Universidade de Aveiro Departamento de Biologia

Ano 2017

Ana Teresa Torre Viana

Antibiotic and metal(loid) resistance of *Pedobacter lusitanus* NL19 and its closely-related species

Resistência de *Pedobacter lusitanus* NL19 a antibióticos, metais e metalóides e das suas espécies estreitamente relacionadas

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Microbiologia, realizada sob a orientação científica de Sónia Alexandra Leite Velho Mendo Barroso, Professora Auxiliar com Agregação do Departamento de Biologia da Universidade de Aveiro e de Tânia Isabel Sousa Caetano, bolseira de Pós-Doutoramento no Departamento de Biologia da Universidade de Aveiro.

Dedico este trabalho aos meus pais e à minha irmã.

o júri

presidente

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agradecimentos

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palavras-chave

Pedobacter spp.; resistência; antibióticos; metais; metalóides; β-lactamases.

resumo

Pedobacter lusitanus NL19 foi isolada de lamas recolhidas numa mina de urânio desativada, na Quinta do Bispo (Viseu, Portugal). O local da recolha das amostras é caracterizado pela presença de elevadas concentrações de metais e radionuclídeos. A análise do genoma da estirpe NL19 revelou a presença de vários genes de resistência a antibióticos (β-lactamases, bombas de efluxo, e outros) e também de determinantes genéticos de resistência a metais e metalóides (arsénio, zinco, cobalto e cádmio).

O principal objetivo deste estudo foi investigar a resistência a antibióticos e a tolerância a metais e metalóides por *P. lusitanus* NL19, por outras quatro espécies próximas, *P. himalayensis* MTCC 6384^T, *P. hartonius* DSM 19033^T, *P. cryoconitis* DSM 14825^T e *P. westerhofensis* DSM 19036^T, e ainda pela estirpe tipo deste género, *P. heparinus* DSM 2366^T.

Os resultados de teste de difusão em disco e a determinação da MIC monstraram que todas as estirpes são resistentes à amoxicilina/ácido clavulânico, ampicilina, apramicina, aztreoname, cefepima, cefoxitina, ceftazidima, cefuroxima, penicilina G, piperacilina, piperacilina/tazobactama, amicacina, gentamicina, netilmicina, streptomicina, tobramicina, ciprofloxacina e colistina. No que se refere aos carbapenemos, todas as estirpes são resistentes ao ertapenemo e a maioria é sensível ao meropenemo e ao imipenemo. As excepções incluem a resistência ao meropenemo de *P. westerhofensis* e diminuição da susceptibilidade ao imipenemo de *P. himalayensis*.

No que se refere à tolerância aos metais, valores de MIC mais elevados foram registados para *P. lusitanus* para o cobre e o cádmio. *P. lusitanus*, *P. himalayensis* e *P. hartonius* são as estirpes mais tolerantes ao zinco e níquel.

A análise do genome de *P. lusitanus* NL19 permitiu a identificação de dois genes que codificam para β-lactamases putativas. Estas proteínas foram consideradas como novos membros das β-lactamases de classe A (LUS-1) e da subclasse B3 metalo-β-lactamases (PLN-1) devido aos seus baixos *scores* com outras β-lactamases. Para compreender se estas enzimas têm a capacidade de hidrolisar β-lactâmicos, os genes *bla*$_{LUS-1}$ e *bla*$_{PLN-1}$ foram expressos em *E. coli* (fundidos com um péptido sinal de *E. coli*). A determinação dos valores da MIC para β-lactâmicos indicam que PLN-1 é capaz de hidrolisar carbapenemos e cefalosporinas de 1ª, 3ª e 4ª geração e cefamicinas. Contudo, não foi detectada nenhuma atividade significativa para a LUS-1.

Este estudo mostra que o fenótipo de multi-resistência identificado em *P. lusitanus* NL19 também é comum a outras espécies estreitamente relacionadas do mesmo género. Para além disso, é possível perceber que as espécies do género *Pedobacter* possuem um *pool* inexplorado de genes de resistência a antibióticos bem como de putativos novos mecanismos de resistência a antibióticos. A tolerância a metais e metalóides é específica para cada estirpe, podendo estar associada ao nicho ecológico que estas estirpes ocupam.

keywords *Pedobacter* spp.; resistance; antibiotics; metal(loid)s; β-lactamases

abstract

Pedobacter lusitanus NL19 was isolated from a sludge collected from a deactivated uranium mine, Quinta do Bispo (Viseu, Portugal). The sampling site is characterized by high levels of metals and radionuclides. *In silico* genome analysis of NL19 disclosed several antibiotic resistance-related genes (β-lactamase, efflux pumps, etc.) and also genetic determinants related with the resistance/tolerance to metal(loid)s (arsenic, zinc, cobalt and cadmium).

The main objective of this study was to investigate the antibiotic resistance and metal(loid)s tolerance of *P. lusitanus* NL19, the closely-related species *P. himalayensis* MTCC 6384^T, *P. hartonius* DSM 19033^T, *P. cryoconitis* DSM 14825^T and *P. westerhofensis* DSM 19036^T as well as the genus type strain, *P. heparinus* DSM 2366^T.

The results of disk-diffusion susceptibility testing and MIC determination showed that all the strains are resistant to amoxicillin/clavulanic acid, ampicillin, apramycin sulfate, aztreonam, cefepime, cefoxitin, ceftazidime, cefuroxime, penicillin G, piperacillin, piperacillin/tazobactam, amikacin, gentamicin, netilmicin, streptomycin, tobramycin, ciprofloxacin and colistin. Regarding the carbapenems all the strains are resistant to ertapenem and the majority were sensitive to meropenem and imipenem. The exceptions included resistance to meropenem by *P. westerhofensis* and decreased susceptibility to imipenem by *P. himalayensis.*

The tolerance to metal(loid)s was tested through the determination of the MIC. *P. lusitanus* exhibited higher MICs than all the other strains, for copper and cadmium. *P. lusitanus*, *P. himalayensis* and *P. hartonius* were the most tolerant to zinc and nickel.

Two genes encoding putative β-lactamases were identified in the genome of *P. lusitanus* NL19. The proteins encoded by these genes are novel members of class A β-lactamases (LUS-1) and subclass B3 metallo-β-lactamases (PLN-1), considering their low scores with other β-lactamases. To futher understand if these two enzymes had the ability to hydrolyse β-lactams, the genes *bla*$_{\text{LUS-1}}$ and *bla*$_{\text{PLN-1}}$ were expressed in *E. coli* (fused to an *E. coli* signal peptide) and MIC to β-lactams were determined. Results indicate that PLN-1 is able to degrade carbapenems and 1st, 3rd and 4th generation cephalosporins and cephamycines. However, no significant activity was detected for LUS-1.

Our study shows that the multiresistance phenotype identified in *P. lusitanus* NL19 is also observed in its closely-related species. In addition, it seems clear that the *Pedobacter* genus have an underexplored pool of antibiotic resistance genes as well as putative novel antibiotic resistance mechanisms. The tolerance to metal(loid)s was more strain-specific and, therefore, it appears to be related to the ecological niches that these strains occupy.

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List of Abbreviations

Al	Aluminium
ARGs	Antibiotic resistant genes
As	Arsenic
Cd	Cadmium
CLSI	Clinical and Laboratory Standards Institute
Co	Cobalt
Cu	Copper
DDH	DNA-DNA hybridization studies
DDT	Disk Diffusion Test
dH_2O	Distilled water
DNA	Deoxyribonucleic acid
ECOFF	Epidemiological cut-off
EUCAST	European Committee on Antimicrobial Susceptibility Testing
HGT	Horizontal gene transfer
IPTG	Isopropyl-β-D-thio-galactoside
LPS	Lipopolysaccharide
LPSN	List of Prokaryotic names with Standing in Nomenclature
MBLs	Metallo-β-lactamases
MCS	Multiple Cloning Sites
MDR	Multi-drug resistant
MIC	Minimum Inhibitory Concentration
Mn	Manganese
Ni	Nickel
PBP	Penicillin binding proteins
PDR	Pandrug-resistant
PFGE	Pulsed–field gel electrophoresis
QC	Quality Control
RAST	Rapid Annotations using Subsystems Technology
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SXT	Sulfamethoxazole/ trimethoprim
U	Uranium
USEPA	United States Environmental Protection Agency
XDR	Extensively drug-resistant
Zn	Zinc

Chapter I. Introduction

1.1 *Pedobacter* spp.

The *Sphingobacteriaceae* family (domain Bacteria, phylum Bacteroidetes, class Sphingobacteria, order Sphingobacteriales [1]) was also described by Steyn *et al.*, in 1998 [2] and comprises nine different genera (*Arcticibacter, Mucilaginibacter*, *Nubsella*, *Olivibacter*, *Parapedobacter*, *Pedobacter*, *Pseudosphingobacterium*, *Solitalea* and *Sphingobacterium*) [3], but it initially included only two of these genera (*Sphingobacterium* and *Pedobacter*) [4]. In last years, in this family has been possible to identify new species and reclassify others by the application of molecular tools became [4]. Several species of *Pedobacter* have been also described [5–8]. Currently, *Pedobacter* genus has 67 species available in the List of Prokaryotic names with Standing in Nomenclature (LPSN) (http://www.bacterio.net/pedobacter.html - accessed on 27.11.2017).

The genus *Pedobacter* was described by Steyn *et al.* in 1998 when two species were reclassified, *Pedobacter heparinus* (genus type strain) and *Pedobacter piscium,* and when two new species were described, *Pedobacter africanus* and *Pedobacter saltans* [2]. *Pedobacter* species have been isolated predominantly from soils [9]. However, they have been also isolated from water [10], fish [11], glaciers, plant rhizosphere, compost [5,11], activated sludge [2], sludge of a deactivated uranium mine [8], chilled food [6] drinking water and other extreme environmentals [12,13]. Therefore, they are ubiquitous in nature [5], from aquatic to terrestrial environments [11]. *Pedobacter* spp. strains are Gram-negative, rod-shaped, aerophilic or microaerophilic [14], and non-motile or motile by gliding [15]. They are catalase-positive, produce phosphatidylethanolamine [3,5] and sphingolipid producers [11] and have a DNA G+C content between 36 and 47.5 mol% [3,5]. All *Pedobacter* strains can produce heparinase except *Pedobacter piscium* [4]. *Pedobacter* is inserted in the Biohazard group 1, which include organisms that probably do not cause human diseases. Regarding antibiotic resistance, the type strain is resistant to gentamicin and kanamycin and susceptible to chloramphenicol, erythromycin and tetracycline [16].

1.1.1 *Pedobacter lusitanus* NL9

Strain NL19 was isolated from a sludge sample collected at the deactivated uranium mine Quinta do Bispo, situated in Viseu district (Portugal) [8,17]. 16S rRNA gene sequencing identified this microorganism as belonging to the genus *Pedobacter* [17], having more than 98% similarity with the 16S rRNA gene of *P. himalayensis* MTCC 6384^T, *P. cryoconitis* DSM 14825^T, *P. westerhofensis* DSM 19036^T and *P. hartonius* DSM 19033^T. However, DNA-DNA hybridization studies (DDH) together with *gyrB* (Figure 1) and ITS sequences classified NL19 strain as a new *Pedobacter* species: *Pedobacter lusitanus* NL19^T [8]. These closely-related species were isolated from different

environments and, therefore, have some differences regarding to their growth conditions (Table 1) [2,8,18–20].

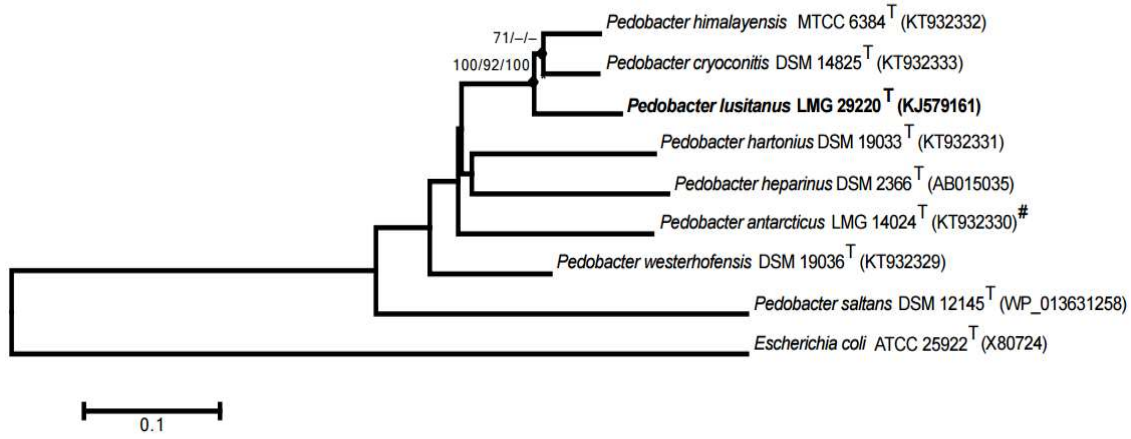

Figure 1 - Phylogenetic tree based on *gryB* showing the phylogenetic position of strain NL19^T and the other strains of the *Pedobacter* genus.

Table 1 - Growth conditions of *P. lusitanus* and its closely-related species and the genus type strain [2,8,18–20]. Strains: 1 - *P. lusitanus* NL19; 2 - *P. hartonius* DSM 19033^T; 3 - *P. himalayensis* MTCC 6384; 4 - *P. heparinus* DSM 2366; 5 - *P. cryoconitis* DSM 14825; 6 - *P. westerhofensis* DSM 19036. () - optimal condition.

	1	2	3	4	5	6
Isolation source	Soil - sludge	Freshwater	Glacial water	Soil	Alpine glacier cryoconite	Freshwater
Temperature (°C)	4-30 (18-30)	6-26 (12-16)	4-25 (22)	5-30	1-25 (20)	9-28 (22-24)
pH	6-8	5.7-7.8	6-10 (7)	-	5-8 (7)	5.3-7.8
Medium culture	NA, TSA, TSB, MacConkey agar, R2A and minimal medium	R2A, TSA, NA	MacConkey, Ayers' agar, R2A, TSA, LB and minimal medium	TSA modified, NA	MacConkey agar, R2A, NA	R2A, TSA, NA

NL19 strain has antibacterial activity against several Gram-positive and Gram-negative bacteria with relevance in the clinical, food, veterinary and aquaculture settings. It does not have antibacterial activity against *Pseudomonas aeruginosa* PAO1 [8]. Its genome sequence shows that it has potencial to produce many secondary metabolites, including lanthipeptides, nonribosomal peptides, siderophores, among others [17]. The RAST annotation of NL19 genome performed by Santos *et al*. 2015 identified several genes encoding antibiotic resistance to β-lactams and fluoroquinolones as well as

multidrug resistance efflux pumps and genetic determinants related with metal(loid)s resistance (arsenic, zinc, cobalt and cadmium) [17].

1.2 Resistance in environmental bacteria

More than 70% of the total biomass of biosphere consists of microbes inhabiting in all the ecosystems on earth. In each particular ecossistem the microbial community is specific and adapts to the different environmental conditions. The ability to survive in these different environments is possible due to the presence of genes in the bacterium´s genome or/and specific genes can also be acquired from that specific environment [21]. As such, there are microorganisms that can thrive in extreme environments, which are intolerably or even lethal to other organisms. Some of these microrganisms adapted to these extreme environments can grow in the presence of toxic waste, organic solvents, metals, or in several other habitats that are considered inhospitable [22]. Toxic compounds in the environment, such as antibiotics and metals, affect bacteria and select for an increase in the number of antibiotic- and metal-resistant bacteria [23].

In the last years, the study of antibacterial resistance in environmental bacteria became of interest because scientists realized that the clinical setting was not the only source for the dissemination of resistance traits [24]. Several studies have demonstrated that some antibiotic resistance genes (ARGs) of high significance could have evolved from environmental bacteria [25]. Therefore, the environment is possibly the largest and oldest reservoir of potential antimicrobial resistance, constituting the environment "resistome" [26]. It is believed that soil is one of the most important reservoirs of antibiotic resistance. Most antibiotics used in human medicine were isolated from soil microorganisms. Thus, the presence of antibiotics in the soil should have promoted the development of highly specific antibiotic resistance mechanisms in antibiotic producing and non-producing bacteria [25,27]. So, study in this reservoir, specially through the understanding of antibiotic resistance evolution by phylogenetic analysis can help predict resistance development to clinically relevant antibiotics [27–29]. This information contributed to accept the fact that resistance to antibiotics does not arise solely from the misuse or overuse of antibiotics [24]. In addition, it also contributed to clarify that the existence of genes conferring resistance to various classes of antibiotics in nature is far before the antibiotic era [29].

Due to the volcanic activity and other natural geological events, for billions of years bacteria have been exposed to metals, both toxic and essential. This contact was probable driver for an evolutionary process that allowed the microorganisms to be able to control the cellular levels of these bioavailable metallic ions. Besides, anthropogenic

activities such as mining, manufacturing, fossil fuel burning and other industrial applications have also contributed for the exposure of bacteria to lethal concentrations of these metals [30]. The increasing dissemination of metallic compounds in the environment promoted the interest to understand their toxicity to bacteria [31], recently assessed in bacteria isolated from different terrestrial habitats [32]. Results show a vast range of metal concentrations are tolerated by soil bacterial communities that also have a role in the speciation and transport of metals in the environment [32,33]. For this reason, the level of tolerance to metals by soil bacteria has been proposed as an indicator of the potential toxicity of metals to other organisms [23].

Also, it has been suggested by researchers that contaminants other than antibiotics, such as metals, can also contribute to the spread of ARGs [34]. It has been shown that microbial communities more exposed to diverse toxic compounds such as hazardous domestic, agricultural, biomedical and industrial wastes, containing toxic organic compounds (e.g. antibiotics) and inorganic chemicals (e.g. metal ions), have an over-abundance of genes conferring defense against various types of environmental stresses. Some of the genes required for the catabolic activities involved in the degradation or accumulation of the toxic compounds are arranged on plasmids. Therefore, the continuous exposure to these compounds puts the microorganisms under selective pressure, triggering point mutations that can result into new resistance phenotypes that can be transferred to the next generation or to other microorganisms in the community by horizontal gene transfer (HGT). Transfers of this type of genes are, therefore, the main driving force in microbial evolution that is influenced by environmental conditions [21].

