Universidade de Aveiro Departamento de Química
2016

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Análise genómica global da resistência a antibióticos e metais em *Pseudomonas* e *Chromobacterium*

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Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biotecnologia Molecular, realizada sob a orientação científica da Doutora Isabel da Silva Henriques Investigadora Auxiliar do Departamento de Biologia da Universidade de Aveiro, e da Doutora Marta Cristina Oliveira Martins Tacão, investigadora em pós-doutoramento no Departamento de Biologia da Universidade de Aveiro.

Apoio financeiro da Fundação para a Ciência e a Tecnologia e Fundo Social Europeu no âmbito do III Quadro Comunitário de Apoio através do projeto de referência IF/00492/2013

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agradecimentos

Antes de tudo, quero agradecer aos meus pais por me terem dado a oportunidade de estudar numa das melhores universidades de Portugal e também pela paciência e apoio incondicional que me deram no dia a dia. Agradeço também aos meus familiares que estiveram sempre presentes e que me motivaram durante todo o percurso académico.

À Doutora Isabel Henriques e à Doutora Marta Tacão, o meu sincero agradecimento pela constante atenção e acompanhamento ao longo do ano. Receberam-me de braços abertos no seu grupo de trabalho e foi realmente uma experiência muito gratificante para mim.

Agradecer a todo o Microlab pelo seu tremendo espírito de equipa e também pela forma como me receberam e me fizeram sentir em casa. Quero agradecer especialmente à Jaqueline Rocha pela ajuda e orientação no laboratório e pela boa disposição e disponibilidade total. Agradeço também à Marta Alves pela a ajuda na submissão dos genomas, e sobretudo pela sua contagiante boa disposição e pelos seus conselhos.

Tenho também de agradecer aos meus amigos de sempre que me apoiaram nos momentos mais complicados, seja nos dias de trabalho na Mediateca ou nos cafés à noite.

palavra-chave

Antibióticos, resistência a antibióticos, *Pseudomonas aeruginosa*, co-selecção, resistência a metais, *Chromobacterium haemolyticum,* elementos genéticos móveis

resumo

Atualmente, a resistência a antibióticos constitui uma grande ameaça para a saúde pública, reconhecida globalmente. O uso excessivo e incorreto de antibióticos é considerado um problema sério que contribui para o aparecimento de bactérias resistentes responsáveis pelo aumento da taxa de mortalidade de pacientes infetados. Para além disso, a contaminação ambiental com estes compostos é também considerada um factor potenciador do aumento da prevalência de resistência a antibióticos.

O objetivo deste estudo foi analisar o perfil de resistência e avaliar a co-seleção para características de resistência a antibióticos e metais em isolados ambientais de *Pseudomonas* e *Chromobacterium* utilizando uma estratégia de sequenciação dos genomas. Para isso, os genomas dos isolados foram sequenciados e a identificação destes foi confirmada através de uma análise filogenética baseada na sequencia de marcadores moleculares. As características fenotípicas obtidas foram consistentes com a afiliação *P. aeruginosa* e *C. haemolyticum.* A análise do genoma efectuada com base em várias ferramentas bioinformáticas permitiu a identificação de genes provavelmente envolvidos na resistência a antibióticos β-lactâmicos , aminoglicosídeos, cloranfenicol e polimixinas. Vários determinantes genéticos associados a sistemas de fluxo responsáveis pela expulsão de vários antibióticos foram também detectados. Determinantes génicos que contribuem para a motilidade destes genes foram também identificados, como por exemplo integrases, relaxases, transposases e recombinases .

Os resultados obtidos mostram que ambos isolados *P. aeruginosa* E67 e *C. haemolyticum* IR17 possuem um vasto arsenal de determinantes de resistência codificados no seu genoma, e que no caso do isolado de *Pseudomonas*, provavelmente contribuem para a sua sobrevivência num ecossistema altamente poluído. Para além disso, alguns destes genes encontram-se associados a estruturas móveis, o que enfatiza o contributo destas plataformas no desenvolvimento de fenótipos de multirresistência.

Desta forma, este trabalho possibilitou um avanço no conhecimento global do resistoma destas duas espécies, reforçando o interesse em estudar isolados ambientais que podem conter mecanismos de resistência com relevância clínica.

keywords

Antibiotics, antibiotic resistance, metal resistance, *Pseudomonas aeruginosa*, co-selection, *Chromobacterium haemolyticum,* mobile genetic elements

abstract

Nowadays, antibiotic resistance is a well-acknowledged global health problem. The overuse and misuse of antibiotics is considered to be a serious threat that contributes to the appearance of clinically relevant resistant bacteria responsible for the increase of infected patients mortality rates. Furthermore, the environmental contamination with these compounds is also considered an enhancer factor to the increase the antibiotic resistance prevalence.

The aim of this study is to analyse the resistance profile and to assess co-selection for antibiotic and metal resistance traits in *Pseudomonas* and *Chromobacterium* environmental isolates using a WGS approach. To do this, the isolates genomes were sequenced and the species identification thereof was confirmed by phylogenetic analysis based on the sequence of molecular markers. The phenotypic characteristics observed were consistent with the affiliation with *P. aeruginosa* and C*. haemolyticum.* Genome analysis using several bioinformatics tools allowed identifying genes probably involved in resistance to β-lactams, aminoglycosides, chloramphenicol and polymyxins. Many genetic determinants associated to efflux systems able to export a variety of compounds were also identified. Genetic determinants contributing to the motility of genes, such as integrases, relaxases, transposases and recombinases were identified, for instance.

Results show that both *P. aeruginosa* E67 and *C. haemolyticum* IR17 have a vast arsenal of resistance determinants encoded in its genome, and in the case of the *Pseudomonas* isolate, it probably contributes to its survival in a highly polluted ecosystem. In addition, some of these genes are associated with mobile structures, which emphasizes the contribution of these platforms in the development of multidrug resistance phenotypes. Thus, this work enabled a breakthrough in the global knowledge of the resistome of these two species, reinforcing the interest in studying environmental isolates that may contain mechanisms of resistance of clinical relevance.

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ABBREVIATIONS

ABC	ATP-binding cassette	**MATE**	Multidrug and toxic compound extrusion
AMC	Amoxicillin/ clavulanic acid	**Mbp**	Mega base pairs
AMP	Ampicillin	**MBL(s)**	metallo-β-lactamase(s)
AMX	Amoxicillin	**MDR**	Multidrug Resistance
ATM	Aztreonam	**MFS**	Major facilitator superfamily
AR	Antibiotic Resistance	**MGAP**	Microbial Genome Annotation Pipeline
ANI	Average Nucleotide Identity	**MIC**	Minimum Inhibitory Concentration
ARDB	Antibiotic Resistance Database	**MEGA**	Molecular Evolutionary Genetics Analysis
ARG	Antibiotic Resistance Genes	**MLST**	Multilocus Sequence Typing
ATP	Adenosine triphosphate	**MRSA**	Methicillin-*Staphylococcus aureus*
BLAST	Basic Local Alignment Search Tool	**NA**	Nalidixic acid
bp	Base pairs	**NCBI**	National Center for Biotechnology Information
C	Chloramphenicol	**NGS**	Next-Generation Sequencing
CARD	Comprehensive Antibiotic Resistance Database	**OD_{600}**	Optical Density at 600 nm
CDS	Coding Sequences	**PABA**	Para-aminobenzoic acid
CRISPR	Clustered regularly interspaced palindromic repeats	**PAGI**	*Pseudomonas aeruginosa* Genomic Island
CTX	Cefotaxime	**PBP**	Penicillin Binding Protein
DMT	Drug/Metabolite transporter	**PCR**	Polymerase Chain Reaction

DHFR	Dihydrofolate reductase	**PEG**	Protein encoding gene
DNA	Deoxyribonucleic acid	**PFGI-1**	*Pseudomonas fluorescens* Genomic Island 1
DOE-JGI	Department of Energy Joint Genome Institute	**RAST**	Rapid Annotation using Subsystem Technology
ESLB(s)	Extended Spectrum β-lactamase(s)	**rDNA**	Ribosomal DNA
EUCAST	European Committee on Antimicrobial Susceptibility Testing	**RGI**	Resistance Gene Identifier
ETP	Ertapenem	**RNA**	Ribonucleic acid
FEP	Cefepime	**rRNA**	Ribosomal RNA
GC	Guanosine and Cytosine	**RND**	Resistance nodulation division
GI	Genomic Island	**SMR**	Small Multidrug Resistance
HTH	Helix-turn-Helix	**SNP**	Single Nucleotide Polymorphism
HGT	Horizontal Gene Transfer	**SRS**	Short Read Sequences
ICE	Integrative Conjugative Element	**ST**	Sequence Type
IMG/ER	Integrated Microbial Genomes/ Expert Review	**SXT**	Sulphamethoxazole/Trimethropim
IPM	Imipenem	**THF**	Tetrahydrofolic acid
K	Kanamycin	**TE**	Tetracycline
KF	Cephalotin	**tRNA**	Transfer RNA
Kb	Kilo base pairs	**WGS**	Whole-genome sequencing
KPC	*Klebsiella pneumoiae* carbapenemase		
LA	Luria-Bertani Agar		
LB	Luria Broth		

INTRODUCTION

1 Antibiotics

When in the year 1928 Alexander Fleming found by chance that a mould contamination of *Penicillium notatum* in a petri dish was responsible for the growth inhibition of staphylococci colonies due to the production of an antibacterial compound called penicillin, he never thought that 12 years later, this new discovery would revolutionise the practices of modern medicine by allowing the treatment of infectious diseases that were till then a death sentence[1,2]. Along with penicillin, other antibiotics such as sulphonamides and streptomycin were also discovered and its usage in surgical interventions and organ transplants would start to be a standard procedure in most hospitals and clinical facilities, marking the dawn of a new era in human healthcare[3,4].

The term antibiotic can be defined as a chemical substance that kills or inhibits the growth of microorganisms which can be produced by the microorganisms themselves as part of their secondary metabolism, by plants and also produced artificially[5,6]. Antibiotics that kill bacteria are termed bactericidal while antibiotics that inhibit the growth with no loss of cellular viability are considered bacteriostatic[7].

Synthetic antimicrobials drugs, such as sulphonamides inhibitors of folate metabolism, are chemicals exclusively produced artificially in the laboratory that started being applied first in the early 1900s with the same purpose of killing or inhibiting microorganisms[5]. Nowadays, sulphonamides are still one of the most widely used classes of antibiotics alongside with other artificial classes of antibiotics that have been developed over time such as quinolones and trimethoprim[8,9]. Despite the success of the naturally occurring antimicrobial drugs, researchers found out that they can be artificially modified to increase their efficiency and thus defined another class named semi-synthetic antibiotics[3].

Over the years, several new antibiotics have been developed through constant research and since the time when penicillin started to be used clinically, the rate of mortality caused by bacterial infections has dropped abruptly[3,10]. Furthermore, the field of application of these antibacterial compounds has expanded to other areas rather then

medicine such as agriculture, animal health and aquaculture, emphasising the great importance of antibiotics in today's society[11,12].

1.1 Classes of antibiotics and mechanisms of action

Currently, several classes of antibiotics have been recognized and described according to their mechanism of action (Figure 1)[9]. Antibiotics can have as target the cell's protein synthesis, transcription, cell wall synthesis, the cytoplasmic membrane structure, DNA replication and folic acid metabolism[5,7].

