, 4 (3), pp.482-501

Rice, L.B., 2012. Mechanisms of Resistance and Clinical Relevance of Resistance to β-Lactams, Glycopeptides, and Fluoroquinolones. *Mayo Clinic Proceedings,* 87 (2), pp.198-208.

Riley, M.A. and Wertz, J.E., 2002. Bacteriocin diversity: ecological and evolutionary perspectives. *Biochimie,* 84 (5), pp.357-364.

Rivera, L., López-Patiño, M.A., Milton, D.L., Nieto, T.P. and Farto, R., 2015. Effective qPCR methodology to quantify the expression of virulence genes in Aeromonas salmonicida subsp. salmonicida. *Journal of Applied Microbiology,* 118 (4), pp.792-802.

Rocha, J P G, Castro, T.L.P., Aguiar, Eric R G R and Pacheco, L.G.C., 2020. Gene Expression Analysis in Bacteria by RT-qPCR. *Methods in molecular biology (Clifton, N.J.),* 2065, pp.119-137.

Rocker, A., Lacey, J.A., Belousoff, M.J., Wilksch, J.J., Strugnell, R.A., Davies, M.R. and Lithgow, T., 2020. Global Trends in Proteome Remodeling of the Outer Membrane Modulate Antimicrobial Permeability in Klebsiella pneumoniae. *mBio,* [e-journal] 11 (2), pp.603.

RODRIGUEZ-VILLALOBOS, H., MALAVIOLLE, V., FRANKARD, J., DE MENDONCA, R., NONHOFF, C., DEPLANO, A., BYL, B. and STRUELENS, M.J., 2005. Emergence of

CTX-M extended spectrum β-lactamase-producing Escherichia coli in Belgium. *Euro surveillance : bulletin européen sur les maladies transmissibles,* 10 (1-3), pp.E050224.3.

Rodríguez-Baño, J., Gutiérrez-Gutiérrez, B., Machuca, I. and Pascual, A., 2018. Treatment of Infections Caused by Extended-Spectrum-Beta-Lactamase-, AmpC-, and Carbapenemase-Producing Enterobacteriaceae. *Clinical microbiology reviews,* 31 (2), pp.15-30

Roier, S., Zingl, F.G., Cakar, F., Durakovic, S., Kohl, P., Eichmann, T.O., Klug, L., Gadermaier, B., Weinzerl, K., Prassl, R., Lass, A., Daum, G., Reidl, J., Feldman, M.F. and Schild, S., 2016. A novel mechanism for the biogenesis of outer membrane vesicles in Gram-negative bacteria. *Nature communications,* 7 (1), pp.10515-10524.

Rolain, J.M., Parola, P. and Cornaglia, G., 2010;New Delhi metallo-beta-lactamase (NDM 1): towards a new pandemia? *Clinical Microbiology and Infections*, 16 (1), pp. 1699–1701.

Rolinson, 1998. Historical perspective. Forty years of β-lactam research. *Journal of Antimicrobial Chemotherapy*, 41: 589-603.

Rovers, M.M., 2008. The burden of otitis media. *Vaccine,* 26, (1), pp.G2-G4.

Rozwandowicz, M., Brouwer, M.S.M., Fischer, J., Wagenaar, J.A., Gonzalez-Zorn, B., Guerra, B., Mevius, D.J. and Hordijk, J., 2018. Plasmids carrying antimicrobial resistance genes in Enterobacteriaceae. *The Journal of antimicrobial chemotherapy,* 73 (5), pp.1121-1137.

Rúbena, F., Paulad, A., Prudêncio C., 2013. β-Lactams: chemical structure, mode of action and mechanisms of resistance. *Reviews in Medical Microbiology*, 24 (1), pp. 7-17.

Rumbo, C., Moreira, E. F., Merino, M., Poza, M., Mendez, J. A., Soares, N. C., Mosquera, A., Chaves, F. and Bou, G., 2011. Horizontal Transfer of the OXA-24 Carbapenemase Gene via Outer Membrane Vesicles: a New Mechanism of Dissemination of Carbapenem Resistance Genes in Acinetobacter baumannii. *Antimicrobial Agents and Chemotherapy,* 55 (7), pp. 3084-3090.

Rupp, M., Kern, S., Weber, T., Menges, T.D., Schnettler, R., Heiss, C. and Alt, V., 2020. Polymicrobial infections and microbial patterns in infected nonunions – a descriptive analysis of 42 cases. *BMC infectious diseases,* 20 (1), pp.1-667.

Russo, T.A., Olson, R., MacDonald, U., Beanan, J. and Davidson, B.A., 2015. Aerobactin, but not yersiniabactin, salmochelin, or enterobactin, enables the growth/survival of hypervirulent (hypermucoviscous) Klebsiella pneumoniae ex vivo and in vivo. *Infection and immunity,* 83 (8), pp.3325-3333.

Rutherford, S.T. and Bassler, B.L., 2012. Bacterial Quorum Sensing: Its Role in Virulence and Possibilities for Its Control. *Cold Spring Harbor perspectives in medicine,* 2 (11), pp. 427-435.

SaadatianFarivar, A., Nowroozi, J., Eslami, G. and Sabokbar, A., 2018. RAPD PCR Profile, Antibiotic Resistance, Prevalence of armA Gene, and Detection of KPC

Enzyme in Klebsiellapneumoniae Isolates. *Canadian Journal of Infectious Diseases and Medical Microbiology,*2018 (6183162) pp.1-7.

Saavedra,S.Y., Diaz, L., Wiesner, M., Correa, A., Arévalo, S.A., Reyes, J., Hidalgo, M., de la Cadena, E., Perenguez, M., Montaño, L.A., Ardila, J., Ríos, R., Ovalle,a Paula Díaz, M.V., Porras, P., Villegas, M., Arias, C.,Beltrán, M., and Duarte, C., 2017. Genomic and Molecular Characterization of Clinical Isolates of EnterobacteriaceaeHarboring mcr-1 in Colombia, 2002 to 2016.*Antimicrobial Agents and Chemotherapy,* 61 (12), e00841-17.

Saga and Yamaguchi.2009. History of Antimicrobial Agents and Resistant Bacteria. *Japan Medical Association,* 52 (5), pp. 103–108.

Sahl, J.W., Morris, C.R., Emberger, J., Fraser, C.M., Ochieng, J.B., Juma, J., Fields, B., Breiman, R.F., Gilmour, M., Nataro, J.P. and Rasko, D.A., 2015. Defining the Phylogenomics of Shigella Species: a Pathway to Diagnostics. *Journal of clinical microbiology,* 53 (3), pp.951-960.

Sahly, H. and Podschun, R.,1997. Clinical, bacteriological, and serological aspects of Klebsiella infections and their spondylarthropathic sequelae. Clinical Diagnostic Lab Immunology, 4 (185), pp. 393-399.

Sahra, 2019. *Antimicrobials, Antibiotic Resistance, Antibiofilm Strategies and Activity Methods.* IntechOpen.

Salipante, S.J., SenGupta, D.J., Cummings, L.A., Land, T.A., Hoogestraat, D.R. and Cookson, B.T., 2015. Application of Whole-Genome Sequencing for Bacterial Strain Typing in Molecular Epidemiology. *Journal of clinical microbiology,* 53 (4), pp.1072-1079.

Sanbongi, Y., Ida, T., Ishikawa, M., Osaki, Y., Kataoka, H., Suzuki, T., Kondo, K., Ohsawa, F. and Yonezawa, M., 2004. Complete Sequences of Six Penicillin-Binding Protein Genes from 40 Streptococcus pneumoniae Clinical Isolates Collected in Japan. *Antimicrobial Agents and Chemotherapy,* 48 (6), pp. 2244-2250.

Sánchez-López, J., García-Caballero, A., Navarro-San Francisco, C., Quereda, C., Ruiz-Garbajosa, P., Navas, E., Dronda, F., Morosini, M.I., Cantón, R. and Diez-Aguilar, M., 2019. Hypermucoviscous Klebsiella pneumoniae: A challenge in community acquired infection. *IDCases,* 17 (56), pp. 540-556.

Sanger, F., Nicklen, S. and Coulson, A.R., 1977. DNA sequencing with chain-terminating inhibitors. *Proceedings National Academy Sciences U.S.A*. 74 (12), pp. 5463–5467.

Sarkar, P., Yarlagadda, V., Ghosh, C. and Haldar, J., 2017. A review on cell wall synthesis inhibitors with an emphasis on glycopeptide antibiotics. *MedChemComm,* 8 (3), pp.516-533.

Sawa, T., Kooguchi, K. and Moriyama, K., 2020. Molecular diversity of extended-spectrum β-lactamases and carbapenemases, and antimicrobial resistance. *Journal of intensive care,* 8 (1), pp.13-18.

Schaar, V., Nordström, T., Mörgelin, M. and Riesbeck, K., 2011. Moraxella catarrhalis Outer Membrane Vesicles Carry β-Lactamase and Promote Survival of Streptococcus pneumoniae and Haemophilus influenzae by Inactivating Amoxicillin. *Antimicrobial Agents and Chemotherapy,* 55 (8), pp. 3845-3853.

Schaar, V., Uddbäck, I., Nordström, T. and Riesbeck, K., 2014. Group A streptococci are protected from amoxicillin-mediated killing by vesicles containing β-lactamase derived from Haemophilus influenzae. *The Journal of antimicrobial chemotherapy,* 69 (1), pp.117-126.

Schimmel,P., 2002. The protein synthesis world. *Trends in Biochemical Sciences*, 27 (10), pp.505-506.

Schirmer, T., Keller, T.A., Wang, Y.F. and Rosenbusch, J.P., 1995. Structural basis for sugar translocation through maltoporin channels at 3.1 A resolution. *Science,* 267 (5197), pp.512-514.

Schlesinger, J., Navon-Venezia, S., Chmelnitsky, I., Hammer-Munz, O., Leavitt, A., Gold, H.S., Schwaber, M.J. and Carmeli, Y., 2005. Extended-Spectrum Beta-Lactamases among Enterobacter Isolates Obtained in Tel Aviv, Israel. *Antimicrobial agents and chemotherapy,* 49 (3), pp.1150-1156.

Schneider, C.A., Rasband, W.S. and Eliceiri, K.W., 2012. NIH Image to ImageJ: 25 years of image analysis. *Nature Methods,* 9 (7), pp. 671-675.

Schrager, L.K., Harris, R.C. and Vekemans, J., 2018. Research and development of new tuberculosis vaccines: a review [version 1; peer review: 3 approved, 1 approved with reservations]. *F1000Research,* 7, pp.1-32.

Schroll, C., Barken, K.B., Krogfelt, K.A. and Struve, C., 2010. Role of type 1 and type 3 fimbriae in Klebsiella pneumoniae biofilm formation. *BMC microbiology,* 10 (1), pp.179-185.

Schwartz, D.C. and Cantor, C.R., 1984. Separation of yeast chromosome-sized DNAs by pulsed field gradient gel electrophoresis. *Cell,* 37 (1), pp.67-75.

Schwarzmann, S. W., Adler, J. L., Sullivan, Jr. R. J., 1971. Marine W. M. Bacterial pneumonia during the Hong Konginfluenza epidemic of 1968–1969. *Archives of Internal Medicine*,127 (6), pp. 1037–1041.

Schwechheimer, C., Kulp, A., Kuehn, M.J., 2014. Modulation of bacterial outer membrane vesicle production by envelope structure and content. *BMC Microbiology*, 14 (324), pp. 40-50.

Schwechheimer, C. and Kuehn, M.J., 2015. Outer-membrane vesicles from Gram-negative bacteria: biogenesis and functions. *Nature reviews. Microbiology,* 13 (10), pp.605-619.

Schweizer, H.P., 2003. Efflux as a mechanism of resistance to antimicrobials in Pseudomonas aeruginosa and related bacteria: unanswered questions. *Genetics and molecular research,* 2 (1), pp.48-62.

Scott, I. U., Matharoo, N., Flynn, H. W., Jr. and Miller, D. (2004). Endophthalmitis caused by Klebsiella species. *American Journal Ophthalmology*, 138 (154), pp. 662-663.

Seale, M., Lee, W. K., Daffy, J., Tan, Y. and Trost, N., 2007. Fulminant endogenous K. pneumoniae endophthalmitis: imaging findings. *Emergence Radiology*, 13 (46), pp.209-212.

Seemann, T., 2014. Prokka: rapid prokaryotic genome annotation. *Bioinformatics (Oxford, England),* 30 (14), pp. 2068-2069.

Segal, N., Leibovitz, E., Dagan, R. and Leiberman, A., 2005. Acute otitis media-diagnosis and treatment in the era of antibiotic resistant organisms: Updated clinical practice guidelines. *International journal of pediatric otorhinolaryngology,* 69 (10), pp.1311-1319.

Sengupta, S., Chattopadhyay, M.K. and Grossart, H., 2013. The multifaceted roles of antibiotics and antibiotic resistance in nature. Frontiers in microbiology, 4, (10), pp.47-51.

Seth, E.C. and Taga, M.E., 2014. Nutrient cross-feeding in the microbial world. *Frontiers in microbiology,* 5, (8), pp.350-355.

Shahbaz, K., 2017. Cephalosporins: pharmacology and chemistry. Pharmaceutical and Biological Evaluations, 4 (6), pp. 234-238.

Shaikh, S., Fatima, J., Shakil, S., Mohd, S., Rizvi, D. and Kamal, M., 2015. Antibiotic resistance and extended spectrum beta-lactamases: Types, epidemiology and treatment. Saudi Journal of Biological Sciences, 22(1), pp. 90-101.

Sharan, 2007. Analysis of Biological Networks:Transcriptional Networks - Promoter Sequence Analysis. *Lecture 11,* pp. 1-15.

Sharma, D., Misba, L. and Khan, A.U., 2019. Antibiotics versus biofilm: an emerging battleground in microbial communities. *Antimicrobial resistance & infection control,* 8 (1), pp.76-90.

Shehadul Islam, M., Aryasomayajula, A. and Selvaganapathy, P., 2017. A Review on Macroscale and Microscale Cell Lysis Methods. *Micromachines,* 8 (3), pp. 1-27.

Shen, Z., Ding, B., Bi, Y., Wu, S., Xu, S., Xu, X., Guo, Q. and Wang, M., 2017. CTX-M-190, a Novel β-Lactamase Resistant to Tazobactam and Sulbactam, Identified in an Escherichia coli Clinical Isolate. *Antimicrobial agents and chemotherapy,* 61 (1), pp. 11-21.

Sheu, C., Chang, Y., Lin, S., Chen, Y. and Hsueh, P., 2019. Infections Caused by Carbapenem-Resistant Enterobacteriaceae: An Update on Therapeutic Options. *Frontiers in microbiology,* 10(9), pp.1-13.

Shoberg, R.J. and Thomas, D.D., 1993. Specific adherence of Borrelia burgdorferi extracellular vesicles to human endothelial cells in culture. *Infection and Immunity,* 61 (9), pp.3892-3900.

Short, F.L., Murdoch, S.L. and Ryan, R.P., 2014. Polybacterial human disease: the ills of social networking. *Trends in Microbiology,* 22 (9), pp.508-516.

Shu, H., Fung, C., Liu, Y., Wu, K., Chen, Y., Li, L., Liu, T., Kirby, R. and Tsai, S., 2009. Genetic diversity of capsular polysaccharide biosynthesis in Klebsiella pneumoniae clinical isolates. *Microbiology (Society for General Microbiology),* 155 (12), pp.4170-4183.

Side, D.D., Nassisi, V., Pennetta, C., Alifano, P., Di Salvo, M., Talà, A., Chechkin, A., Seno, F. and Trovato, A., 2017. Bacterial bioluminescence onset and quenching: a dynamical model for a quorum sensing -mediated property. *Royal Society open science,*4 (12), pp.171586-171610.