1.3 Antibiotics

Antibiotics are complex molecules that can be natural, synthetic and semi-synthetic [35] and can be defined as pharmacological agents able to selectively kill (bactericidal) or inhibit (bacteriostatic) the growth of bacterial cells, with little or no effect in the host [36]. The ancient Chinese, Greeks and Egyptians used the antimicrobial properties of plants, herbs and molds to treat infections [36], as in traditional/alternative medicine. Thus, the use of antibiotics is an ancient practice [29]. The properties of these natural products were studied in laboratory and compounds with antibacterial activity were discovered, serving as the basis for semi-synthetic antibiotics [37]. In 1897, Ernest Duchesme was the first to discover and describe the antibacterial properties in a strain of *Penicillium* genus. Alexander Fleming, in 1928, observed the antibacterial properties of *Penicillium notatum* [29,36,38]. Fleming, believed that his discovery could make a difference, and worked for 12

years trying to get chemists interested in solving problems with the purification and stability of the active substance. In 1940, when he abandoned the idea, an Oxford team led by Howard Florey and Ernest Chain published a paper with the purification process of penicillin [29]. The first commercially successful broad-spectrum antibiotics were the synthetic sulphonamides, back in 1935 [36]. Nevertheless, Paul Ehrlich proposed the idea of a “magic bullet” that chemical compounds could be synthesized to exert its action in the microrganisms without affecting their host many years before. This idea led him to discover Salvarsan in 1909, a compound active against *Treponema pallidum* that causes syphilis [29].

The best period of discovery of new antibiotics occurred between the 1950s and 1970s [29]. After this period, several new molecules were introduced in human and veterinary medicines, in agriculture and aquaculture. However, the development of antibiotic resistance led to the reduction of therapeutic activity of these compounds [37]. Therefore, there is an urgent need to develop new antibiotics to control drug-resistant pathogens [39].

The most common way to classify these compounds is based on their mode of action, grouping them in differents classes (Table 3) [37,40].

1.3.1 Antibiotics mode of action

For the antibiotics currently in use, the antibacterial drug-target interactions have been very well studied. It is well known which antibiotics can inhibit essential cellular functions such as, cell wall biosynthesis, transcription, translation, folate biosynthesis or DNA supercoiling (Table 2) [41]. The three of the main antibacterial drug-target interations are: 1) inhibition of cell-wall synthesis; 2) inhibition of protein synthesis; and 3) inhibition of nucleic acids replication and repair [42,43].

Inhibition of cell wall synthesis

Peptidoglycan is a constituint of the bacterial cell wall which is essential for bacteria cell morphology and fundamental to the mechanical resistance that control osmotic pressure [44]. Peptidoglycan is composed by the polysaccharides with alternating N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc). The MurNAc groups have the L-Ala-γ-D-Glu-L-lysine (or -meso-diaminopimelic acid)-D-Ala-D-Ala amino acids attached [45]. This covalently crosslinked polymer matrix is preserved by the activity of transglycosylases and penicillin binding proteins (PBPs, also known as transpeptidases) [44].

β-lactams and glycopeptides are two of several classes of antibiotics that affect the synthesis of peptidoglycan. β-lactams have a cyclic amide ring that interact with PBPs, acting as analogous substrates. This blocks the cross-linking of peptidoglycan units through the inhibition of the peptide bond formation reaction catalysed by PBPs [44]. Glycopeptides, like vancomycin, interact with peptidoglycan building blocks, at the D-alanyl-D-alanine dipeptide, blocking transglycosylase and PBP activity [43]. Although β-lactams can be used to treat infections caused by Gram-positive and Gram-negative bacteria, glycopeptides are only effective against Gram-positive bacteria, due to their low permeability through the outer membranes of Gram-negative bacteria. The contact with these cell wall synthesis inhibitors can result in changes to cell shape and size, induction of cell stress responses and, ultimately, cell lysis. As for lipopeptides, they also affect the structural integrity of peptidoglycan. These compounds are inserted into the cell membrane and induce membrane depolarization [44].

Table 2 - Antibiotic classes based on their mode of action, and respective targets (Adapted from [35,37,40,44]).

Antibiotic Classes	Mode of Action	Target	Example(s)
Aminocyclitols	Inhibition of the protein synthesis	Translation - 30S ribosome	Amikacin, apramycin, streptomycin, kanamycin
Amphenicols	Inhibition of the protein synthesis	Translation - 50S ribosome	Chloramphenicol
β-Lactams	Inhibition of cell wall synthesis (peptidoglycan biosynthesis)	Penicillin-binding proteins	Amoxicillin, meropenem, cephalosporins, aztreonam
Lipopeptides	Inhibition of cell wall synthesis	Cell membrane	Colistin, Daptomycin
Glycopeptides	Inhibition of cell wall synthesis	Peptodoglycan units (terminal D-Ala-D-Ala dipeptide)	Vancomycin
Lincosamides	Inhibition of the protein synthesis	Translation - 50S ribosome	Lincomycin
Macrolides	Inhibiion of the protein synthesis	Translation - 50S ribosome	Erythromycin
Oxazolidinones	Inhibiion of the protein synthesis	Translation - 50S ribosome	Linezolid
Pyrimidines	Inhibition of purine and pyrimidine synthesis	C_1 metabolism	Trimethoprim
Quinolones	Inhibition of DNA replication	Topoisomerase II (DNA gyrase), topoisomerase IV	Ciprofloxacin
Rifamycins	Inhibition of RNA synthesis	Transcription - DNA-dependet RNA polymerase	Rifampicin
Streptograins	Inhibition of the protein synthesis	Translation - 50S ribosome	Synercid
Sulfonamides	Inhibition of the folic acid synthesis	C_1 metabolism	Sulfamethoxazole
Tetracyclines	Inhibition of the protein synthesis	Translation - 30S ribosome	Tetracycline

Inhibition of the protein synthesis

The bacterial ribosome is constituted of two ribonucleoprotein subunits: the 50S and 30S. Thus, antibiotics that inhibit protein synthesis can be divided into two subclasses: the 50S inhibitors and 30S inhibitors [44].

Macrolides, lincosamides, streptogramins, amphenicols and oxazolidinones target the 50S subunit and block the initiation of mRNA translation or the translocation of peptidyl-tRNAs. Tetracyclines and aminocyclitols (including spectinomycin and aminoglycosides) interact with the 30S subunit. Tetracyclines inibith the access of aminoacyl-tRNAs to the ribosome and aminocyclitols bind to the 16S rRNA component of the 30S ribosome subunit. Spectinomycin alters the stability of peptidyl-tRNA, inhibiting elongation factor-catalysed translocation and aminoglycosides can change the conformation of the complex formed between an mRNA codon and respective charged aminoacyl-tRNA at the ribosome. This can produce mistranslated proteins [44].

Inhibition of nucleic acids synthesis

Clinically relevant antimicrobials, such as fluoroquinolones, a class of synthetic quinolones, use the DNA-topoisomerase complex as targets. The targets of these nalidixic acid derivates are topoisomerase II (gyrase) and topoisomerase IV, which are essential for DNA replication [44]. The gyrase, induces negative supercoils into the DNA. After replication, topoisomerase IV is able to decatenate daughter chromosomes [46]. The formation of the quinolone-topoisomerase-DNA complex blocks the replication forks, trapping the DNA replication machinery and leading to the inhibition of DNA synthesis. In addition, these enzymes are also essential for mRNA transcription and cell division [44].

The synthesis of mRNA can be disturbed by the semi-synthetic rifamycin that binds to RNA polymerase when a RNA polymerase-DNA complex is formed. Thus, this antibiotic inhibits RNA strand initialization [44].

New bacterial targets were screened when bacterial genomes began to be sequenced [46]. This were important to understand how antibiotics can lead to the death or to inhibition of growth of the bacterial cells, focusing on essential bacterial cell function [44]. Thus, the selection of new targets encoded by single genes, which are homologous in different bacterial species, can be used to develop new antibiotics with the desired spectrum, without affecting human cells [46]. However, the most successful approaches cannot involve the target of one sigle enzyme. This is because some studies have suggested that development of bacterial resistance can be delayed when the antibiotic has more than one target [46].

1.4 Antibiotic resistance

Bacteria can be intrinsically resistant to specific antibiotics or they can acquire resistance through other means [36,47]. Intrinsic resistance is the ability of bacterial species to escape the action of specific antibiotics as a result of innate structural or functional characteristics [27,47]. For example, Gram-negative bacteria are intrinsically resistant to vancomycin because the compound is unable to penetrate the outer membrane [47]. In addition to the intrinsic resistance, bacteria can become multidrug-resistant or totally resistant to antibiotics, through the acquisition of exogenous DNA by HTG, or they can become resistant by mutations occurring in chromosomal genes [21,47]. Antimicrobial resistant bacteria can be classified as multidrug-resistant (MDR), extensively drug-resistant (XDR) or pandrug-resistant (PDR) according to patterns of resistance identified. A MDR bacteria is "resistant to three or more antimicrobial classes"; bacteria classified as XDR are epidemiologically significant in terms of their resistance to multiple antimicrobial agents and their likelihood of being resistant to all antimicrobial agents; PDR bateria are "resistant to all antimicrobial agents" [48]. On the other hand, ARGs are also associated with antibiotic biosynthetic gene clusters in antibiotic-producing microorganisms as a self-protection mechanisms [49].

The emergence of pathogenic bacteria resistant to antibiotics has today become a major global health problem [26]. The review published by O'Neill in 2014 estimated that antimicrobial resistance could cause 10 million deaths a year by 2050 [50]. Comparing the number of deaths caused by to other major causes of death, such as cancer, antimicrobial resistance will be the first cause of death [51].

Antibiotic misuse and overuse, among other factors mentioned above, influence the selection and dissemination of resistance [49]. In fact, environmental contamination with antibiotics exerths selective pressure to the microorganisms, contributing to the emergence, persistence and dissemination of ARGs. In addition, recent studies showed that high levels of ARGs can occurs in environmental in the absence of antibiotics [34], indicating that ARGs can persist even in the absence of selective antibiotic pressure [35]. However, other factors, such as the presence of metals, may promote the dissemination of ARGs [34].

The determination of the minimum inhibitory concentrations (MICs) is considered the "gold standard" to test antimicrobial susceptibility of bacteria. MIC is defined as the lowest concentration of an antibiotic capable of inhibiting the visible growth of an organism after incubation. This method is used to assess the performance of the other methods of susceptibility testing [52]. The disk diffusion assay is another approach that is also widely used. This versatile method allows testing almost all antimicrobial agents

without requiring special equipment and, at the same time, is reproducible and accurate [53]. The inhibitory effect was reported by Fleming when he observed an area of growth inhibition on the staphylococcal agar plate caused by *Penicillium* contamination. Fleming also contributed to the development of minimum inhibitory concentration methodology with the development of a broth dilution technique which used turbidity as an end-point determination. Diffusion methods were developed in the 1940s, however, many variables that influenced the results of this methodology. The first standardization of the disc method was proposed by Bauer, Kirby and co-workers. This method became the basis of the National Committee for Clinical Laboratory Standards (NCCLS) disc diffusion standards and was followed by many societies in several countries [54] developing their own disk diffusion methods. Since there was no common method calibrated to European breakpoints, the need to create a standardized disk diffusion method calibrated with European Committee on Antimicrobial Susceptibility Testing (EUCAST) clinical MIC breakpoints emerged. Thus, through the analysis of distributions, EUCAST determined a large series of MIC and disk diffusion tests for several microorganisms and antibiotics to establish diameter zone breakpoints correlated to the EUCAST clinical MIC breakpoints. The same process was performed using wild-type isolates to distinguish them from the resistant isolates, establishing epidemiological cut-offs (ECOFFs) [53]. At the clinical setting, the results obtained allow the classification of clinical bacteria as susceptible, intermediate or resistant to the tested antibiotic based on the breakpoints determined by EUCAST and the Clinical and Laboratory Standards Institute (CLSI) [55]. EUCAST and CLSI have quality control tables available for MIC and zone diameter values [56,57] that are used to monitor assay performance [53]. Regarding non-clinical bacteria, the methodologies required to study them and standardize the definition of antibiotic resistance is needed. The most appropriate approach would be to establish ECOFFs. However, to achieve this, it would be necessary to collect and test the MICs of several different strains of a certain species that were collected from different environments, in order to have a representative sample [27].

1.4.1 Antibiotic resistance mechanisms

Bacteria can overcome the action of antibiotics through four main mechanisms: 1) reduction of membrane permeability to antibiotics, 2) efflux of the antibiotic, 3) antibiotic inactivation and 4) target modification (Figure 2 and Table 3). This antibiotic resistance can be encoded by acquired or intrinsic genes which are present in the chromosome or in mobile genetic elements [58].

Reduction of membrane permeability

Antibiotics that have periplasmic or intracellular bacterial targets need to penetrate the outer (Gram-negative) or cytoplasmic/inner membrane. Bacteria have developed mechanisms to prevent this contact with the target by decreasing the uptake of the antimicrobial molecule. Gram-negative bacteria are intrinsically less permeable than Gram-positive bacteria, once Gram-negative outer membrane forms a selective permeability barrier. In fact, the outer membrane works as the first-line defense to many toxic compounds. The reduction of permeability of the outer membrane and, consequently, the reduction of antibiotic entry into the bacterial cell occurs by the down-regulation of porins or by the replacement of porins with more-selective channels. Hydrophilic molecules such as β-lactams, tetracyclines and some fluoroquinolones use these porins to cross this barrier. So, they are affected by alterations in the permeability of the outer membrane [47,59].

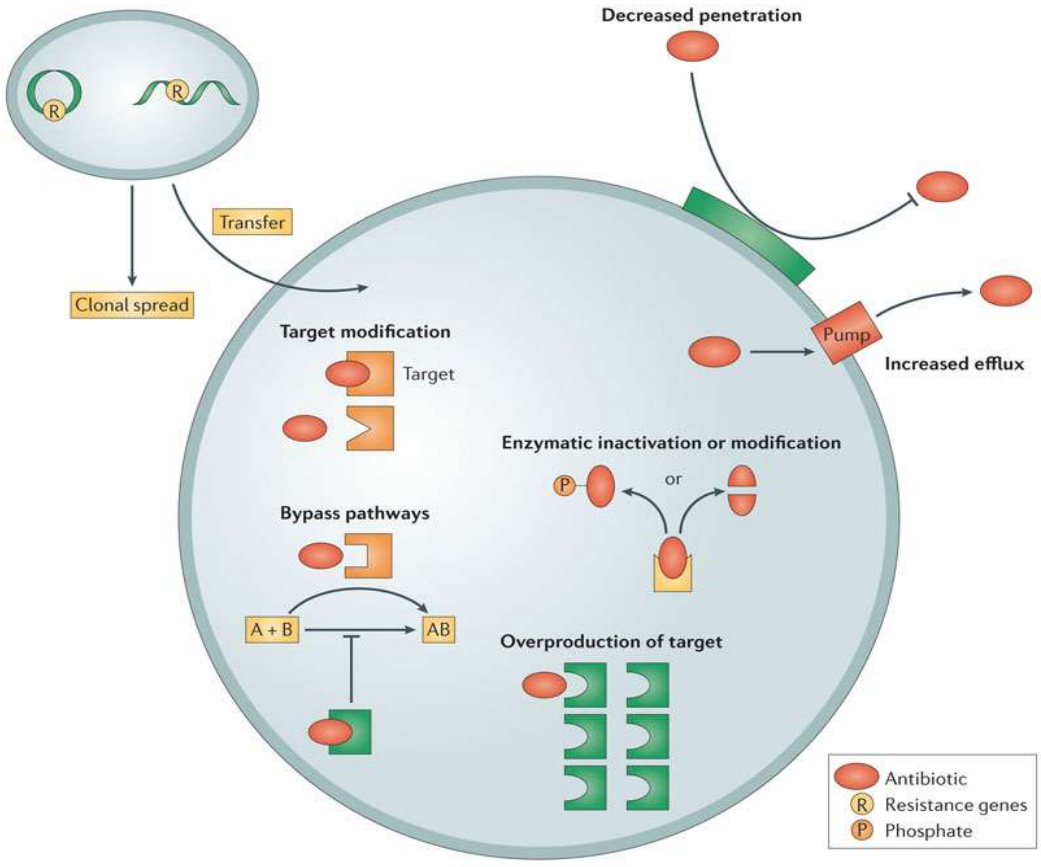

Figure 2 - Schematic representation of microbial mechanisms of resistance to antibiotics [128].

Table 3 - Antibiotic resistance mechanisms in microorganisms (Adapted from [40]).

Class of Antibiotic	Resistance Mechanisms
Aminocyclitols	Phosphorylation, acetylation, nucleotidylation, efflux, altered target
Amphenicols	Acetylation, efflux, altered target
β-Lactams	Hydrolysis, efflux, altered target
Lipopeptides	Altered target, efflux
Glycopeptides	Reprogramming peptidoglycan biosynthesis
Lincosamides	Nucleotidylation, efflux, altered target
Macrolides	Hydrolysis, glycosylation, phosphorylation, efflux, altered target
Oxazolidinones	Efflux, altered target
Pyrimidines	Efflux, altered target
Quinolones	Acetylation, efflux, altered target
Rifamycins	ADP-ribosylation, efflux, altered target
Streptograins	C-O lyase (type B streptogramins), acetylation (type A streptogramins), efflux, altered target
Sulfonamides	Efflux, altered target
Tetracyclines	Monooxygenation, efflux, altered target

Increase of antibiotic efflux

Efflux pumps are naturally present in all bacteria [42] to actively transport many antibiotics out of the cell [47]. In antibiotic producer bacteria, they are a protective mechanism that allows pumping the produced antibiotic out of the cells which prevents them for being killed by their own compounds [42]. These multidrug resistance (MDR) efflux pumps can be extended to a wide range of antimicrobial classes, resulting in antimicrobial resistance [47,59].

The genes encoding efflux pumps can be carried by plasmids or in other mobile genetic elements present in plasmids or in the chromosome. Chromosomal genes encoding these pumps can justify the inherent resistance of some bacterial species to a specific antibiotic and some of these genes can be mobilized for plasmids, favoring the transference between bacteria [47,59].

Antibiotic inactivation

Antibiotics can be degradated or modified by enzymes that inactivate them. This can occur by hydrolysis or transfer of a chemical group and these mechanisms have been highly relevant since the first use of antibiotics. The chemical alteration of the molecule will prevent the antibiotic from binding to its target. The most frequent reactions involving the transfer of a chemical group include i) acetylation (aminoglycosides, chloramphenicol and streptogramins), ii) phosphorylation (aminoglycosides and chloramphenicol), and iii) adenylation (aminoglycosides, lincosamides). Aminoglycosides are more susceptible to these modifications since they have many exposed hydroxyl and amide groups. There are many enzymes able to degrade different classes of antibiotics, including β-lactams, aminoglycosides, phenicols and macrolides. Among the most important are the β-lactamase enzymes that hydrolyse the amide bond of the β-lactam ring, making β-lactams ineffective [47,59]. These enzymes are classified based on their molecular characteristics and functional properties (Table 4). Molecular classification is based on the amino acid sequence and conserved motifs. Accordingly, β-lactamases are divided into four classes: A, C, and D classes that include enzymes which utilize serine for β-lactam hydrolysis and class B metalloenzymes which require zinc ions to hydrolyze substrates. Functional groups 1, 2, and 3 is based on the proposal of Bush *et al*., that consider preferential substrate and inhibitor profiles, correlating phenotype of isolates. Major groupings are correlate with the molecular classification. The group 1 cephalosporinases are correlated with class C; group 2 serine β-lactamases are associated with classes A and D; and group 3 are metallo-β-lactamases. Within each group there are several subgroups, such as the extended-spectrum-β-lactamases, ESBLs (group 2 serine β-lactamases) [60,61].