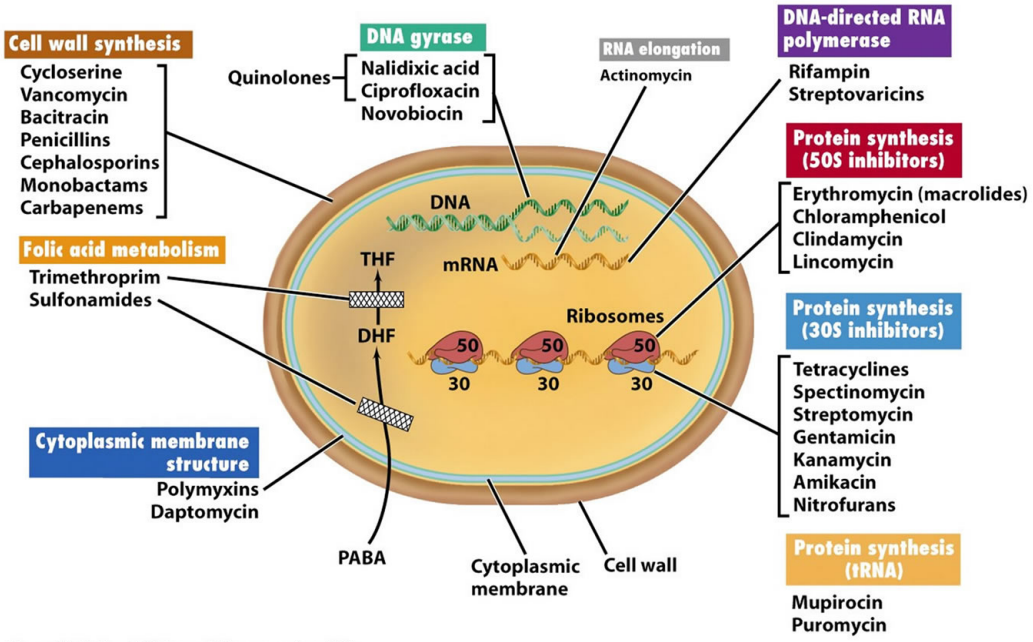

Figure 1 Representation of the cellular structures that constitute the main targets of the major antimicrobial compounds[5].

1.1.1 Antibiotics targeting cell wall synthesis

The bacterial cell wall is a rigid structure composed by layers of peptidoglycan, a covalently crossed-linked polymer matrix composed of peptide-linked β-(1–4)-N-acetyl hexosamine, which is located outside the cytoplasmic membrane and has the important role of conferring structural strength to the cell and protection from osmotic lysis[5,7]. Although peptidoglycan is common to all bacterial cell wall composition, its abundance varies when looking at Gram-positive bacteria, which have a relatively thick layer of this

complex polysaccharide, and Gram-negative bacteria, which possess only a thin layer of peptidoglycan but have an outer-bilayer[13].

β-Lactams antibiotics are among the oldest groups of antibiotics and however, they remain one of the most used group of antibiotics in clinical settings[14]. Since the discovery of penicillin, other β-Lactam antibiotics have been discovered, possessing some molecular structure variations that characterize all the β-Lactam sub-classes **(**Table 1**).** However, all the antibiotics belonging to the β-Lactam family have in common one characteristic structural component, the β-Lactam ring[5,14].

Table 1 Brief description of the different β -lactam antibiotics sub-classes[15–17].

Sub-classes	Description	Examples
Penicillins	Short range antibiotics that can be divided in five categories: natural penicillins; penicillinase-resistant penicillins, aminopenicillins, extended spectrum penicillins and aminopenicillin/β lactamase inhibitor combinations	Penicillin G; Ampicilin; Amoxicillin; Ticarcillin; Piperacillin;
Cephalosporins	They share the same mechanism of action and have a similar structure of penicillins, having an additional atom in the side ring. These antibiotics are generally more resistant to β-lactamases than penicillins and have been grouped into generations according to their spectrum of activity	**1st Generation:** Cefaloridin; Cefalotin **2nd Generation:** Cefaclor; Cefamandol **3rd Generation:** Ceftazidime; Cefotaxime **4th Generation:** Cefepine
Carbapenems	Broad-spectrum antibiotics used many times as last line agents, which differ structurally from penicillins and carbapenems by the absence of a acylamino substituent on the β-Lactam ring.	Ertapenem; Imipenem; Meropenem
Monobactams	Aztreonam is the only monobactam available and structurally, it contains only the four membered ring of the basic β-lactam structure.	Aztreonem

All β-Lactams share a common mechanism of action, which is the binding of the transpeptidases known as penicillin binding proteins (PBP's) and consequent inhibition of the peptidoglycan layer synthesis[18]. Consequently, the cell wall is debilitated and loses its integrity, resulting in cell lysis also due to the contribution of osmotic pressure differences[7].

Glycopeptides, which include vancomycin, are another class of antibiotics that also affect the cell wall biosynthesis[19]. They inhibit the trans-glycosylation reaction by steric hindrance, having as molecular target the D-alanyl-D-alanine terminus of the cell wall peptidoglycan precursor[9]. It is also relevant the fact that glycopeptides are only effective against Gram-positive bacteria due to low permeability whilst β-Lactams can be used to treat Gram-negative and Gram-positive bacterial infections[7].

Still included in the cell wall biosynthesis-targeting category is fosfomycin, a broad-spectrum antibiotic belonging to the class of phosphonic antibiotics that inhibits phosphoenolpyruvate transferase, the first enzyme involved in the synthesis of peptidoglycan[20].

1.1.2 Antibiotics targeting protein synthesis

In order to stop cell protein synthesis, these antibiotics disrupt the translation process by interacting specifically with the cells ribosomes[5]. Since proteins play a major role in cell function, these drugs have broad activity spectrum and can be divided in two classes: the 50S inhibitors and the 30S inhibitors (Table 2)[7]. The majority of these antibiotics are medically relevant and also important research tools since they block specific steps of protein synthesis such as protein chain initiation and chain elongation[5].

Table 2 Major subclasses of antibiotics targeting cell protein synthesis, and some of the more well known examples, having in to account the classes that inhibit the 30S or the 50S ribosomal subunit[5,7,9].

50S inhibitors (These antibiotics work in general by physically blocking either initiation of protein translation or translocation of peptidyl-tRNA)	-Macrolides (Erythromycin; Dirithromycin; Clarithromycin)
	-Lincosamides (Clindamycin)
	-Streptogramins (Dalfopristin)
	-Amphenicols (Chloramphenicol)
	-Oxazolidinones (Linezolid)
30S inhibitors (Tetracycline's block the access of aminoacyl-tRNA to the ribosome while amynoglicosides bind the 16S rRNA component of the 30S ribosome subunit)	-Tetracyclines (Tetracyclin; Oxytetracyclin)
	-Aminoglycosides (Streptomycin; Gentamicin; Kanamycin; Neomycin; Amikacin)

1.1.3 Antibiotics targeting nucleic acids synthesis

Currently, quinolones and ansamycins are the main antibiotic classes which mechanism of action affects the cell's DNA and RNA synthesis, having a devastating effect on prokaryotic nucleic acid metabolism that most of the times results in bacterial cell death[10]. Fluoroquinolones such as ciprofloxacin and levofloxacin, derive from the quinolone nalidixic acid and like cephalosporins, can be divided in four generations according to their chemical structure and slight differences in the mechanism of action[7]. They interfere with DNA replication and transcription by targeting the bacterial DNA gyrase in Gram-negative bacteria and type IV topoisomerase in Gram-positive, preventing the supercoiling of DNA and thus leading to bacterial death[21].

Ansamycins such as Rifamycin and Naphthomycin, inhibit cell's transcription by acting at the RNA synthesis level. These antibiotics bind with high affinity to the subunit of the RNA polymerase enzyme, leading to the inhibition of nascent RNA strand initializition[7,22].

1.1.4 Antibiotics targeting metabolic pathways

Sulfonamides were the first antimicrobial synthetic drugs to be used systematically and marked the beginning of the antibiotic revolution in medicine[23]. Their classification as antimetabolic drugs comes from the ability to compete with PABA (para-aminobenzoic acid) molecules, which are crucial in the synthesis of nucleotides required for DNA and RNA synthesis[13]. They compete for the active site of the enzyme involved in the production of dihydrofolic acid, resulting in a THF (tetrahydrofolic acid) production decrease and thus of DNA and RNA[13]. Of the several sulphonamides that have been developed so far, sulfamethoxazole is currently the most used in combination with trimethoprim (termed co-trimoxazole), which exerts a synergetic bacterial effect[9].

Trimethoprim is a completely synthetic drug with anti-metabolic activity that also interferes with nucleic acid synthesis through the inhibition of the enzyme dihydrofolate reductase (DHFR) by competitively binding to its active site[13].

2 Antibiotic Resistance

As previously stated in section 1, the discovery and introduction of antibiotics in the human healthcare system became one of the most important achievements of the 20th century[24]. Effectively, during the first couple of years when these drugs were used in medical practices, the bacterial infection diseases that were once almost a death sentence seemed to be a solved situation[4]. However, when Fleming first discovered penicillin, he also predicted that the misuse and overuse of antibiotics could lead to a problematic situation where these compounds would no longer be effective due to the appearance of resistant pathogenic microorganisms[2]. This prediction would turn out to be true, since ten years after the wide scale introduction of penicillin, resistance to antibiotics started to emerge[25].

However, the emergence of resistance occurred long before the first antibiotic was characterized[9]. In fact, it is presently known that antibiotic resistance (AR) is an ancient, naturally occurring phenomenon widespread in the environment and antibiotic resistance genes (ARG) predate our use of antibiotics[26]. Nonetheless, it is presently well known that the mishandling and misprescription of these drugs have transformed bacterial populations in such a way that many antibiotics have partially or entirely lost their effectiveness, making this a serious and growing global public health concern[25,27].

2.1 Bacterial resistance strategies

Antimicrobial drug resistance can be defined as the ability of a microorganism to resist the effects of an antimicrobial agent to which it is normally susceptible[5]. This ability can be carried out by a multitude of mechanisms, being that the nature and efficiency of these resistance tools depend on the species and the particular antibiotic[28]. Thus, the main strategies used by bacteria for protection against the various antibiotic effects are enzymatic modification and degradation, efflux and alterations in the cell wall permeability, target modification and development of alternative resistance biochemical pathways[9,28].

2.1.1 Enzymatic inactivation or modification of the antibiotic

One of the main types of biochemical mechanisms of resistance used by bacteria is the enzyme-catalysed inactivation of antibiotics through structural modifications that result in the inability of the antibiotics to act in general[28,29]. These enzymes can be divided in two general classes: the class where β-lactamases are included, which includes enzymes that degrade antibiotics through hydrolytic activity and the class that include aminoglycoside-modifying enzymes and chloramphenicol acetyltransferases, which perform chemical transformations[19,30].

β-lactamases are among the most well-known and clinically relevant resistance enzymes and they act by hydrolysing the β-Lactam ring of β-Lactam antibiotics (Figure 2), preventing the binding of these antibiotics to the active site of PBP's enzymes[13].

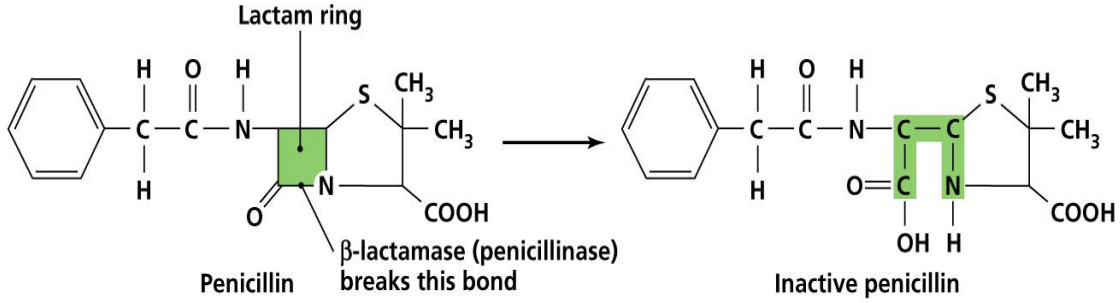

Figure 2 Disruption of penicillin's β-lactam ring by the action of a β-lactamase, resulting in the antibiotic inactivation.[13]

These enzymes were first discovered in the 1940's in penicillin G-resistant isolates of *E. coli* and in the meantime hundreds of β-lactamases have been discovered and characterized[31]. In order to counter the action of these enzymes, β-lactamases inhibitors such as clavulanic acid, sulbactam and tazobactam, were developed and started to be used in combination with β-Lactam antibiotics in order to enhance their efficacy[23].