Sievers, F. and Higgins, D.G., 2014. Clustal Omega, accurate alignment of very large numbers of sequences. *Methods in molecular biology (Clifton, N.J.),* 1079, pp.105-116.

Silhavy,T.J., Kahne, D. and Walker, S., 2010. The Bacterial Cell Envelope. *Cold Spring Harbor perspectives in biology,* 2 (5), pp.414-425.

Silveira, M.C., Azevedo da Silva, R., Faria da Mota, F., Catanho, M., Jardim, R., R Guimarães, A.C. and de Miranda, A.B., 2018. Systematic Identification and Classification of β-Lactamases Based on Sequence Similarity Criteria: β-Lactamase Annotation. *Evolutionary bioinformatics online,* 14, (1), pp. 1-11.

Silver, P.A. and Wintermute, E.H., 2010. Emergent cooperation in microbial metabolism. *Molecular Systems Biology,* 6 (1), pp.55-70.

Simoons-Smit, A.M., Verweij-Van Vught, A. M. J. J and Maclaren, D.M., 1986. The role of K antigens as virulence factors in Klebsiella. *Journal of medical microbiology,* 21 (2), pp.133-137.

Simpson, I. N., P. B. Harper, and C. H. O'Callaghan. 1980. Principal b-lactamases responsible for resistance to b-lactam antibiotics in urinary tract infections. *Antimicrobial Agents Chemotherapy*, 17 (6), pp.929–936

Sirot, D., Sirot, J., Labia, R., Morand, A., Courvalin, P., Darfeuille-Michaud, A., Perroux, R. andCluzel, R., 1987. Transferable resistance to third-generation cephalosporins in clinical isolates of Klebsiellapneumoniae: identification of CTX-1, a novel beta-lactamase. *Journal of Antimicrobial Chemotherapy,* 20 (3), pp.323–334.

Siu, L.K., Yeh, K., Lin, J., Fung, C. and Chang, F., 2012. Klebsiella pneumoniae liver abscess: a new invasive syndrome. *The Lancet infectious diseases,* 12 (11), pp.881-887.

Sivaramakrishnan, A., Mack, D., El-Mugamar, H., Jacques, J., Paget, S., Phee, L. and Carter, Y., 2020. Epidemiology and control measures of an OXA-48-producing Enterobacteriaceae hospital outbreak. *Infection Prevention in Practice,* 2 (3), pp.100021-100026.

Sköld, O., 2010. Sulfonamides and trimethoprim. *Expert Review of Anti-infective Therapy,* 8 (1), pp.1-6.

Smith, H., 1982. The role of microbial interactions in infectious disease. Philosophical Transactions of Royal Society of London, B, 3 (297), pp. 551–561.

Smith, G.R., Barton, S.A. and Wallace, L.M., 1991. Further observations on enhancement of the infectivity ofFusobacterium necrophorum by other bacteria. Epidemiology and Infections, 106 (2), pp.305–310.

Smith, A., Lovelace, A.H. and Kvitko, B.H., 2018. Validation of RT-qPCR Approaches to Monitor Pseudomonas syringae Gene Expression During Infection and Exposure to Pattern-Triggered Immunity. *Molecular plant-microbe interactions : MPMI,* 31 (4), pp.410-419.

Smith, P. and Schuster, M., 2019. Public goods and cheating in microbes. *Current biology,* 29 (11), pp.R442-R447.

Smith, N.W., Shorten, P.R., Altermann, E., Roy, N.C. and McNabb, W.C., 2019. The Classification and Evolution of Bacterial Cross-Feeding. *Frontiers in ecology and evolution,* 7(153), pp. 1-13.

Snitkin, E.S., Zelazny, A.M., Thomas, P.J., Stock, F., Henderson, D.K., Palmore, T.N. and Segre, J.A., 2012. Tracking a Hospital Outbreak of Carbapenem-Resistant Klebsiella pneumoniae with Whole-Genome Sequencing. *Science translational medicine,* 4 (148), pp.116-126.

Soares, N.C., Bou, G. and Blackburn, J.M., 2016. Editorial: Proteomics of Microbial Human Pathogens. *Frontiers in microbiology,* 7, (5), pp.1742-1747.

Solano, G. I., Segales, J., Collins, J. E., Molitor, T. W. and Pijoan, C., 1997. Porcine reproductive and respiratory syndromevirus (PRRSv) interaction with Haemophilus parasuis. Veterinary. Microbiology, 55 (9), pp. 247–257.

Sougakoff, W., Goussard, S. and Courvalin, P., 1988. The TEM-3 β-lactamase, which hydrolyzes broad-spectrum cephalosporins, is derived from the TEM-2 penicillinase by two amino acid substitutions. *FEMS Microbiology Letters,* 56 (3), pp.343-348.

Souli, M., Galani, I., Antoniadu, A., 2009. An outtbreak of Infectioun due to β-lactamase *K. pneumoniae* carbapenemase 2-producing *K. pneumoniae* in a Greek hospital. Molecular Characterization Epidemiology and Outcomes. Clinical Infectious Diseases, 50 (6), pp. 364- 373.

Spellberg, B. and Gilbert, D.N., 2014. The Future of Antibiotics and Resistance: A Tribute to a Career of Leadership by John Bartlett. *Clinical infectious diseases,* 59 (suppl 2), pp.S71-S75.

Spellberg, B., Morris, A.M., Ovens, H., Bellovin, B., Wunderink, R.G. and Waterer, G.W., 2014. Community-Acquired Pneumonia. *The New England Journal of Medicine,* 370 (19), pp.1861-1863.

Srinivasan, V.B., Singh, B.B., Priyadarshi, N., Chauhan, N.K. and Rajamohan, G., 2014. Role of novel multidrug efflux pump involved in drug resistance in Klebsiella pneumoniae. *PloS one,* 9 (5), pp.e96288-e96297.

Sriram, A., Kalanxhi, E., Kapoor, G., Craig, J., Balasubramanian, R., Brar, S., Criscuolo, N., Hamilton, A., Klein, E., Tseng, K., Van Boeckel, T. and Laxminarayan, R., 2021. The State of the World's Antibiotics Report in 2021. The Center For Disease Dynamics, Economics & Policy.

Stapleton, P.D. and Taylor, P.W, 2002. Methicillin resistance in Staphylococcus aureus. Science Progress, 85 (1), pp.57-72.

Stentz, R., Horn, N., Cross, K., Salt, L., Brearley, C., Livermore, D.M. and Carding, S.R., 2015. Cephalosporinases associated with outer membrane vesicles released by Bacteroides spp. protect gut pathogens and commensals against β-lactam antibiotics. *The Journal of antimicrobial chemotherapy,* 70 (1), pp. 701-709.

Stewart, E.J., 2012. Growing Unculturable Bacteria. *Journal of bacteriology,*194 (16), pp.4151-4160.

Stewart, N.K., Smith, C.A., Frase, H., Black, D.J. and Vakulenko, S.B., 2015. Kinetic and Structural Requirements for Carbapenemase Activity in GES-Type β-Lactamases. *Biochemistry,* 54 (2), pp.588-597.

Strampfer, M. J., Schoch, P. E. & Cunha, B. A., 1987. Cerebral abscess caused by *Klebsiella ozaenae*. Journal of Clinical Microbiology, 25 (30), pp. 1553-1554.

Strassmann, J.E. and Queller, D., 2011. Evolution of cooperation and control of cheating in a social microbe. *Proceedings of the National Academy of Sciences - PNAS,* 108 (Supplement_2), pp.10855-10862.

Straus, D.C., 1987. Production of an extracellular toxic complex by various strains of Klebsiella pneumoniae. *Infection and Immunity,* 55 (1), pp.44-48.

Struve, C., Bojer, M. and Krogfelt, K.A., 2009. Identification of a Conserved Chromosomal Region Encoding Klebsiella pneumoniae Type 1 and Type 3 Fimbriae and Assessment of the Role of Fimbriae in Pathogenicity. *Infection and immunity,*77 (11), pp.5016-5024.

Stubbendieck, R.M. and Straight, P.D., 2016. Multifaceted Interfaces of Bacterial Competition. *Journal of bacteriology,* 198 (16), pp.2145-2155.

Stubbendieck, R.M., Vargas-Bautista, C. and Straight, P.D., 2016. Bacterial Communities: Interactions to Scale. *Frontiers in microbiology,* 7, pp.1-19.

Su, L. and Chiu, C., 2007. Salmonella: clinical importance and evolution of nomenclature. *Chang Gung medical journal,* 30 (3), pp.210-219.

Sugawara, E., Kojima, S. and Nikaido, H., 2016. Klebsiella pneumoniae Major Porins OmpK35 and OmpK36 Allow More Efficient Diffusion of β-Lactams than Their Escherichia coli Homologs OmpF and OmpC. *Journal of bacteriology,* 198 (23), pp.3200-3208.

Sullivan, J.A., Lewis, M.J., Nikko, E. and Pelham, H.R.B., 2007. Multiple Interactions Drive Adaptor-Mediated Recruitment of the Ubiquitin Ligase Rsp5 to Membrane Proteins In Vivo and In Vitro. *Molecular Biology of the Cell*, 18 (7), pp. 2429-2440.

Sun, J., Deng, Z. and Yan, A., 2014a. Bacterial multidrug efflux pumps: Mechanisms, physiology and pharmacological exploitations. *Biochemical and Biophysical Research Communications,* 453 (2), pp.254-267.

Sun, S., Selmer, M. and Andersson, D.I., 2014b. Resistance to β-Lactam Antibiotics Conferred by Point Mutations in Penicillin-Binding Proteins PBP3, PBP4 and PBP6 in Salmonella enterica. *PloS one,* 9 (5), pp.1-10.

Suzuki, S., Horinouchi, T., and Furusawa, C., 2014. Prediction of antibiotic resistance by gene expression profiles. *Nature Communications,* 5 (5792), pp. 57-68.

Szabo, O., Kocsis, B., Szabo, N., Kristof, K. and Szabo, D., 2018. Contribution of OqxAB Efflux Pump in Selection of Fluoroquinolone-Resistant Klebsiella pneumoniae. *The Canadian journal of infectious diseases & medical microbiology,* 20 (15), pp.1-5.

Tamilselvi, A. and Mugesh, G., 2008. Zn and antibiotic resistance: metallo beta-lactamases and their synthetic analogues. *Journal of Biological Inorganic Chemistry,*13, (1), pp. 1039-1053.

Tan, S.C. and Yiap, B.C., 2009. DNA, RNA and Protein extraction: The past and the present. *Biomed Research International*, 10 (574398), pp. 1-11.

Tan, S.Y. and Tatsumura, Y., 2015. Alexander Fleming (1881–1955): Discoverer of penicillin. *Singapore medical journal,* 56 (7), pp.366-367.

TASHIRO, Y., INAGAKI, A., SHIMIZU, M., ICHIKAWA, S., TAKAYA, N., NAKAJIMA-KAMBE, T., UCHIYAMA, H. and NOMURA, N., 2014. Characterization of Phospholipids in Membrane Vesicles Derived from Pseudomonas aeruginosa. *Bioscience, biotechnology, and biochemistry,* 75 (3), pp.605-607.

Thacker E. L., Halbur P. G., Ross R. F., Thanawongnuwech R., Thacker B. J., 1999. *Mycoplasma hyopneumoniae*potentiation of porcine reproductive and respiratory syndrome virus-induced pneumonia. *Journal of Clinical Microbiology*, 37 (6), pp. 620–627.

Thibodeau, S.N., 1987. Use of restriction fragment length polymorphism analysis for detecting carriers of "fragile X" syndrome. *Clinical Chemistry,* 33 (10), pp.1726-1730.

Todd, R., Donoff, R.B., Kim, Y. and Wong, D.T.W., 2001. From the chromosome to DNA: Restriction fragment length polymorphism analysis and its clinical application. *Journal of Oral and Maxillofacial Surgery,* 59 (6), pp.660-667.

Tooke, C.L., Hinchliffe, P., Bragginton, E.C., Colenso, C.K., Hirvonen, V.H.A., Takebayashi, Y. and Spencer, J., 2019. β-Lactamases and β-Lactamase Inhibitors in the 21st Century. *Journal of molecular biology,* 431 (18), pp.3472-3500.

Toyofuku, M., Cárcamo-Oyarce, G., Yamamoto, T., Eisenstein, F., Hsiao, C., Kurosawa, M., Gademann, K., Pilhofer, M., Nomura, N. and Eberl, L., 2017. Prophage-triggered membrane vesicle formation through peptidoglycan damage in Bacillus subtilis. *Nature communications,* 8 (1), pp.1-10.

Tristram, S., Jacobs, M.R. and Appelbaum, P.C., 2007. Antimicrobial Resistance in Haemophilus influenzae. *Clinical microbiology reviews,* 20 (2), pp.368-389.

Tsai, Y., Fung, C., Lin, J., Chen, J., Chang, F., Chen, T. and Siu, L.K., 2011. Klebsiella pneumoniae Outer Membrane Porins OmpK35 and OmpK36 Play Roles in both Antimicrobial Resistance and Virulence. *Antimicrobial Agents and Chemotherapy,* 55 (4), pp.1485-1493.

Toussaint , K.D., and Gallagher, J.C., 2015. β-Lactam/β-Lactamase Inhibitor Combinations: From Then to Now. *Annals of Pharmacotherapy*, 1 (31), pp. 1–13.

Turnbull, L., Toyofuku, M., Hynen, A.L., Kurosawa, M., Pessi, G., Petty, N.K., Osvath, S.R., Cárcamo-Oyarce, G., Gloag, E.S., Shimoni, R., Omasits, U., Ito, S., Yap, X., Monahan, L.G., Cavaliere, R., Ahrens, C.H., Charles, I.G., Nomura, N., Eberl, L. and Whitchurch, C.B.,2016. Explosive cell lysis as a mechanism for the biogenesis of bacterial membrane vesicles and biofilms. Nature Communications, 14 (7), pp.11220-11234.

Turner, L., Bitto, N.J., Steer, D.L., Lo, C., D'Costa, K., Ramm, G., Shambrook, M., Hill, A.F., Ferrero, R.L. and Kaparakis-Liaskos, M., 2018. Helicobacter pylori Outer Membrane Vesicle Size Determines Their Mechanisms of Host Cell Entry and Protein Content. *Frontiers in immunology,* 9 (35), pp.1466-1473.

Urbach, C., Fastrez, J. and Soumillion, P., 2008. A new family of cyanobacterial penicillin-binding proteins. A missing link in the evolution of class A beta-lactamases. *The Journal of biological chemistry,* 283 (47), pp.32516-32526.

Valguarnera, E., Scott, N.E., Azimzadeh, P. and Feldman, M.F., 2018. Surface Exposure and Packing of Lipoproteins into Outer Membrane Vesicles Are Coupled Processes in Bacteroides. *mSphere,* 3 (6), pp.559.

van der Putten, Boas C L, Remondini, D., Pasquini, G., Janes, V.A., Matamoros, S. and Schultsz, C., 2019. Quantifying the contribution of four resistance mechanisms to ciprofloxacin MIC in Escherichia coli : a systematic review. *Journal of antimicrobial chemotherapy,* [e-journal] 74 (2), pp.298-310.

van Duin, D. and Doi, Y., 2017. The global epidemiology of carbapenemase-producing Enterobacteriaceae. *Virulence,* 8 (4), pp.460-469.

van Eijk, E., Wittekoek, B., Kuijper, E.J. and Smits, W.K., 2017. DNA replication proteins as potential targets for antimicrobials in drug-resistant bacterial pathogens. *Journal of antimicrobial chemotherapy,* 72 (5), pp.1275-1284.

van Hoek, Angela H. A. M, Mevius, D., Guerra, B., Mullany, P., Roberts, A.P. and Aarts, H.J.M., 2011. Acquired Antibiotic Resistance Genes: An Overview. *Frontiers in microbiology,* 2, pp.1-203.