Target modification

Bacteria can resist the action of antibiotics by protecting or modifying their cellular targets, resulting in decreased affinity for the antibiotic molecule. This is a common mechanism of antibiotic resistance that affect almost all classes of antimicrobial compounds and can consist of i) point mutations in the genes encoding the target site, ii) enzymatic alterations of the binding site, and/or iii) replacement or bypass of the original target [59]. There are some examples of proteins that mediate target protection, such as Qnr, that belongs to the pentapeptide repeat protein family, confering quinolone resistace. These protein act as DNA homologue, competing for the DNA binding site of the DNA gyrase and topoisomerase IV [59], protecting them from the lethal action of quinolones [47].

Table 4 - Classification schemes for bacterial β-lactamases (Adapted from [60]).

Funcional Group	Molecular Class	Substrate Preference
1	C	Cephalosporins
2a	A	Penicillins
2b	A	Penicillins, early cephalosporins
2be	A	Extended-spectrum cephalosporins and monobactams
2br	A	Penicillins
2ber	A	Extended-spectrum cephalosporins, monobactams
2c	A	Carbenicillin
2ce	A	Carbenicillin, cefepime
2d	D	Cloxacillin
2de	D	Extended-spectrum cephalosporins
2df	D	Carbapenems
2e	A	Extended-spectrum cephalosporins
2f	A	Carbapenems
3a	B1 B2 B3	Carbapenems

1.5 Metal(loid)s

Metals can be defined accoording to the physical properties of the elemental state as “elements with metallic luster, the capacity to lose electrons to form positive ions and the ability to conduct heat and electricity”. They can be also defined in terms of density, atomic weight, atomic number and other chemical properties. Metalloids are defined as elements that have the “physical appearance and properties of a metal but chemically behaves like a non-metal”. However, there is no consensus on the classification of these elements, and there is no authoritative definition in the literature [62]. Since heaviness and toxicity are inter-related, metalloids are included in the metals group, once both are capable of inducing toxicity at a low level of exposure [63].

Metals are naturally present in the environment, being found in minerals or organometallic compounds [64]. Physical factors, such as temperature and adsorption, and chemical factors, such as thermodynamic equilibrium and lipid solubility, have influence on their bioavailability [63]. Metals, such as cobalt (Co), copper (Cu), chromium (Cr), iron (Fe), magnesium (Mg), manganese (Mn), nickel (Ni), potassium (K), sodium (Na) and zinc (Zn) are essential nutrients for life processes of microorganisms. These metals are used as micronutrients [31,63–65] and play important roles in biochemical catalysis, specially acting as protein and nucleic acid stabilizers, and also as enzyme cofactors. They influence several biological processes such as the osmotic balance and the regulation

of membrane channels activity, among others [66]. However, at high concentrations, these metals can be toxic. Other metals, such as aluminium (Al), cadmium (Cd), mercury (Hg) and arsenic (As), are not biologically relevant and are toxic to microorganisms even at very low concentrations [31,63–65].

In order to group metals based on their ligand affinity or toxicity, several classifications schemes were created. The two classifications that are the most widely accepted are: i) the Irving–Williams series that describe the divalent metal ion ligand affinities with biological molecules and ii) the classification of metals into Lewis acids that measure the toxicity of metal ions considering their strength as Lewis acids [30]. The wide range of chemical properties of metal allows these elements to react differently with living cells. Depending on their chemical properties, the biocide behavior can be triggered mainly by i) donor atom selectivity, ii) reduction potential (redox reactions) and iii) speciation [31,67].

Metal(loid)s have been used by humans in medicine, as chemotherapeutics, anti-depressants and antimicrobials drugs and in agriculture as pesticides [30] They are also important to other industries [31] to produce clothes, cosmetics, computer keyboards and other materials used daily [30]. This have been contributing to an increasing exposure of environment and humans to metal(loid)s [63] because they are not easily degraded to harmless products and, thus, can persist in the environment indefinitely [68]. Therefore, metal(loid)s contamination is creating an ecological and global public health problem [63].

1.5.1 Mechanisms of metal toxicity

When metal(loid)s are present in toxic doses they can disrupt processes needed for cell growth [31] i) by binding to or blocking functional groups in biological molecules, ii) by displacing essential metals in enzymes, iii) by binding to the cellular thiol pool or iv) by participating in chemical reactions harmful to the cell [30]. Thus, the mechanisms of metal(loid)s toxicity described in microorganisms (Table 5) include, i) production of reactive oxygen species (ROS) and depletion of antioxidants, ii) protein dysfunction, such as loss of enzyme activity, iii) damage of membrane function, iv) interfere with nutrient assimilation and v) mutagenic effects. Each of these mechanisms has been associated with specific metal(loid) species. In some circumstances, the growth inhibition and cellular death results from an association of different mechanisms [31].

Table 5 - Mechanisms of metal(loid)s toxicity in microorganisms (Adapted from [31]).

Mechanisms of Metal Toxicity	Metal(loid)s
Production of ROS and antioxidant depletion	Cr (VI), As (III), Te (IV), Fe (II), Cu (II)
Protein dysfunction and loss of enzyme activity	Cr (VI), Ag (I), Hg (II), Cd (II), Zn (II), Pb (II), Cu (I), Ni (II)
Impaired membrane function	Ag (I), Al, Cu (II, Cd (II)
Inhibition of enzyme activity	Hg, Pb, Cd, Ni, Ag, Cu and Cd
Interference with nutrient assimilation	Cr (VI), Ga (III)
Genotoxicity	Mn (II), Cr (VI), Co (II), Cd (II), Mo (IV), Sb (III), As (III)

1.5.2 Uranium mine - Quinta do Bispo and Cunha Baixa

Uranium is naturally present in Earth's crust [65,69] and it is used for production of nuclear energy, weapons [70] and phosphate fertilizers [65]. This radioactive element can be extracted conventionally from open pits and underground mines or it can be obtained by treating mined ore in hydrometallurgical plants near the mine site or by *in situ leach* operation [71]. Uranium ores have changeful high metal(loid)s concentrations such as As, Cu, Mo, Ni, Pb, Ra, Re, Sc, Se, Th, V, Y, Zr [71], Pb, Cd, Zn and Mn [72]. Therefore, its processing produced and still produces large quantities of toxic waste commonly known as uranium tailings or uranium mill tailings [71]. This is considered a global problem [65,69,70], because these toxic compounds are contaminating the soil and groundwater [70] causing environmental and health problems [72]. The uranium tailings can be solid or liquid and have different chemical, mineralogical and radiochemical properties. Solid tailings incorporate radionuclides, metals and metalloids that can be released into pore waters. This mobilization can be induced through several factors, including bacterial reduction [71] by bacteria populations that live in repositories as planktonic cells and biofilms [69]. To grow and survive, these bacteria are adapted to these environments, using different mechanisms to uptake essential metals and drive their metabolism and, at same time, protect themselves against toxic elements [69]. Bacteria isolated from uranium contaminated sites play an important role in regulating uranium mobility and toxicity [73]. They can modify the redox state of uranium, thereby altering its solubility, a processes that has been described in several species [33].

In Portugal, during the first half of the last century, the exploration of radioactive ore had a profound importance at the economic level. Today, all of these mining explorations closed down their activity and only some of them are being monitored to reduce environmental impacts [74]. The Quinta do Bispo and Cunha Baixa were some of the most

important uranium exploitations in Portugal. These deactivated uranium mines are 2 km away from each other [75], and therefore share the same geological environment and are situated in the same drainage basin. The exploration activity of these mines produced large amounts of waste and therefore high levels of metals and radionuclides can be found on site [76]. For these reasons, since 2000, Quinta do Bispo is included in remediation projects and environmental characterization studies [77]. The open-pit exploration, which were abandoned in 1992, led to the accumulation of acidic water effluents in the open pit. These waters have been treated in treatment ponds using standard procedures generally applied to uranium mines effluents. This involves the use of calcium hydroxide and barium chloride to precipitate radionuclides, which allows the removal of a large fraction of the radioactivity from water before its discharge in a nearby stream [78]. *P. lusitanus* NL19, the bacterium that is object of the present study, was isolated from the sludge deposited in the ponds that resulted from this type of chemical treatment [8]. Studies carried out in Quinta do Bispo evaluated its contamination by radionuclides [76] and the amount of metals in sediment elutriates [78]. However, regarding Cunha Baixa mine, a study performed assessed the pseudo-total metal soil concentrations [72]. One of the sampling sites of the study conducted in Cunha Baixa, sampling site B, has a pH value similar to that of the sediment elutriates of Quinta do Bispo [72,78]. In these mines several metals such as, aluminium (Al), manganese (Mn), uranium (U), zinc (Zn), nickel (Ni), cadmium (Cd), copper (Cu) and cobalt (Co) were extracted in different concentrations (Table 6) [72]. Quality guideline values determinated by different governmental entities, such as, USEPA (United States Environmental Protection Agency) and Danish Government were used in Cunha Baixa uranium mine study to access whether sites are considered or not as risks to ecological systems (Table 6) [72].

Table 6 - Values of metals obtained in determination of pseudo-metal concentrations in B sample site from Cunha Baixa uranium mine and in metals determination of elutriate soft sediment from Quinta do Bispo, in comparison with soil quality guideline values (Adapted from [72,78,(a),(b)])

Metals	Cunha Baixa (mg/kg soil)	Quinta do Bispo (µg/L)	USEPA (mg/kg soil) [(a)]	Denmark (mg/kg soil) [(b)]
Aluminium	40 000	<14,8	71 000	50
Manganese	5 000	5	600	NA
Uranium	400	914	1	NA
Zinc	300	12	50	100
Copper	90	-	30	30
Nickel	90	13	40	10
Cobalt	50	-	8	NA
Cadmium	4	<3,21	0,06	0.3

[(a)] - the average concentration values of metals in natural soils determined by USEPA Office of Solid Waste and Emergency Response, Hazardous Waste Land Treatment, revised at 09/2010 (https://cdn.shopify.com/s/files/1/0979/5626/files/FS25_-_Metal_Concentrations_In_Natural_Soils.pdf);
[(b)] - soil quality criteria for inorganic compounds determined by Ministry of Environment and Energy and Danish Environmental Protection Agency in 1995 [72]. NA – not available.

1.6 Metal(loid)s tolerance

Microorganisms have created alternatives that allow them to survive in environments containing toxic concentrations of metal(loid)s. These ability comes from highly modified genetic systems, through which they synthesize proteins which allow them to thrive in these environments [79]. These proteins can be encoded in the chromosome or in plasmids, and are frequently associated with antibiotic resistance genetic determinants [80]. Their defense mechanisms can be further exploited for applications, such as, the cleaning of contaminated environments [79] through bioremediation approaches [23].

The definition of bacteria as sensitive or resistant to metal(loid)s is ambiguous. Therefore, some authors consider more appropriate to use the term tolerant instead of resistant [81]. The ambiguity is mainly due to the absence of standard procedures to test bacterial metal(loid)s sensitivity and the absence of breakpoints established so far that allow to classify them as resistant or sensitive. This is a difficult task for several reasons, namely: i) there are different forms of metal(loid)s that can be tested, ii) the media components can influence their bioavailability and iii) the pH to which they are soluble can be incompatible with the growth of most bacterial species [82]. Most of the studies performed so far select the medium based on its ability to encourage bacterial growth when incubated at specific time and temperature. However, high levels of metal(loid) tolerance by bacteria have been associated with media containing undefined organic components, such as peptone, tryptone, yeast extract, and beef extract or containing a

high amount (2–10 g/L) of carbon sources (e.g. glucose, gluconate, mannitol, etc) [32]. In these media, the chelation of metal(loid)s to the organic constituents form complexes that can reduce their activity as well as their binding capacity. Consequently, one can overestimate the metal(loid) levels tolerated because the concentration added to the media can not correspond to its activity as a free ion. Despite of these limitations, the minimal inhibitory concentration (MIC) method is considered a valid approach to evaluate the action of metals in microorganisms [32,81]. There are some microorganisms that are usually used as quality controls in studies testing metal(loid) susceptibility including *Thiobacillus ferrooxidans* and *Escherichia coli. T. ferrooxidans* which can tolerate concentrations of Zn, Ni, Cu, Co, Mn, and Al up concentrations of 10 g/L. However, it does not grow in the presence of Ag, As, and other metals at concentrations ranging from 50 to 100 mg/L [82]. MICs for *E. coli* have also been determined under specific conditions for some metals: 0,01 mM for Hg^{2+}, 0,02 mM for Ag^{+}, 0,05 mM for Cd^{2+}, 1 mM to Co^{2+}, Ni^{2+}, Cu^{2+} and Zn^{2+}, 2 mM for UO_2^{2-}, Al^{3+} and 20 mM for Mn^{2+} [83].

The association between the type and levels of metal(loid)s contamination and the patterns of antibiotic resistance suggests that the mechanisms involved in metal(loid)s tolerance can also contribute to the bacterial resistance to some antibiotics (Table 7). In this way, metal(loid)s contamination also exerts a selective pressure towards antibiotic resistant bacteria and, therefore, leads to the co-selection processes. This is worrisome considering that, unlike antibiotics, metals produced in anthropogenic activities are not easily degraded [58].

Table 7 - Well-characterized examples of shared antibiotic- and metal-resistance mechanisms in prokaryots [58].

Resistance mechanism	Metal ions	Antibiotics
Reduction in permeability	As, Cu, Zn, Mn, Co, Ag	Ciprofloxacin, tetracycline, chloramphenicol, β-lactams
Drug and metal alteration	As, Hg	β-lactams, chloramphenicol
Drug and metal efflux	Cu, Co, Zn, Cd, Ni, As	Tetracycline, β-lactams, chloramphenicol
Alteration of cellular target	Hg, Zn, Cu	Ciprofloxacin, β-lactams, trimethoprim, rifampicin
Drug and metal sequestration	Zn, Cd, Cu	Coumermycin A

1.6.1 Metal(loid)s tolerance mechanisms

Bacteria have developed several defense mechanisms against metal(loid)s. Their removal from cell and the decrease of their toxicity are the basis of many resistance mechanisms. Therefore, the main mechanisms involved in bacterial tolerance to metal(loid)s include: 1) active efflux of metal ions, 2) transformation of the metal ion into a less toxic form and 3) metal-complex formation by precipitation, either intracellularly or extracellularly (Figure 3) [66,84].

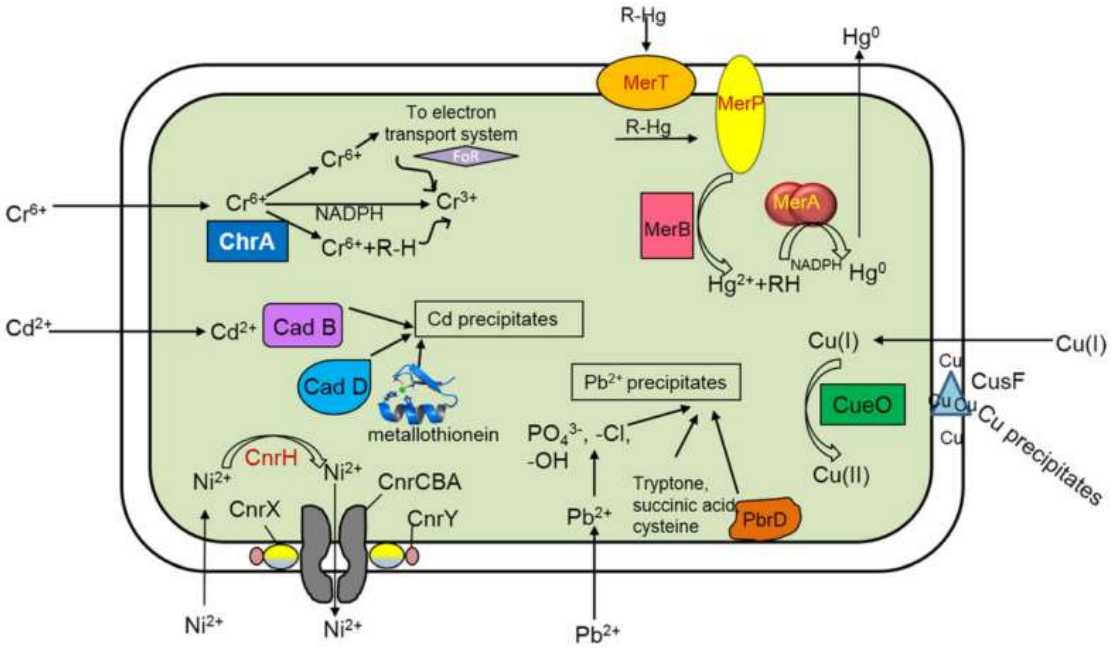

Figure 3 - General scheme of genetic mechanisms of resistance to toxic metals by bacteria [79].

Copper is an essential metal in cellular processes that is toxic at high concentrations. This duality has forced different bacteria to develop several mechanisms in order to keep the appropriate copper concentration inside the cells. For instance, the CueO enzyme is involved in periplasmic copper detoxification by oxidizing Cu(I) to a less toxic Cu(II). Other mechanism involves the transport of excedent monovalent Cu from the cytoplasm by CopA. Other mechanisms involving efflux and periplasmic chaperones that remove the excess of copper from the periplasm by sequestration were also described [85].

Several efflux pumps have been associated with the tolerance of metals such as Ni, Co, Cd and Zn, including the CnrCBA (cobalt-nickel resistance), the NccCBA (nickel-cobalt-cadmium), the NreB (nickel resistance) and the CznABC (cadmium-zinc-nickel resistance) [86]. For Zn, there are mechanisms involving extracellular accumulation, sequestration by metallothioneins and intracellular physical sequestration were also characterized [87]. Additionally to efflux (Cad systems), bacteria can avoid Cd toxicity by

i) accumulation, ii) cell wall impermeabilization, iii) transformation of Cd to less toxic forms or by iv) ion sequestration [88].

The maintenance of manganese ion homeostasis involves many proteins. Examples include i) the Nramp H+-Mn^{2+} transporters, ii) the ATP-binding cassette (ABC) Mn^{2+} permeases [89], iii) the efflux pump MntX, iv) the cation diffusion facilitator (CDF) protein MntE and v) the MnxG enzyme that catalyzes oxidations of Mn(II) to Mn(III) and from Mn(III) to Mn(IV)[90]. Regarding aluminium, some bacteria are able to exude Al as an insoluble precipitate [91].