Currently, there are two valid classifications that complement each other, one based on functional characteristics related to substrate and inhibitor profiles and the other based on molecular characteristics related to amino acid sequences[14]. The molecular classification resulted in the definition of four Ambler-classes, whereas classes A, C and D include the β-lactamases with serine at their active site such as AmpC, class B β-lactamases are metallo-proteases that require zinc as a metal cofactor for their catalytic activity such as NMD-1[9]. On the other hand, the functional classification divides β-

lactamases in group 1 cephalosporinases (class C); group 2, which include broad-spectrum, inhibitor-resistant, extended spectrum β-lactamases (ESBL) and serine carbapenemases (class A and D) and finally group 3 metallo- β-lactamases (MBL) (class B)[14].

Among the class A β-lactamases, ESBL hold a particular significance since, in addition to their broad spectrum activity in antibiotics such as penicillins, cephalosporins and monobactam aztreonam, the occurrence of infections caused by ESBL-producing bacteria has been constantly rising and constitutes a serious threat to human health[12]. Additionally, a family of Class A carbapenemases named as *K. pneumonia* carbapenemases (KPC's) also represent a clinical challenge due to the β-lactam resistance they mediate[32]. Carbapenem resistance as a result of KPC production was first described in a *K. pneumoniae* recovered in North Carolina in 1996[32] and since then twenty three KPC variants have been described, with KPC-2 being the most prevalent worldwide[32,33]. Also, the production of KPC enzymes has been reported in other *Enterobacteriaceae* and the corresponding genes are often associated with transposons and insertion sequences, which contributes to the cross-species transmission of bla_{KPC} genes[34,35]. These enzymes mediate resistance to both carbapenems and extended-spectrum cephalosporins[36], which are considered last line antimicrobials for human medicine, and thus constitute a threat to public health[37]. However, the organism from which KPC has originated is still unknown and recent research shows that bla_{KPC} genes mobilization to plasmids and its further insertion in different open reading frames may be due to the action of a Tn3-based transposon[38].

On the other hand, the group of enzymes responsible for the inactivation of aminoglycosides, chloramphenicol, streptogramin, macrolides and rifampicin are transferases that act not by hydrolysis but through the binding of adenylyl, phosphoryl or acetyl groups to the periphery of the antibiotic molecule, therefore blocking the interaction of these drugs with their target[19].

2.1.2 Membrane permeability and active efflux

Although all bacteria possess a cell wall that constitutes a first line of defence against the entry of small molecules, Gram-negative bacteria's outer membrane confers a higher level of protection, rendering them impermeable to some antibiotics such as

penicillin G and platensimycin[5,8]. However, some antibiotics are able to penetrate the outer membrane by passive diffusion through porin proteins such as OmpF and OprD[8]. In order to restrict the antibiotic influx, some bacteria have developed a low-level resistance mechanism based on the infliction of modifications on the internal eyelet of porins that result in a slow drug entry into the cell[28].

In contrast with the low efficiency and poor specificity of antibiotics influx control, another resistance mechanism consisting of the active expelling of harmful agents present in the cell interior has been proved to have a high efficiency and is able to deal with a wide-range of unrelated antibiotics[19]. The task of active removal of antibiotics is carried out by membrane spanning efflux proteins that can be divided into five different families: the major facilitator superfamily (MFS); the ATP-binding cassette (ABC) superfamily; the small multidrug resistance (SMR) family; the resistance-nodulation-division (RND) superfamily and the multidrug and toxic compound extrusion (MATE) family (Figure 3**)** [23].

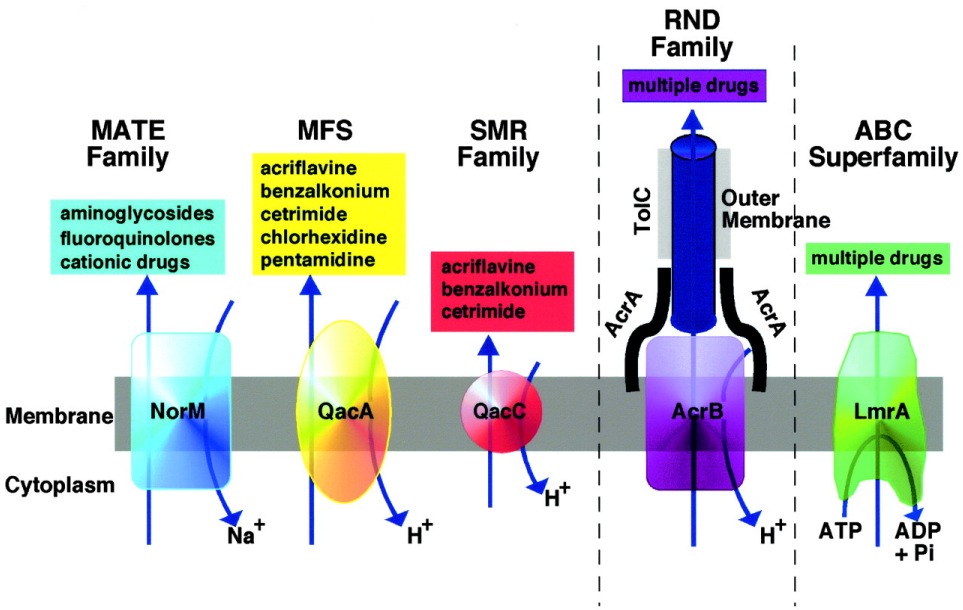

Figure 3 Constitution and targeted antibiotics of the five families of efflux pumps[39].

The RND efflux pumps are the most well studied of all the five families due to their particular relevance in Gram-negative bacteria intrinsic resistance. In fact, evidence suggests that these pumps play a crucial role not only in the bacterial virulence that contributes to the multidrug resistance profile but also bacterial survival, colonization and biofilm formation, which are considered to be their primary physiological function[40].

An alternative resistance mechanism has also been evoked to explain resistance against third generation cephalosporins in some Gram-negative bacteria that cannot inactivate or expel the antibiotic, recurring to the sequestration of the drug in stable intracellular complexes that end up neutralizing its effects[28].

2.1.3 Target modification and molecular bypass

The affinity of antibiotics to their cellular targets can be altered as a result of two different events: point mutations that introduce amino acid substitutions in the target molecules or enzymatic alteration of antibiotics target[41]. The mutational target alteration is well known to be the basis of resistance to quinolones and fluoroquinolones through mutations in *gyrA* and *gyrB* genes that codify for DNA gyrase and also in *parC* and *parE* genes that codify for topoisomerase IV, causing the failure in the replication of these enzymes and therefore, quinolones cannot bind[19]. There is also the case where antibiotics need to be modified by bacterial enzymes to be in its active form, so mutations in the genes coding for these enzymes are a variation of this resistance mechanism[28].

In concern to enzymatic target alteration, a well known resistance mechanism is the enzymatic methylation of certain adenine residues of the 23S rRNA subunit that prevents antibiotics belonging to the macrolides, lincosamides, streptogramins classes to correctly position in the peptidyltransferase domain and consequently the protein synthesis occurs normally[28].

Finally, some bacteria possess a different resistance mechanism that consists in the production of an alternative target or metabolic pathway that is resistant to the antibiotic inhibitory effect and thus allows the bacteria to survive.[19,29]

2.2 Intrinsic and acquired antibiotic resistance

Bacterial AR can be achieved through intrinsic or acquired mechanisms[23]. Intrinsic resistance involves no genetic alterations and implies that the resistance mechanisms are naturally occurring in the genes of the host's chromosome or plasmids, being always present in a given phylogenetic group[30]. This type of resistance is common in entire bacterial species and includes strategies like the resistance of all Gram-negative bacteria to glycopeptides due to the non permeability of the outer membrane, in Gram-negative

bacteria to hydrophilic antibiotics and the resistance of anaerobic bacteria to aminoglycosides since their movement across the cytoplasmic membrane is an oxygen–dependent process[23,28]. Additionally, intrinsic resistance is not affected by the misuse of antibiotics and represents a major contribution to antibiotic research and the pursuit for specific therapies[25].

On the other hand, acquired resistance is based on microorganism's ability to interchange genetic material and also chromosomal mutations[19]. Chromosomal mutations can result in reduced target affinity, alterations in regulatory networks and also reduced access of the antibiotic to its target[28]. Several of these mutation-based resistance events have been reported and constitute a relevant contribution to the bacteria resistance profile: rifampin resistance can be the consequence of point mutations on highly conserved regions of the *rpoB* gene[30], synthetic drugs such as quinolones, sulphonamides and trimethoprim can be ineffective due to single nucleotide polymorphisms (SNPs), macrolide resistance can derive from nucleotide base substitutions in the 23S rRNA gene[9], β-lactam antibiotics resistance can occur owing to chromosomal mutations that result in the overproduction of the AmpC β-lactamase and also efflux pumps can be overexpressed due to mutational events[42], among other examples. However, these point mutations in specific genes typically only confer moderate levels of resistance to antibiotics, since sequential mutations must be accumulated in order to have a sufficient expression that can be considered clinically significant[28].

In contrast, most clinically relevant antimicrobial resistance is a result of the acquisition of exogenous DNA coding for resistance determinants[28]. This genetic material codes for a variety of resistance mechanisms that were referred in section 2.1 and its transfer can be mediated by different mobile genetic elements between organisms belonging to the same genus and also between evolutionary distant organisms, for instance Gram-positive and Gram-negative bacteria[30].

2.2.1 Horizontal gene transfer of resistance genes

The process by which prokaryotes acquire genes from other microbes is entitled Horizontal Gene Transfer (HGT) and involves a donor cell that contributes part of its genome and a recipient cell, which may belong to a different species from the donor[13]. AR

genes (ARG) and other determinants that confer a selective advantage to bacteria can be transferred horizontally between bacteria in the form of different genetic elements through three main mechanisms: transduction, conjugation and transformation **(**Figure 4**)** [4,19].

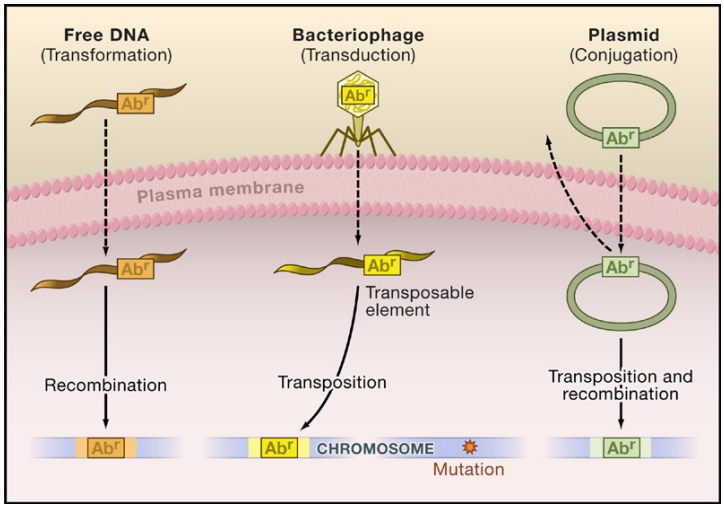

Figure 4 Main strategies used by bacteria for the acquisition of AR[30]**.**

Transformation can occur in both Gram-negative and Gram-positive bacteria who are termed "competent" and consists in the capacity to acquire free DNA from the environment and integrate it into its genome across regions of relative or complete homology through recombination processes[28].