Vanaja, S. K., Russo, A. J., Behl., B., Banerjee, B., Yankova, M., Deshmuckh, S. D., Ratinam, V. A. K., 2016. Bacterial Outer Membrane Vesicles Mediate Cytosolic Localization of LPS and Caspase-11 Activation. *Cell,* 165 (5), pp. 1106-1119.

Venter, H., Mowla, R., Ohene-Agyei, T. and Ma, S., 2015. RND-type drug efflux pumps from Gram-negative bacteria: molecular mechanism and inhibition. *Frontiers in microbiology,* 6, (377), pp.1-11.

Ventola, C.L., 2015. The Antibiotic Resistance Crisis. Part 1: Causes and Threats. Pharmacy and Therapeutics, 40 (15), 277-283.

Vickers, A.A., Potter, N.J., Fishwick, C.W.G., Chopra, I. and O'Neill, A.J., 2009. Analysis of mutational resistance to trimethoprim in Staphylococcus aureus by genetic and structural modelling techniques. *Journal of antimicrobial chemotherapy*, 63 (6), pp.1112-1117.

Vinothkumar, K.R. and Henderson, R., 2010. Structures of membrane proteins. *Quarterly reviews of biophysics,* 43 (1), pp.65-158.

Vogel, L., Jones, G., Triep, S., Koek, A. and Dijkshoorn, L., 1999. RAPD typing of *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *Serratia marcescens* and *Pseudomonas aeruginosa* isolates using standardized reagents. *Clinical Microbiology and Infections*, 5 (5), pp. 270-276.

Voss, G., Casimiro, D., Neyrolles, O., Williams, A., Kaufmann, S.H.E., McShane, H., Hatherill, M. and Fletcher, H.A., 2018. Progress and challenges in TB vaccine development [version 1; peer review: 2 approved]. *F1000Research,* 7 (199), pp.1-14.

Yan, S. and Wu, G., 2019. Can Biofilm Be Reversed Through Quorum Sensing in Pseudomonas aeruginosa? *Frontiers in microbiology,* 10 (15), pp.1582-1600.

Yanagawa, T., Nakamura, H., Takei, I., Maruyama, H., Kataoka, K., Saruta, T. & Kobayashi, Y., 1989. *K. pneumoniae* meningitis associated with liver abscess: a case report. *Journal of Antibiotics,* 42 (5), pp. 2135-2140.

Yang, N.J. and Hinner, M.J., 2016. Getting across the cell membrane: An overview for small molecules, peptides, and proteins. *Methods in molecular biology (Clifton, N.J.),* 1266 (14), pp.29-53.

You, H.S., Kim, M.J., Ok, Y.J., Lee, E.J., Kang, S.S. and Hyun, S.H., 2017. CHARACTERISTICS OF OUTER-MEMBRANE VESICLES DERIVED FROM KLEBSIELLA PNEUMONIAE ATCC 13883. International Journal of Current Medical and Pharmaceutical Research, 4 (3), pp. 3171-3176.

Yurtsev, E.A., Chao, H.X., Datta, M.S., Artemova T., and Gore, J., 2013. Bacterial ch eating drives the population dynamics of cooperative antibiotic resistance plasmids. *Molecular Systems Biology,* 9, pp.683-686.

Waksman, S.A.; Reilly, H.C.; Harris, D. 1948. *Streptomyces griseus* (Krainsky) Waksman and Henrici. Journal of Bacteriology, 56 (18), pp. 259-269.

Walker, T.M., Camilla, L.C., Harrell, Ruth, H., Evans, J.T., Kapatai, G., Dedicoat, M.J., Eyre, D.W., Wilson, D.J., Hawkey, P.M., Crook, P., Derrick, W., Parkhill, J., Harris, D., Walker, A.S., Bowden, R., Monk, P., Smith, E.G., Peto, M. Tim, E.A., 2013. Whole-genome sequencing to delineate Mycobacterium tuberculosis outbreaks: a retrospective observational study. *Lancet Infectious Diseases,*13 (2), pp.137-146.

Walkiewicz, K., Benitez Cardenas, A.S., Sun, C., Bacorn, C.,Saxer, G. and Shamoo, Y., 2012. Small changes in enzyme function can lead to surprisingly large fitness effects during adaptive evolution of antibiotic resistance. *Proceedings of the National Academy of Sciences,* 109 (52), pp.21408-21413.

Wallace, R.J., Steingrube, Jr, V. A., Nash, D. R., Hollis, D. G., and Weaver, R. E., 1989. BRO beta-lactamases of Branhamella catarrhalis and Moraxella subgenus Moraxella, including evidence for chromosomal beta-lactamase transfer by conjugation in B. catarrhalis, M. nonliquefaciens, and M. lacunata. *Antimicrobial Agents and Chemotherapy,* 33 (11), pp.1845-1854.

Wang, X., Thompson, C.D., Weidenmaier, C. and Lee, J.C., 2018. Release of Staphylococcus aureus extracellular vesicles and their application as a vaccine platform. *Nature communications,* 9 (1), pp.1379-13.

Wang, Y., Sun, X., Kong, F., Xia, L., Deng, X., Wang, D. and Wang, J., 2020. Specific NDM-1 Inhibitor of Isoliquiritin Enhances the Activity of Meropenem against NDM-1-positive Enterobacteriaceae in vitro. *International journal of environmental research and public health,* 17 (6), pp.1-10.

Waterer, G., 2017. Recovery from community acquired pneumonia: the view from the top of the iceberg. *The European respiratory journal,* 49 (6), pp. 56-67.

Waterhouse, A.M., Procter, J.B., Martin, D.M.A., Clamp, M. and Barton, G.J., 2009. Jalview Version 2-a multiple sequence alignment editor and analysis workbench. *Bioinformatics*, 25, pp. 1189-1191.

Weber, B. S., Kinsella, R. L., Harding, C. M., Feldman, M. F., 2017. The Secrets of Acinetobacter Secretion. *Trends in Microbiology,* 25 (7), pp. 532-545.

Weisburg, W.G., Barns, S.M., Pelletier, D.A. and Lane, D.J., 1991. 16S ribosomal DNA amplification for phylogenetic study. *Journal of bacteriology,* 173 (2), pp.697-703.

Wen, X., Langevin, A. and Dunlop, M.j., 2018. Antibiotic export by efflux pumps affects growth of neighboring bacteria. Nature, 8 (15120), pp. 1-9.

West, S.A., 2002. Cooperation and Competition Between Relatives. *Science (American Association for the Advancement of Science),* 296 (5565), pp.72-75.

West, S.A. and Buckling, A., 2003. Cooperation, virulence and siderophore production in bacterial parasites. *Proceedings of the Royal Society. B, Biological sciences,* 270 (1510), pp.37-44.

West, S.A., Griffin, A.S., Gardner, A. and Diggle, S.P., 2006. Social evolution theory for microorganisms. *Nature reviews. Microbiology,* 4 (8), pp.597-607.

West, S.A., Diggle, S.P., Buckling, A., Gardner, A. and Griffin, A.S., 2007. The Social Lives of Microbes. *Annual review of ecology, evolution, and systematics,* 38 (1), pp.53-77.

Whitehead, N.A., Barnard, A.M.L., Slater, H., Simpson, N.J.L. and Salmond, G.P.C., 2001. Quorum-sensing in Gram-negative bacteria. *FEMS Microbiology Reviews,* 25 (4), pp.365-404.

Wilke, M.S., 2005. beta-lactam resistance: a current structural peprspective. Current Opinions in Microbiology, 8 (5), pp. 525-33

Wilson, W.R. and Cockerill, F.R., 1987. Tetracyclines, Chloramphenicol, Erythromycin, and Clindamycin. *Mayo Clinic Proceedings,* 62 (10), pp.906-915.

Wilson, K., 2001. Preparation of Genomic DNA from Bacteria. *Current protocols in Molecular Biology*, 56 (1), Unit 2.4.

Winey, M, Meehl, J.B., Toole, E.T. and Giddings, T.H., 2014. Conventional transmission electron microscopy. *Journal of Molecular Biology,* 25 (3), pp..319-323.

Wingreen, N.S. and Levin, S.A., 2006. Cooperation among Microorganisms. *PLoS biology,* 4 (9), pp.e299-305.

Winkelmann, G., 2002. Microbial siderophore-mediated transport. *Biochemical Society transactions,* 30 (4), pp.691-696.

Witney, A.A., Gould, K.A., Pope, C.F., Bolt, F., Stoker, N.G., Cubbon, M.D., Bradley, C.R., Fraise, A., Breathnach, A.S., Butcher, P.D., Planche, T.D. and Hinds, J., 2014. Genome sequencing and characterization of an extensively drug-resistant sequence type 111 serotype O12 hospital outbreak strain of Pseudomonas aeruginosa. *Clinical microbiology and infection,* 20 (10), pp.O609-O618.

Wiuff, C., Zappala, R.M., Regoes, R.R., Garner, K.N., Baquero, F. and Levin, B.R., 2005. Phenotypic Tolerance: Antibiotic Enrichment of Noninherited Resistance in Bacterial Populations. *Antimicrobial agents and chemotherapy,* 49 (4), pp.1483-1494.

Wong, J. S., Chan, T. K., Lee, H. M. & Chee, S. P., 2000. Endogenous bacterial endophthalmitis: an East Asian experience and a reappraisal of a severe ocular affliction. *Ophthalmology*, 107 (18), pp. 1483-1491.

Wong, M. L. and Medrano, J.F., 2005. Real-time PCR for mRNA quantitation. *BioTechniques,* 39 (1), pp.75-85.

Woodruff, H. B., 2014. Selman A. Waksman, Winner of the 1952 Nobel Prize for Physiology or Medicine. *Applied and Environmental Microbiology,* 80 (1), pp. 2-8.

Worthington, R.J. and Melander, C., 2013. Overcoming Resistance to β-Lactam Antibiotics. *Journal of Organic Chemistry*, 78(9), pp. 4207–4213.

Wright, M.S., Haft, D.H., Harkins, D.M., Perez, F., Hujer, K.M., Bajaksouzian, S., Benard, M.F., Jacobs, M.R., Bonomo, R.A. and Adams, M.D., 2014. New Insights into Dissemination and Variation of the Health Care-Associated Pathogen Acinetobacter baumannii from Genomic Analysis. *mBio,* 5 (1), pp.e00963-e00913.

Wu, W., Feng, Y., Tang, G., Qiao, F., McNally, A. and Zong, Z., 2019. NDM Metallo-β-Lactamases and Their Bacterial Producers in Health Care Settings. *Clinical microbiology reviews,* 32 (2), pp. 1-45.

Yan, J.J., Ko, W.C., Tsai, S.H., Wu, H.M., and Wu, J.J. (2001). Outbreak of infection with multidrug-resistant Klebsiella pneumoniae carrying bla(IMP-8) in a university medical center in Taiwan. *Journal of Clinical Microbiology*, 39, (12), pp. 4433–4439.

Yu, J., Tan, K., Rong, Z., Wang, Y., Chen, Z., Zhu, X., Wu, L., Tan, L., Xiong, W., Sun, Z. and Chen, L., 2016. Nosocomial outbreak of KPC-2- and NDM-1-producing Klebsiella pneumoniae in a neonatal ward: a retrospective study. *BMC infectious diseases,* 16 (1), pp.563-566.

Zaman, S.B., Hussain, M.A., Nye, R., Mehta, V., Mamun, K.T. and Hossain, N., 2017. A Review on Antibiotic Resistance: Alarm Bells are Ringing. *Curēus (Palo Alto, CA),* 9 (6), pp.1-9.

Zango, U.U., Ibrahim, M., Shawai, A.A.M. and Shamsuddin, I.M., 2019. A review on β-lactam antibiotic drug resistance. *MOJ Drug Design Development & Therapy,* 3(2), pp. 52-58.

Zankari, E., Hasman, H., Cosentino, S., Vestergaard, M., Rasmussen, S., Lund, O., Aarestrup, F. M and Voldby, L. M., 2012. Identification of acquired antimicrobial resistance genes. *The Journal of Antimicrobial Chemotherapy,* 67 (11), pp. 2640-2644.

Zapun, A., Contreras-Martel, C. and Vernet, T., 2008. Penicillin-binding proteins and [beta]-lactam resistance. *FEMS Microbiology Reviews,* 32 (2), pp.361-385.

Zavascki, A.P., Goldani, L.Z., Li, J. and Nation, R.L., 2007. Polymyxin B for the treatment of multidrug-resistant pathogens: a critical review. *Journal of antimicrobial chemotherapy,* 60 (6), pp.1206-1215.

Zeynudin, A., Pritsch, M., Schubert, S., Messerer, M., Liegl, G., Hoelscher, M., Belachew, T. and Wieser, A., 2018. Prevalence and antibiotic susceptibility pattern of CTX-M type extended-spectrum β-lactamases among clinical isolates of Gram-negative bacilli in Jimma, Ethiopia. *BMC infectious diseases,* 18 (1), pp.1-10.

Zhang, S., Shen, J., Xu, P., Li, D., Sun, Z., Li, L., Yang, Z. and Sun, Q., 2007. A novel genotypic test for rapid detection of multidrug-resistant Mycobacterium tuberculosis isolates by a multiplex probe array. *Journal of Applied Microbiology,* 103 (4), pp.1262-1271.

Zhang, J., Zheng, B., Zhao, L., Wei, Z., Ji, J., Li, L. and Xiao, Y., 2014. Nationwide high prevalence of CTX-M and an increase of CTX-M-55 in Escherichia coli isolated from patients with community-onset infections in Chinese county hospitals. *BMC infectious diseases,* 14 (1), pp.659-666.

Ziervogel, B. and Roux, B., 2013. The Binding of Antibiotics in OmpF Porin. *Structure,* ] 21 (1), pp.76-87.

Zhang, Z., Du, C., de Barsy, F., Liem, M., Liakopoulos, A., van Wezel, G.P., Choi, Y.H., Claessen, D. and Rozen, D.E., 2020. Antibiotic production in Streptomyces is organized by a division of labor through terminal genomic differentiation. *Science advances,* 6 (3), pp. 1-10.