The strategies developed by microorganisms to avoid the effects of arsenic include the oxidation or methylation and the active efflux of arsenite (As III). The arsenite detoxification machinery is generally encoded by three to five *ars* genes, usually arranged in a single unit of transcription. There are other families of arsenite carriers although they are not well-charactrized [92].

Uranium can be immobilized by bacteria through i) reductive precipitation of U(VI) to U(IV), ii) U uptake followed by accumulation, iii) biosorption and complexation with proteins, polysaccharides and other microbial biomolecules and iv) biomineralization of U(VI) with phosphates or carbonates [93].

1.7 Objectives

Pedobacter lusitanus NL19 was isolated from sludge collected from a deactivated uranium mine, in central Portugal, characterized by high levels of metals. As so, it is expected that NL19 has mechanisms allowing it to survive and thrive in this type of environment. The analyses of the bacterium's genome revealed the presence of various genetic determinants associated with metal(loid)s and antibiotic resistance. These traits can be strain specific but they can also be characteristic of the species or even the genus. Therefore, the main aim of this thesis was to characterize the antibiotic and metal(loid) susceptibility of i) *Pedobacter lusitanus* NL19, ii) its closely-related species (*P. hartonius* DSM 19033^T, *P. himalayensis* MTCC 6384^T, *P. cryoconitis* DSM 14825^T and *P. westerhofensis* DSM 19036^T) and the type strain of *Pedobacter* genus (*P. heparinus* DSM 2366^T). To achieve this goal, the following specific objectives were defined:

1. Determine the susceptibility of these strains to different antibiotics;
2. Determine the susceptibility of these strains to different metal(loid)s;
3. Analyse, *in silico*, the genomes of these strains that are available at databases, and identify genes that can be involved in antibiotic and/or metal(loid) tolerance;
4. Characterize, at the molecular level, the activity of some genes found in *P. lusitanus* NL19 genome and that are involved in antibiotic resistance;
5. Investigate the presence of plasmids in these strains.

Chapter II. Materials and Methods

2.1 Strains

Pedobacter lusitanus NL19^T and its four closely-related species *Pedobacter himalayensis* DSM 16196^T, *Pedobacter hartonius* DSM 19033^T, *Pedobacter cryoconitis* DSM 14825^T and *Pedobacter westerhofensis* DSM 19036^T as well as the genus type strain, *Pedobacter heparinus* DSM 2366^T, were used in this study. The strains were grown at 20ºC, in CMP medium (3,75 g of casein extract, 1,25 g of soy peptone and 1,25 g of sodium chloride per liter). Medium was solidified with agar (15 g per liter).

2.2 Disk diffusion test

Activity of different antibiotics towards *Pedobacter* spp. strains was assessed by the standard method of disk diffusion on a Mueller–Hinton (MH) agar (OXOID). Testing was performed according to the EUCAST guidelines [94] using *E. coli* ATCC 25922 as quality control strain. The following antibacterial disks (OXOID) were applied with antimicrobial susceptibility disk dispenser (OXOID): amikacin (30 µg), amoxicillin/clavulanic acid (30 µg), ampicillin (10 µg), aztreonam (30 µg), cefepime (30 µg), cefoxitin (30 µg), ceftazidime (10 µg), cefuroxime (30 µg), chloramphenicol (30 µg), ciprofloxacin (5 µg), colistin sulfate (10 µg), gentamicin (10 µg), imipenem (10 µg), netilmicin (10 µg), nitrofurantoin (100 µg), penicillin G (10 µg), piperacillin (30 µg), piperacillin/tazobactam (30-6 µg), streptomycin (10 µg), sulfamethoxazole/trimethoprim (25 µg), tetracycline (30 µg), ticarcillin (75 µg), tobramycin (10 µg) and vancomycin (30 µg). The quality control (QC) breakpoint tables used to validate the assay was based on version 6.1 (valid from 2016-03-01) of EUCAST [56]. The QC guidelines of CLSI were used for colistin sulfate, streptomycin and tetracycline [57]. Breakpoint tables of EUCAST or CLSI (for colistin sulfate, streptomycin and tetracycline) for *Enterobacteriaceae* were used to classify *Pedobacter* spp. in three different categories: resistant, intermediate and sensitive, based on diameter of the inhibition zones [57,95]. *Pedobacter* spp. strains were tested in triplicate and plates were incubated at 20°C for 48 hours. *E. coli* ATCC 25922 was also tested in triplicate and plates were incubated at 37°C for 24 hours.

2.3 Determination of minimum inhibitory concentration of antibiotics

The minimal inhibitory concentrations (MICs) of some antibiotics were determined by broth microdilution method according to International Standard ISO 20776-1:2006(E) as recommended by EUCAST [96]. The stock solutions of antibiotics were sterilized by filtration with cellulose acetate membrane filters (0,2 µm). 12 different antibiotics were tested: imipenem, meropenem, ertapenem, cefetazidime, tetracycline, ampicillin,

apramycin sulfate, streptomycin, kanamycin, chloramphenicol, colistin sulfate and ciprofloxacin. All antibiotic solutions were prepared in Mueller-Hinton broth (MHB - Oxoid). Bacterial suspension was prepared as follows: *Pedobacter* spp. strains were grown at 20°C in CMP broth with aeration (180 rpm) until OD_{625nm} > 0,13. The cultures were adjusted to an OD_{625nm} of approximately 0,1 with CMP broth that corresponds to a turbidity equivalent to the 0,5 McFarland standard (OD_{625nm} 0,08-0,13). To obtain an inoculum with a final concentration equal to 5×10^6 CFU/mL, these cultures were diluted 1:100 proportion using CMP broth. 50 µl of this inoculum was added to 50 µl of each antibiotic dilution, reaching a final concentration of 5×10^5 CFU/mL in each well of the microdilution test. In order to use recommended the correct final inoculum size, 5 x 10^5 colony-forming units per millilitre (CFU/mL), were counted viable cells by platting a microbial suspension for all isolates using CMP agar, following the recommendations given by ISO [96].

A positive and a negative control were included in each test and growth was visually checked after 24 hours of incubation at 20°C for all strains tested in triplicate. The classification of the strains as resistant, intermediate and sensitive was performed according to breakpoints established by EUCAST for *Enterobacteriaceae* [95]. For the tetracycline, the ranges given by CLSI were applied and for streptomycin there are no interpretative standards [57]. The reference strain *E. coli* ATCC 25922 was used as a quality control, using quality control guidelines for MIC determination as recommended by EUCAST available in version 6.1 (valid from 2016-03-01) [56], except for the apramycin, streptomycin and tetracycline where quality control guidelines for veterinary applications [97] and quality control guidelines of CLSI were applied [57]. *E. coli* ATCC 25922 was incubated at 37°C for 24 hours.

2.4 Determination of minimum inhibitory concentration of metal(loid)s

The minimal inhibitory concentrations (MICs) of metal(loid)s were also determined by broth microdilution according to ISO 20776-1:2006(E) standards [96]. The medium used was CMP broth in order to avoid complexation or chelation of metals to the medium unspecified organic constituents [32]. 9 different metals and one metalloid were tested: zinc ($ZnSO_4.7H_2O$), cobalt ($CoCl_2.6H_2O$), aluminium ($AlCl_3$), manganese ($MnSO_4.H_2O$), nickel ($NiCl_2.6H_2O$), cadmium ($CdCl_2$), uranium ($UO_2(NO_3)_2.6H_2O$), copper ($CuSO_4.5H_2O$) and arsenic ($Na_2HAsO_4.7H_2O$). The stock solutions were prepared with autoclaved ddH_2O and all the working solutions were serially diluted in CMP broth. Solutions were prepared in glass tubes previously washed with acidic solution (10% HCl) and autoclaved. The solubility limit at pH 7 ± 0,5 was determined for each metal(loid) and therefore the range of concentrations (mM) tested were: Zn: 0,03 to 8; Co: 0,03 to

4; Al: 0,0016 to 2; Mn: 0,03 to 8; Ni: 0,03 to 4; Cd: 0,03 to 2; U: 0.002 to 0.5; Cu: 0,03 to 4; and As: 0.25 to 32. The bacterial suspensions were prepared as described in section 2.3. Visible growth was checked after 48 hours at 20°C and the minimal inhibitory concentration was defined as the lowest concentration without visible growth. The sensitivity of strain *E. coli* ATCC 25922 was also determined. All strains were tested in triplicate.

2.5 *in silico* detection of antibiotic and metal(loid) resistance genes

Presently, genomes for the strains *P. lusitanus* (NZ_JXRA00000000; strain NL19), *P. cryoconitis* (NZ_CP014504; strain PAMC 27485), *P. himalayensis* (NZ_MPZO00000000; strain DSM 16196), *P. heparinus* (NC_013061; strain DSM 2366) and *P. hartonius* (NZ_FNRA01000001.1; DSM 19033) are available. All these strains were used in this study, except for *P. cryoconitis* since we tested DSM 14825 strain instead. The presence of antibiotic resistance genes in the genomes of the were surveyed using the bioinformatic Comprehensive Antibiotic Resistance Database (CARD) [98]. Data curated in CARD combine detected genes with functional categories and respective cut-offs, based on BLAST Expect values (*E*) [99]. The genes with an *E* value greater than or equal to e^{-10} were selected and organized in different categories. The pool of putative metal(loid) resistance/tolerance genes in the same strains was obtained with the automatic genome annotation software Rapid Annotations using Subsystems Technology (RAST) [100] and was based on the categorie virulence, disease and defense subsystems – resistance to antibiotics and toxic compounds. Data was visualized with Circos Circular Genome Data Visualization software (http://circos.ca/software/) [101].

2.6 Analysis of LUS-1 and PLN-1 β-lactamases

Two genes encoding a novel family of β-lactamases (*bla*$_{LUS-1}$ and *bla*$_{PLN-1}$) were identified with CARD analysis of the *P. lusitanus* NL19 strain. The sequences of the two encoded enzymes were aligned with representative β-lactamases of different molecular classes using Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/) in order to identify their conserved residues. The presence of genes encoding β-lactamases identical to LUS-1 and PLN-1 in the other *Pedobacter* spp. strains was performed by tblastp in the Microbial BLAST platform. The similarity of the genetic environment of *bla*$_{LUS-1}$ and *bla*$_{PLN-1}$ and their homologues in other *Pedobacter* spp. strains was analysed and visualized with Easyfig software [102]. The putative function of proteins encoded by all genes analyzed was predicted with InterPro [103] and PSORTb v.3.0 [104].

2.7 Cloning of β-lactamases produced by *P. lusitanus* NL19

bla$_{LUS-1}$ and *bla*$_{PLN-1}$ were amplified using total DNA of NL19 strain previously purified with DNeasy Tissue Kit (Qiagen) using primers containing *NcoI* and *NotI* restriction sites (Table 8). The forward primer (Table 8) applied to the reaction depended on the cloning vector used: pCDFDuet™-1 or pET-26b(+). The last vector was selected in order to clone *bla*$_{LUS-1}$ and *bla*$_{PLN-1}$ (without the region encoding their signal peptide) fused to *E. coli pelB* signal peptide sequence that assures periplasmic localization. The signal peptide sequences of *bla*$_{LUS-1}$ and *bla*$_{PLN-1}$ were predicted with the SignalP 4.1 Server (http://www.cbs.dtu.dk/services/SignalP/).

PCR amplification of *bla*$_{LUS-1}$ and *bla*$_{PLN-1}$ was performed with NZYProof DNA polymerase (NZYTech) in a final volume of of 50µl containing 10x reaction buffer, 0,2 mM dNTPs, 10 µM of each primer, 1,25 U NZYProof DNA Polymerase and 10 µg of template DNA. The parameters of amplification were: initial denaturation at 95°C for 3 min, followed by 30 cycles of 95°C for 30 sec, variable annealing temperature (60°C for *bla*$_{PLN-1}$ and 63°C for *bla*$_{LUS-1}$ with pCDF primers and 60°C for both genes with pET primers) for 30 sec, 72°C for 1 min and a final extension step at 72°C for 7 min.

After amplification, the PCR products were purified with NZYGelpure kit (NZYTech) for further digestion. The plasmids pCDFDuet™-1 and pET-26b(+) were purified from *Escherichia coli* strain DH5α (*fhu*A2Δ(*arg*F-*lacZ)* U169 *pho*A *gln*V44 *Φ80* Δ*(lacZ)*M15 *gyr*A96 *rec*A1 *rel*A1 *end*A1 *thi-1 hsd*R17) using the GeneJET Plasmid Miniprep Kit (Thermo Fisher Scientific). 1µg of each PCR product and plasmid was digested with FastDigest *NcoI* and *NotI* at 37°C for one hour. Following digestion, the products were subjected to electrophoresis and the DNA was purified from the agarose gel with the QIAquick Gel Extraction Kit (QIAGEN). Digested PCR products and plasmids were ligated in a 3:1 molar ratio proportion with 1 U of T4 DNA ligase (Thermo Fisher Scientific). The reaction was performed at 22°C for 1 hour and 5 µl was used to transform of chemically competent *E. coli* DH5α by heat shock [105]. Clones were selected from LB agar plates containing streptomycin (50 mg/mL) for pCDFDuet™-1 or kanamycin (30 mg/mL) for pET-26b(+) after overnight growth at 37°C. Colonies were screened by colony PCR using NZYTaq with 5x Gel Load Reaction Buffer (NZYtech) and primers ACYCDuetUP1 and DuetDOWN1 for pCDFDuet™-1 and T7prom and T7 term for pET-26b(+).The reactions were performed in a final volume of 50µl containing 5× Gel Load Reaction Buffer, 1,5 mM $MgCl_2$, 0,3 mM dNTPs, 10 µM of each primer and 1,25 U NZYTaq DNA polymerase. The parameters of amplification were: initial denaturation at 95°C for 2 min, followed by 30 cycles of 95°C for 30 sec, annealing temperature of 52°C for 30 sec, 72°C for 1:10 min and a final extension step at 72°C for 7 min. A positive

clone from each transformation was selected and grown in LB broth supplemented with the respective selective marker at 37°C, overnight, for plasmid extraction. The four plasmids with inserts (Table 9) were sent for Sanger sequencing at Stabvida (Portugal), in order to confirm the absence of mutations in the genes cloned.

Table 8 - Primer list.

Target	Identification	Sequence (5' → 3')	Reference
bla$_{PLN-1}$	pCDF_PLN_Fw	CGATCCATGGGCCTTAAACAGAGTATCAAC	This study
	pET_PLN_Fw	CGTACCATGGATTGTGCTCAAAAAGTAGCAGAA	This study
	PLN_Rv	AGTAGCGGCCGCTTATTCTTTCCTGAGAAATTTATC	This study
bla$_{LUS-1}$	pCDF_LUS_Fw	CGTACCATGGGCAAAGGATTTTTTAGAATAAAGAGC	This study
	pET_LUS_Fw	TGCTCCATGGATCAGAAAAGTGAACTACGGGAA	This study
	LUS_Rv	AGTAGCGGCCGCTTATGGTTTCCCTGTGGTCAG	This study
pCDFDuet MCS1	ACYCDuetUP1	GGATCTCGACGCTCTCCCT	Novagen
	DuetDOWN1	GATTATGCGGCCGTGTACAA	Novagen
pET26 MCS	T7 prom (Fw)	TAATACGACTCACTATAGGG	Novagen
	T7 term (Rv)	GCTAGTTATTGCTCAGCGG	Novagen

2.8 Whole-cell protein analysis by SDS-PAGE

Each plasmid constructed, as well as the empty vectors, were transformed into the expression strain *E. coli* BL21-Gold (DE3) by heat shock [105]. Production of LUS-1 and PLN-1 β-lactamases was confirmed by whole-cell protein analysis by SDS-PAGE. The strains with and without the *bla*$_{LUS-1}$ and *bla*$_{PLN-1}$ genes in pCDFDuet™-1 were grown in LB broth supplemented with streptomycin (50 mg/mL) at 37°C overnight. 300µl of these pre-cultures were used to inoculate 30 mL of LB broth supplemented with the appropriate selective marker. The cultures were incubated at 37°C, 180 rpm, until OD_{600} was between 0,5-0,6. At this point, the cultures were induced with isopropyl-β-D-thio-galactoside (IPTG) at a final concentration of 0,5 mM. The cultures were further grown at 37°C and 1 mL of each culture was collected after 4 and 18 hours of incubation and centrifuged at 10 000 g for 3 minutes. The supernatant was discarded and the cell pellets were stored at -80°C until further use [106,107]. The pellets were dissolved in denaturing buffer containing 3:1 of PBS and 4X denaturing buffer (0,25 M Tris-HCl pH 7, 40% glycerol, 20% β-mercaptoethanol, 0,4% bromophenol blue, 10% SDS). After the addition of protein loading buffer (final concentration 1X), the samples were boiled for 5 minutes and applied in a 10% polyacrylamide gel. Preparation of the gel was carried out

according to CSH (Cold Spring Harbor) protocols (http://cshprotocols.cshlp.org). The proteins were separated at 15 V/cm for 1 hour and the gels were stained according to Caetano (2011) [108].

2.9 MIC determination of *E. coli* expressing LUS-1 and PLN-1 β-lactamases

The minimum inhibitory concentration of some β-lactams was determined for *E. coli* BL21-Gold (DE3) strains containing pET-26b(+) and pCDFDuet™-1 with and without bla_{LUS-1} and bla_{PLN-1} genes (Table 9) as described in section 2.3. The selective marker and IPTG at a final concentration of 0,5 mM were always added to the culture media [25,60].

Table 9 - List of plasmids used in this study.

Plasmid	Description	Selective Marker	Source
pCDFDuet™-1	General vector with two multiple cloning sites (MCS1 and MCS2), preceded by T7lac promoter	Streptomycin	Novagen
pCPLN-1	pCDFDuet™-1 with bla_{PLN-1} cloned in the first MCS	Streptomycin	This study
pCLUS-1	pCDFDuet™-1 with bla_{LUS-1} in first MCS	Streptomycin	This study
pET-26b(+)	General vector with a N-terminal *pelB* signal sequence	Kanamycin	Novagen
pETPLN-1	pET-26b(+) with bla_{PLN-1} fused to *pelB* signal sequence	Kanamycin	This study
pETLUS-1	pET-26b(+) with bla_{LUS-1} fused to *pelB* signal sequence	Kanamycin	This study

2.10 Identification of plasmid in *Pedobacter* spp. strains

The identification of plasmids in the *Pedobacter* spp. strains was performed using i) plasmid extraction by alkaline lysis and ii) PFGE analysis of S1 digested plugs.