Transduction implies the action of replication virus such as bacteriophages that have the capacity to transfer its DNA to spatially distant bacteria communities[9].

Conjugation is one of the most relevant mechanisms used by bacteria in the exchange of ARG and is performed with the involvement of physical contact between two bacteria mediated by structures named *pili*[13]. Although in transformation and transduction DNA can also be transferred in this form, in conjugation, mobile genetic elements such as plasmids and integrative conjugation elements are of great importance[4].

Plasmids are autonomously replicating circular or linear segments of extra chromosomal DNA that contain their own origin of replication and can be found in many microorganisms in the *Bacteria*, *Archaea*, and *Eukaryota* domains[43]. Plasmids that own conjugation genes are called conjugative and generally carry complete sets of genes necessary for transfer[9]. Most of the so-called R-Plasmids are an example of these plasmids that encode for the exchange functions themselves and are given this designation since

they encode genes that confer resistance to the main classes of antibiotics, toxic metals and virulence determinants that contribute to bacterial survival[19].

Integrative conjugation elements are generally termed conjugative transposons and consist of mobile genetic segments that encode a site-specific transposase that allows them to move from one replicon to another within or between cells[28]. Transposons have been proved to have the ability to mediate the spread of AR and there are several classes of transposons based on their mechanisms of transposition, although they all have common features: they have palindromic sequences known as inverted repeats at each end, they don't contain an origin of replication and have to be integrated into a plasmid or chromosome in order to be maintained[9,42].

In addition to transposons and plasmids, integrons are another example of mobile genetic elements that function as gene capture systems through a specific recombination mechanism and can be transferred both in plasmids and in transposons[19]. These elements are known for capturing and expressing gene cassettes, which can encode AR determinants and they all have a mutual gene coding for a site-specific tyrosine recombinase called integrase which can excise an integrate these gene cassettes into the integrin[4].

Genomic Islands (GI's) can be defined as clusters of genes of probable horizontal origin, usually with a size higher than 8 kb, which are present in archaeal and bacterial genomes and who are also known to have a role in the evolution of microbial genomes through the means of horizontal gene transfer[44,45]. These mobile elements normally encode genes involved in notable adaptations of medical and/or environment interest such as virulence factors and ARG[45],whose ultimate goal is to confer the carrier of GI's a fitness increase and a selective advantage over other bacteria[46]. Also, they are frequently integrated adjacent to tRNA genes, have a G+C content distinct from that of the host core chromosome and contain components of mobile genetic elements[47].

All the previously referred mobile genetic elements that primarily reside in the host cell's chromosome and also have the ability to be transferred between cells by conjugation are termed Integrative and conjugative elements (ICEs)[48]. These modular mobile genetic elements include conjugative transposons, integrative plasmids and other elements that are excise to form a circular molecule, which is then transferred by conjugation and integrated into the genome by site-specific recombination[44]. Structurally, ICEs contain a core region of genes that encode the function necessary for transfer between bacteria, including the

conjugation apparatus and integrase[49]. ICEs size range varies approximately from 18 kb to more than 500 kb and can confer various phenotypes, such as antibiotic and metal resistance, carbon-source utilization, symbiosis, pathogenesis, restriction modification, bacteriocin synthesis and biofilm formation[50].

The content of these structures reveals a concerning way in which AR and virulence factors can be combined and transferred[49]. Although ICEs are being identified in increasing numbers as sequenced genomes databases expand exponentially, still only a few of these elements are precisely characterized and delineated within sequenced bacterial genomes[48,51,52].

The ability of mobile genetic elements containing AR genes to spread depends on a range of factors including, selective pressures in the environment, host factors and properties of the genetic elements themselves[9]. Regarding specific host encoded factors, CRISPR Cas systems comprise bacterial adaptative immune systems that function as variable genetic elements[53]. Multiple reports have suggested that these systems may play a major role in controlling horizontal gene transfer events and, consequently, the dynamics of AR gene acquisition in some bacteria such as *Enterococcus faecalis* and *P. aeruginosa*[54].

3 Metal Resistance

Metal ions and metalloids such as arsenic, zinc, mercury, silver and copper have been proven to have an antimicrobial effect due to their capacity to induce oxidative stress and to combine with sulphur atoms in molecules of cysteine, leading to the denaturation and/or inhibition of proteins[13]. In fact, antimicrobial properties of metals have been used in the past fourth century as a treatment for wounds and in even earlier dates as a method for food and water decontamination[55]. However, alongside with antibiotics, bacteria have adapted to the presence of metals through a variety of chromosomal, transposon and plasmid-mediated resistance systems as a result of selective pressures from metal containing environments[56]. The resistance mechanisms employed by bacteria in order to survive in metal contaminated environments are analogous to AR mechanisms[57].

Table 3 Resistance mechanisms applied to both metal ions and antibiotics by prokaryotes[57].

Resistance mechanism	Metal ions	Antibiotics
Reduction in permeability	As, Cu, Zn, Mn, Co, Ag	Chloramphenicol, Tetracycline, Ciprofloxacin, β-lactams
Drug and metal alteration	As, Hg	β-lactams, Chloramphenicol
Drug and metal efflux	Cu, co, Zn, Cd, Ni, As	Tetracycline, Chloramphenicol, β-lactams
Alteration of cellular targets	Hg, Zn, Cu	Ciprofloxacin, β-lactams, Trimethoprim, Rifampicin
Drug and metal sequestration	Zn, Cd, Cu	Coumermycin

Despite of the use of all these resistance mechanisms by bacteria to counteract metal derived stress, efflux systems are the most used method for dealing with metals and have been a continuous subject of study due to their ability to expel a wide range of structurally dissimilar compounds using the same mechanism[58,59].

4 Co-selection of antibiotic and metal resistance

Recently, some studies done in the antibiotic and metal resistance area have proved that besides their antimicrobial action, these compounds also act as selective forces for bacterial evolution in a process termed co-selection where the natural susceptible bacteria present in a community are eliminated and only the ones that own the resistance mechanisms are able to survive[60].

The proliferation of AR and the co-selection process are a result of two distinct mechanisms: the accumulation of two or more genetic determinants on the same mobile genetic element through mutational or gene acquisition processes which is called co-resistance and the presence of mutated or acquired genetic determinants that provide tolerance to one or more antimicrobial agents, which is termed cross-resistance[60,61].

Currently, it is commonly accepted that co-selection and the presence of metal ions in the environment contributes to increasing resistance mechanisms due to the localization of ARG in close neighbourhood on genetic mobile elements[62]. Besides co-selection, metals are also known to co-regulate genes responsible for AR and decrease antibiotic susceptibility[63].

5 Propagation of antibiotic and metal resistance in the environment

The impact on human health resultant of global spread of AR is becoming a major concern in our society and much progress has already been achieved in order to understand the complex mechanisms behind selective pressures that are imposed by antimicrobial compounds or other contaminants and its contribute to the maintenance and spread of AR[57]. However, most of the investigation done in the past few years on this subject has been restricted to clinical settings[4].

Only recently researchers are beginning to realize that some pathogenic strains of several bacteria genera have established environmental reservoirs of ARG that can ultimately be laterally transferred from environmental microorganisms to human commensals, posing a threat to human health[12,57,64].

Indeed, the understanding of the ecological and environmental processes involved in resistance gene acquisition has become a growing concern[4].

5.1 Impact of anthropogenic pressure in the environmental spread of ARG

Besides hospitals and medical settings, AR can also be found in environmental settings that are subjected to the products of anthropogenic activities such as municipal and hospital wastewaters, pharmaceutical manufacturing effluents and aquaculture and animal husbandry facilities (Figure 5) [64].

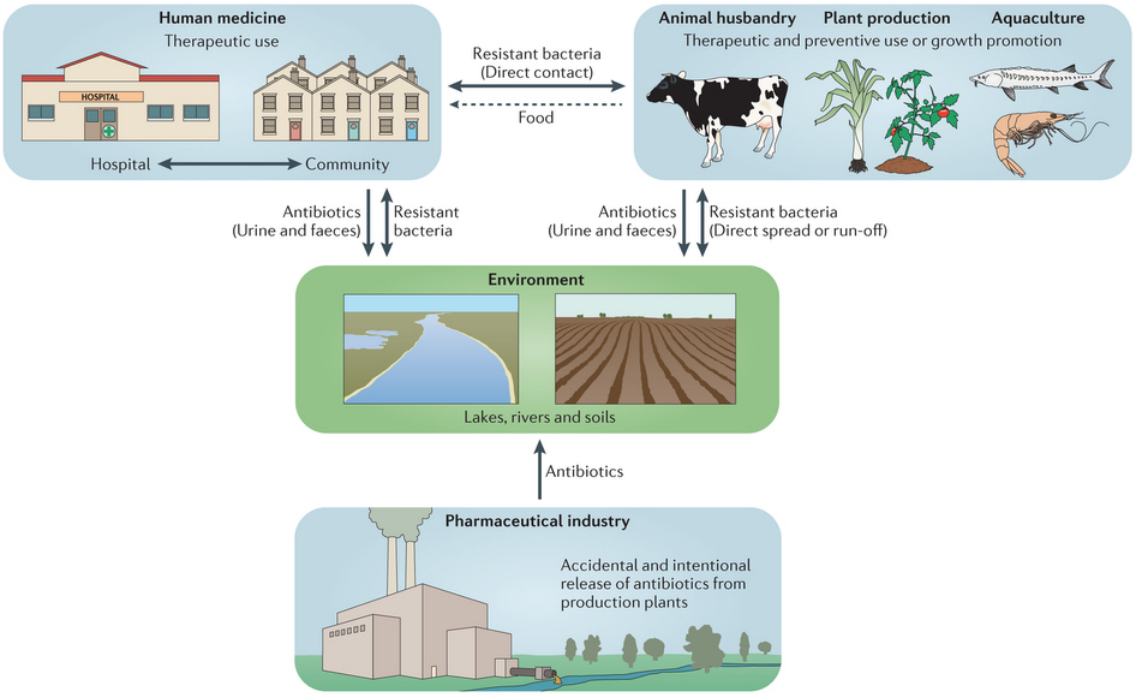

Figure 5 Dissemination of ARG and resistance bacteria in the environment and its correlation to anthropogenic contamination with antibiotics[65].

The contribution of anthropogenic pressure to the persistence and dissemination of ARG and resistant bacteria is aggravated by the consequences of uncontrolled use of antibiotics both in human and veterinary settings, since the antibiotics can be excreted in an active form in both human and animals and persist in the environment, creating an opportunity for resistance selection within bacterial populations[11,25]. These activities and malpractices result in a continuous release of antibiotics in the environment that leads to the creation of resistance hotspots where antibiotics, resistant bacteria and the environmental bacterial flora interact and new resistant strains appear by the means of HGT processes[4]. Once present in the environment, humans may come into contact with resistant bacteria through various ways such as consumption of contaminated crops or drinking of water drawn from contaminated soils, and once when they enter our organism, they may be able to spread the genes that render them resistance to certain antibiotics to the human microbiome[25,66].

Currently, some large-scale studies are being made in order to try to understand the relationships between the consumption of antibiotics and the occurrence of antimicrobial resistance in humans and food-producing animals[67]. However, even with the recent knowledge increase regarding the reservoir of ARG in environmental landscapes and the

effects of anthropogenic activities, there is still a need to understand the complex interactions involved in AR proliferation in environmental microbial communities[4,61].

5.2 Multidrug-resistant bacteria: a threat to human healthcare

Co-selection processes constitute the foundation of another relevant phenomenon termed multidrug-resistance (MDR), which consists in the non-susceptibility to at least one agent in three or more antimicrobial categories[60]. The continued selective pressure by different drugs over the years has resulted in bacteria resistant to more then one type of antibiotic due the possession of multiple resistance mechanisms such as novel penicillin-binding proteins (PBP's), enzymatic mechanisms of drug modification, mutated drug targets, enhanced efflux pump expression and altered membrane permeability[23].