# 8 Appendices

## 8.1 Abbreviations

| | |
|---|---|
| Amp | Ampicillin |
| APS | Ammonium persulfate |
| Cefe | Cefepime |
| Cefo | Cefotaxime |
| Ceft | Ceftazidime |
| Cfu | Colony forming unit |
| Chlor | Chloramphenicol |
| Cipr | Ciprofloxacin |
| ConA | ConcanavalinA |
| CTAB | Cetyl Trimethylammonium Bromide |
| $DdH_2O$ | Double distilled water |
| $DIH_2O$ | Deionised water |
| DMSO | Dimethyl sulfoxide |
| DNA | Deoxyribonucleic acid |
| EDTA | Ethylenediaminetetraacetic acid |
| Ert | Ertapenem |
| ESBLs | extended-spectrum β-lactamases |
| GFP | Green fluorescent protein |
| HGT | Horizontal gene transfer |

| | |
|---|---|
| IPTG | Isopropyl-Beta-D-Thiogalactoside |
| Kan | Kanamycin |
| Lpp | Braun’s lipoprotein |
| LPS | Lypopolisaccharide |
| MATE | Multidrug and toxic compound extrusion |
| MBLs | Metallo-β-lactamases |
| Mer | Meropenem |
| MFS | major facilitator superfamily |
| MIC | Minimul inhibitory concentration |
| MOPS | 3-(N-morpholino)propanesulfonic acid |
| MD | Multidrug |
| MDR | Multidrug resistance |
| MSA | Mannitol Salt Agar |
| OM | Outer membrane |
| OMP | Outer membrane protein |
| OMV | Outer membrane vesicle |
| OXA | Oxacillinase |
| PBS | Phosphate Buffered Saline |
| PCR | Polymerase Chain reaction |
| PG | Peptidoglycan |
| PK | Proteinase Kinase |
| PVDF | Polyvinylidene Fluoride |
| RAPD | Random Amplified Polymorphic DNA |

| | |
|---|---|
| Rb | Rose bengal |
| RND | Resistance-nodulation-cell division |
| Rpm | Revolutions for minute |
| SDS | Sodium Dodecyl Sulphate |
| Sf | Supernatant fraction |
| SM | Specialized bioactive metabolite |
| T7t | T7 terminator |
| TAE | Tris-Acetate-EDTA |
| TBS | Tris-Buffered saline |
| TCA | Trichloroacetic acid |
| TE | Tris EDTA |
| TEM | Transmission Electron Microscopy |
| TEMED | Tetramethylethylenediamine |
| TfbI | Transformation Buffer I |
| TfbII | Transformation Buffer II |
| TR | Triton X-100 |
| w/v | weight/volume |

## 8.2 Buffer recipes

**TE buffer**: 1 L

10 mM Tris base pH 7.5

1 mM EDTA pH 8.0

**TAE buffer preparation**: 50X

2 M Tris base

57.1 ml of Glacial acetic acid

50 mM EDTA, pH 8.0

**PBS**:1X

1 tablet (OXOID) dissolved in 100 ml dd$H_2O$

**Transformation Buffer I (TfbI)**

30 mM Potassium Acetate

50 mM MnCl2 *4H20

100 mM KCl

10 mM CaCl *2H2O

15 ml glycerol

Filtered through 0.22 µm filters

**Transformation Buffer II (TfbII)**

10 mM MOPS

75 mM CaCl2 * 2H2O

10 mM KCl

15 mL glycerol

**Buffer A**: 1 L

700 mM TRIS

7 mM

pH 8.8

**Buffer B**: 1 L

300 mM Tris

7 Mm SDS

pH 8.8

**Buffer C**: 1 L

300 mM

2 M

35 mM SDS

**Western Transfer buffer:** 1 L

50 mM Tris pH 7.5

400  mM Glycine

20% v/v methanol

0.1% w/v SDS

**TBS:** 1 L: 10X

1.5 M NaCl

100 mM Tris base

pH 8

**Lysis buffer**

50 mM sodium hydroxide

100 µl β-mercaptoethanol

## 8. 3 Tables

**Table 8.1 Descriptive statistics of the maximum distance values of a single furthest colony**. For each of the 15 resistant isolates inoculated the maximum distance value of the furthest colony detected on three different plates, the mean of these three values and the Standard deviation (Std deviation) are reported.

| Resistant isolate | Plate 1 | Plate 2 | Plate 3 | Mean | Std Deviation |
|---|---|---|---|---|---|
| K1 | 0.42 | 1.47 | 0.25 | 0.71 | 0.66 |
| K2 | 0.40 | 1.02 | 1.56 | 0.99 | 0.58 |
| K3 | 3.56 | 3.63 | 2.72 | 3.31 | 0.51 |
| K7 | 0.52 | 1.50 | 0.85 | 0.96 | 0.50 |
| K10 | 1.51 | 2.25 | 1.12 | 1.63 | 0.58 |
| K11 | 3.56 | 2.38 | 1.79 | 2.58 | 0.90 |
| K13 | 0.30 | 0.88 | 0.56 | 0.58 | 0.29 |
| K17 | 3.08 | 3.72 | 2.72 | 3.17 | 0.50 |
| K18 | 0.75 | 0.91 | 1.50 | 1.05 | 0.39 |
| K19 | 3.11 | 3.56 | 3.22 | 3.30 | 0.24 |
| K20 | 1.58 | 3.30 | 2.92 | 2.60 | 0.90 |
| K21 | 0.88 | 1.06 | 0.86 | 0.93 | 0.11 |
| K23 | 0.85 | 0.93 | 0.95 | 0.91 | 0.05 |
| K25 | 0.85 | 0.80 | 0.97 | 0.87 | 0.09 |
| K30 | 1.37 | 0.99 | 1.16 | 1.17 | 0.19 |

**Table 8.2 Descriptive statistics of the average maximum distance values of 20 furthest colonies**. The average maximum distance values (mean) and the Std deviation of the 20 furthest colonies detected on three different plates was calculated. The number of colonies (N colonies) detected for each plate and the number of missing colonies (N missing values) are also reported for each plate. With the exception of 12 plates where 20 colonies were countable, in all the other cases the number of countable colonies was less than 20, thus resulting in a number of missing values.

| Resistant isolate | Plate | Mean | Std Deviation | N colonies | N missing values |
|---|---|---|---|---|---|
| K1 | 1 | 0.30 | 0.17 | 2 | 18 |
| | 2 | 0.78 | 0.33 | 14 | 6 |
| | 3 | 0.23 | 0.02 | 2 | 18 |
| K2 | 1 | 0.37 | 0.03 | 2 | 18 |
| | 2 | 0.60 | 0.16 | 10 | 10 |
| | 3 | 0.89 | 0.48 | 8 | 12 |
| K3 | 1 | 0.25 | 0.5 | 20 | 0 |
| | 2 | 0.28 | 0.36 | 20 | 0 |
| | 3 | 0.17 | 0.42 | 20 | 0 |
| K7 | 1 | 0.48 | 0.05 | 2 | 18 |
| | 2 | 0.97 | 0.31 | 15 | 5 |
| | 3 | 0.82 | 0.04 | 2 | 18 |
| K10 | 1 | 0.94 | 0.32 | 13 | 7 |
| | 2 | 1.54 | 0.32 | 20 | 0 |
| | 3 | 0.82 | 0.29 | 5 | 15 |
| K11 | 1 | 1.83 | 1.01 | 20 | 0 |
| | 2 | 1.69 | 0.32 | 20 | 0 |
| | 3 | 1.29 | 0.28 | 12 | 8 |
| K13 | 1 | 0.23 | 0.10 | 2 | 18 |
| | 2 | 0.8 | 0.09 | 5 | 15 |
| | 3 | 0.52 | 0.06 | 2 | 18 |
| K17 | 1 | 2.06 | 0.43 | 20 | 0 |
| | 2 | 2.64 | 0.45 | 20 | 0 |
| | 3 | 2.04 | 0.41 | 20 | 0 |
| K18 | 1 | 0.55 | 0.22 | 4 | 16 |
| | 2 | 0.69 | 0.15 | 13 | 7 |
| | 3 | 1.28 | 0.3 | 2 | 18 |
| K19 | 1 | 2.22 | 0.48 | 17 | 3 |
| | 2 | 2.85 | 0.35 | 20 | 0 |
| | 3 | 2.42 | 0.4 | 20 | 0 |
| K20 | 1 | 1.14 | 0.3 | 14 | 6 |
| | 2 | 1.94 | 0.69 | 20 | 0 |
| | 3 | 2.46 | 0.32 | 14 | 6 |
| K21 | 1 | 0.66 | 0.23 | 6 | 14 |
| | 2 | 0.75 | 0.17 | 8 | 12 |
| | 3 | 0.54 | 0.44 | 2 | 18 |
| K23 | 1 | 0.45 | 0.25 | 5 | 15 |
| | 2 | 0.8 | 0.13 | 5 | 15 |
| | 3 | 0.59 | 0.31 | 5 | 15 |
| K25 | 1 | 0.8 | 0.06 | 2 | 18 |
| | 2 | 0.7 | 0.14 | 2 | 18 |
| | 3 | 0.64 | 0.45 | 2 | 18 |
| K30 | 1 | 0.7 | 0.4 | 11 | 9 |
| | 2 | 0.76 | 0.18 | 5 | 15 |
| | 3 | 0.84 | 0.27 | 3 | 17 |

**Table 8.3 Descriptive statistics of the average maximum distance values of the 10 furthest colonies**. The average maximum distance values (mean) and the Std deviation of the 10 furthest colonies detected on three different plates was calculated. The number of colonies (N colonies) detected for each plate and the number of missing colonies (N missing values) are also reported for each plate. With the exception of 22 plates where 10 colonies were countable, in all the other cases the number of countable colonies was less than 10, thus resulting in a number of missing values.

| Resistant isolate | Plate | Mean | Std Deviation | N colonies | N missing values |
|---|---|---|---|---|---|
| K1 | 1 | 0.29 | 0.17 | 2 | 8 |
| | 2 | 0.91 | 0.29 | 10 | 0 |
| | 3 | 0.23 | 0.28 | 2 | 8 |
| K2 | 1 | 0.37 | 0.03 | 2 | 8 |
| | 2 | 0.59 | 0.16 | 10 | 0 |
| | 3 | 0.89 | 0.48 | 8 | 2 |
| K3 | 1 | 2.85 | 0.45 | 10 | 0 |
| | 2 | 3.07 | 0.24 | 10 | 0 |
| | 3 | 2.01 | 0.35 | 10 | 0 |
| K7 | 1 | 0.48 | 0.048 | 2 | 8 |
| | 2 | 1.13 | 0.2 | 10 | 0 |
| | 3 | 0.82 | 0.036 | 2 | 8 |
| K10 | 1 | 1.04 | 0.28 | 10 | 0 |
| | 2 | 1.79 | 0.25 | 10 | 0 |
| | 3 | 0.81 | 0.29 | 5 | 5 |
| K11 | 1 | 2.65 | 0.66 | 10 | 0 |
| | 2 | 1.92 | 0.23 | 10 | 0 |
| | 3 | 1.37 | 0.22 | 10 | 0 |
| K13 | 1 | 0.23 | 0.09 | 2 | 8 |
| | 2 | 0.8 | 0.09 | 5 | 5 |
| | 3 | 0.51 | 0.06 | 2 | 8 |
| K17 | 1 | 2.38 | 0.34 | 10 | 0 |
| | 2 | 2.96 | 0.38 | 10 | 0 |
| | 3 | 2.38 | 0.23 | 10 | 0 |
| K18 | 1 | 0.55 | 0.22 | 4 | 6 |
| | 2 | 0.75 | 0.11 | 10 | 0 |
| | 3 | 1.28 | 0.3 | 2 | 8 |
| K19 | 1 | 2.53 | 0.26 | 10 | 0 |
| | 2 | 3.13 | 0.29 | 10 | 0 |
| | 3 | 2.77 | 0.23 | 10 | 0 |
| K20 | 1 | 1.28 | 0.18 | 10 | 0 |
| | 2 | 2.48 | 0.42 | 10 | 0 |
| | 3 | 2.61 | 0.2 | 10 | 0 |
| K21 | 1 | 0.66 | 0.23 | 6 | 4 |
| | 2 | 0.75 | 0.17 | 8 | 2 |
| | 3 | 0.55 | 0.44 | 2 | 8 |
| K23 | 1 | 0.45 | 0.22 | 5 | 5 |
| | 2 | 0.8 | 0.25 | 5 | 5 |
| | 3 | 0.59 | 0.13 | 5 | 5 |
| K25 | 1 | 0.8 | 0.62 | 2 | 8 |
| | 2 | 0.7 | 0.14 | 2 | 8 |
| | 3 | 0.64 | 0.46 | 2 | 8 |
| K30 | 1 | 0.73 | 0.4 | 10 | 0 |
| | 2 | 0.76 | 0.17 | 5 | 5 |
| | 3 | 0.85 | 0.27 | 3 | 7 |

**Table 8.4 Descriptive statistics of the average maximum distance values of 5 furthest colonies**. The average maximum distance values (mean) and the Std deviation of the 5 furthest colonies detected on three different plates was calculated. The number of colonies (N colonies) detected for each plate and the number of missing colonies (N missing values) are also reported for each plate. With the exception of 31 plates where 5 colonies were countable, in all the other cases the number of countable colonies was less than 5, thus resulting in a number of missing values.

| Resistant isolate | Plate | Mean | Std Deviation | N colonies | N missing values |
|---|---|---|---|---|---|
| K1 | 1 | 0.29 | 0.18 | 2 | 3 |
| | 2 | 1.15 | 0.19 | 5 | 0 |
| | 3 | 0.23 | 0.03 | 2 | 3 |
| K2 | 1 | 0.37 | 0.04 | 2 | 3 |
| | 2 | 0.7 | 0.18 | 5 | 0 |
| | 3 | 1.14 | 0.4 | 5 | 0 |
| K3 | 1 | 3.23 | 0.33 | 5 | 0 |
| | 2 | 3.25 | 0.23 | 5 | 0 |
| | 3 | 2.28 | 0.3 | 5 | 0 |
| K7 | 1 | 0.48 | 0.05 | 2 | 3 |
| | 2 | 1.29 | 0.13 | 5 | 0 |
| | 3 | 0.82 | 0.03 | 2 | 3 |
| K10 | 1 | 1.26 | 0.2 | 5 | 0 |
| | 2 | 1.2 | 0.19 | 5 | 0 |
| | 3 | 0.81 | 0.3 | 5 | 0 |
| K11 | 1 | 3.19 | 0.38 | 5 | 0 |
| | 2 | 2.1 | 0.19 | 5 | 0 |
| | 3 | 1.53 | 0.18 | 5 | 0 |
| K13 | 1 | 0.23 | 0.95 | 2 | 3 |
| | 2 | 0.8 | 0.93 | 5 | 0 |
| | 3 | 0.51 | 0.62 | 2 | 3 |
| K17 | 1 | 2.66 | 2.64 | 5 | 0 |
| | 2 | 3.28 | 2.75 | 5 | 0 |
| | 3 | 2.57 | 0.14 | 5 | 0 |
| K18 | 1 | 0.55 | 0.22 | 4 | 1 |
| | 2 | 0.84 | 0.71 | 5 | 0 |
| | 3 | 1.28 | 0.3 | 2 | 3 |
| K19 | 1 | 2.72 | 0.26 | 5 | 0 |
| | 2 | 3.4 | 0.16 | 5 | 0 |
| | 3 | 2.93 | 0.2 | 5 | 0 |
| K20 | 1 | 1.43 | 0.14 | 5 | 0 |
| | 2 | 2.8 | 0.32 | 5 | 0 |
| | 3 | 2.77 | 0.14 | 5 | 0 |
| K21 | 1 | 0.72 | 0.21 | 5 | 0 |
| | 2 | 0.83 | 0.17 | 5 | 0 |
| | 3 | 0.54 | 0.44 | 2 | 3 |
| K23 | 1 | 0.45 | 0.25 | 5 | 0 |
| | 2 | 0.8 | 0.13 | 5 | 0 |
| | 3 | 0.6 | 0.27 | 5 | 0 |
| K25 | 1 | 0.8 | 0.06 | 2 | 3 |
| | 2 | 0.7 | 0.14 | 2 | 3 |
| | 3 | 0.64 | 0.46 | 2 | 3 |
| K30 | 1 | 1.1 | 0.18 | 5 | 0 |
| | 2 | 0.76 | 0.17 | 5 | 0 |
| | 3 | 0.85 | 0.27 | 3 | 2 |

**Table 8.5 Comparison of the results obtained with the four variants of the distance-based method.** For each resistant isolate the mean values of the distances calculated with the different variants (V1: average maximum distance of the 20 furthest colonies; V2: average maximum distance of the 10 furthest colonies; V3: average maximum distance of the 5 furthest colonies; V4: maximum distance of one furthest colony) and the corresponding standard errors are reported. For each resistant isolate the P-value (P) obtained with one-way ANOVA for the comparison of the four variants is also reported.