For plasmid extraction, the strains were grown in 10 mL of CMP broth at 20°C overnight and 10 mL of culture was centrifuged. Plasmids were extracted with the GeneJET Plasmid Miniprep kit according to manufacturer's instructions. The purified DNA was visualized after electrophoresis using a 1% agarose gel.

For PFGE analysis, the plugs were prepared according to the protocol established by PulseNet (https://www.cdc.gov/pulsenet/pathogens/protocols.html), using colonies picked directly form CPM agar plates, after grown at 20°C for 72 hours. Digestion of the plugs was performed by adding S1 nuclease (Promega) at the final concentration of

1U/µL to convert existing supercoiled plasmids into full-length linear molecules [109]. The plugs were loaded in a 1% of Pulsed Field Certified™ agarose (Bio-Rad) gel and electrophoresis was performed at 6V with 5s-45s pulses for 20h with 0,5X TBE buffer using the CHEF DR II system (BioRad, Hercules, USA). Gel was stained with ethidium bromide (10 mg/mL) for 30 minutes and washed three times with distilled water for 20 minutes, before visualization in a UV transilluminator

Chapter III. Results and Discussion

3.1 Disk diffusion test

According to the clinical breakpoints established by EUCAST and CLSI, it was possible to classify all strains as resistant to amikacin, amoxicillin/clavulanic acid, ampicillin, aztreonam, cefepime, cefoxitin, ceftazidime, cefuroxime, ciprofloxacin, colistin sulfate, gentamicin, netilmicin, penicillin G, piperacillin, piperacillin/tazobactam, streptomycin, tobramycin and vancomycin, but susceptible or intermediate to sulfamethoxazole/trimethoprim (SXT) and imipenem, respectively. The results are shown in Table 10.

Table 10 - Results of disk diffusion test for *Pedobacter* spp. strains under study. Strains: 1 - *P. lusitanus* NL19; 2 - *P. hartonius* DSM 19033; 3 - *P. himalayensis* MTCC 6384; 4 - *P. heparinus* DSM 2366; 5 - *P. cryoconitis* DSM 14825; 6 - *P. westerhofensis* DSM 19036. R - resistant; I - intermediate; S - susceptible.

Antibiotics	µg	1	2	3	4	5	6
Amikacin	30	R	R	R	R	R	R
Amoxicillin-clavulanic acid	30	R	R	R	R	R	R
Ampicillin	10	R	R	R	R	R	R
Aztreonam	30	R	R	R	R	R	R
Cefepime	30	R	R	R	R	R	R
Cefoxitin	30	R	R	R	R	R	R
Ceftazidime	10	R	R	R	R	R	R
Cefuroxime	30	R	R	R	R	R	R
Chloramphenicol	30	R	R	R	**S**	R	R
Ciprofloxacin	5	R	R	R	R	R	R
Colistin sulfate	10	R	R	R	R	R	R
Gentamicin	10	R	R	R	R	R	R
Imipenem	10	**S**	**S**	**I**	**S**	**S**	**S**
Netilmicin	10	R	R	R	R	R	R
Nitrofurantoin	100	**S**	**S**	R	R	R	R
Penicillin G	10	R	R	R	R	R	R
Piperacillin	30	R	R	R	R	R	R
Piperacillin/tazobactam	30-6	R	R	R	R	R	R
Streptomycin	10	R	R	R	R	R	R
Sulfamethoxazole/trimethoprim	25	**S**	**S**	**S**	**S**	**S**	**S**
Tetracycline	30	R	R	R	S	R	R
Ticarcillin	75	R	R	R	R	R	S
Tobramycin	10	R	R	R	R	R	R
Vancomycin	30	R	R	R	R	R	R

All *Pedobacter* spp. strains were resistant to almost all β-lactams, to all aminoglycosides, lipopeptides, quinolones and glycopeptides tested (Figure 4A). On the other hand, no strains were resistant to imipenem and SXT (Figure 4A). Their pattern of resistance was the same, except for chloramphenicol, imipenem, nitrofurantoin, tetracycline and ticarcillin (Table 10). Among all strains, *P. heparinus* DSM 2366 was the

most susceptible (susceptible to 4 antibiotics, out of 24) and *P. cryoconitis* DSM 14825 and *P. himalayensis* MTCC 6384 were the more resistant (only susceptible to 2 antibiotics, out of 24) (Figure 4B).

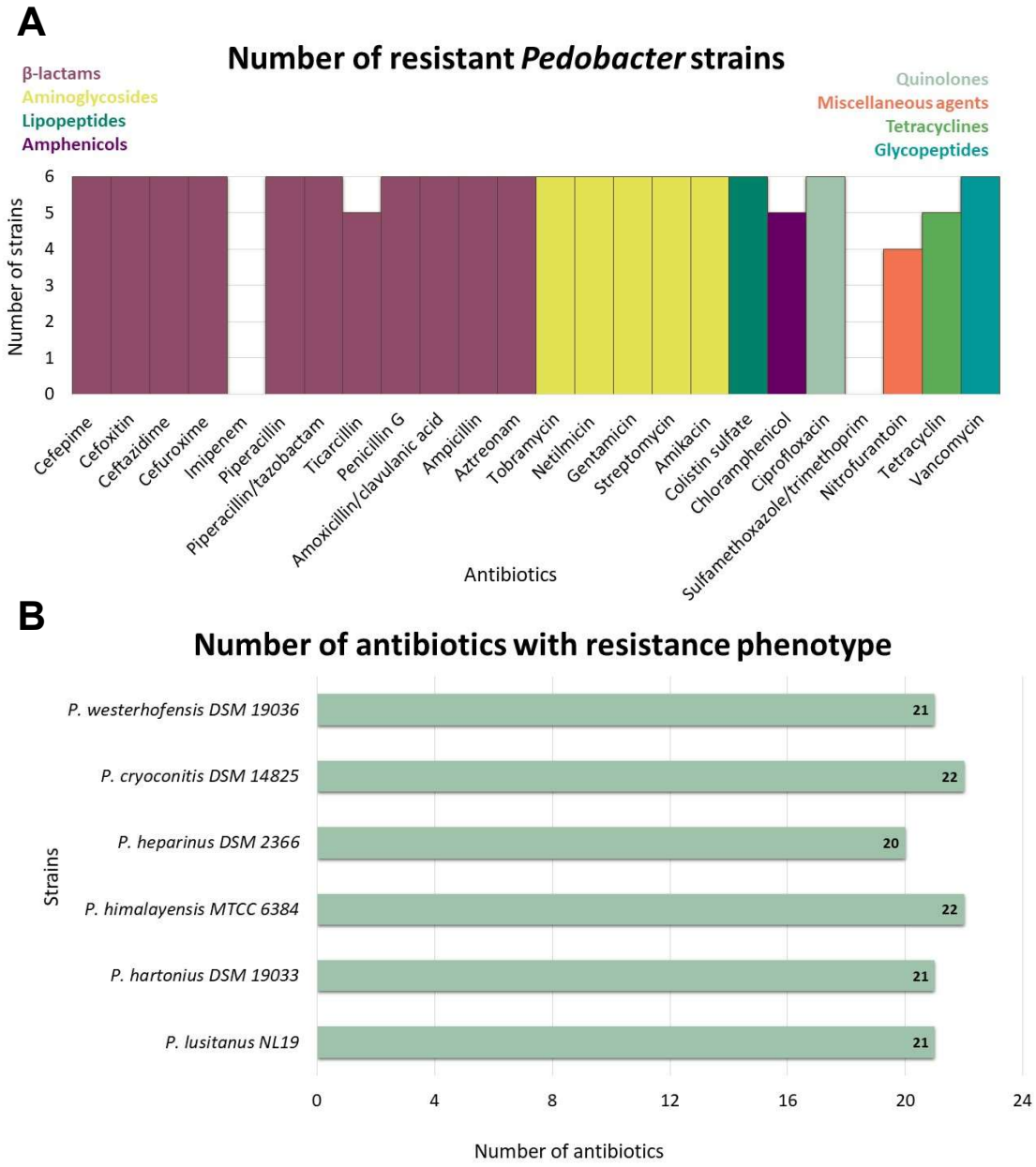

Figure 4 - Number of *Pedobacter* spp. strains resistant to each antibiotic tested (A) and number of antibiotics with resistance phenotype for each *Pedobacter* spp. strain (B).

Some of our results differed from those obtained in previous studies (Appendix 1). For instance, herein, *P. hartonius* presented a resistant phenotype to tetracycline, whereas it was previously described as sensitive to this compound. *P. westerhofensis* strain susceptibility to chloramphenicol, ticarcillin and tetracycline was previously

classified as intermediate, resitant and susceptible, respectively [19]. However, here, it is classified as resistant to chloramphenicol and tetracycline and as susceptible to ticarcillin. *P. himalayensis* was described as being sensitive to amikacin and vancomycin [20] and according to our results this strain is resistant. For cefoxitin and penicillin G, *P. heparinus* was expected to be susceptible [2], and that was not confirmed in our study. Yet, our methodology was different from that of Stein *et al.* (1998) [2]. Finally, *P. cryoconitis* was considered resistant to imipenem using ATB strips [18], whereas in the present study, by disk diffusion testing, the results indicate that it is susceptible to this carbapenem.

3.2 Determination of minimum inhibitory concentration of antibiotics

MICs results were interpreted by clinical breakpoints since environmental breakpoints to non-clinical bacteria are not yet established [27]. MICs confirmed all the results obtained by disk diffusion testing, except for chloramphenicol and tetracycline (Table 11). For chloramphenicol, it was expected that *P. westerhofensis* would be resistant, but its MIC was 4 mg/L that is within the susceptible range. For tetracycline, by disk diffusion, only *P. heparinus* was susceptible and by MIC determination, *P. heparinus*, *P. cryoconitis* and *P. westerhofensis* are susceptible and *P. hartonius* is intermediate. In fact, the range of tetracycline MICs were very different between strains and varied between 0,5 mg/L (*P. westerhofensis*) and 16 mg/L (*P. lusitanus* and *P. himalayensis*). Moreover, we determined MICs to four antibiotics that were not tested by the disk diffusion assay. These included apramycin, ertapenem, meropenem and kanamycin. All the strains were resistant to ertapenem and kanamycin and survived to high concentrations of apramycin. Only *P. westerhofensis* showed resistance to meropenem (Figure 5).

The majority of the strains tolerate high concentrations (MIC ≥ 128 mg/L) of ampicillin, apramycin, ceftazidime, colistin, kanamycin and streptomycin. For streptomycin, it is not possible classify strains as resistant, intermediate or sensitive because there are no interpretative standards [57], but we verified that steptomycin MIC is high, ranging from 64 to 512 mg/L. Albeit all strains were resistant to quinolone criprofloxacin, the MICs greatly varied from 4 mg/L (*P. himalayensis*, *P. cryoconitis* and *P. westerhofensis*) to 256 mg/L (*P. hartonius*). It is particularly interesting that all *Pedobacter* spp. strains are resistant to colistin (except *P. westerhofensis*) and are able to grow in the presence of 512 mg/L (except *P. heparinus* and *P. westerhofensis*). Colistin and other polymyxins specifically bind to lipopolysaccharide (LPS), targeting efficiently Gram-negative bacteria. These antibiotics are cyclic antimicrobial peptides with long hydrophobic tails. This hydrophobic chain binds to LPS and can disrupt both cell membranes. The polymyxin resistance is

commonly associated to alterations in the expression of regulators that affect LPS production. This results in the target modification and reduces antibiotic binding [47].

Table 11 - Results of MIC (mg/L) values obtained for several antibiotics, for the following *Pedobacter* spp. strains: 1 - *P. lusitanus* NL19; 2 - *P. hartonius* DSM 19033; 3 - *P. himalayensis* MTCC 6384; 4 - *P. heparinus* DSM 2366; 5 - *P. cryoconitis* DSM 14825; 6 - *P. westerhofensis* DSM 19036. R - resistant; I - intermediate; S - susceptible.

Antibiotics	1	2	3	4	5	6
Ampicillin	> 512 (R)	> 512 (R)	> 512 (R)	128 (R)	> 512 (R)	128 (R)
Apramycin sulfate	> 512	> 512	> 512	512	> 512	512
Ceftazidime	> 512 (R)	> 512 (R)	> 512 (R)	64 (R)	> 512 (R)	512 (R)
Chloramphenicol	32 (R)	32 (R)	32 (R)	**4 (S)**	16 (R)	**4 (S)**
Ciprofloxacin	8 (R)	256 (R)	4 (R)	128 (R)	4 (R)	4 (R)
Colistin sulfate	> 512 (R)	> 512 (R)	> 512 (R)	512 (R)	> 512 (R)	32 (R)
Ertapenem	16 (R)	32 (R)	8 (R)	128 (R)	16 (R)	32 (R)
Imipenem	**2 (S)**	**2 (S)**	**4 (I)**	**0,25 (S)**	**1 (S)**	**0,5 (S)**
Meropenem	**2 (S)**	**1 (S)**	**1 (S)**	**0,5 (S)**	**2 (S)**	16 (R)
Kanamycin	> 512 (R)	> 512 (R)	> 512 (R)	256 (R)	> 512 (R)	512 (R)
Streptomycin	256	512	64	128	512	64
Tetracycline	16 (R)	**8 (I)**	16 (R)	**4 (S)**	**4 (S)**	**0,5 (S)**

Focusing on carbapenems susceptibility, it was surprising to verify that all strains were resistant to ertapenem, whereas only *P. westerhofensis* was resistant to meropenem and no resistance was detected for imipenem. Nevertheless, decreased susceptibility for the latter was observed for *P. himalayensis*. *P. heparinus* had the highest MIC to ertapenem (128 mg/L) and *P. himalayensis* had the lowest (8 mg/L). The ertapenem-resistance and imipenem- and meropenem-susceptibility phenotype was previously described for *Klebsiella pneumoniae* due to the non-production of the OmpK35 caused by a nonsense mutation and to the expression of a novel OmpK36 variant. In fact, the sequence analysis of the *ompK36* gene in different resistant clinical isolates demonstrated the importance of this porin in the specific uptake of β-lactams [110].

The MIC of ampicillin, chloramphenicol, kanamycin, meropenem and tetracycline were previously determined for *P. lusitanus* NL19 [8] and they are in accordance for ampicillin and kanamycin. For chloramphenicol and meropenem, the described MIC's were > 32 mg/L and for tetracycline was > 64 mg/L [8]. However, lower MIC to chloramphenicol, meropenem and tetracycline were obtained in the present study (Table

11). Even so, NL19 is still resistant to chloramphenicol and tetracycline, according to EUCAST breakpoint tables. However, the same is not applied for meropenem, where a MIC of 2 mg/L is within the susceptible range. The main difference between the procedure described by Covas *et al.* (2017) [8] and the MIC procedure here adopted was the temperature of incubation tested that was 26°C and 20°C, respectively. Could this factor influence the differences in MIC to these antibiotics, especially for meropenem? This seems to be a possibility, since the influence of the temperature in the MIC determination by microdilution broth methodology was also described for a dimorphic fungus, *Paracoccidioides brasiliensis* [111].

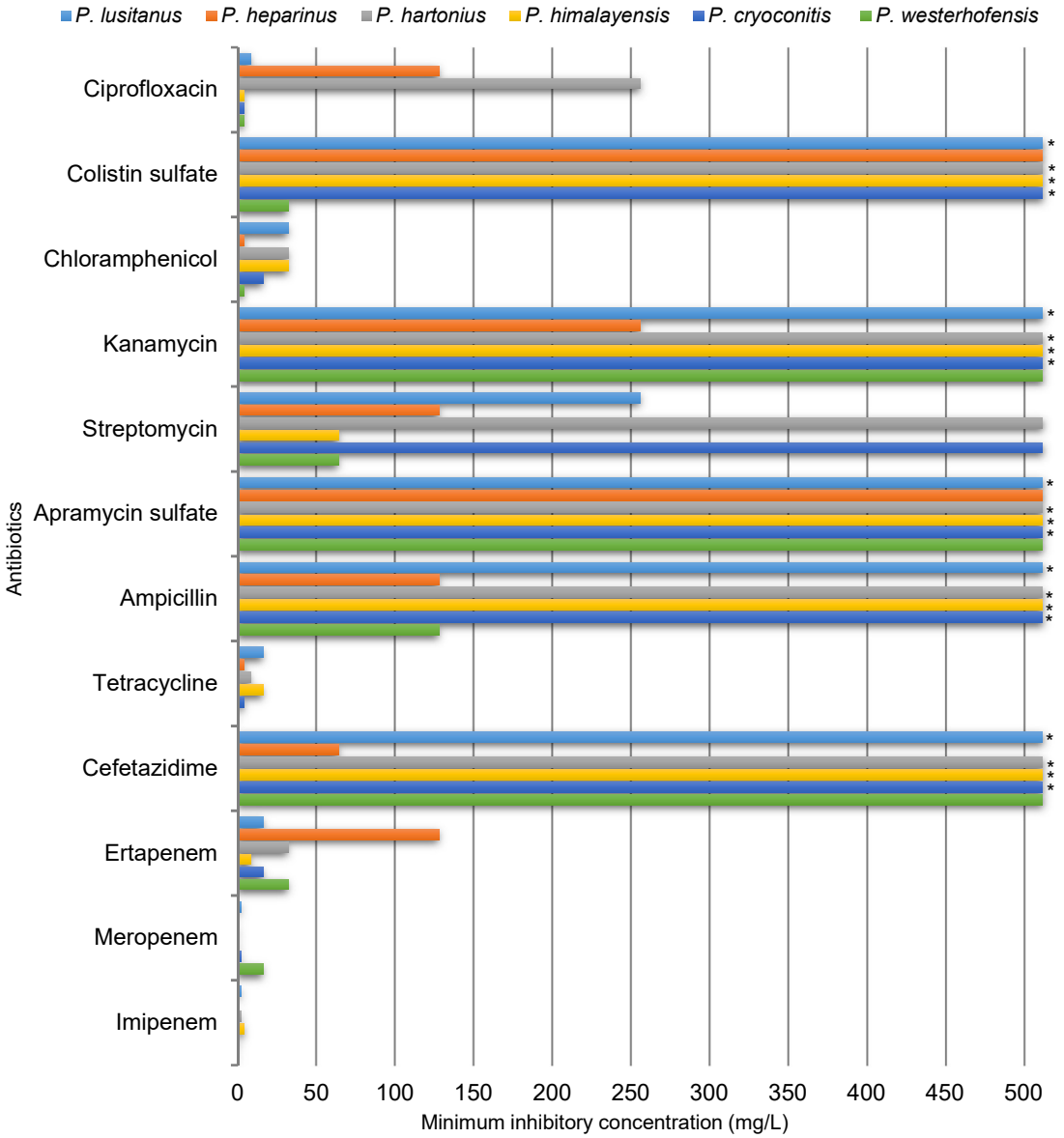

Figure 5 - Results of minimum inhibitory concentration (mg/L) of all antibiotics tested for each *Pedobacter* spp. strain (* - MIC value is >).