One of the most famous cases of MDR was the appearance of methicillin-resistant *Staphylococcus aureus* (MRSA), which is resistant not only to methicillin but usually also to aminoglycoside, macrolides, tetracycline, chloramphenicol and lincosamides[18]. MRSA, along with MDR organisms such as *Pseudomonas aeruginosa*, *Acinetobacter baumanii* and extensively drug-resistant *Mycobacterium tuberculosis,* gave rise to infections for which there are almost no effective antimicrobial agents and turn out to be deadly, especially in developing countries that don't have access to basic medical cares (Figure 6**)** [4,30].

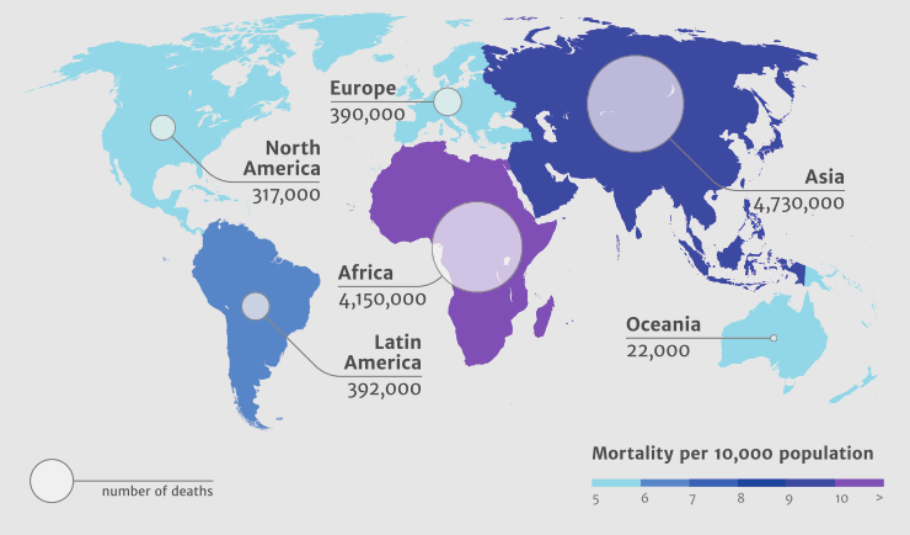

Figure 6 Estimated deaths to AR every year by 2050[1].

Also, there is a considerable lack of data regarding the consumption of antibiotics, their persistence in the environment and also there is a need for the efficient monitoring of infections with MDR bacteria, especially in these countries[1,67].

Thus, AR, and more specifically, MDR is considered to be a growing worldwide problem and the international community is now starting to develop strategies to fight this phenomenon, since it is the source of millions of hospital infections all over the world that frequently end up in the patient's death[27].

5.3 Molecular and Bioinformatic tools role in ARG screening

Since the beginning of the genomic era in 1995, much progress has been achieved in regard to sequencing technologies which has been accompanied by a decrease in its monetary costs[68]. These technological breakthroughs led to the emergence of massive parallel sequencing platforms like Roche/454, Illumina and Ion Torrent, which allow the production of an incredible amount of data associated to entire bacterial genomes that exceed by far the original Sanger sequencing capacity[69]. To store the resulting genomic information, there was a need for the development of various biological databases *in silico* and bioinformatic tools that permit the access and management of massive amount of the generated data[70]. However, before being deposited in databases, the sequencing data generated by Next-Generation Sequencing (NGS) technologies must first be submitted to an assembly and annotation phase that includes standardized and well-defined steps[71]. First, the short read sequences (SRSs) produced must be assembled, using software programs, into contigs, which consist in sets of overlapping joined DNA segments that once placed in the right order form scaffolds (Figure 7) [72].

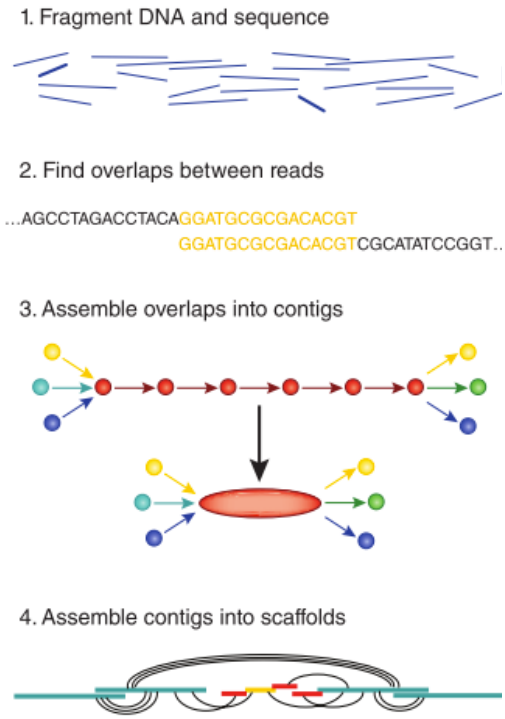

Figure 7 Graphic representation of the different genome assembly steps[73].

Then, some metric parameters are determined and analysed in order to access the quality of the assembly. These parameters include the assembly size and the N50 value, a median weighted statistic that is calculated by sorting all contigs from smallest to largest and determining the contig size at which 50 % off all bases in the assembly are contained in contig or scaffolds larger and smaller then this value[72].

Finally, the assembly is submitted to gene prediction/annotation, a computational process in which regions of the DNA containing coding genes are identified[71]. Automated annotation systems such as RAST (Rapid Annotation using Subsystem Technology) and IMG (Integrated Microbial Genomes) allow one to browse the annotated genomes, also supporting the use of external complementary and comparative tools to analyse them such as Glimmer for coding-sequence prediction, tRNA-Scan-SE and RNAmmer for RNA prediction, among others[68]. Nonetheless, automatic pipelines are prone to the introduction and propagation of poor annotation and errors and a manual curation step is often necessary to remove these[74]. Finally, after further processing, annotations are submitted to public databases such as Genbank or Embl, which give an enormous contribute to the globalization and exchange of scientific research knowledge[64].

Concerning AR, NGS technologies have permitted the creation of AR databases like ResFinder, ARDB (antibiotic resistance database) and CARD (comprehensive antibiotic resistance database), which constitute excellent ARG screening tools[64]. In order to confirm the presence of ARG in a genome, there are several techniques that can be used such as AR phenotypic testing based on antibiograms and MIC (minimum inhibitory concentration) determination, targeted PCR and Sanger sequencing[28,75]. Eventually, genetic engineering procedures and proteomic analysis contribute to a more comprehensive and detailed understating of the bacterial AR framework, since they offer insights on the level of the gene expression and response to a variety of environmental influences[57,76].

6 Antibiotic Resistance in *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is a rod-shaped Gram-negative obligatory aerobic bacteria belonging to the family of *Pseudomonadaceae* and the *Gamma-Proteobacteria* class[5,75]. It has the ability to use many different organic compounds as carbon and energy source by employing Entner-Doudoroff pathway and can also thrive in conditions of partial oxygen depletion using alternative external electron acceptors or/and by fermentation of arginine or pyruvate[5]. This metabolic diversity is reflected by a large genome with an average size of 6.6 Mbp, being one of the largest amongst bacteria[77]. It is composed by a conserved core genome containing the majority of genes with housekeeping functions, which is common to nearly all members of the species and accessory genomic elements consisting of variable length stretches of DNA separated core genome elements[77]. Together, the accessory genomic elements form the accessory genome, which has a preponderant role in *P. aeruginosa* biology by conferring specific phenotypes, such as AR, that are advantageous under certain selective pressures[78].

P. aeruginosa can be found ubiquitously in nature, particularly in moist environments, and is well known for its high potential to form biofilms[62]. In fact, the versatility of this bacteria and its capacity to adapt to various environmental settings such as hospital wastewaters, respiratory equipment, solutions, medicines, and food products, makes it a remarkable opportunistic pathogen whose infections are nosocomial in nature and have a more abundant incidence in patients in intensive care or suffering from predisposing conditions such as cystic fibrosis, burn wounds and immunodeficiencies[14,62].

By examining all the sequenced genomes deposited in the *Pseudomonas* Genome Database (*http:// www.pseudomonas.com*)[79] and performing a global statistical analysis (Figure 8), we can infer that more than half of the host organisms or isolation sources are human patients while environmental *Pseudomonas* isolates represent a small part of the sequenced genomes.

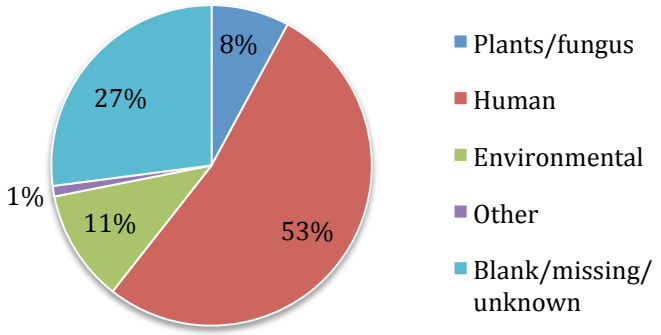

Figure 8 Distribution of all the sequenced *Pseudomonas* genomes present in the *Pseudomonas* Genome Database according to their isolation source and host organism. This analysis was made according to the database update done in 28/09/15.

The increasing morbidity and mortality associated with *P. aeruginosa* infections has become a worldwide problem which is aggravated by the emergence of strains resistant to multiple classes of antibiotics[76]. Actually, only a few antibiotics are effective for treatment of these infections since this species is intrinsically resistant to a large number of antibiotics and also, under selective pressure it can acquire other resistance mechanisms through chromosomal mutations or HGT of resistance gene determinants[14].

In regards to resistance determinants possessed by *P. aeruginosa,* studies have shown the existence of intrinsic RND multidrug efflux pumps (e.g. mexAB-OprM) capable of exporting a broad range of substrates[80], porin mutations (OprD) that result in lower outer membrane permeability[34], a chromosomally encoded AmpC β-lactamase[14] and also acquired resistance determinants such as ESBL's (PER and OXA)[35] and MBL (IMP and VIM)[81], which, all together compromise the use of several varieties of penicillins, carbapenems and extended-spectrum cephalosporins[62]. Also, *Pseudomonas* is known for the common occurrence of spontaneous mutations that result in the overexpression of some of these resistance mechanisms[42] and the dynamic propensity to acquire new resistance mechanisms from other genera such as *Acinetobacter bumannii, Klesibella pneumonie* and

Salmonella spp.[62,82] Together, these resistance determinants can result in the appearance of MDR isolates that impose a huge limitation on the therapeutic choices available for treatment of *Pseudomonas* infections.[18]

P.aeruginosa is also known to contain in its genome a large number of genomic islands, which are found in some strains and constitute an accessory genome that may account for 10 % of an individual isolates genetic material[47]. Many of the *P. aeruginosa* genomic islands are ICEs or are derived from such elements[78]. Some of the identified and characterized islands in *P. aeruginosa* are PAGI-1 (*P. aeruginosa* Genomic Island 1), a 49 kb island identified in a urinary tract infection isolate[47]; PAGI-2 and PAGI-3, which are large genomic islands that were identified by sequencing of a hyper variable region in two different strains: a cystic fibrosis lung isolate and an environmental aquatic isolate[47]; and PAPI-1, which is representative of a large family of genomic islands derived from an ancestral pKLC102-like plasmid[47].

Additionally, the contributions of restriction modification systems, clusters of randomly interspersed palindromic repeats (CRISPR) and phage mediated acquired resistance in *P. aeruginosa* are still unkown, but the combination of these events probably restricts uptake and acquisition of exogenous elements[83].