| **Resistant isolate** | **V1** | | **V2** | | **V3** | | **V4** | | **P** |
|---|---|---|---|---|---|---|---|---|---|
| | *Mean* | *Std. error* | *Mean* | *Std. error* | *Mean* | *Std. error* | *Mean* | *Std. error* | |
| K1 | 0.44 | 0.17 | 0.48 | 0.22 | 0.56 | 0.30 | 0.71 | 0.38 | 0.90 |
| K2 | 0.62 | 0.15 | 0.62 | 0.15 | 0.74 | 0.22 | 0.99 | 0.33 | 0.64 |
| K3 | 2.33 | 0.32 | 2.65 | 0.32 | 2.92 | 0.32 | 3.31 | 0.29 | 0.23 |
| K7 | 2.33 | 0.32 | 2.65 | 0.32 | 2.92 | 0.32 | 3.31 | 0.29 | 0.93 |
| K10 | 1.10 | 0.23 | 1.22 | 0.29 | 1.36 | 0.34 | 1.63 | 0.33 | 0.66 |
| K11 | 1.61 | 0.16 | 1.99 | 0.37 | 2.28 | 0.85 | 2.58 | 0.52 | 0.43 |
| K13 | 0.52 | 0.16 | 0.52 | 0.16 | 0.52 | 0.16 | 0.58 | 0.17 | 0.99 |
| K17 | 2.25 | 0.20 | 2.58 | 0.19 | 2.84 | 0.22 | 3.17 | 0.29 | 0.10 |
| K18 | 0.84 | 0.22 | 0.86 | 0.22 | 0.89 | 0.21 | 1.05 | 0.23 | 0.90 |
| K19 | 2.50 | 0.19 | 2.81 | 0.17 | 3.01 | 0.20 | 3.30 | 0.14 | 0.052 |
| K20 | 1.85 | 0.39 | 2.13 | 0.42 | 2.34 | 0.45 | 2.43 | 0.43 | 0.78 |
| K21 | 0.65 | 0.06 | 0.65 | 0.06 | 0.70 | 0.08 | 0.93 | 0.06 | 0.06 |
| K23 | 0.62 | 0.10 | 0.62 | 0.10 | 0.62 | 0.10 | 0.91 | 0.03 | 0.11 |
| K25 | 0.72 | 0.05 | 0.72 | 0.05 | 0.72 | 0.05 | 0.87 | 0.05 | 0.12 |
| K30 | 0.77 | 0.04 | 0.78 | 0.03 | 0.90 | 0.10 | 1.03 | 0.19 | 0.37 |

**Table 8.6. Descriptive statistics of data obtained by using the area-based method.** For each resistant isolate inoculated, the total area, the sheltering area and the sheltered area are reported for three plates inoculated with the same resistant isolate. The mean of the three values of the sheltered areas and the Standard deviation (Std deviation) are also reported.

| Resistant isolate | Plate | Total area | Sheltering area | Sheltered area | Mean | St dev |
|---|---|---|---|---|---|---|
| K1 | 1 | 48.728 | 40.123 | 8.605 | | |
| | 2 | 43.802 | 27.723 | 16.079 | | |
| | 3 | 26.891 | 22.721 | 4.17 | 9.6 | 6.018779 |
| K2 | 1 | 54.465 | 48.48 | 5.985 | | |
| | 2 | 43.419 | 31 | 12.419 | | |
| | 3 | 56.046 | 45.984 | 10.062 | 9.45 | 3.255092 |
| K3 | 1 | 73.828 | 23.557 | 50.271 | | |
| | 2 | 86.355 | 24.665 | 61.69 | | |
| | 3 | 49.767 | 19.505 | 30.262 | 47 | 15.90845 |
| K7 | 1 | 46.254 | 34.422 | 11.832 | | |
| | 2 | 63.065 | 37.966 | 25.099 | | |
| | 3 | 35.485 | 26.891 | 8.594 | 15 | 8.745598 |
| K10 | 1 | 48.968 | 31.182 | 17.786 | | |
| | 2 | 58.671 | 21.676 | 36.995 | | |
| | 3 | 50.969 | 43.496 | 7.473 | 20.8 | 14.98272 |
| K11 | 1 | 54.365 | 20.834 | 33.531 | | |
| | 2 | 51.72 | 20.75 | 30.97 | | |
| | 3 | 52.586 | 21.692 | 30.894 | 31.8 | 1.501014 |
| K13 | 1 | 39.739 | 34.845 | 4.894 | | |
| | 2 | 57.589 | 49.017 | 8.572 | | |
| | 3 | 34.63 | 28.914 | 5.716 | 6.4 | 1.930462 |
| K17 | 1 | 64.377 | 18.877 | 45.5 | | |
| | 2 | 94.918 | 25.487 | 69.431 | | |
| | 3 | 53.924 | 13.188 | 40.736 | 52 | 15.37743 |
| K18 | 1 | 38.117 | 29.504 | 8.613 | | |
| | 2 | 51.411 | 36.582 | 14.829 | | |
| | 3 | 32.79 | 27.827 | 4.963 | 9.5 | 4.988305 |
| K19 | 1 | 52.257 | 19.098 | 33.159 | | |
| | 2 | 84.165 | 17.845 | 66.32 | | |
| | 3 | 50.814 | 13.301 | 37.513 | 45.7 | 18.0206 |
| K20 | 1 | 61.215 | 33.913 | 27.302 | | |
| | 2 | 80.666 | 32.838 | 47.828 | | |
| | 3 | 58.58 | 19.294 | 39.286 | 38 | 10.31099 |
| K21 | 1 | 42.699 | 33.455 | 9.244 | | |
| | 2 | 50.892 | 31.408 | 19.484 | | |
| | 3 | 34.557 | 22.68 | 11.877 | 13.5 | 5.31753 |
| K23 | 1 | 45.761 | 36.031 | 9.73 | | |
| | 2 | 41.291 | 29.749 | 11.542 | | |
| | 3 | 40.254 | 27.651 | 12.603 | 11.3 | 1.452767 |
| K25 | 1 | 42.413 | 32.425 | 9.988 | | |
| | 2 | 40.984 | 33.247 | 7.737 | | |
| | 3 | 34.396 | 25.098 | 9.298 | 9.01 | 1.153243 |
| K30 | 1 | 48.584 | 33.666 | 14.918 | | |
| | 2 | 70.923 | 53.291 | 17.632 | | |
| | 3 | 40.032 | 25.345 | 15.087 | 15 | 1.543802 |

**Table 8.7 Results of the Tukey's test for the pairwise comparison of the *E. coli* NCTC 10418 sheltered areas obtained with the incubation of all the *K. pneumoniae* isolates.** The significance level (S. level)and the P Value obtained for each pairwise comparison (Pair compared) are reported. For convenience, only the pairwise comparisons that generated significant P value (≤ 0.05) are reported.

| Pair compared | S. level | P Value | Pair compared | S. level | P Value |
|---|---|---|---|---|---|
| K1 vs. K3 | **** | <0.0001 | K12 vs. K17 | **** | <0.0001 |
| K1 vs. K17 | *** | 0.0001 | K12 vs. K19 | *** | 0.0008 |
| K1 vs. K19 | ** | 0.0061 | K13 vs. K17 | **** | <0.0001 |
| K2 vs. K3 | *** | 0.0002 | K13 vs. K19 | ** | 0.0037 |
| K2 vs. K17 | *** | 0.0005 | K15 vs. K17 | ** | 0.0019 |
| K2 vs. K19 | * | 0.0218 | K16 vs. K17 | ** | 0.0027 |
| K3 vs. K4 | **** | <0.0001 | K17 vs. K18 | **** | <0.0001 |
| K3 vs. K5 | **** | <0.0001 | K17 vs. K21 | *** | 0.0004 |
| K3 vs. K7 | *** | 0.0009 | K17 vs. K22 | **** | <0.0001 |
| K3 vs. K8 | **** | <0.0001 | K17 vs. K23 | *** | 0.0004 |
| K3 vs. K9 | **** | <0.0001 | K17 vs. K24 | **** | <0.0001 |
| K3 vs. K10 | * | 0.0362 | K17 vs. K25 | **** | <0.0001 |
| K3 vs. K12 | **** | <0.0001 | K17 vs. K26 | **** | <0.0001 |
| K3 vs. K13 | **** | <0.0001 | K17 vs. K27 | **** | <0.0001 |
| K3 vs. K15 | *** | 0.0009 | K17 vs. K28 | **** | <0.0001 |
| K3 vs. K16 | ** | 0.0013 | K17 vs. K29 | **** | <0.0001 |
| K3 vs. K18 | **** | <0.0001 | K17 vs. K30 | ** | 0.003 |
| K3 vs. K21 | *** | 0.0002 | K17 vs. K41 | **** | <0.0001 |
| K3 vs. K22 | **** | <0.0001 | K17 vs. K47 | **** | <0.0001 |
| K3 vs. K23 | *** | 0.0002 | K17 vs. K48 | **** | <0.0001 |
| K3 vs. K24 | **** | <0.0001 | K17 vs. K51 | *** | 0.0001 |
| K3 vs. K25 | **** | <0.0001 | K17 vs. K56 | **** | <0.0001 |
| K3 vs. K26 | **** | <0.0001 | K18 vs. K19 | ** | 0.0052 |
| K3 vs. K27 | **** | <0.0001 | K19 vs. K21 | * | 0.0192 |
| K3 vs. K28 | **** | <0.0001 | K19 vs. K22 | **** | <0.0001 |
| K3 vs. K29 | **** | <0.0001 | K19 vs. K23 | * | 0.0205 |
| K3 vs. K30 | ** | 0.0014 | K19 vs. K24 | ** | 0.0018 |
| K3 vs. K41 | **** | <0.0001 | K19 vs. K25 | ** | 0.0019 |
| K3 vs. K47 | **** | <0.0001 | K19 vs. K26 | **** | <0.0001 |
| K3 vs. K48 | **** | <0.0001 | K19 vs. K27 | *** | 0.0004 |
| K3 vs. K51 | **** | <0.0001 | K19 vs. K28 | **** | <0.0001 |
| K3 vs. K56 | **** | <0.0001 | K19 vs. K29 | **** | <0.0001 |
| K4 vs. K17 | **** | <0.0001 | K19 vs. K41 | **** | <0.0001 |
| K4 vs. K19 | **** | <0.0001 | K19 vs. K47 | **** | <0.0001 |
| K4 vs. K20 | * | 0.0269 | K19 vs. K48 | **** | <0.0001 |
| K5 vs. K17 | **** | <0.0001 | K19 vs. K51 | ** | 0.0067 |
| K5 vs. K19 | **** | <0.0001 | K19 vs. K56 | **** | <0.0001 |
| K5 vs. K20 | * | 0.0269 | K20 vs. K22 | * | 0.0269 |
| K7 vs. K17 | ** | 0.0018 | K20 vs. K26 | * | 0.0269 |
| K8 vs. K17 | *** | 0.0002 | K20 vs. K28 | * | 0.0269 |
| K8 vs. K19 | * | 0.0118 | K20 vs. K29 | * | 0.0269 |
| K9 vs. K17 | **** | <0.0001 | K20 vs. K41 | * | 0.0269 |
| K9 vs. K19 | **** | <0.0001 | K20 vs. K47 | * | 0.0269 |
| K9 vs. K20 | * | 0.0269 | K20 vs. K48 | * | 0.0269 |
| | | | K20 vs. K56 | * | 0.0269 |

**Table 8.8 Results of the Tukey's test for the pairwise comparison of the *S. aureus* NCTC 6571 sheltered areas obtained with the incubation of all the *K. pneumoniae* isolates.** The significance level (S. level) and the P Value obtained for each pairwise comparison (Pair compared) are reported. For convenience, only the pairwise comparisons that generated significant P value (≤ 0.05) are reported.