3.3 Determination of minimum inhibitory concentration of metal(loid)s

As above referred, there are no breakpoints established for metal(loid)s susceptibility or quality control [82]. Therefore, in our study we determined MICs of metal(loid)s and we also included *E. coli* ATCC 25922 for comparison purposes (Table 12).

Some of the metals tested, such as Co, Cu, Ni, Mn and Zn are essential for cells but they can be toxic in high concentrations. However, As, Al, Cd and U are toxic to microbial cells even at low concentrations [63–65,73,112]. Overall, *P. cryoconitis* and *P. lusitanus* were the *Pedobacter* spp. strains with higher MICs values to the metal(loid)s tested, whereas *P. heparinus* and *P. westerhofensis* had the lowest ones. The metal(loid) As was most well tolerated metal(loid) by all strains tested (Figure 6).

Focusing on the essential metals, the levels tolerated by *Pedobacter* spp. strains were very different (Table 12). The MIC values of Co ranged from 0,25 to 1 mM, where the highest concentration was only tolerated by *P. cryoconitis*. *P. lusitanus*, *P. hartonius* and *P. himalayensis*, were the strains with the highest MIC value for Ni (4 mM), in contrast with *P. heparinus* and *P. westerhofensis* that had the lowest (1 mM). A study focused on bacteria isolated from uranium mine wastes reported MIC values for cobalt between 0.1 and 2.5 mM and for Ni between 0,1 and 6 mM [73]. Co and Ni can be toxic when accumulated in cells, causing oxidative stress and their mechanisms of resistance seem to be related [86,113]. *P. cryoconitis* was the strain that tolerated the highest concentration of Co, but which was not the strain with higher MIC to Ni. Therefore, in this study, a direct relation between Co and Ni tolerance was not evident (Table 12). *P. lusitanus* survived to 8 mM of Zn, but the other strains did not have the same capacity. *P. hartonius* and *P. himalayensis* were almost as much tolerant as *P. lusitanus*, but the MICs for the other strains were lower than 4 mM. The study focused on uranium mine waste that was abovementioned [73] detected MIC of Zn between 0,1 to 10 mM. The same report identified MIC for Cu between 0,1 to 2,5 mM [73] and *P. lusitanus* had a higher MIC for this metal (4 mM). *P. hartonius*, *P. himalayensis* and *P. cryoconitis* do not tolerate copper as *P. lusitanus*, showing a MIC value of 2 mM. *P. heparinus* was the most susceptible strain to Cu, with an MIC of 0,5 mM that is even lower than the MIC of *E. coli* (1 mM). *P. lusitanus* and *P. cryoconitis* could grow when exposed to at least 8 mM of Mn. Manganese plays an important role in antioxidant systems, improving cellular ROS resistance [89]. Some microorganisms such as *E. coli* and many lactobacilli tolerate very well manganese and can accumulate it to millimolar concentrations [90]. This may justify the MIC value obtained for *E. coli*, higher than that obtained for *P. himalayensis* (4 mM), *P. heparinus* (2 mM) and *P. westerhofensis* (2 mM).

Table 12 - Results of minimum inhibitory concentration (mM) of several metal(loid)s obtained in this study. Strains: 1 - *P. lusitanus* NL19; 2 - *P. hartonius* DSM 19033; 3 - *P. himalayensis* MTCC 6384; 4 - *P. heparinus* DSM 2366; 5 - *P. cryoconitis* DSM 14825; 6 - *P. westerhofensis* DSM 19036; 7 - *E. coli* ATCC 25922.

Metal(loid)s	1	2	3	4	5	6	7
Cobalt	0,5	0,25	0,5	0,25	**1**	0,25	0,5
Zinc	**> 8**	**8**	**8**	1	**4**	1	1
Manganese	**> 8**	8	4	2	**> 8**	2	8
Nickel	**4**	**4**	**4**	1	**2**	1	1
Copper	**4**	**2**	**2**	0,5	**2**	1	1
Arsenic	**16**	**32**	**16**	**4**	**32**	**4**	0,5
Aluminium	> 2	> 2	> 2	> 2	> 2	> 2	> 2
Cadmium	**2**	0,25	**0,5**	0,06	**0,5**	0,125	0,25
Uranium	**> 0,5**	0,125	**> 0,5**	0,25	**> 0,5**	**0,5**	0,25

Regarding the toxic metal Al, all the strains tested, including *E. coli*, could grow in the presence of 2 mM. This was the highest concentration possible to test for Al since it exists as oxide and complex aluminosilicates at pH 7, which makes it poorly soluble in aqueous media [114]. Also, due to the solubility at neutral pH, the highest concentration of U tested was 0,5 mM and was tolerated by *P. lusitanus*, *P. himalayensis* and *P. cryoconitis*. Uranium is present in contaminated sites predominantly in two stable valence states. The U(VI) is the most oxidized valence state, being the most soluble and toxic uranyl specie (UO_2^{2-}) [93]. Since uranyl nitrate hexahydrate ($UO_2(NO_3)_2.6H_2O$) was used in this study, the strains were exposed to the most toxic uranyl species. In the case of As, despite being a toxic metalloid regardless of its concentration, all strains tolerated higher concentrations than *E. coli* ATCC 25922. Arsenic enters in cells via phosphate membrane transport systems and can disrupt metabolic reactions that require phosphorylation. One of the most common oxidation states for soluble arsenic in nature is As(V), as oxyanions arsenate (AsO_4^{3-}) [92]. So, *Pedobacter* spp. strains could tolerate high concentrations of the most common state of As present in nature with disruptive capabilities. The resistance to As is being commonly identified in Gram-negative and Gram-positive bacteria maybe because of its wide distribution in the environment and its use as an antimicrobial [30]. Cd was tolerated by microorganisms of this study at very different concentrations and the MICs ranged from 0,06 to 2 mM, where the highest concentration was tolerated by *P. lusitanus*. Diverse extremophilic strains of genus *Pseudomonas* with bioremediation potential were found to be tolerant up to 5 mM of $CdCl_2$ [115].

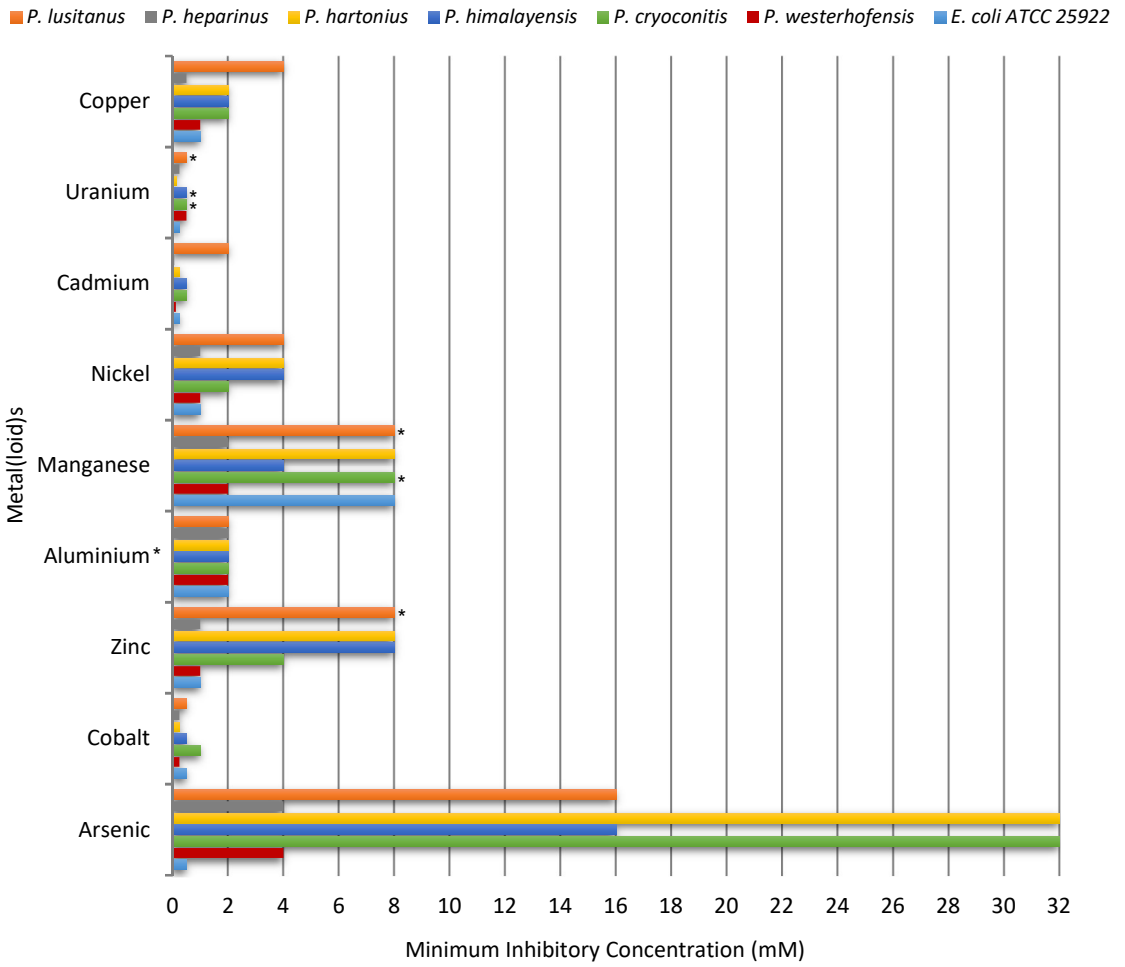

Figure 6 - Results of minimum inhibitory concentration (mM) of all metal(loid)s tested for each *Pedobacter* spp. strain (* - MIC value is >).

The comparison of metal(loid) tolerances between studies is very difficult because the methodologies are not standardized and, therefore, are always different. This is worrisome since the bioavailability of metal(loid)s is very depend on the type of media components. For instance, Paul and Sinha (2015) concluded that MIC values for some metals were higher when determined on agar instead of broth medium [116]. Moreover, the concentration of carbon source and phosphate buffer in most of the media are associated with the overestimation of metal tolerance [32]. So, and as highlighted by other authors, the interpretation of the toxic effects of metals on microorganisms is not an easy task [82]. To overcome this, Rathnayake *et al.* (2013) proposed the use of MBMM medium that allows a greater availability of the metals. MBMM has the minimal nutritional requirements to bacteria with 0,2% of carbon source (glucose) [32]. However, it is possible that this medium does not support the growth of all/the great majority bacterial strains. Therefore, here we used the CMP medium since it is the most suitable medium to grow

all the strains used in the study. Since this medium has nutrients in minimum concentrations and it also allows fair amounts of free metal ion in solutions.

Strains from the *Pedobacter* genus have been isolated from several different environments. A study carried out by Rastogi *et al.* (2010) [70] detected different *Pedobacter* species (*Pedobacter africanus*, *Pedobacter ginsengisoli*, *Pedobacter piscium* and *Pedobacter cryoconitis*) as part of the microbial community of soils of a uranium mine. This suggests that this group of organisms could have evolved with the ability to tolerate high concentrations of metals. However, we verified that the profile of metal(loid) tolerance is heterogeneous among the strains, suggesting that it can be more strain-specific and more related with their original environmental niches.

3.4 *in silico* detection of antibiotic and metal(loid) resistance genes

The genomes available for the *Pedobacter* spp. strains used in this study were analysed by CARD, to detect genes associated with antibiotic resistance. All the genes with a *E* value superior or equal to e^{-10} were selected and organized into six different categories (Figure 7 and Appendix 2): 1) inactivation enzyme, 2) target modification, 3) target replacement, 4) target protection, 5) efflux systems and 6) others. The first category includes enzymes that catalyse the inactivation reactions of antibiotics, triggering resistance. The target modification category embrace genes that are involved in resistance by target mutation or enzymatic modification. The target replacement is a category that comprehends genes confering resistance by replacement or substitution of antibiotic action target. This includes, for example, proteins that have the same functions of antibiotic target proteins, substituting them and leading to antibiotic resistance. The target protection consists of genes encoding proteins that bind to the antibiotic target, preventing the action of the antibiotic. Efflux systems include genes that encode efflux proteins that are responsible for pumping the antibiotics out of a cell. Finally, the other category is composed of several other genes that can trigger resistance to antibiotics through various mechanisms such as reduction of permeability or genes that are involved in the regulation (overexpression or repression) of the mechanisms of resistance abovementioned [98]. The analysis revealed that genes possibly associated with antibiotic resistance represent 6% to 8% of the total protein-encoding genes of *Pedobacter* spp. strains (Appendix 2): *P. himalayensis* and *P. lusitanus* have the higher percentage (8%), followed by *P. cryoconitis* (7%) and *P. heparinus* and *P. hartonius* (6%). Among the different categories analyzed, the majority of these genes encode antibiotic efflux mechanisms (except for *P. hartonius*), whereas a few enconde target replacement and target protection proteins (Figure 7). All the strains also have a pool of

genes encoding target modification and inactivation enzymes. *P. himalayensis* is the strain with a higher number of efflux systems and target modification genes. *P. lusitanus* is the strain with more percentage of genes that encode proteins involved in the inactivation of antibiotics (approximately 10%).

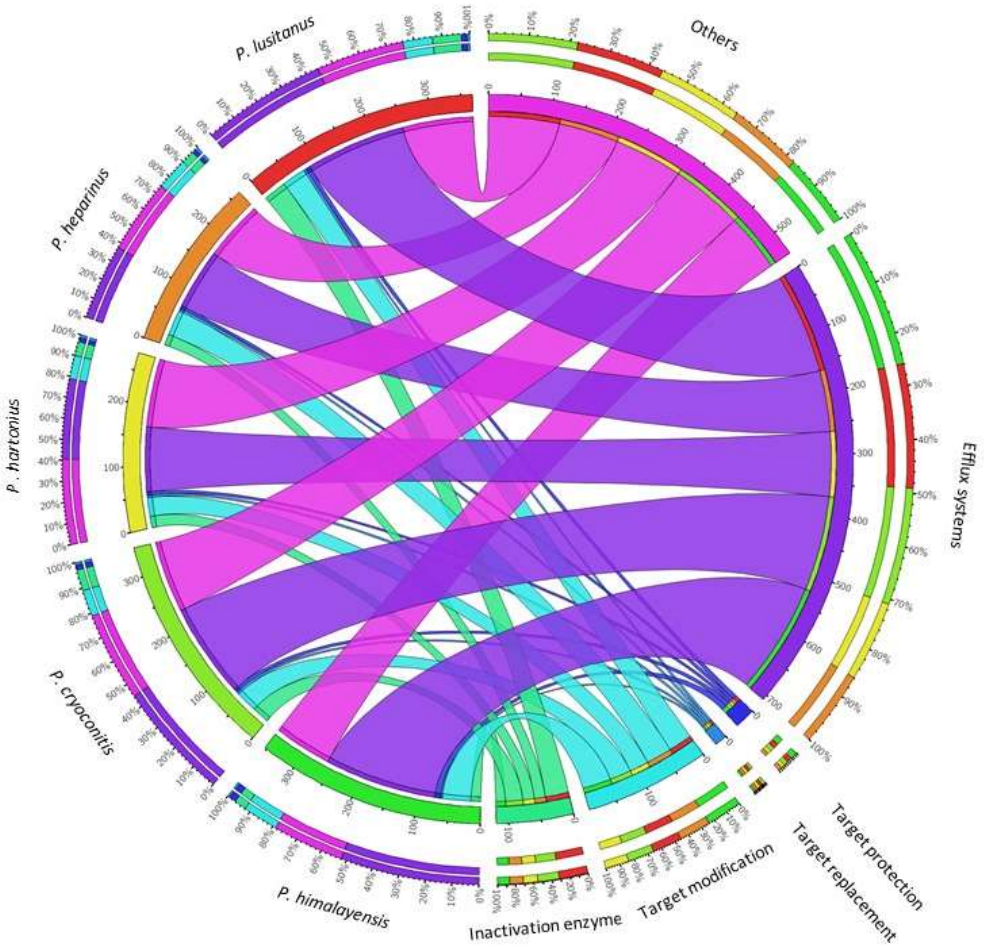

Figure 7 - Number of genes in the genomes of *Pedobacter* spp. strains possible involved in antibiotic resistance identified by CARD analysis.

The RAST annotation of the same genomes allowed to recognize genes possibly involved in metal(loid) resistance/tolerance (Figure 8 and Appendix 3). The categories identified by RAST include: 1) copper homeostasis/tolerance, 2) cobalt-zinc-cadmium resistance, 3) zinc resistance and 4) arsenic resistance. The genes putatively involved in these mechanisms represent 0,6% to 1,20% of the total protein-encoding genes of *Pedobacter* spp. strains (Appendix 3): *P. cryoconitis* and *P. lusitanus* have the higher percentage (1,20%), followed by *P. heparinus* (0,88%), *P. hartonius* (0,75%) and *P. himalayensis* (0,62%). The data shows that all the strains have several genes that can be involved in the tolerance to cobalt-zinc-cadmium complex. Albeit present, mechanisms related exclusively with zinc or arsenic resistance are the less abundant.

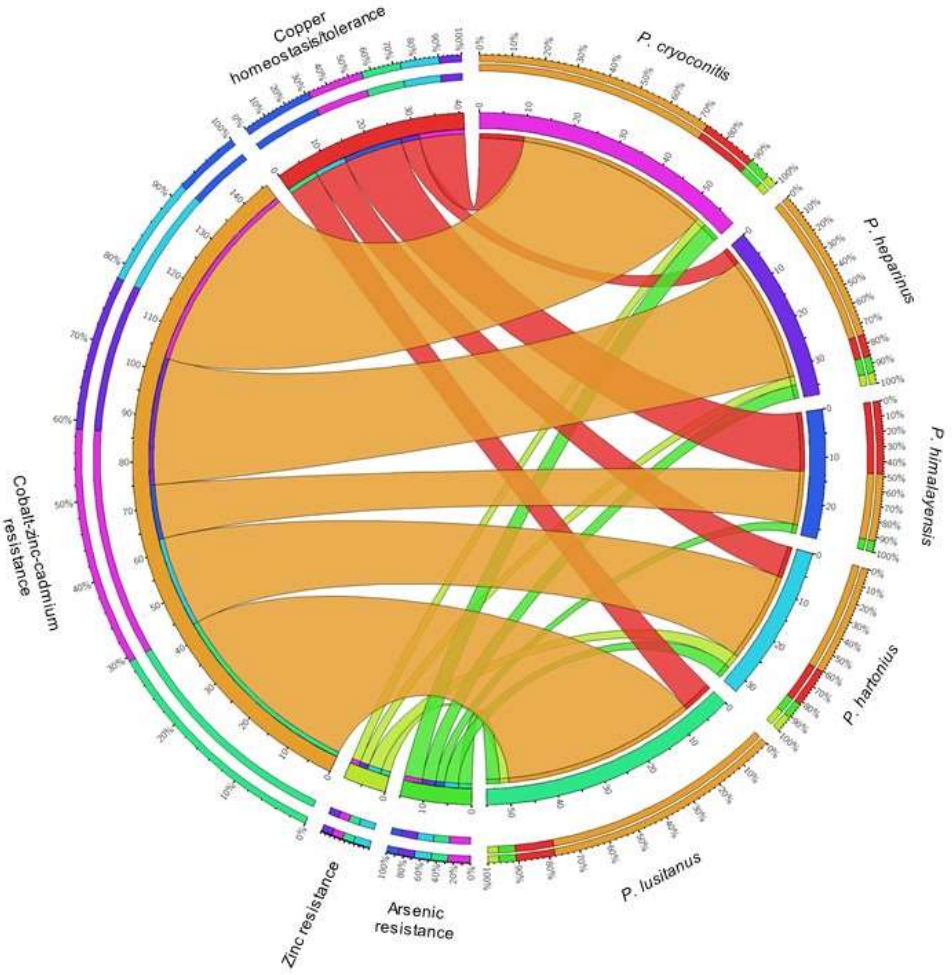

Figure 8 - Number of genes in the genomes of *Pedobacter* spp. strains possible involved in metal(loid) resistance/tolerance identified by RAST analysis.