7 Antibiotic Resistance in *Chromobacterium haemolyticum*

Chromobacterium is a genus of gram-negative rod-shaped bacteria belonging to the family of *Neisseriaceae* and the *B-Proteobacteria* class[5]. It includes 8 recognized species, some of which are considered opportunistic pathogens to humans, as for example *C. violaceum* and *C. haemolyticum*[84–86]. These two species in particular are closely related phylogenetically, which makes them hard to distinguish through the means of biochemical tests in cases of human infections[86]. In the *Chromobacterium* genus, cells are rod-shaped in morphology and are also facultative aerobes, growing fermentatively on sugars and aerobically on various carbon sources[5]. Furthermore, *C. violaceum* is the best-known *Chromobacterium* species and, like some few other *Chromobacteria*, it produces the purple pigment violacein, a water-insoluble pigment with both antimicrobial and antioxidant proprieties[5,87].

Information on *C. haemolyticum* natural habit is scarce, although it has already been isolated from both environmental[88–90] and clinical samples[86,90,91]. Like *C. violaceum,* it can be found in tropical and subtropical ecosystems, primarily in water and soil[87]. Only recently some studies have been focused in providing valuable phenotypic characterizations of the species, since there is still a lack of information on genotypic and phenotypic features as well as its diversity in the environment[92,93]. Also, only two full genomes of *C. haemolyticum* are available in Genbank, from the strains *C. haemolyticum* DSM19808 *and C. haemolyticum* T124*,* which emphasises the amount of genomic information that is still unexplored.

Carbapenems and ciprofloxacin are, nowadays, the most frequently used drugs to combat infections caused by *Chromobacterium* spp.[85,90] and also, *C. haemolyticum* infections sepsis was in some cases reversed with antibacterial therapy[90].

Detailed knowledge on *C. haemolyticum* resistance profile is also scarce, though some studies suggest that it may be more resistant to antibiotics than *C. violaceum*[93], exhibiting resistance to penicillin, ceftriaxone, cefepime and ticarcillin/clavulanate[90,93]. This resistance profile may be the result of the expression of genes coding for various β-lactam, such as class A carbapenemases, which have been recently reported in *C. haemolyticum*, and multidrug resistance genes such as efflux pumps[33,94].

8 Scopes and objectives

Antimicrobial resistance within a wide range of infectious agents such as bacteria, parasites, viruses and fungi is a growing public health problem of broad concern to countries, which threatens the effective prevention and treatment of a broad range of infections.

The exposure of environmental settings to clinically relevant ARG and AR bacteria that derive from anthropogenic sources, together with the excessive use of antibiotics is currently considered to be a serious problem and ever-increasing evidence shows that the evolution and spread of AR in the environment contributes to its occurrence in clinical and urban settings.

Some bacteria that belong to the *Pseudomonas* genus are an example of pathogens that can be commonly found in hospital settings and are also known for its high potential to develop multi-drug and metal resistance. Due to their clinical relevance, it is important to understand the impact of human contaminants in the co-selection for antibiotic and metal resistance traits in environmental *Pseudomonas*. Also, the sudden increase in the number of *Chromobacterium* severe infection reports, especially in tropical and subtropical regions, and the existent lack of knowledge regarding this genus has raised the need to study their AR arsenal.

Taking this into account, the major aims of this work are:

- to analyse the genomic resistance profile of two environmental isolates;
- to confirm the AR traits through phenotypic tests and find evidence of co-selection phenomenon's;
- to identify and characterize new significant resistance determinants;

For this, the genome of environmental strains will be sequenced, annotated and resistance determinants of interest will be selected for detailed analysis.

MATERIALS AND METHODS

1 Strain selection

Pseudomonas E67 was part of a collection of bacterial isolates previously obtained from *Halimione portulacoides*[95], a small metal-accumulator halophyte greyish-shrub. Plant samples were collected from Ria de Aveiro salt marshes, in an area contaminated with high levels of metals both in sediments and accumulated in *H. portulacoides* tissues[96]. The bacterial isolation and identification procedures were described previously[95]. Briefly isolates were cultivated on MacConkey agar plates and incubated at 37 ºC for 16 h. The isolate was identified at the genus level by sequencing the 16S rRNA gene.

On the other hand, *Chromobacterium* IR17 was isolated from river water from River Alfusqueiro located in the Vouga River hydrographic basin. River water was filtered in sterile 0.45-µm-pore-size cellulose ester filters, and the membranes placed on MacConkey agar plates (Merck) supplemented with carbapenem and imipenem[89]. These antibiotics are considered last-line drugs for the treatment of serious infections caused by multiresistant bacteria[32,89]. Imipenem resistant bacteria were incubated at 37 ºC for 16h and colonies were then purified and stored in 20 % glycerol at -80 ºC.

2 Optimal growth conditions

The determination of the isolates optimal temperature, salinity and pH growth conditions was conducted. For this, isolates were grown on LA (Luria Agar) medium and subjected to different temperatures - 25 ºC, 30 ºC and 37 ºC -, salt (NaCl) concentrations – 0.5 %, 1.0 %, 2.0 %, 4.0 % and 8.0 % - and pH conditions of 4.5, 6.0, 7.0 and 8.0. Three replicates and four dilutions of culture with an OD_{600} (optical density at 600 nm) of 0,6 of both *Pseudomonas* E67 and *Chromobacterium* IR17 were cultured and left to grow overnight before results were assessed.

During this study, *Pseudomonas* E67 and *Chromobacterium* IR17 were then cultivated in the optimal conditions, in Tryptic Soy Agar, LA, LB or Mueller Hinton media. Agar plates were stored at 4 ºC for further use. Cultures were also stored in 20 % glycerol at -80 ºC.

3 Phenotypic resistance profile

Previously, the susceptibility profiles to some antibiotics (amoxicillin, amoxicillin/clavulanic acid, ampicillin, aztreonam, cefepime, cefotaxime, ceftazidime, cephalothin, ciprofloxacin, chloramphenicol, gentamicin, imipenem, meropenem, kanamycin, nalidixic acid, sulfamethoxazole/trimethoprim, rifampin) were obtained by the agar disc diffusion method and metal susceptibility testing (As, Cr, Cu, Hg, Ni, Zn) was evaluated for the *Pseudomonas* isolate[95]. In the present study, additional substances were tested using an identical methodology, namely piperacillin/tazobactam (30 μg/6 μg), ticarcillin (75 μg), ticarcillin/clavulanic acid (75 μg/10 μg), and tetracycline (30 μg) (Oxoid, United Kingdom). Briefly, the profiles were determined by the agar disc diffusion method on Mueller–Hinton agar, after 24 h of incubation at 37 ºC, according to the Clinical Laboratory Standards Institute guidelines[97]. *Escherichia coli* ATCC 25922 was used as quality control.

Antibiotic susceptibility tests were also previously performed for *Chromobacterium* IR17 by the agar disc diffusion method on Mueller–Hinton agar, with antibiotics from 6 classes (amoxicillin, amoxicillin/clavulanic acid, cefotaxime, cefepime, imipenem, ertapenem, aztreonam, nalidixic acid, ciprofloxacin, kanamycin, gentamicin, sulfamethoxazole/trimethoprim, tetracycline and chloramphenicol), following the EUCAST (European Committee on Antimicrobial Susceptibility Testing) guidelines[98]. Metal (loid) susceptibility testing was performed in this study by determining minimal inhibitory concentrations (MICs) for 4 metals(loids). MIC of each metal(loid) was recorded as the lowest concentration at which the isolate did not grow. For that, the culture optical density was first adjusted so it would fit in the interval between 0.4 and 0.6, and then 5 μl were inoculated, in triplicate, on Luria-Bertani (LA) medium supplemented with As (5, 10 and 20 mM As as Na_2AsO_4), Cu (2.5 and 10 mM Cu as $CuSO_4.5H_2O$), Ni (2, 5 and 10 mM Ni as $NiSO_4.6H_2O$) and Zn (2, 5 and 10 mM Zn as $ZnCl_2$).

4 Whole Genome Sequencing, assembly and annotation

Pseudomonas E67 and *Chromobacterium* IR17 genomic DNA was isolated from pure cultures using the Wizard Genomic DNA purification Kit (Promega, USA), following manufacture's instructions. Next, the two genomes were sequenced by STAB VIDA using

the Ion torrent Sequencing Technology (Life Technologies, Portugal) and resulting reads were then subjected to a trimming process using the CLC Genomic Workbench version 6.5, in order to remove potential vector contamination and take care of the reads with poor quality[100]. The quality of reads was determined with the FastQC program (v3.4.1.1)[101]. CLC Bio software package was then used to assemble the genomes sequence data using the sequencing reads of Phred quality score Q20 and higher.

The genome drafts were annotated using the Rapid Annotation using System Technology (RAST)[86], an automatic annotation server for microbial genomes built upon the framework provided by the SEED system[103]. The tRNA genes were predicted using the tRNAscan-SE [104] while rRNA genes were predicted using RNAmmer 1.2 [105]. A secondary annotation was performed using the DOE-JGI Microbial Genome Annotation Pipeline (MGAP v.4) [106], which allowed to refine the results obtained with the RAST annotation by performing a comparative analysis.

5 Phylogenetic analysis

In order to confirm the phylogenetic affiliation of the E67 isolate, the DNA sequences of two housekeeping genes, 16S rRNA and g*yrB*, were obtained from the RAST annotation and submitted to a phylogenetic analysis where the sequences of other 20 type strains included in *Pseudomonas* genus were also used. In case of *Chromobacterium* IR17, the 16S rRNA gene sequence was sufficient to confirm its phylogenetic affiliation by comparison with other 8 type strains belonging to the *Chromobacterium* genus.

The phylogenetic analysis was carried out by Molecular Evolutionary Genetics Analysis (MEGA) software version 6.0[107] and the concatenated nucleotide sequences were initially aligned using the ClustalW[108], application which is integrated in the MEGA6 software. Subsequently, one phylogenetic neighbour joining tree was constructed for each isolate, using bootstrap values for accessing reliability of the branches that were calculated by resampling 1000 times.

The average nucleotide identity (ANI) value was also calculated between the *Pseudomonas* E67 draft genome obtained in this study and the available reference genome of *P. aeruginosa* PAO1 [109]. This method represents a reliable and robust measure of genetic and evolutionary relatedness between two genomes from closely related species or

different strains within a species that share 80-100 % ANI, since it shows strong correlation to DNA-DNA reassociation values and the mutation rate of the genome[110,111].

Additionally, the RAST pipeline was also used to infer the phylogenetic affiliation of the isolates, since it is able to generate an estimate of the thirty closest phylogenetic neighbours in the SEED by comparing *ab initio* GLIMMER3 gene-candidates with a set of universal proteins plus up to two hundred unduplicated proteins[103].

5.1 Multilocus Sequence Typing

Multilocus Sequence Typing (MLST) is a genetic fingerprinting method based in the sequence analysis of a number of housekeeping genes and the identification of mutational differences in order to characterize strains within species.[112] Thus, to determine the sequence type, multilocus sequence typing (MLST) of E67 was performed according to the protocol of Curran *et al.* [113] and modified by Van Mansfeld *et al.* [114], using the internal fragments of a group of seven housekeeping genes: *acsA, aroE, guaA, mutL, nuoD, ppsA* and *trpE*. The complete sequences of these genes were obtained by browsing the RAST annotation and using the incorporated BLAST search tool.