| Pair compared | Summary | P Value | Pair compared | Summary | P Value | Pair compared | Summary | P Value |
|---|---|---|---|---|---|---|---|---|
| K1 vs. K3 | **** | <0.0001 | K9 vs. K10 | **** | <0.0001 | K17 vs. K26 | **** | <0.0001 |
| K1 vs. K11 | ** | 0.0071 | K9 vs. K11 | **** | <0.0001 | K17 vs. K27 | **** | <0.0001 |
| K1 vs. K17 | **** | <0.0001 | K9 vs. K15 | ** | 0.0063 | K17 vs. K28 | **** | <0.0001 |
| K1 vs. K19 | **** | <0.0001 | K9 vs. K17 | **** | <0.0001 | K17 vs. K29 | **** | <0.0001 |
| K1 vs. K20 | * | 0.0156 | K9 vs. K19 | **** | <0.0001 | K17 vs. K41 | **** | <0.0001 |
| K1 vs. K30 | * | 0.0367 | K9 vs. K20 | **** | <0.0001 | K17 vs. K47 | **** | <0.0001 |
| K2 vs. K3 | **** | <0.0001 | K9 vs. K21 | * | 0.0415 | K17 vs. K48 | **** | <0.0001 |
| K3 vs. K4 | **** | <0.0001 | K9 vs. K25 | ** | 0.0035 | K17 vs. K51 | **** | <0.0001 |
| K3 vs. K5 | **** | <0.0001 | K9 vs. K30 | **** | <0.0001 | K17 vs. K56 | **** | <0.0001 |
| K3 vs. K7 | **** | <0.0001 | K10 vs. K12 | * | 0.0133 | K18 vs. K19 | ** | 0.0013 |
| K3 vs. K8 | **** | <0.0001 | K10 vs. K16 | * | 0.0112 | K19 vs. K21 | ** | 0.005 |
| K3 vs. K9 | **** | <0.0001 | K10 vs. K22 | **** | <0.0001 | K19 vs. K22 | **** | <0.0001 |
| K3 vs. K10 | **** | <0.0001 | K10 vs. K26 | **** | <0.0001 | K19 vs. K23 | ** | 0.0017 |
| K3 vs. K11 | * | 0.0145 | K10 vs. K28 | **** | <0.0001 | K19 vs. K24 | **** | <0.0001 |
| K3 vs. K12 | **** | <0.0001 | K10 vs. K29 | **** | <0.0001 | K19 vs. K26 | **** | <0.0001 |
| K3 vs. K13 | **** | <0.0001 | K10 vs. K41 | **** | <0.0001 | K19 vs. K27 | **** | <0.0001 |
| K3 vs. K15 | **** | <0.0001 | K10 vs. K47 | **** | <0.0001 | K19 vs. K28 | **** | <0.0001 |
| K3 vs. K16 | **** | <0.0001 | K10 vs. K48 | **** | <0.0001 | K19 vs. K29 | **** | <0.0001 |
| K3 vs. K18 | **** | <0.0001 | K10 vs. K56 | **** | <0.0001 | K19 vs. K41 | **** | <0.0001 |
| K3 vs. K20 | ** | 0.0066 | K11 vs. K12 | **** | <0.0001 | K19 vs. K47 | **** | <0.0001 |
| K3 vs. K21 | **** | <0.0001 | K11 vs. K13 | *** | 0.0003 | K19 vs. K48 | **** | <0.0001 |
| K3 vs. K22 | **** | <0.0001 | K11 vs. K16 | **** | <0.0001 | K19 vs. K51 | **** | <0.0001 |
| K3 vs. K23 | **** | <0.0001 | K11 vs. K22 | **** | <0.0001 | K19 vs. K56 | **** | <0.0001 |
| K3 vs. K24 | **** | <0.0001 | K11 vs. K24 | *** | 0.0002 | K20 vs. K22 | **** | <0.0001 |
| K3 vs. K25 | **** | <0.0001 | K11 vs. K26 | **** | <0.0001 | K20 vs. K24 | *** | 0.0005 |
| K3 vs. K26 | **** | <0.0001 | K11 vs. K27 | *** | 0.0006 | K20 vs. K26 | **** | <0.0001 |
| K3 vs. K27 | **** | <0.0001 | K11 vs. K28 | **** | <0.0001 | K20 vs. K27 | ** | 0.0014 |
| K3 vs. K28 | **** | <0.0001 | K11 vs. K29 | **** | <0.0001 | K20 vs. K28 | **** | <0.0001 |
| K3 vs. K29 | **** | <0.0001 | K11 vs. K41 | **** | <0.0001 | K20 vs. K29 | **** | <0.0001 |
| K3 vs. K30 | ** | 0.0025 | K11 vs. K47 | **** | <0.0001 | K20 vs. K41 | **** | <0.0001 |
| K3 vs. K41 | **** | <0.0001 | K11 vs. K48 | **** | <0.0001 | K20 vs. K47 | **** | <0.0001 |
| K3 vs. K47 | **** | <0.0001 | K11 vs. K51 | *** | 0.0002 | K20 vs. K48 | **** | <0.0001 |
| K3 vs. K48 | **** | <0.0001 | K11 vs. K56 | **** | <0.0001 | K20 vs. K51 | *** | 0.0005 |
| K3 vs. K51 | **** | <0.0001 | K12 vs. K17 | **** | <0.0001 | K20 vs. K56 | **** | <0.0001 |
| K3 vs. K56 | **** | <0.0001 | K12 vs. K19 | **** | <0.0001 | K21 vs. K22 | * | 0.0415 |
| K4 vs. K11 | ** | 0.0014 | K12 vs. K20 | **** | <0.0001 | K21 vs. K26 | * | 0.0415 |
| K4 vs. K17 | **** | <0.0001 | K12 vs. K30 | *** | 0.0002 | K21 vs. K28 | * | 0.0415 |
| K4 vs. K19 | **** | <0.0001 | K13 vs. K17 | **** | <0.0001 | K21 vs. K29 | * | 0.0415 |
| K4 vs. K20 | ** | 0.0033 | K13 vs. K19 | **** | <0.0001 | K21 vs. K41 | * | 0.0415 |
| K4 vs. K30 | ** | 0.0085 | K13 vs. K20 | *** | 0.0008 | K21 vs. K47 | * | 0.0415 |
| K5 vs. K9 | * | 0.0172 | K13 vs. K30 | ** | 0.0023 | K21 vs. K48 | * | 0.0415 |
| K5 vs. K17 | ** | 0.0014 | K15 vs. K17 | ** | 0.0042 | K21 vs. K56 | * | 0.0415 |
| K5 vs. K19 | * | 0.0131 | K15 vs. K19 | * | 0.0341 | K22 vs. K25 | ** | 0.0035 |
| K5 vs. K22 | * | 0.0172 | K15 vs. K22 | ** | 0.0063 | K22 vs. K30 | **** | <0.0001 |
| K5 vs. K26 | * | 0.0172 | K15 vs. K26 | ** | 0.0063 | K24 vs. K30 | ** | 0.0014 |
| K5 vs. K28 | * | 0.0172 | K15 vs. K28 | ** | 0.0063 | K25 vs. K26 | ** | 0.0035 |
| K5 vs. K29 | * | 0.0172 | K15 vs. K29 | ** | 0.0063 | K25 vs. K28 | ** | 0.0035 |
| K5 vs. K41 | * | 0.0172 | K15 vs. K41 | ** | 0.0063 | K25 vs. K29 | ** | 0.0035 |
| K5 vs. K47 | * | 0.0172 | K15 vs. K47 | ** | 0.0063 | K25 vs. K41 | ** | 0.0035 |
| K5 vs. K48 | * | 0.0172 | K15 vs. K48 | ** | 0.0063 | K25 vs. K47 | ** | 0.0035 |
| K5 vs. K56 | * | 0.0172 | K15 vs. K56 | ** | 0.0063 | K25 vs. K48 | ** | 0.0035 |
| K7 vs. K11 | * | 0.0444 | K16 vs. K17 | **** | <0.0001 | K25 vs. K56 | ** | 0.0035 |
| K7 vs. K17 | **** | <0.0001 | K16 vs. K19 | **** | <0.0001 | K26 vs. K30 | **** | <0.0001 |
| K7 vs. K19 | *** | 0.0007 | K16 vs. K20 | **** | <0.0001 | K27 vs. K30 | ** | 0.0037 |
| K8 vs. K11 | ** | 0.004 | K16 vs. K30 | *** | 0.0002 | K28 vs. K30 | **** | <0.0001 |
| K8 vs. K17 | **** | <0.0001 | K17 vs. K18 | *** | 0.0001 | K29 vs. K30 | **** | <0.0001 |
| K8 vs. K19 | **** | <0.0001 | K17 vs. K21 | *** | 0.0005 | K30 vs. K41 | **** | <0.0001 |
| K8 vs. K20 | ** | 0.0089 | K17 vs. K22 | **** | <0.0001 | K30 vs. K47 | **** | <0.0001 |
| K8 vs. K30 | * | 0.0218 | K17 vs. K23 | *** | 0.0001 | K30 vs. K48 | **** | <0.0001 |
| | | | K17 vs. K24 | **** | <0.0001 | K30 vs. K51 | ** | 0.0013 |
| | | | K17 vs. K25 | ** | 0.0075 | K30 vs. K56 | **** | <0.0001 |

**Table 8.9 Identification of the β-lactamase genes by ResFinder.** For each isolate the β-lactamase genes found are reported, as well as the percentage of identity (% Id) and the Query coverage (%) with the corresponding known genes and their variant. The expected β-lactamase genes that were not found by ResFinder are indicated with "NF".

| Isolates | Genes found | % Id | Query Coverage (%) |
|---|---|---|---|
| | $bla_{OXA-48}$ | 100 | 100 |
| | $bla_{TEM-1B}$ | 99.84 | 100 |
| | $bla_{SHV}$ | NF | NF |
| K1 | $bla_{CTX-M-15}$ | 99.66 | 100 |
| | $bla_{OXA-48}$ | 100 | 100 |
| | $bla_{SHV-27}$ | 99.88 | 100 |
| | $bla_{TEM-1B}$ | 100 | 100 |
| K2 | $bla_{CTX-M-15}$ | 100 | 100 |
| | $bla_{OXA-48}$ | 100 | 100 |
| | $bla_{SHV-1}$ | 100 | 100 |
| | $bla_{TEM-1B}$ | 100 | 100 |
| K3 | $bla_{CTX-M-15}$ | 100 | 100 |
| | $bla_{OXA-48}$ | 100 | 100 |
| K4 | $bla_{SHV-1}$ | 99.88 | 100 |
| | $bla_{OXA-48}$ | 100 | 100 |
| | $bla_{TEM-1B}$ | 99.88 | 100 |
| K5 | $bla_{SHV}$ | NF | NF |
| | $bla_{NDM}$ | NF | NF |
| | $bla_{SHV}$ | NF | NF |
| | $bla_{TEM}$ | NF | NF |
| K7 | $bla_{CTX-M}$ | NF | NF |
| | $bla_{OXA-48}$ | 100 | 100 |
| | $bla_{SHV-27}$ | 99.88 | 100 |
| | $bla_{TEM-1B}$ | 100 | 100 |
| K8 | $bla_{CTX-M-15}$ | 100 | 100 |
| | $bla_{TEM-1B}$ | 100 | 100 |
| | $bla_{SHV-1}$ | 99.88 | 100 |
| K9 | $bla_{NDM-1}$ | 100 | 100 |
| | $bla_{NDM}$ | NF | NF |
| | $bla_{SHV}$ | NF | NF |
| | $bla_{TEM}$ | NF | NF |
| K10 | $bla_{CTX-M}$ | NF | NF |
| | $bla_{OXA-48}$ | 99.62 | 100 |
| | $bla_{SHV-1}$ | 100 | 100 |
| | $bla_{TEM-1B}$ | 99.88 | 100 |
| K11 | $bla_{CTX-M-14}$ | 88.89 | 100 |

| Isolates | Genes found | % Id | Query cov (%) |
|---|---|---|---|
| | *bla*$_{OXA-48}$ | 100 | 100 |
| | *bla*$_{SHV-1}$ | 99.65 | 100 |
| | *bla*$_{TEM-1B}$ | 100 | 100 |
| K12 | *bla*$_{CTX-M-15}$ | 99.89 | 100 |
| | *bla*$_{OXA-48}$ | 100 | 100 |
| | *bla*$_{SHV-1}$ | 99.65 | 100 |
| | *bla*$_{TEM-1B}$ | 100 | 100 |
| K15 | *bla*$_{CTX-M-15}$ | 100 | 100 |
| | *bla*$_{OXA-48}$ | 100 | 100 |
| | *bla*$_{SHV-1}$ | 99.65 | 100 |
| | *bla*$_{TEM-1B}$ | 100 | 100 |
| K16 | *bla*$_{CTX-M-15}$ | 100 | 100 |
| | *bla*$_{OXA-48}$ | 100 | 100 |
| | *bla*$_{SHV-1}$ | 99.65 | 100 |
| | *bla*$_{TEM-1B}$ | 100 | 100 |
| K17 | *bla*$_{CTX-M-15}$ | 100 | 100 |
| | *bla*$_{OXA-48}$ | 100 | 100 |
| | *bla*$_{SHV-1}$ | 100 | 100 |
| | *bla*$_{TEM-1B}$ | 100 | 100 |
| K18 | *bla*$_{CTX-M-15}$ | 100 | 100 |
| | *bla*$_{OXA-48}$ | 100 | 100 |
| | *bla*$_{SHV-1}$ | 99.65 | 100 |
| | *bla*$_{TEM-1B}$ | 100 | 100 |
| K19 | *bla*$_{CTX-M-15}$ | 100 | 100 |
| | *bla*$_{OXA-48}$ | 99.65 | 100 |
| | *bla*$_{SHV-1}$ | 100 | 100 |
| | *bla*$_{TEM-1B}$ | 99.88 | 100 |
| K20 | *bla*$_{CTX-M-14}$ | 100 | 100 |
| | *bla*$_{OXA-48}$ | 100 | 100 |
| | *bla*$_{SHV-1}$ | 99.65 | 100 |
| | *bla*$_{TEM-1B}$ | 100 | 100 |
| K21 | *bla*$_{CTX-M-15}$ | 100 | 100 |
| | *bla*$_{TEM-1B}$ | 100 | 100 |
| | *bla*$_{SHV-1}$ | 100 | 100 |
| K22 | *bla*$_{NDM-1}$ | 100 | 100 |
| | *bla*$_{OXA-48}$ | 100 | 100 |
| | *bla*$_{SHV-1}$ | 99.65 | 100 |
| | *bla*$_{TEM-1B}$ | 100 | 100 |
| K24 | *bla*$_{CTX-M-15}$ | 100 | 100 |

| Isolates | Genes found | % Id | Query cov (%) |
|---|---|---|---|
| | *bla*$_{OXA-48}$ | 100 | 100 |
| | *bla*$_{SHV-1}$ | 99.65 | 100 |
| | *bla*$_{TEM-1B}$ | 100 | 100 |
| K25 | *bla*$_{CTX-M-15}$ | 100 | 100 |
| | *bla*$_{OXA-48}$ | 99.87 | 100 |
| K26 | *bla*$_{SHV-1}$ | 99.88 | 100 |
| | *bla*$_{OXA-48}$ | NF | NF |
| | *bla*$_{SHV}$ | NF | NF |
| | *bla*$_{TEM}$ | NF | NF |
| K28 | *bla*$_{CTX-M}$ | NF | NF |
| | *bla*$_{OXA-48}$ | 100 | 100 |
| K29 | *bla*$_{SHV-1}$ | 99.65 | 100 |
| | *bla*$_{OXA-181}$ | 100 | 100 |
| | *bla*$_{SHV}$ | NF | NF |
| | *bla*$_{TEM-1B}$ | 99.88 | 100 |
| K30 | *bla*$_{CTX-M-15}$ | 100 | 100 |
| | *bla*$_{TEM-1B}$ | 100 | 100 |
| | *bla*$_{SHV-1}$ | 100 | 100 |
| | *bla*$_{NDM-1}$ | 100 | 100 |
| K41 | *bla*$_{CTX-M-15}$ | 100 | 100 |
| | *bla*$_{TEM-1B}$ | 100 | 100 |
| | *bla*$_{SHV-1}$ | 100 | 100 |
| | *bla*$_{NDM-1-1}$ | 100 | 100 |
| K47 | *bla*$_{CTX-M-15}$ | 100 | 100 |
| | *bla*$_{TEM-1B}$ | 100 | 100 |
| | *bla*$_{SHV-1}$ | 99.88 | 100 |
| | *bla*$_{NDM-5}$ | 100 | 100 |
| K48 | *bla*$_{CTX-M-15}$ | 100 | 100 |
| | *bla*$_{TEM-1B}$ | 100 | 100 |
| | *bla*$_{SHV-1}$ | 100 | 100 |
| | *bla*$_{NDM-5}$ | 100 | 100 |
| K51 | *bla*$_{CTX-M-15}$ | 100 | 100 |
| | *bla*$_{TEM-1B}$ | 100 | 100 |
| | *bla*$_{SHV-1}$ | 100 | 100 |
| | *bla*$_{NDM-1}$ | 100 | 100 |
| K56 | *bla*$_{CTX-M-15}$ | 100 | 100 |

## 8.4 Agarose gel images

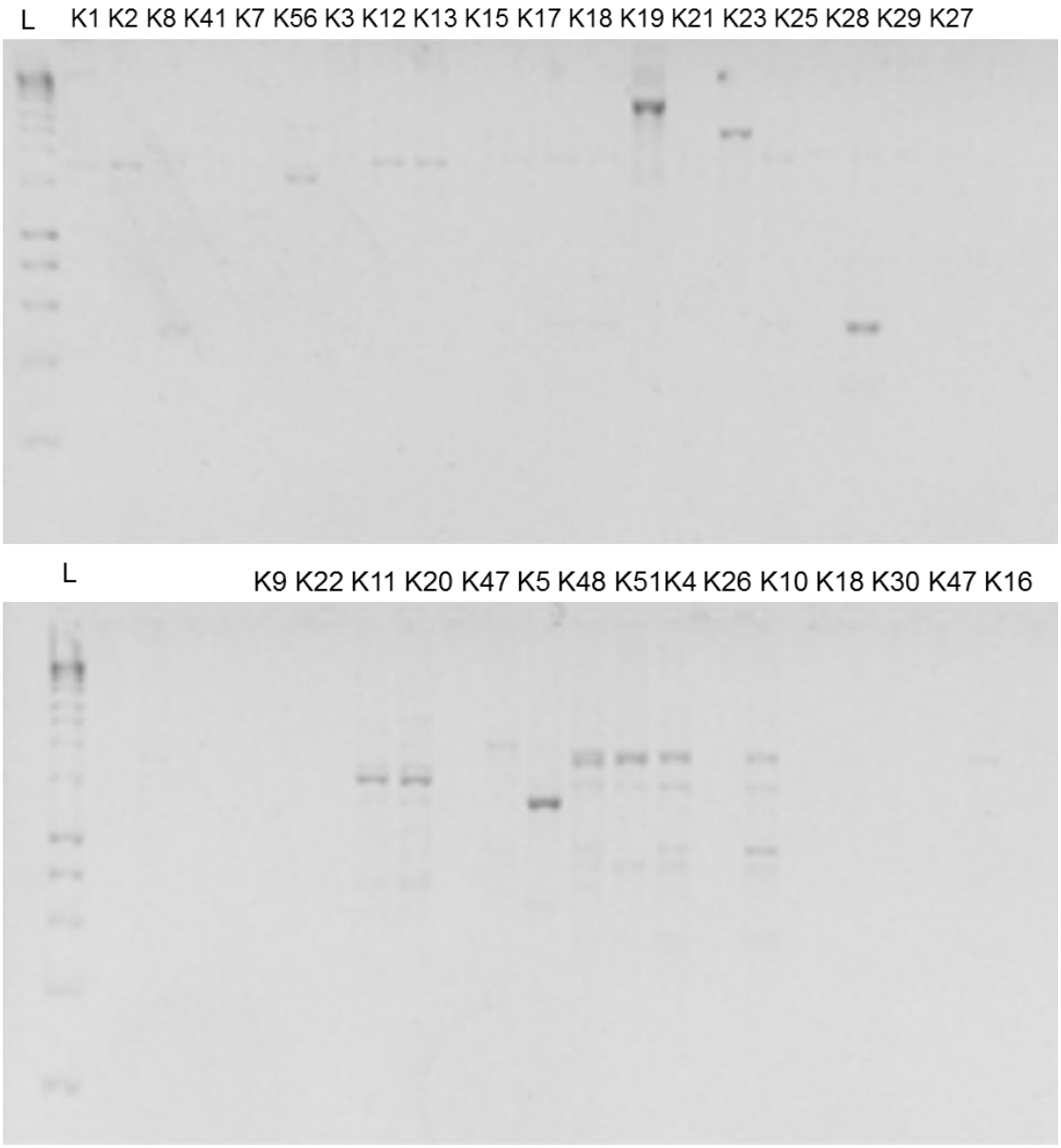



**Figure 8.1 RAPD results obtained with AP4 primer on the DNA of the isolates.** L: ladder

The amplification of the DNA with AP4 was expected to produce a pattern of bands of different sizes specific for each isolate when the DNA is run on agarose gel. However, with the exception of K4 and K10 , a pattern of bands specific for each of the other isolates was not detectable. L: ladder