It is difficult to establish a direct relationship between the results obtained by *in silico* analysis of genomes and the results obtained by resistance phenotype determination. Moreover, the prediction of resistance genes is based on the similarity with genes that have already been described. In the case of antibiotics, there is a high number of known genes. However, for metal(loid)s the information is more restricted. Moreover, once the analysis is based on similarity scores, it can overestimate the genes that are involved in resistance mechanisms *in vivo*. However, it is possible to realize that these strains have a great pool of resistance genes that should be further exploited.

3.5 Analysis of *P. lusitanus* NL19 β-lactamases

As abovementioned, *P. lusitanus* NL19 is the *Pedobacter* spp. strain with the higher number of genes encoding proteins putatively associated with antibiotic inactivation. Among these, two genes encoding putative β-lactamases were identified. One protein had the highest similarity (54%) with TLA-1 (WP_032494759.1) and TLA-3 (WP_032494759.1) β-lactamases, whereas the other had 66% similarity with LRA-12 (WP_063842707.1) β-lactamase. Following the recommendations of the Bacterial Antimicrobial Resistance Reference Gene Database (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA313047), the β-lactamases of *P. lusitanus* were considered novel enzymes with the designation of LUS-1 (KIO77103.1; similar to TLA-3) and PLN-1 (KIO75746.1; similar to LRA-12). LUS-1 is a class A β-lactamase that is encoded in the contig 45 and PLN-1 is a subclass B3 metallo-β-lactamase encoded in the contig 85. According to ExPasy (http://web.expasy.org/compute_pi/), LUS-1 has a molecular mass of 33,10 kDa and a pI of 8.29 and PLN-1 has a molecular mass of 32,07 kDa and a pI of 9,24. They have 303 and 289 amino acids, respectively.

Classe A β-lactamases generally have less than 310 amino acids and include members of the functional group 2, except for group 2d (2a, 2b, 2be, 2br, 2ber, 2c, 2ce, 2e and 2f). These subgroups have one or more representative enzymes and were created based on substrate profile and response to clavulanic acid [61]. The enzymes belonging to this class are inhibited by clavulanic acid, sulbactam and tazobactam [117]. At the molecular level, the typical sequence motifs of class A β-lactamases are: S^{70}TSK (also present in classes C and D - Ser-x-x-Lys, S^{130}DN, E^{166}xxLN and K^{234}TG (where x's represent variable amino acids) [61]. Recently, Philippon *et al*. proposed the creation of two subclasses, A1 and A2, based on diversity found among class A β-lactamases. These two subgroups contain very different conserved residues, which clearly distinguished enzymes belonging to A1 or A2 subclasses. One of these differences is observed in position 136: subclass A1 has an asparagine (N) and subclass A2 has an aspartate (D) [118]. In the reanalysis of the SxxK motif published in 2017, Brandt *et al*., sugested that this conserved region could be expanded upstream by 5 amino acid residues, since there is an amino acid perference. This study shown that the conserved active site of the class A1 has the R^{65}FxxxS^{70}xxK motif but in class A2, the arginine (R) at position 65 is replaced with histidine (H) or lysine (K) [119]. The serine of SxxK motif corresponds to the active site serine [61] and the SDN motif is present in class A β-lactamases that are efficiently inhibited by clavulanic acid [120]. The ExxLN motif, located in the omega loop, is associated with the active-site pocket [121] and is crucial for the

definition of the molecular class A β-lactamases. Its E residue is critical for the fast hydrolysis of penicillins. The Asp residue is involved in the positioning of the active-site water molecule, that acts together with the highly conserved E^{166} and S^{70} residues [118].

The alignment of LUS-1 with random sequences of representative enzymes of subgroups and A1 and A2 subclasses (PC1, SHV-1 and CARB-3 belong to subclass A1 and are 2a, 2b and 2c, respectively, representatives of subgroups of the functional group; TLA-1 and PER-1 belong to subclass A2 and PER-1 belong to 2be subgroup of the functional group [60,118]), shows the three typical motifs of class A, SxxK, SDN and KTG (Figure 9). In $R^{65}FxxxS^{70}xxK$ motif our enzyme has a histidine (H) instead a arginine (R) at position 65. At position 136 the β-lactamases herein described an aspartic acid (D) (highlighted with an arrow in figure 9) is present instead of an asparagine (N). In LUS-1, the last two residues of ExxLN motif are Met and His. The substitution Asn^{170}→His was observed in this motif for some class A β-lactamases subclass A2, including PER-1, TLA-1, TLA-2 and VEB-1 [118]. Moreover, this substitution was also related with a higher ceftazidime-hydrolytic activity in PenA from *Burkholderia thailandensis* [122]. Given these molecular characteristics LUS-1 is included in class A2.

Figure 9 - Protein sequence alignment with representative enzymes of class A β-lactamase and LUS-1. Conserved motifs are shown.

Class B of β-lactamases (metallo-β-lactamases - MBLs) are divided, according to molecular characteristics, into the subclasses, B1, B2 and B3. Subclass B1 and B3 MBLs required the binding of two zinc ions (Zn1 and Zn2) to their active sites. Subclass B2 MBLs only need one zinc ion and their activity is inhibited upon the binding of a second zinc ion. Subclass B1 and B3 exhibit broad-spectrum activity (penicillins, cephalosporins, and carbapenems) while subclass B2 have a strong preference for carbapenems. In general, the MBLs have a reduced hydrolytic capability for monobactams and are not inhibited by clavulanic acid or tazobactam. In contrast, metal ion chelators such as EDTA inhibit MBLs [25,60]. The active site of B3 metallo-β-lactamases have the following consensus amino acids: His/Gln^{116}, His^{118}, His^{196}, Asp^{120}, His^{121} and His^{263}. Zn1 bind to His/Gln^{116}, His^{118} and His^{196,} whereas Zn2 bind to Asp^{120}, His^{121} and His^{263} residues [61]. The alignment of PLN-1 with representative B3 metallo-β-lactamases (L1, CAU-1, GOB-1, FEZ-1) [60,61], allows to identify the amino acids of its active site (Figure 10). This supports the classification assigned by NCBI to this enzyme as a B3 metallo-β-lactamase.

```
L1 (CRD51542)          MRLQTLAITLAALLPAAASAAEPALPQLRAYTVDASWLQPMAPLQIADHTWQIGTEDLTA
CAU-1 (CAC87665)       ---------MKRLILAAAASLLALASAAHADDMPANWTKPTKPYRVVGNIYYVGTEGISS
FEZ-1 (WP_058468956)   --------MKKVLSLTALMMVLNHSSFAYPMPN------PFPPFRIAGNLYYVGTDDLAS
PLN-1 (KIO75746.1)     MLKQSINTVVLLLFLTFSSLFACAQKVAEPTRNPPEWTQPYQPFRIAGNLYYVGTSDLAS
GOB-1 (AAF04458.1)     ------MRNFATLFFMFICLGLNAQVVKEPENMPKEWNQAYEPFRIAGNLYYVGTYDLAS
                                      *                          * ::..: : :** .:::

L1 (CRD51542)          LLVQTPQGAVLLDGGM-PQMADHLLRNMKARGVAPQDLHLILLSHAHADHAGPVAALKRS
CAU-1 (CAC87665)       WLITSSEGHVVLDGGPNAETGKLVERNITALGFQLADVKILINTHAHYDHAGGLAQLKAD
FEZ-1 (WP_058468956)   YLIVTPRGNILINSDL-EANVPMIKASIKKLGFKFSDTKILLISHAHFDHAAGSELIKQQ
PLN-1 (KIO75746.1)     YLITTPKGHILINTGL-SSSLSSIKANVKTLGFKFSDIKILLTTQAHFDHMGAMAAIKKL
GOB-1 (AAF04458.1)     YLIVTDKGNILINTGT-AESLPIIKANIQKLGFNYKDIKILLLTQAHYDHTGALQDFKTE
                        *: : .* :::: .        :  .:   *.   * :::: ::** ** .    :*

L1 (CRD51542)          TAAQIVANAESAVLLARGGSN-DLHFGD--DITYPPATADRIIMDGEVVSLGGVDFTAHF
CAU-1 (CAC87665)       TGAKLWISRDDAPAMAAGHHIGDNIYGP---TPMPAAKPDRSFGDQTKLKLGEIAMVAHL
FEZ-1 (WP_058468956)   TKAKYMVMDEDVSVILSGGKS-DFHYANDSSTYFTQSTVDKVLHDGERVELGGTVLTAHL
PLN-1 (KIO75746.1)     TGAKFMVDEKDAKVAADGGRS-DYALGG-HRSTYVPVKADRILHDKDKITLGGMELVMLH
GOB-1 (AAF04458.1)     TAAKFYADKADVDVLRTGGKS-DYEMGK-YGVTFKPVTPDKTLKDQDKIKLGNITLTLLH
                       * *:      ..     *   *   .          . *: : *   : **   :.

L1_(CRD51542)          VPGHTPGSTAWTWTDTRGGKPVRMVYADSLSA-PGYQLQANPRYPHLIEDYRRSFATVRA
CAU-1 (CAC87665)       TPGHTIGCTSWTTAVVEKGRPLTVTFPCSLSVAGN-VLVGNKTHRTIVADYRASFAKLRA
FEZ-1 (WP_058468956)   TPGHTRGCTTWTMKLKDHGKQYQAVIIGSIGVNPGYKLVDNITYPKIAEDYKHSIKVLES
PLN-1 (KIO75746.1)     HPGHTQGSCSFLFNVKDESRVYSVLIANMPTIVTEKKFSEVTTYPGIAKDYAYTLNAMKK
GOB-1 (AAF04458.1)     HPGHTKGSCSFIFETKDEKRKYRVLIANMPSVIVDKKFSEVTAYPNIQSDYAYTFGVMKK
                        **** *. ::       :                :     :  :  **  ::  :.

L1 (CRD51542)          LPCDVLLTPHPGASGWN------YAAGTSAGAKAMTCQDYADSAERNFDAQLKKQREKQR
CAU-1 (CAC87665)       IPTDVMLPAHEEQGNLLAKRQKQLRGDPNAFVDPGELARFVDASEAAFNKELARQQAAGP
FEZ-1 (WP_058468956)   MRCDIFLGSHAGMFDLKNKYVLLQKGQNNPFVDPTGCKNYIEQKANDFYTELKKQETA--
PLN-1 (KIO75746.1)     LKFDMWLSSHASQFGLLTKHKPGDAYNPAAFIDQKGYDSAIRDLEDKFLRKE*-------
GOB-1 (AAF04458.1)     LDFDIWVASHASQFDLHEKRKEGDPYNPQLFMDKQSYFQNLNDLEKSYLDKIKKDSQDK-
                       :  *: :  *    .                  .              :  :

L1 (CRD51542)          --
CAU-1 (CAC87665)       NR
FEZ-1 (WP_058468956)   --
PLN-1 (KIO75746.1)     --
GOB-1 (AAF04458.1)     --
```

Figure 10 - Protein sequence alignment with representative enzymes of subgroups of B3 metallo-β-lactamases and PNL-1. The amino acids of their active site are highlighted.

3.6 Analysis of *bla*$_{LUS-1}$ and *bla*$_{PLN-1}$ genetic environment

The gene *bla*$_{LUS-1}$ is located in contig 45 of *P. lusitanus* NL19 genome. The gene downstream of *bla*$_{LUS-1}$ (TH53_RS11255; Figure 11) encodes an outer membrane protein involved in transcription regulation (Appendix 4). The upstream region has a number of genes encoding several proteins of unkown function, a protease (TH53_RS11295), an ABC transporter and transcriptional regulators (Appendix 4). The presence of LUS-1 homologues in the genome of the other *Pedobacter* spp. strains revealed that *P. cryoconitis* has a gene (AY601_RS03400) encoding a protein with 78% identity to LUS-1 (Figure 11). Curiously, the proteins encoded by the upstream genes of AY601_RS03400 also have homology with the proteins encoded by the upstream genes of *bla*$_{LUS-1}$ (Figure 11; Appendix 5).

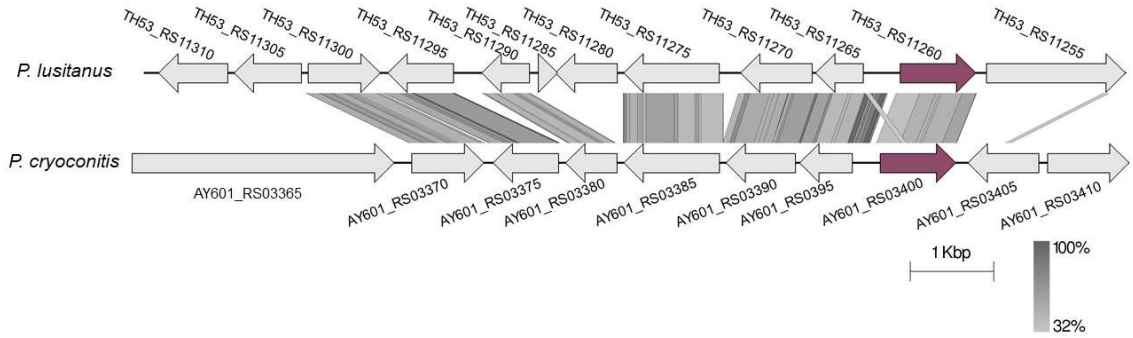

Figure 11 - Schematic presentation of genetic environment of *bla*$_{LUS-1}$ of *P. lusitanus* genome and its homologe in *P. cryoconitis* PAMC 27485 genome and percentage of similarity between both clusters.

Regarding *bla*$_{PLN-1}$, it is located in contig 85 of *P. lusitanus* NL19 genome. The genes downstream of *bla*$_{PLN-1}$ (Figure 12 and Appendix 6) encode an unknown protein (TH53_RS18920) and a protein putatively involved in the metabolism of carbohydrates (TH53_RS18925). The upstream region has genes encoding proteins of unknown function and a transcriptional regulator (TH53_RS18900). The genomes of the other *Pedobacter* spp. strains were screened for the presence of PLN-1 homologues. The results showed that *P. hartonius* has an encoded protein with 70% identity with PLN-1 (SAMN05443550_101322). Contrary to the observed for *bla*$_{LUS-1}$ the genetic environments of *bla*$_{PLN-1}$ and SAMN05443550_101322 are not similar. In *P. hartonius*, genes encoding proteins of unknown function and proteins related with nucleoside and oxidation-reduction metabolisms surround the β-lactamase gene (Figure 12 and Appendix 7).

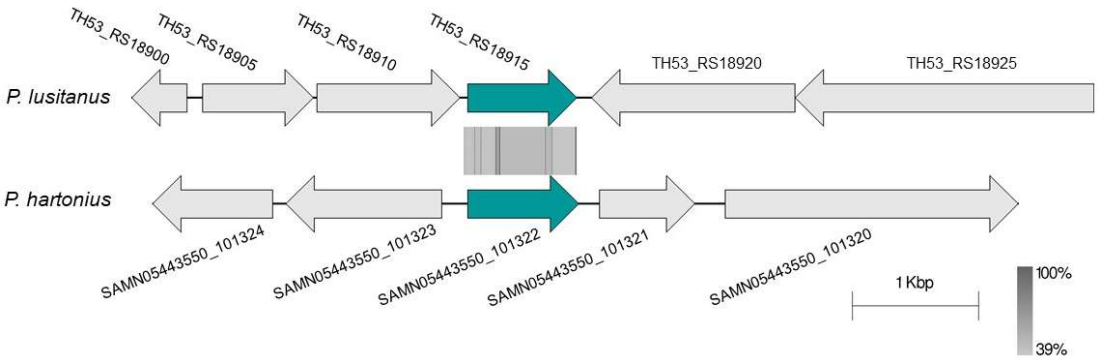

Figure 12 - Schematic presentation of genetic environment of *bla*$_{PLN-1}$ of *P. lusitanus* genome and its homologe in *P. hartonius* DSM 19033 genome and percentage of similarity between both clusters.