6 Annotation of Antibiotic and Metal Resistance Genes

The genomic resistance profile of the *Pseudomonas* and *Chromobacterium* isolates were obtained by browsing the RAST subsystems related to virulence, disease and defence, and also transposable elements. In addition, IMG annotation was accessed as well as three additional web-based tools: Resfinder 2.1[24], which uses BLAST for identification of acquired ARG in whole-genome data, the Resistance Gene Identifier (RGI) provided by CARD (Comprehensive Antibiotic Resistance Database)[115], the Antibiotic Resistance Genes Database (ARDB)[116] and BLASTn in Genbank. Additionally, IslandViewer 3 [45] was used to predict and analyze the presence of GI's in the E67 and IR17 isolates genome using *P. aeruginosa PAO1* and *C. haemolyticum* ATCC 12472 as reference genomes. This tool is a widely used web-based resource that can be used in the prediction and analysis of GI's present in bacterial and archaeal genomes[45].

RESULTS AND DISCUSSION

Characterization of *P. aeruginosa* E67: phenotypic and whole genome sequence analysis

1 Phenotypic analysis

P. aeruginosa E67 optimal temperature for growth is 37 ºC and although growth also occurs at 30 ºC, at 25 ºC no growth is observed. The optimal pH for growth is 6.0, being that growth occurs at pH 7 and 8, but not at pH 4.5. Regarding the NaCl concentration, growth occurs in the presence of 0.5-4 % (w/v) NaCL and optimal growth is observed in the presence of 0.5 % (w/v) NaCl.

Regarding the AR phenotype, isolate E67 displayed resistance or intermediate resistance to several antibiotics, namely amoxicillin, the combination of amoxicillin with clavulanic acid, ampicillin, cefotaxime, kanamycin, cefalotin, nalidixic acid, chloramphenicol, rifampicin, aztreonam, imipenem, meropenem and tetracycline. However, it was susceptible to cefepime, ceftazidime, ciprofloxacin, gentamicin, ticarcillin, sulphamethoxazole with trimethoprim and piperacillin with tazobactam.

The isolates optimal growth conditions are summarized on Table 4, together with the previously obtained antibiotic and metal phenotypic resistance profile.

Table 4 Phenotypic caracteristics of the isolate E67

Characteristics*	
AR phenotype	AMX AMC AMP ATM CTX KF IPM NA C K TE SXT
Metal(loid) resistance phenotype	As Cr Cu Hg Ni
Temperature optimum	37 °C
pH range for growth	6 - 8
NaCl concentration range for growth	0.5 – 4.0 %

* AMP –ampicillin, AMX -amoxicillin, AMC –amoxicillin/clavulanic acid, ATM –aztreonam, KF – cephalotin, CTX –cefotaxime, IPM –imipenem, C –chloramphenicol, K-kanamycin, TE –tetracycline, SXT – sulfamethoxazole-trimethoprim, NA –nalidixic acid;

P. aeruginosa has intrinsic resistance to many antimicrobial agents including most penicillins, narrow-spectrum cephalosporins, cefotaxime, and also other compounds such as tetracycline and chloramphenicol, which in part explains the isolates resistance phenotype obtained[14,117]. However, E67 was found to have intermediate resistance to

aztreonam, imipenem and meropenem, antibiotics which are considered to be clinically relevant antipseudomonal drugs[118].

Considering the AR phenotype obtained and contemplating the antimicrobial categories and agents used by the European Centre for Disease Prevention and Control to define MDR in *Pseudomonas*, we cannot infer that the isolate E67 exhibits a MDR profile [118]. However, there is no established and consensual definition for MDR and if we take into account both the intrinsic and acquired resistance profile, *P. aeruginosa* E67 can be considered MDR, since it exhibits resistance to more then three classes of antibiotics[119]. Also, *P. aeruginosa* E67 exhibits a metal(loid) resistance phenotype, including resistance to arsenic, chromium, copper, mercury and nickel, that allows it to survive in metal contaminated environments such as largo do laranjo.

Given the relevant resistance phenotype detected, a Whole Genome Sequencing (WGS) approach was adopted to gain more detailed information on the *P. aeruginosa* E67 antibiotic and metal resistance genetic basis, which may explain co-selection of resistance to both classes of compounds.

2 Whole Genome Sequencing, assembly and annotation

From the whole genome sequencing process, a total of 643.09 Mbp were generated comprised in 2,313,586 reads. After trimming, 2,277,184 reads were generated with an average length of 239,2 bp, which were then assembled *de novo* using CLC Genomics workbench software.

From the assembly process resulted 316 contigs with a G+C content of 66.0 % and a N_{50} of 102 kb. Also based on assembly data, the estimated genome size of the isolate was 6,876,232 bp, from which a genome coverage value of 79x was obtained, taking into account the average read length and the total number of reads. Sequencing and assembly information is summarized in Table 5.

Table 5 General genomic features of the whole genome sequence of *P. aeruginosa E67*

Feature	
Accumulated lenght (bp)	6,876,232
Number of Q20 bases (Mbp)	572.28
Total number of reads	2,277,184
Mean read length (bp)	239.23
Number of contigs	316
N75 (bp)	56,447
N50 (bp)	102,009
N25 (bp)	148,574
Average contig length (bp)	21,830
Average GC content	66.0 %
Aligned reads count	2,277,184
Number of CDS	6760
Number of tRNA genes	62
Number of rRNA genes	5

Due to the constant and quick changes in sequencing technologies, it is difficult to access the quality of sequencing and assembly[73]. Nevertheless, there are some common metrics such as N50, assembly size and genome coverage that are typically used by researchers in order to gain insight on projects quality.[72] However, when researching for sequencing and assembly results from other *P. aeruginosa* sequenced genomes, the data found was divergent and didn't allow to make a comparative study with the data obtained on the *E67* isolate.

The RAST server predicted a total of 261 contigs containing protein coding genes (PEG) and 6760 coding sequences (CDS). From the annotation also resulted 563 RAST subsystems that covered 48.0 % of the genomic features, while 52.0 % did not belong to any subsystem.

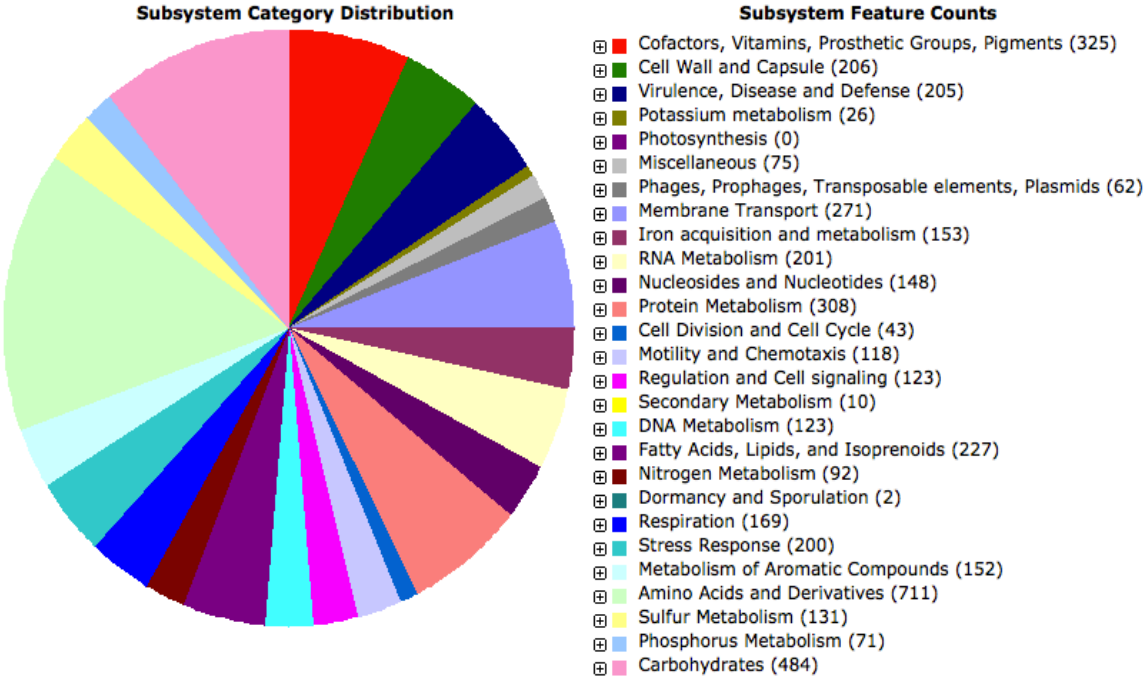

Figure 9 Main subsystem categories resulting from RAST annotation of the *P. aeruginosa E67* genome

In Table 6 and Figure 9, it is noticeable that among the 563 subsystems identified, the main ones were related to biosynthesis and degradation of amino acids and derivatives followed by carbohydrates metabolism, cofactors metabolism and pigment biosynthesis.

These results are in concordance with metabolomics studies of *P. aeruginosa* strains since metabolites related to amino acid and sugar metabolism are shown to represent approximately half of the bacteria metabolome.[120,121] Also, the importance of amino acids and sugar metabolism is reflected by the broad metabolic capacity of this organism, rendering it able to adapt to multiple niches, many times in low nutrition conditions.[122]

Additionally, tRNA genes were predicted using the tRNAscanSE program version 1.21[123] while rRNA genes were predicted using RNAmmer version 1.2[105], which together detected 62 tRNA and 5 rRNA (three relative to the 5S subunit, one to the 23S subunit and another one relative to the 16S subunit). Also, no plasmids were found in the genome sequence of this bacterium.

By comparing the sequencing and annotation results of *P. aeruginosa E67* to those of completed *Pseudomonas* genomes described in literature[121] and in the *http://www.www.pseudomonas.com* database, it is clear that, although variations exist

between different species and strains, the sequenced genome fits the profile of the *Pseudomonas* genus.

Table 6 Functional classes of predicted genes

Functional Class	Genomic features	Genes (%)
Amino acids and Derivatives	711	8.5
Carbohydrates	484	5.8
Cell Division and Cell Cylce	43	0.5
Cell Wall and Capsule	206	2.5
Cofactors, Vitamins, Prosthetic Groups, Pigments	325	3.9
DNA Metabolism	123	1.5
Dormancy and Sporulation	2	0.02
Fatty Acids, Lipids and Isoprenoids	227	2.7
Iron acquisition and metabolism	153	1.8
Metabolism of Aromatic Compounds	152	1.8
Membrane Transport	271	3.2
Miscellaneous	75	0.9
Motility and Chemotaxis	118	1.4
Nitrogen Metabolism	92	1.1
Nucleosides and Nucleotides	148	1.8
Phages, Prophages, Transposable elements, Plasmids	62	0.7
Phosphorous Metabolism	71	0.8
Potassium Metabolism	26	0.3
Protein Metabolism	308	3.7
Regulation and Cell signalling	123	1.5
Respiration	169	2.0
RNA Metabolism	201	2.4
Secondary Metabolism	10	0.1
Stress Response	200	2.4
Sulfur Metabolism	131	1.6
Virulence, Disease and Defence	205	2.4
Hyopthetical	223	2.7
Unknown (conserved hypothetical)	3523	42.0
Total	8382	100

3 Phylogenetic analysis

By analysing the resulting phylogenetic tree (Figure 10), we can observe that the isolate *E67* shares one clade cluster with *P. aeruginosa* DSM50071 with a bootstrap value of 100 %, which means the node relationship is consistent and supports the inclusion of the isolate in the *Pseudomonas* genus and the *P. aeruginosa* species[124].