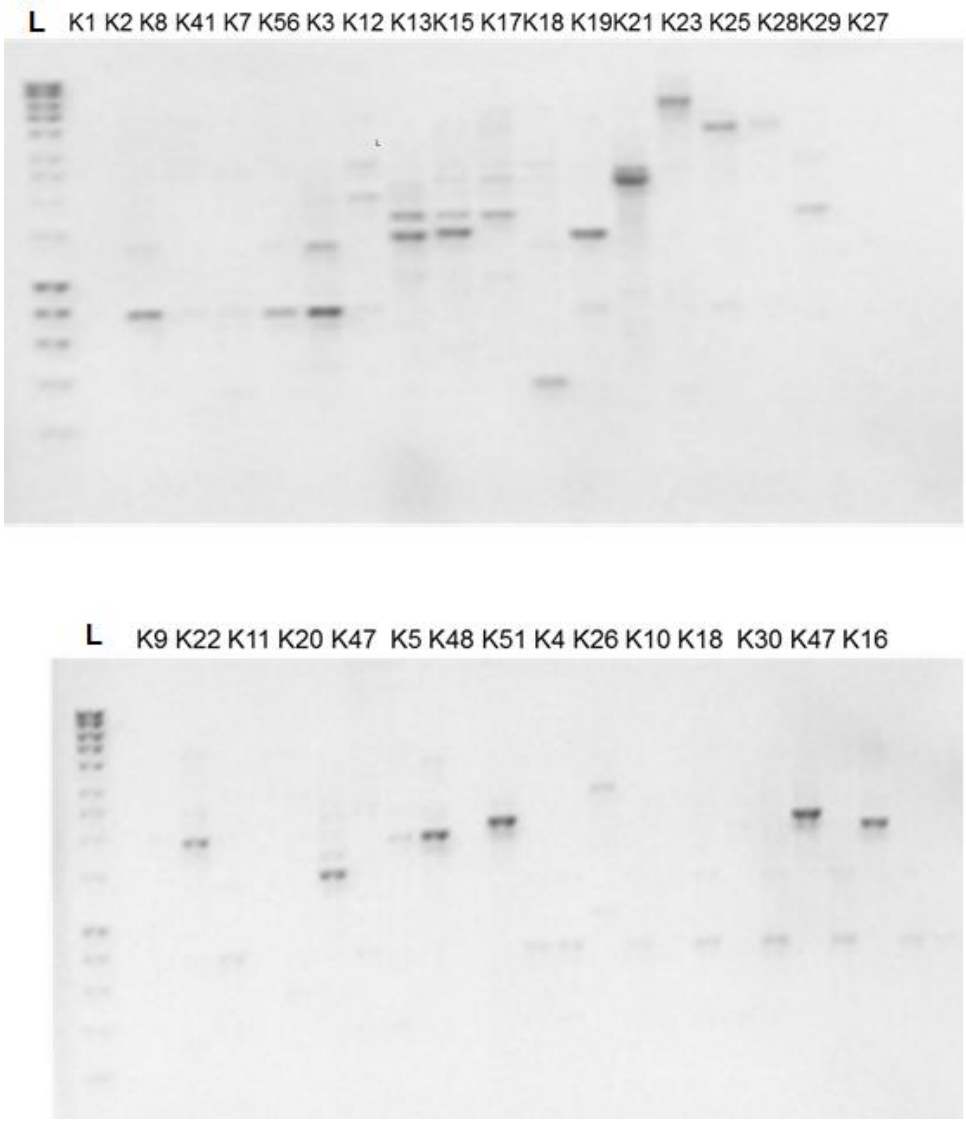



**Figure 8.2 RAPD results obtained with RAPD-7 primer on the DNA of the isolates.**

The amplification of the DNA with RAPD-7 was expected to produce a pattern of bands of different sizes specific for each isolate when the DNA is run on agarose gel. However, a RAPD profile characteristic for each isolate was not detectable due to the lack of a patterns of bands specific for each isolate. L: ladder

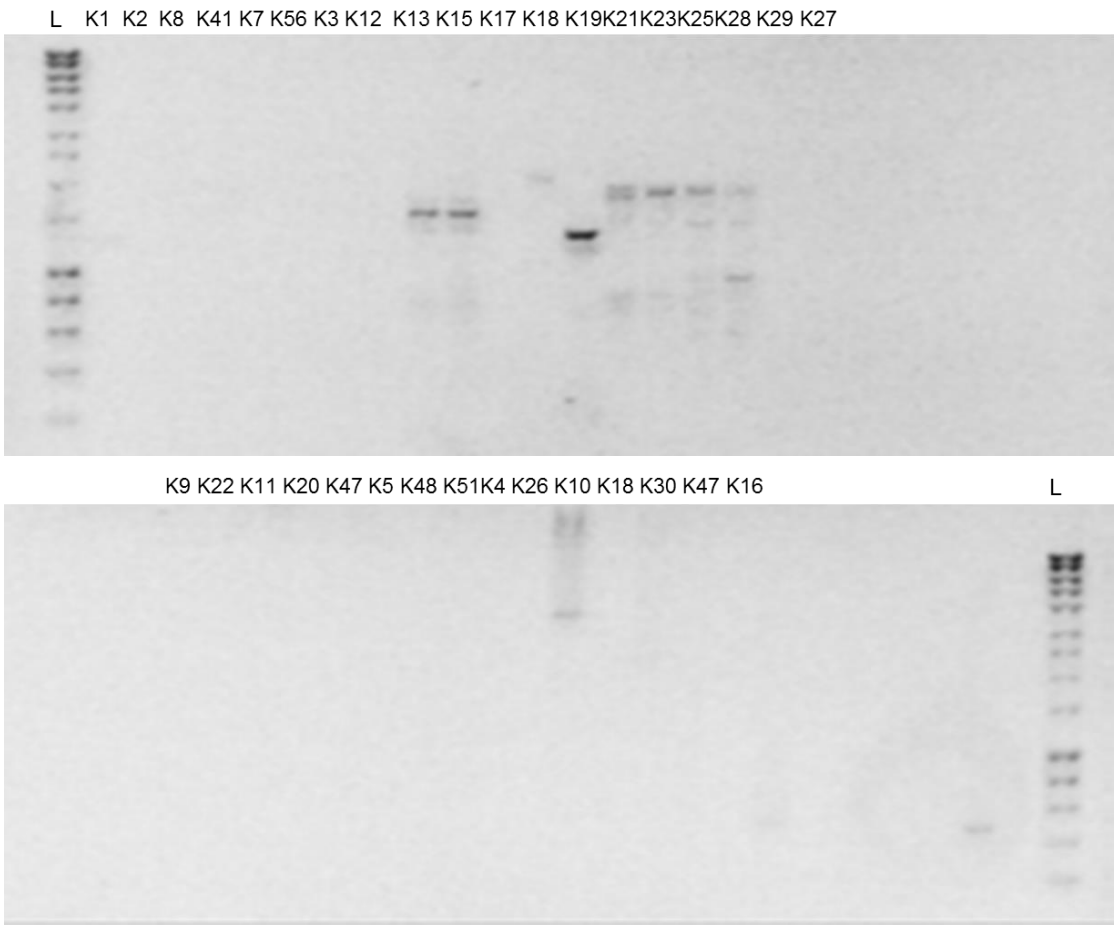



**Figure 8.3 RAPD results obtained with Primer 640 on the DNA of the isolates.**

The amplification of the DNA with Primer 640 was expected to produce a pattern of bands of different sizes specific for each isolate when the DNA is run on agarose gel. However, a RAPD profile characteristic for each isolate was not detectable due to the lack of a patterns of bands specific for each isolate. L: ladder

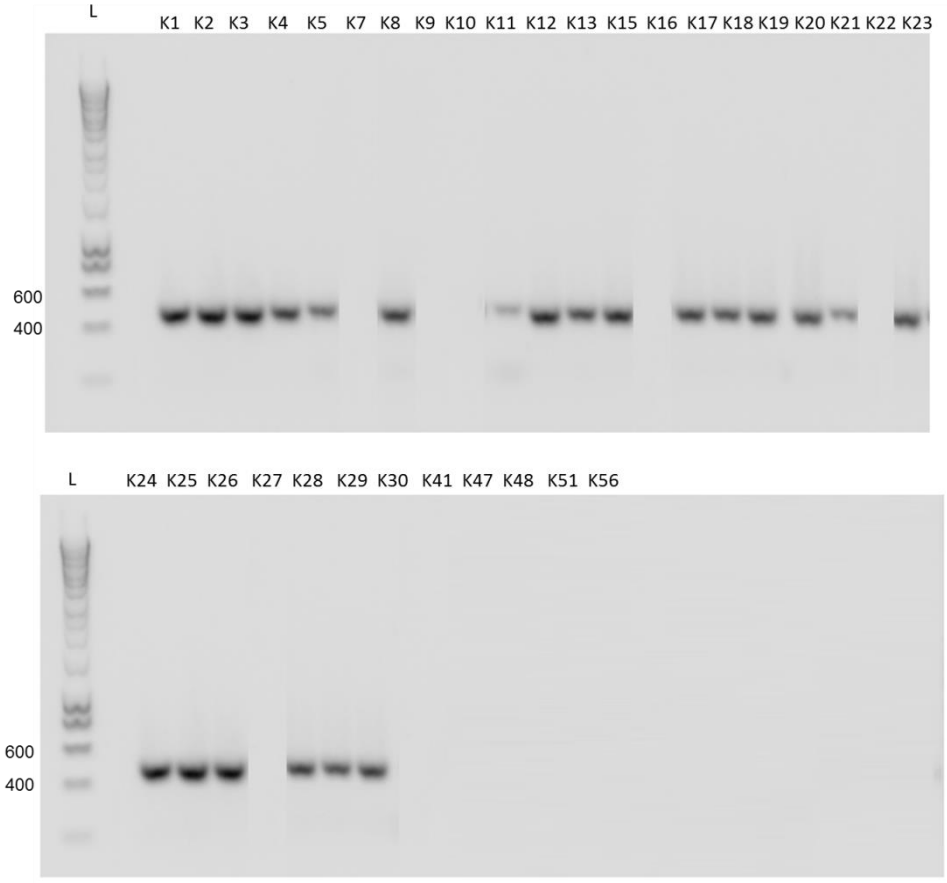



**Figure 8.4 Agarose gel electrophoresis for the separation of PCR products coming from the amplification of *bla*$_{OXA-48}$ in the *K. pneumoniae* isolates** The size of the reference ladder bands used to identify the bands corresponding to the amplification of *bla*$_{OXA-48}$ are reported on the left side of the DNA ladder (L). The presence of a band of 438 bp indicates the amplification of this gene in the *K .pneumoniae* isolates.

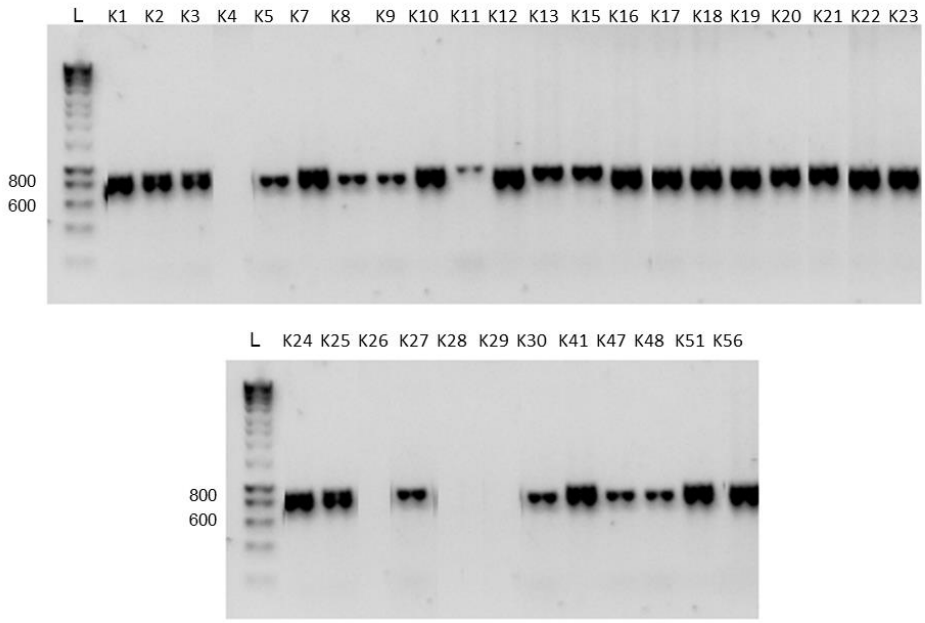



**Figure 8.5 Agarose gel electrophoresis for the separation of PCR products coming from the amplification of *bla*$_{TEM}$ in the *K. pneumoniae* isolates** The size of the reference ladder bands used to identify the bands corresponding to the amplification of *bla*$_{TEM}$ are reported on the left side of the DNA ladder (L). The presence of a band of approximately 800 bp indicates the amplification of this gene in the *K .pneumoniae* isolates.