3.7 Analysis of *bla*$_{LUS-1}$ and *bla*$_{PLN-1}$ expression and resistance phenotype in *E. coli*

In order to study the resistance phenotype of *bla*$_{LUS-1}$ and *bla*$_{PLN-1}$, the genes were cloned pCDFDuet and MICs to several β-lactams were determined. There were no differences in MICs of *E. coli* containing the empty pCDFDuet and pCDFDuet containing *bla*$_{LUS-1}$ or *bla*$_{PLN-1}$. SDS-PAGE whole-cell protein analysis showed that both proteins are expressed after IPTG induction (Figure 13). It is known that mature β-lactamases are located in the periplasmic space, where they can hydrolyze β-lactams. In Gram-negative bacteria, there are two main export systems (Sec and Tat) that translocate proteins to the periplasm along with the cleavage of a signal sequence. Bacteria use these system to transport their β-lactamases [123]. Therefore, the results obtained suggest that the

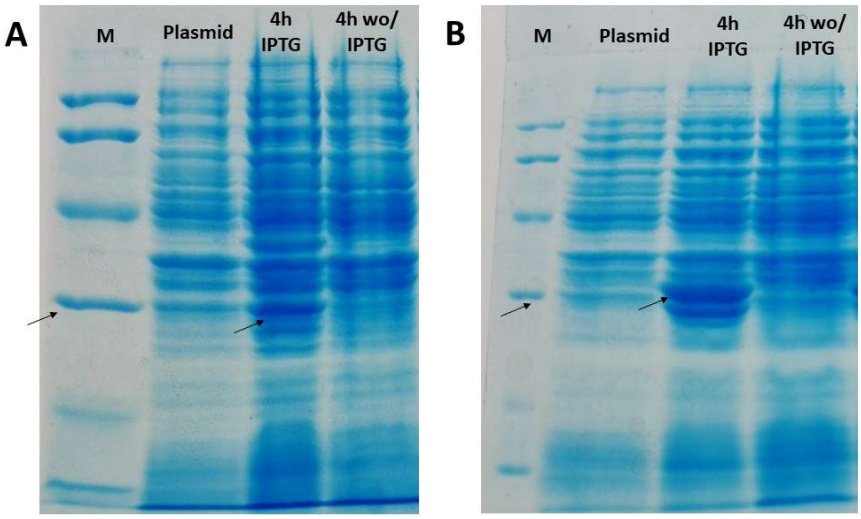

Figure 13 - Whole cell protein analysis of *E. coli* expressing native PLN-1 (A) and native LUS-1 (B) with and without IPTG induction. The extract of *E. coli* transformed with the empty pCDFDuet was also included in the analysis (plasmid). M – molecular weight standards (kDa): lysozyme (14 400); soybean trypsin inhibitor (21 500); carbonic anhydrase (31 000); ovalbumin (45 000); bovine serum albumin (66 200); and phosphorylase b (97 400).

transport machinery of *E. coli* is not able to correctly recognize and cleave the signal sequences of *P. lusitanus* β-lactamases.

To overcome the transport constraint, both genes were cloned in pET-26b(+). With this expression vector, the native signal sequences of LUS-1 and PLN-1 and enzymes were replaced by an *E. coli* signal sequence. The *E. coli* expressing fused LUS-1 had an increased MIC only to cefotaxime (Table 13) when compared with the MICs of *E. coli* transformed with the empty vector. Nevertheless, the difference detected was very low. The MICs of the strains expressing the fused PLN-1 carbapenemase increased for all β-lactam tested (Table 13). Therefore, PLN-1 hydrolise carbapenesms, penicillins, cephamycines and 1st, 3rd and 4th generation cephalosporins. Moreover, PLN-1 activity was inhibited by EDTA (Table 13). Taken together, these results support the assigned classification of this enzyme, since subclass B1 and B3 β-lactamases have a broad-spectrum activity against carbapenems and cephalosporins [25,60]. These results also demonstrate that the inactivity of LUS-1 should not be due to lack of protein translocation to the periplasm. Other possibilities can be: i) the amino acid changes observed in ExxLN motif can originate an inactive β-lactamase or ii) LUS-1 requires other folding and/or post-translational modifications to achieve its function.

Table 13 - Minimum inhibitory concentrations of β-lactams for *E. coli* expressing fused LUS-1 and PLN-1 β-lactmases and for *E. coli* containing the empty pET-26b(+) vector. Results are in mg/L.

Antibiotics	**pET-26b(+)**	**pET-26 LUS-1**	**pET-26 PLN-1**	
			Without EDTA	**With EDTA**
Ampicillin	1	1	> 64	-
Imipenem	0,25	0,25	4	0,25
Meropenem	0,03	0,03	> 2	0,03
Ertapenem	0,008	0,008	> 1	0,008
Cefazolin*	2	2	32	-
Ceftazidime*	0,06	0,06	> 4	-
Cefotaxime*	< 0,008	0,03	> 1	-
Cefepime*	0,03	0,03	0,25	-
Cefoxitin*	1	1	32	-

* Cephalosporins: cefazolin (1st generation), cefoxitin (cephamycines), cefotaxime and ceftazidima (3rd generation) and cefepime (4th generation) [124].

3.8 Presence of plasmids in *Pedobacter* spp. strains

Plasmids are described as genetic elements that very frequently contain genes confering resistance to antibiotics and metal tolerance [125]. Genes of this nature are often present in the same plasmid contributing to co-resistance [21]. Therefore, the presence of plasmids in *Pedobacter* spp. strains was investigated by alkaline lysis extraction and by PFGE. For PFGE DNA plugs were previously digested with S1 nuclease (Figure 14). Plasmids were not detected with any of the approaches. A cryptic plasmid from a *P. cryoconitis* strain (BG5) [125] was already characterized, nonetheless it could not be detected in the *P. cryoconitis* DSM 14825 strain herein analysed.

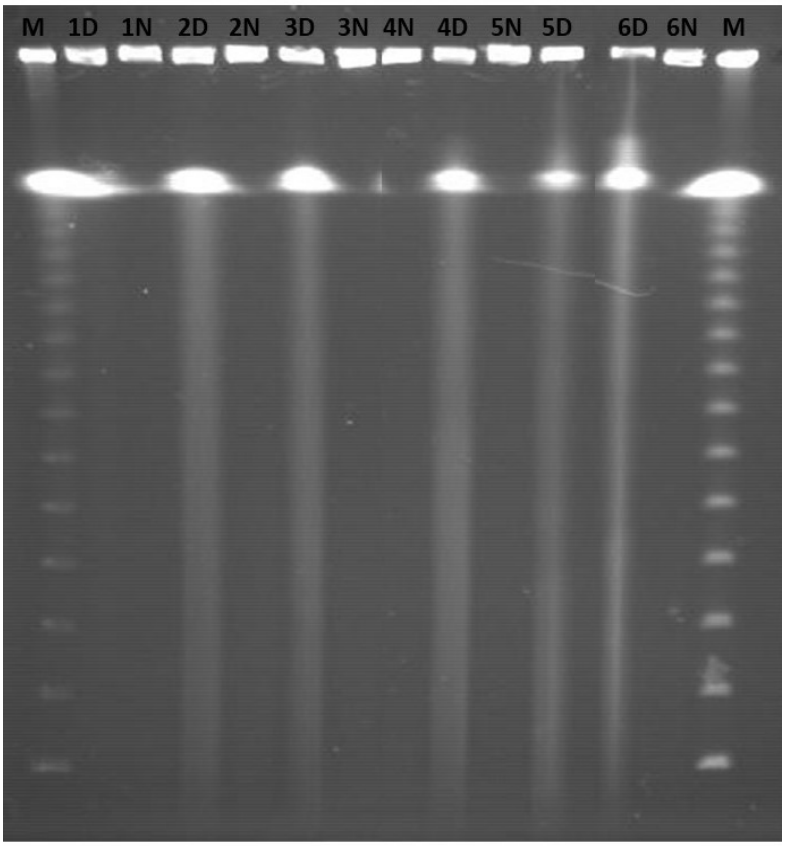

Figure 14 - PFGE of *Pedobacter* spp. strains plugs digested (D) or undigested (N) with S1 enzyme. M - molecular weight; 1 - *P. lusitanus* NL19; 2 - *P. hartonius* DSM 19033; 3 - *P. himalayensis* MTCC 6384; 4 - *P. heparinus* DSM 2366; 5 - *P. cryoconitis* DSM 14825; 6 - *P. westerhofensis* DSM 19036.

Chapter IV. Conclusion

Clinical antibiotic resistance depends on how bacteria respond to therapy, which allows to classify them as susceptible or resistant to a given compound. From the microbiological point of view, resistant microorganisms are "those that possess any type of resistance mechanism or resistance gene" [27]. The results obtained in this study showed a common resistance phenotype for *P. lusitanus* NL19 and its closely-related species as well as for the genus type strain. All the strains are resistant to more than three antibiotics of different classes. Thus, we can consider that the strains of the genus *Pedobacter* herein analysed are MDR strains. Possibly, some of this resistance is innate to the genus *Pedobacter*. In fact, *Pedobacter* spp. strains possess a high number of genes putatively associated with antibiotic resistance, as shown by the *in silico* analysis of their genomes. There were some differences in the MICs obtained for *P. lusitanus* NL19 in our work and those that are described in literature. Therefore, in the future, it would be important to understand if the temperature of growing can be a factor to be considered, that is behind these differences.

Microorganisms adapt to the conditions of the environment to survive [22]. Thus, in the presence of metal(loid)s, microorganisms have also created ways to be able to cope with this toxicity [79]. *P. heparinus* and *P. westerhofensis* were the strains exhibiting the lowest MICs to metal(loid)s and *P. heparinus* and *P. hartonius* were those with the lower number of genes associated with their tolerance mechanisms. *P. lusitanus* was isolated from the sludge of an uranium mine. Because of this, it was expected that this strain was more tolerant to metal(loid)s than the other strains. However, its MICs for non-essential metal(oid)s were similar to those of *P. himalayensis* and *P. cryoconitis*, except for cadmium that was higher. Therefore, at least some sub-groups of *Pedobacter* spp. seem to be well adapted to the presence of metal(loid)s in the environment. Bioremediation by microorganisms with the capacity to degrade or accumulate contaminants in order to remove or reduce them from the environment can be important in areas highly contaminated [21]. In fact, it has been suggested that psychrophiles belonging to the genus *Pedobacter* have potential for biotechnological applications, including bioremediation, detergent production and in the textile and food industries [13]. Therefore, in the future, it would be important to investigate the potencial of the strains herein analyzed for bioremediation of contaminated sites. Also, associations between types and level of metal contamination and the antibiotic resistance patterns have been described for various species [58]. Thus, it would be interesting to verify which mechanisms of antibiotic resistance of *Pedobacter* spp. strains are related with the exposure to different metal(loid)s. Other relevant topic is the lack of standardization of the procedures employed to determine the MICs of metal(loid) elements. Differences in MIC values of metals between broth and agar media were already described [116]. Thus, it would be

important to standardize procedures and guidelines to the determination of the MIC values in agarized medium.

For many years, the antibiotic action and resistance was almost exclusively studied in clinical bacteria. Nowadays, this is changing and more attention is being drawn to environmental bacteria [27]. In this context, the effect of antibiotics and antibiotic resistance in soil bacteria urges because in the future, resistance genes that already exist in these microorganims may arise in the clinical setting [126]. Thus, the identification of the two new β-lactamases described in this study may be important from the clinical point of view to predict mechanisms of resistance that may appear. From an evolutionary perspective, the identification of the two new β-lactamases could also contribute to a better understanding of the origin of β-lactamases, since the origin of resistance genes that encode metallo-β-lactamases are still unknown [25]. It is only known that serine- and metallo-β-lactamases are enzymes that originated more than two billion years ago [29]. Also, it would be interesting to follow the analysis of antibiotic hydrolysis *in vitro*, similar to what was done in TLA-1 β-lactamases identification study [127]. Moreover, the function of the genes upstream of LUS-1 should be investigated in order to understand if LUS-1 function is dependent of some unknown post-translational modification. Additionally, the results of the present study revealed that all *Pedobacter* spp. strains analyzed were resistant to colistin. Thus, it would be interesting to investigate which mechanism(s) are contributing to this phenotype.

Overall, the main aim of the present thesis, which was to charactecterize the antibiotic and metal(loid) susceptibility of *Pedobacter lusitanus* NL19, its closely-related species (*P. hartonius* DSM 19033, *P. himalayensis* MTCC 6384, *P. cryoconitis* DSM 14825 and *P. westerhofensis* DSM 19036) and the type strain of *Pedobacter* genus (*P. heparinus* DSM 2366) was successfully achieved. In my perspective, this study contributed with knowledge and valuable information to a more in-depth understand of the phenotype and associated molecular mechanisms, of *P. lusitanus* NL19, its closely-related species and also the type strain of *Pedobacter* genus. The results of the study showed that these strains have a large pool of resistance genes and their function/relevance should be further exploited.

Chapter V. References

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Chapter VI. Appendices

Appendix 1 – Results of disk diffusion testing available in the literature for closely-related species of *P. lusitanus* NL19 and genus type strain [18–20]. Cells from antibiotics without data were left empty.

Antibiotic (µg per disc)	Strains				
	P. himalayensis	*P. cryoconitis*	*P. westerhofensis*	*P. hartonius*	*P. heparinus*
Fusidic acid		R			
Nalidixic Acid (30)	R				
Amikacin (16)		R			R
Amikacin (30)	S		R	R	
Amoxicillin (100)	R	R			
Ampicillin (10)	R	R	R	R	
Ampicillin (8)		R			R
Aztreonam (30)		R	R	R	
Bacitracin (10 IU)			S	S	
Carbenicillin (128)					S
Cephalexin (16)					S
Cefalotin (16)					S
Cefalotin (30)			R	R	
Cefalotin (8)		R			
Cefamandole (16)		R			S
Cefazolin (30)	R	R	R	R	
Cefetazidime		R			
Cefoperazone (75)	R				
Cefotaxime (30)		R	R	R	
Cefoxitin (10)					S
Cefuroxime (20)	R				
Ciprofloxacin (30)	R	R			
Clindamycin (50)	S	S			
Chloramphenicol (30)	R		I	R	
Chloramphenicol (8)		R			S
Colistin (10)	R		R	R	
Cotrimoxazol (160)					S
Cotrimoxazol (25)	S				
Defotaxime (30)	R				
Doxicicline (100)	S	S			
Doxicicline (30)			S	S	
Erythromycin (15)	R	R	I	I	
Erythromycin (4)					S
Gentamicin (10)		R	R	R	
Gentamicin (4)		R			R
Imipenem (10)		R	S	S	
Kanamycin (2)					R
Kanamycin (30)	R		R	R	
Lincomycin (2)	R		I	R	
Lomefloxacin (30)	S				
Methicillin (4)					S
Mezlocillin (30)		R	R	R	
Neomycin (30)			R	R	
Nitrofurantoin (100)			R	R	
Nitrofurantoin (300)	R				
Norfloxacin (10)	S	R	R	R	
Novobiocin (30)	R				
Ofloxacin (5)			R	R	
Oxacillin (5)		R	R	R	
Penicillin (0.03)					S
Penicillin (0.3)		R			
Penicillin (10)	R				
Penicillin G (6)			R	R	
Polimixine B (300 IU)			R	R	
Roxithromycin (30)	R				
Sisomycin (10)					R
Streptomycin (10)	R	R			
Sulfamethoxazole (128)					
Sulfamethoxazole (16)		S			
Sulfamethoxazole (50)	S				
Tetracycline (2)		R			S
Tetracycline (30)	R		S	S	
Ticarcillin (75)			R	R	
Tobramicina (15)	S				
Tobramicina (4)					
Trimethoprim (50)	S				
Vancomycin (30)	S		R	R	

Appendix 2 – CARD results: number of predicted genes selected and organized by different categories for strains of *Pedobacter* genus with available genomes.

Categories	1	2	3	4	5
Inactivation enzymes	38	19	16	18	28
Target modification	39	31	47	42	35
Target replacement	4	4	3	4	4
Target protection	7	5	9	5	6
Efflux systems	164	106	183	100	155
Others	116	112	95	98	118
Total	368	277	353	267	346
Proportion of total genes	8,00%	5,98%	8,14%	6,35%	7,02%

Strains: 1 - *P. lusitanus* NL19; 2 - *P. hartonius* DSM 19033; 3 - *P. himalayensis* MTCC 6384; 4 - *P. heparinus* DSM 2366; 5 - *P. cryoconitis* PAMC 27485.

Appendix 3 – RAST results: number of predicted genes involved in different resistance/tolerance mechanisms for strains of *Pedobacter* genus with available genomes.

Resistance mechanisms	1	2	3	4	5
Copper homeostasis/tolerance	7	7	13	4	10
Cobalt-zinc-cadmium resistance	43	20	12	28	43
Zinc resistance	2	3	0	2	2
Arsenic resistance	3	3	2	3	4
Total	55	33	27	37	59
Proportion of total genes	1,19%	0,75%	0,62%	0,88%	1,20%

Strains: 1 - *P. lusitanus* NL19; 2 - *P. hartonius* DSM 19033; 3 - *P. himalayensis* MTCC 6384; 4 - *P. heparinus* DSM 2366; 5 - *P. cryoconitis* PAMC 27485.

Appendix 4 – Predicted biological process and cellular localization of genes of *P. lusitanus* flanking class A β-lactamase.

Gene	Biological Process Prediction	Cellular Localization Prediction
TH53_RS11255	Regulation of transcription	Outer Membrane
TH53_RS11260	β-lactam antibiotic catabolic process	Periplasmic
TH53_RS11265	Regulation of transcription	Cytoplasmic
TH53_RS11270	None predicted	Cytoplasmic
TH53_RS11275	Transmembrane transport	Cytoplasmic Membrane
TH53_RS11280	Regulation of transcription	Cytoplasmic
TH53_RS11285	None predicted	Unknown
TH53_RS11290	None predicted	Unknown
TH53_RS11295	Proteolysis	Non-Cytoplasmic
TH53_RS11300	None predicted	Cytoplasmic Membrane
TH53_RS11305	Regulation of transcription	Unknown
TH53_RS11310	None predicted	Unknown

Appendix 5 – Predicted biological process and cellular localization of genes of *P. cryoconitis* PAMC 27485 flanking class A β-lactamase.

Gene	Biological Process Prediction	Cellular Localization Prediction
AY601_RS03410	Catalytic activity	Periplasmic
AY601_RS03405	Regulation of transcription	Cytoplasmic
AY601_RS03400	β-lactam antibiotic catabolic process	Periplasmic
AY601_RS03395	Regulation of transcription	Cytoplasmic
AY601_RS03390	None predicted	Cytoplasmic
AY601_RS03385	Transmembrane transport	Cytoplasmic Membrane
AY601_RS03380	None predicted	Unknown
AY601_RS03375	Proteolysis	Unknown
AY601_RS03370	None predicted	Cytoplasmic Membrane
AY601_RS03365	Transport	Cytoplasmic

Appendix 6 – Predicted biological process and cellular localization of genes of *P. lusitanus* flanking metallo-β-lactamase.

Gene	Biological Process Prediction	Cellular Localization Prediction
TH53_RS18900	Regulation of transcription	Cytoplasmic
TH53_RS18905	Catalytic activity	Extracellular
TH53_RS18910	Peptidase	Unknown
TH53_RS18915	Metallo-β-lactamase	Non-Cytoplasmic
TH53_RS18920	Metallopeptidase activity	Extracellular
TH53_RS18925	Carbohydrate metabolic process	Periplasmic

Appendix 7 – Predicted biological process and cellular localization of genes of *P. hartonius* flanking metallo-β-lactamase.

Gene	Biological Process Prediction	Cellular Localization Prediction
SAMN05443550_101320	Transport	Outer Membrane
SAMN05443550_101321	Nucleoside metabolic process	Cytoplasmic
SAMN05443550_101322	Metallo-β-lactamase	Periplasmic
SAMN05443550_101323	Catalytic activity	Non-Cytoplasmic
SAMN05443550_101324	Oxidation-reduction process	Cytoplasmic