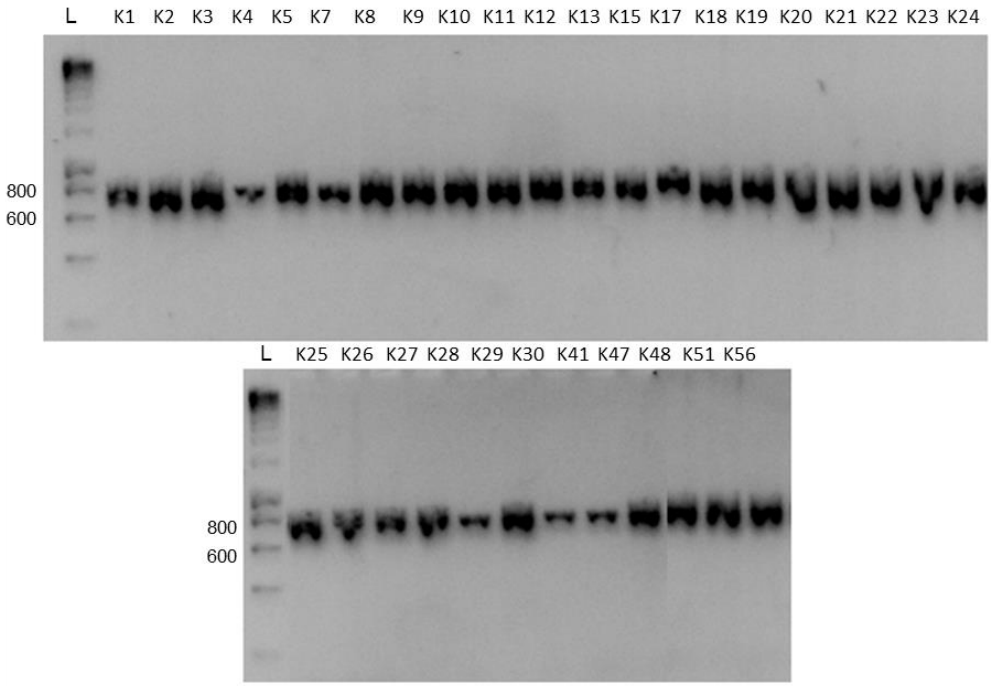



**Figure 8.6 Agarose gel electrophoresis for the separation of PCR products coming from the amplification of *bla*$_{SHV}$ in the *K. pneumoniae* isolates** The size of the reference ladder bands used to identify the bands corresponding to the amplification of *bla*$_{SHV}$ are reported on the left side of the DNA ladder (L). The presence of a band of 713 bp indicates the amplification of this gene in the *K .pneumoniae* isolates.

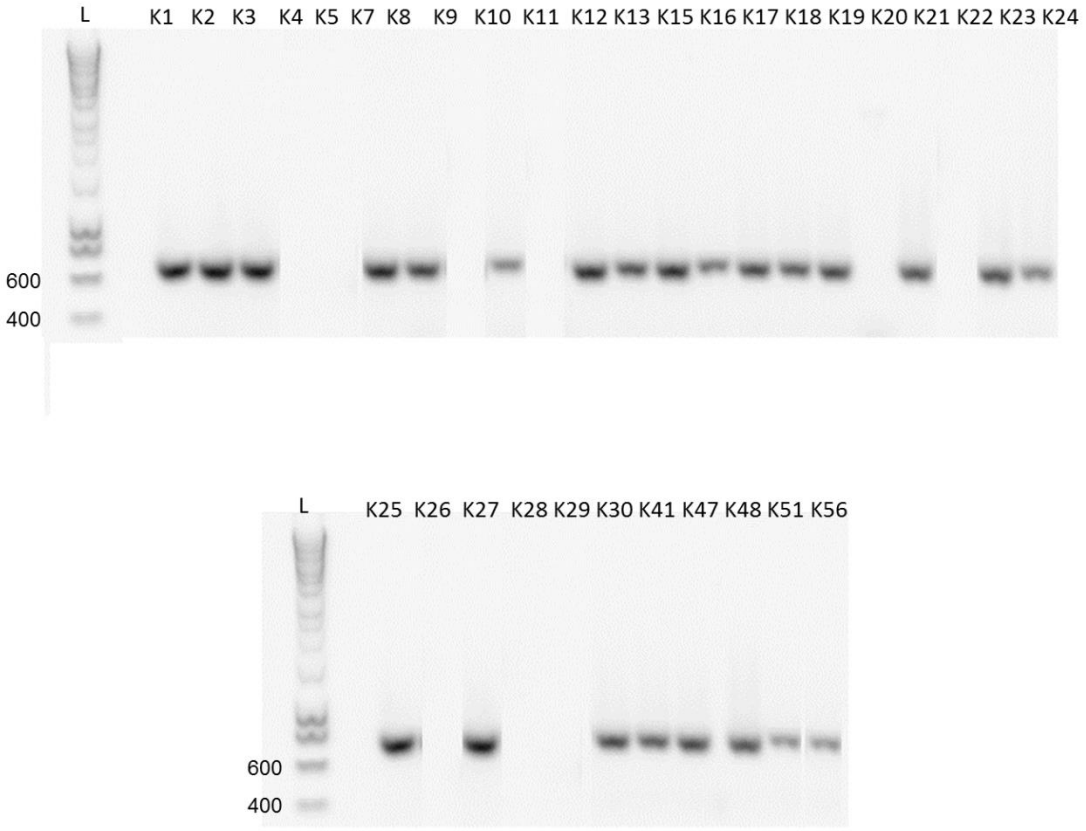



**Figure 8.7 Agarose gel electrophoresis for the separation of PCR products coming from the amplification of *bla*$_{CTXM}$ group 1 in the *K. pneumoniae* isolates** The size of the reference ladder bands used to identify the bands corresponding to the amplification of *bla*$_{CTXM\ group\ 1}$ are reported on the left side of the DNA ladder (L). The presence of a band of 688 bp indicates the amplification of this gene in the *K .pneumoniae* isolates.

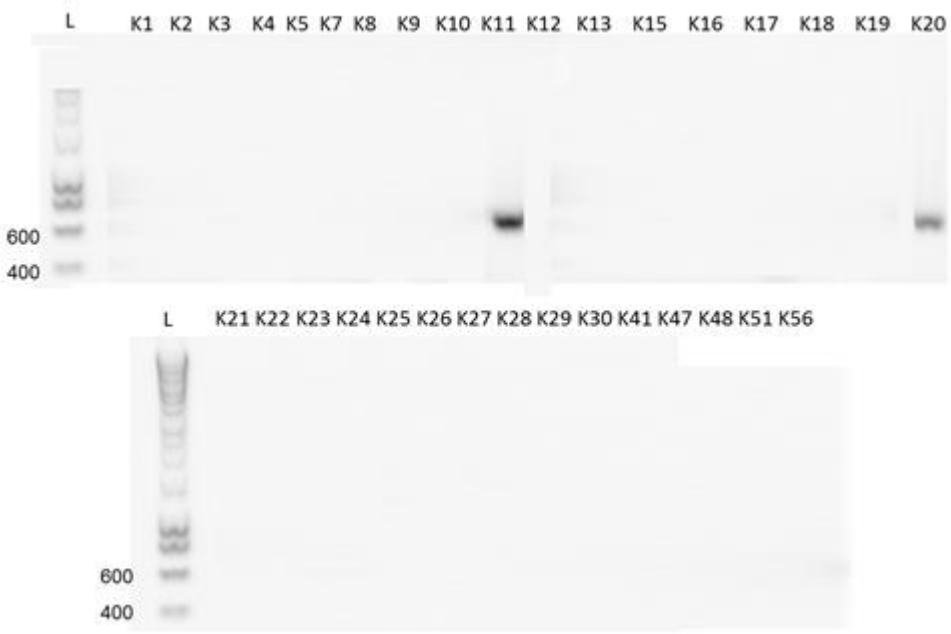



**Figure 8.8. Agarose gel electrophoresis for the separation of PCR products coming from the amplification of *bla*$_{CTXM}$ group 9 in the *K. pneumoniae* isolates** The size of the reference ladder bands used to identify the bands corresponding to the amplification of *bla*$_{CTXM\ group\ 9}$ are reported on the left side of the DNA ladder (L). The presence of a band of 561 bp indicates the amplification of this gene in the *K .pneumoniae* isolates.

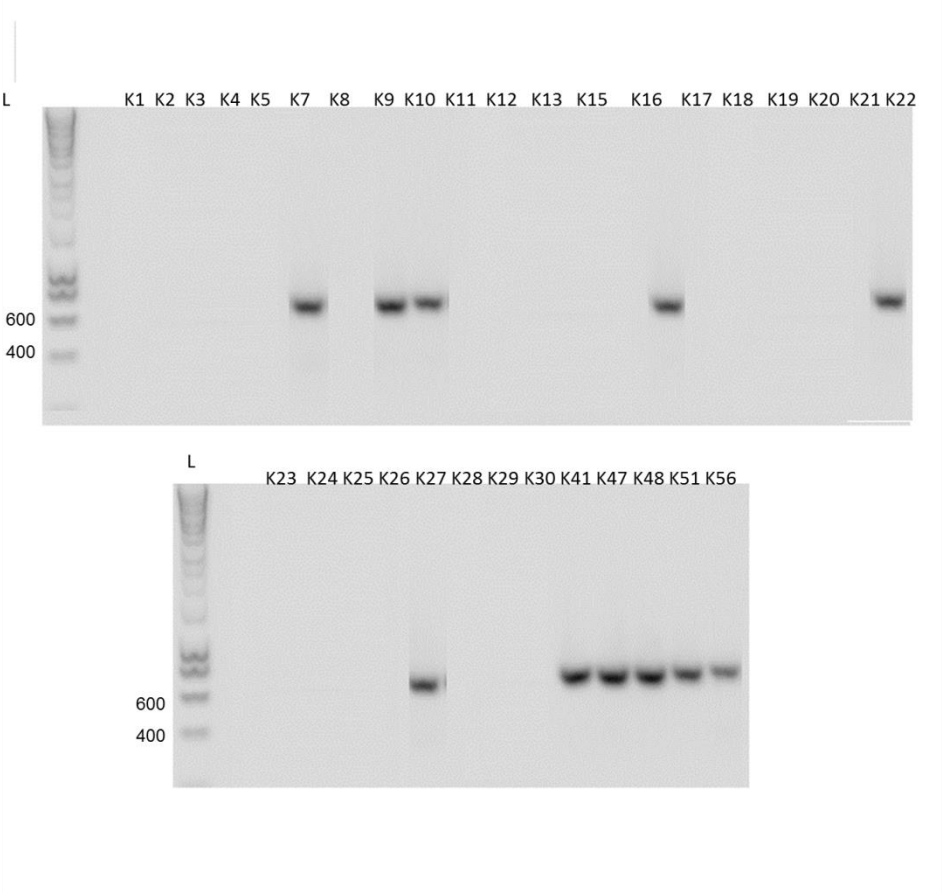



**Figure 8.9 Agarose gel electrophoresis for the separation of PCR products coming from the amplification of *bla*$_{NDM}$ in the *K. pneumoniae* isolates** The size of the reference ladder bands used to identify the bands corresponding to the amplification of *bla*$_{NDM}$ are reported on the left side of the DNA ladder (L). The presence of a band of 621 bp indicates the amplification of this gene in the *K .pneumoniae* isolates.

## 8.5 Protein alignments

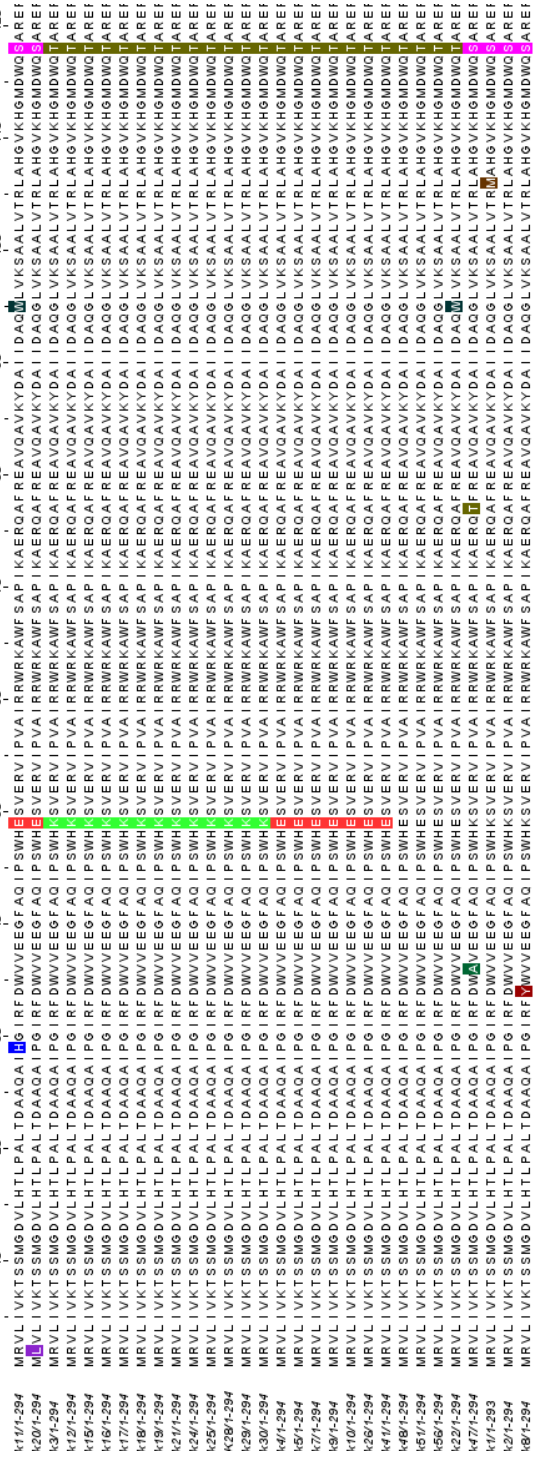


**Figure 8.10 Multiple alignment of the waaC amino acid sequences of the *K. pneumoniae* Isolates.**
For clarity only the aligned regions including the different amino acids are reported. The different aminoacids are distinguished by different colours.

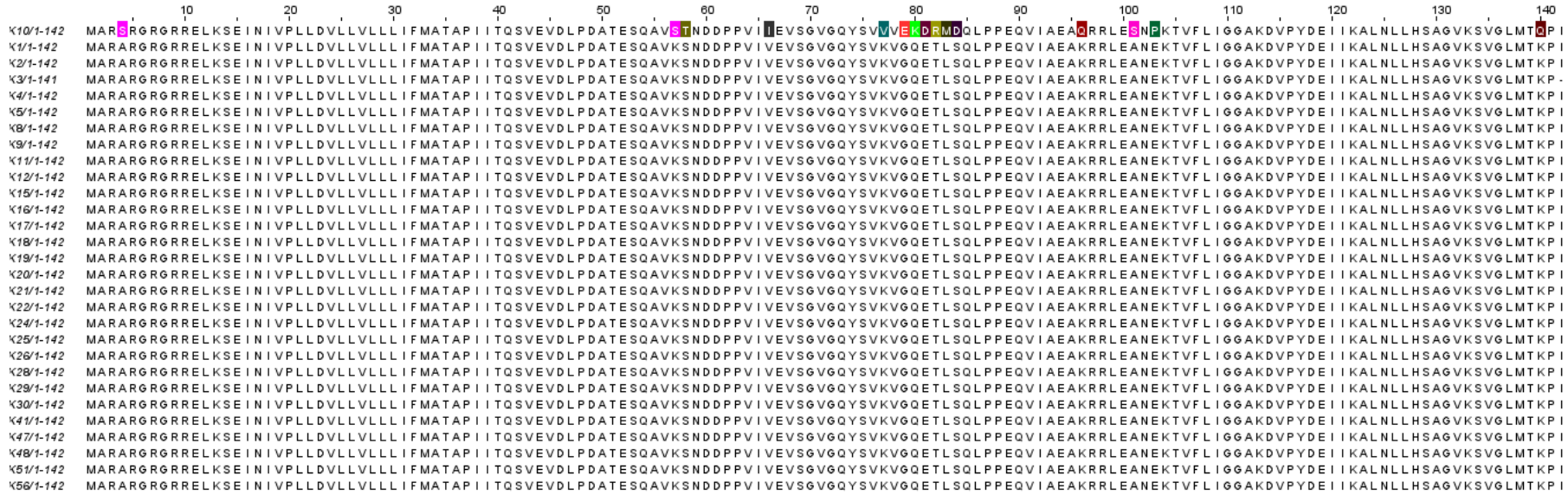



**Figure 8.11 Multiple alignment of the tolR amino acid sequence of the *K. pneumoniae* isolates.**

For clarity only the aligned regions including the different amino acids are reported. The different amino acids are distinguished by different colours.