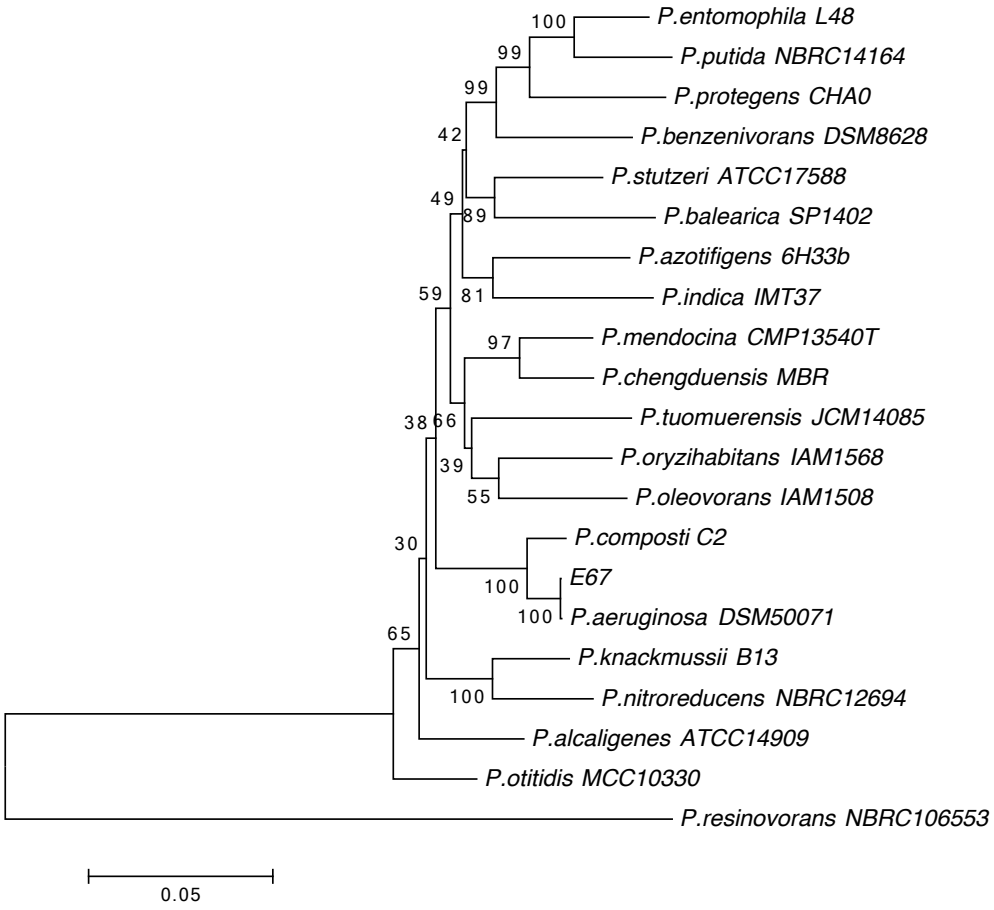

Figure 10 Phylogenetic tree based on 16S and *gyrB* sequences of 20 type strains of *Pseudomonas* species and the E67 isolate generated by the neighbour-joining method. The numbers at nodes represent levels (%) of bootstrap support from 1000 resampled datasets.

The ANI value of 99.43 % obtained corroborates the species identification inferred by the analysis of the phylogenetic tree and also, using the RAST pipeline, it was possible to confirm the identification of the isolate as *P. aeruginosa* with the generation of the closest phylogenetic neighbours [103].

3.1 Multilocus Sequence Typing

The combination of the different alleles at each of the loci resulted in the definition of the ST395 by the Curran scheme for MLST[113] (Table 7). This ST is commonly found in hospitals, urban wastewaters and wastewater treatment plants and is considered to be a high-risk clone due to its ability to produce ESBL such as PER-1, MBL such as IMP-29, various AmpC's and *aadB* determinants responsible for aminoglycoside resistance, which makes it resistant to several different antibiotics.[125] In fact, this ST is associated with a

MDR phenotype and has been reported in hospital wastewaters, water treatment plants and rivers in Hungary [126], eastern France [125], Spain [127] and also in the UK [128]. However, most of the existing reports describing *P. aeruginosa* ST395 isolates refer to clinical cases associated to infections in cystic fibrosis patients[127] and other hospital infections in the previously referred countries[126,127,128,130].Thus, there is little information regarding the AR profile of ST395 isolates prevenient from environmental sceneries, which can be easily transferred from its natural environment to clinical settings[83].

Table 7 Allelic profile and subsequent ST of *P. aeruginosa* E67 obtained from the PubMLST database

MLST profile	
acsA (Acetyl coenzyme A synthase)	6
aroE (Shikimate dehydrogenase)	5
guaA (GMP synthase)	1
mutL (DNA mismatch repair protein)	1
nuoD (NADH dehydrogenase I chain C,D)	1
ppsA (Phosphoenolpyruvate synthase)	12
trpE (Anthralite synthase component I)	1
Sequence Type	**ST395**

4 Antibiotic and metal resistance profile

The search of *P. aeruginosa* E67 genome for AR determinants revealed that genes encoding for components of efflux pumps occupy a significant part of the isolates resistance arsenal, which emphasizes these pumps contribution to a reduced susceptibility towards antibiotics[130,131]. RND efflux pumps that are well characterized in this species[39], such as MexAB-OprM, MexCD-OprJ, MexEF-OprN and MexXY-OprM multidrug efflux systems were identified, as well as the regulator genes *merR*, *nefxB*, *mexT* and *mexG*. The overexpression of these pumps in *P. aeruginosa* is a recognized cause of antibiotic cross-resistance, being responsible for the exportation of fluoroquinolones, tetracycline, chloramphenicol, some β-lactams and also other unrelated compounds involved in the quorum sensing process [39,62]. This overexpression derives from mutations in the repressor genes *nfx*B and *mer*R and results in an increased resistance to those antibiotics that act as substrates for the respective pump[42]. Genes related to other efflux pumps superfamilies such as MFS, ABC, MATE, DMT and SMR superfamily are also well represented in the isolates genome and are known to play a predominant role in the resistance to certain

antibiotics (e.g. tetracyclines, fluoroquinolones, erythromycin and macrolides, amongst others) and other unrelated compounds such as organic solvents[39,132,133]. In fact, some specific components of these efflux systems were identified which confer resistance to specific antibiotics, namely two MFS permeases exhibiting 32.1 % and 36.5 % similarity with chloramphenicol efflux pump components, a MFS permease with 40.7 % similarity with a tetracycline efflux pump component, two ABC transporter exhibiting 32.0 % and 29.5 % similarity with components of a Macrolide-Lincosamide-Streptogramin B efflux system and a membra fusion protein of the RND superfamily with 34.5 % similarity with a component of a macrolide-specific efflux system. We also detected a high number of genes coding for ABC transporter proteins that are involved in the secretion of several virulence factors in addition to antimicrobial compound efflux[39].

Additionally, several genes coding for components of the outer membrane porin OprD were identified. Although this porin's physiological role is the transport of basic amino acids, it is also the main porin used by carbapenems for diffusion in *P. aeruginosa*[16]. However, mutations and negative regulation of the transcription of these genes may result in the loss of this porin and ultimately in cross-resistance to carbapenems like imipenem and meropenem[42,119].

Genes conferring resistance to specific antibiotics were identified and are summarized in Table 8.

Resistance to β-lactam antibiotics also occupies a great portion of the isolates resistance arsenal. Five genes encoding for hydrolases belonging to the β-lactamase class C were annotated by RAST and confirmed with a second annotation based on IMG/ER. This is a characteristic feature described in ST395 *Pseudomonas* isolates[125,126]. One of these genes has high similarity 98.66 % with the chromosomal drug-inducible gene bla_{AmpC}, which encodes a wide-spectrum class C β-lactamase that contributes to the intrinsic β–lactam resistance of *P. aeruginosa*[14]. Also, directly upstream from bla_{AmpC}, the HTH-type transcriptional activator AmpR was detected. When overproduced as a result of mutations, AmpC expression may become a major cause of resistance to widely used antipseudomonal penicillins (ticarcillin and piperacillin), monobactams (aztreonam), and third-generation (ceftazidime) and fourth-generation (cefepime) cephalosporins[134]. The other genes identified encoded putative class C β-lactamases that according to the ARDB database display from 27.5 % to 35.0 % similarity with previously described β-lactamases.

The hydrolytic pattern of these proteins has never been investigated and future studies are needed to assess their contribute to the pseudomonads resistance profiles. Additionally, gene encoding a class B β-lactamase was identified and displayed 23.4 % of similarity with a previously described metallo-β-lactamase. Also, a naturally occurring oxacillinase gene in *P. aeruginosa*, $bla_{\text{OXA-50}}$, was detected with 99.0 % amino acid similarity with the one encoded in the genome of *P. aeruginosa* PAO1. The expression of this gene has been previously described to promote decreased susceptibility to ampicillin, ticarcillin, moxalactam and meropenem [135], though its contribution to the resistance phenotype has poor significance[28].

P. aeruginosa E67 resistome also harbors other relevant AR determinants such as genes putatively expressing phosphotransferases (24.8 % to 99.3 % similarity in terms of amino acid sequence with aminoglycoside O-phosphotransferases present in *P.aeruginosa* PAO1 genome), a chloramphenicol acetyltransferase (98.7 % similarity in terms of amino acid sequence with a group B acetyltransferase found in the *Pseudomonas* genus), responsible for both enzymatic modification and inactivation of aminoglycosides and chloramphenicol respectively. These genes have been previously characterized by Schwartz and his co-workers in *P. aeruginosa*[62]. Also, genes were identified which expression may be responsible for polymyxin resistance (e.g. genes 3580 to 3583 and 4225/4226 in contig 35 and 43, respectively) through the induction of modifications in the LPS structure that results in reduced binding to polymyxins[136]. Finally, a gene coding for a Virginiamycin B lyase, an enzyme responsible for the inactivation of type B streptogramin antibiotics[137], was identified (gene 4095 in contig 42)., exhibiting 21.4 % with streptogramin B lyase detected in *Staphylococcus cohnii*.

Additionally, a gene coding for a vancomycin B-type resistance protein was identified, which displayed 32.3 % similarity with a gene present in a VanG type vancomycin resistance operon characteristic of Enterococcus and Eubacterium[138,139]. This type of resistance in *Pseudomonas* is still poorly described in literature since this antibiotic is not a choice for treatment in cases of *P. aeruginosa* infections. However, the presence of this gene may be related to functions other than AR.

Table 8 Genes conferring AR found in *P. aeruginosa* E67

Antibiotics	Genes	Contig location	Products
Aminoglycosides	*aph*	87_6184	Aminoglycoside phosphotransferase
	aph	92_6490	Aminoglycoside 3'-phosphotransferase type IIb
	aph	65_5285	Predicted aminoglycoside phosphotransferase
	aph	65_5286	Predicted aminoglycoside phosphotransferase
B-Lactams	*bla*	3_2989	B-lactamase class C and other penicillin binding proteins
	bla_{Oxa-50}	3_3019	B-lactamase OXA-50-like
	bla	21_2044	B-lactamase class C
	bla	3_2898	B-lactamase Class B
	bla	48_4456	B-lactamase class C
	ampG	12_768	Permease
	bla	65_5322	B-lactamase class C
	ampR	84_6081	HTH-type transcriptional activator *ampR*
	ampC	84_6082	B-lactamase class C
Chloramphenicol	*catb4*	23_2260	Type-B-Chloramphenicol O-acetyltransferase
	qac	3_2983	Arabinose ABC transporter permease
	rarD	42_4113-4114	Chloramphenicol-sensitive protein
	rarD	93_6551	Chloramphenicol-sensitive protein
Lincosamide	*uup*	91_6459	ABC transporter ATP-binding protein
Polymyxin	*arnT*	35_3580	Undecaprenyl phosphate-alpha-4-amino-4-deoxy-L-arabinose arabinosyl transferase
	arnD	35_3581	4-deoxy-4-formamido-L-arabinose-phosphoundecaprenol deformylase
	arnA	35_3582	UDP-4-amino-4-deoxy-L-arabinose formylase
	arnC	35_3583	Glycosyl transferase
	eptA	52_4839	Phosphoethanolamine transferase
	arnC	43_4225	Glycosyl transferase
	arnT	43_4226	Undecaprenyl phosphate-alpha-L-Ara4N transferase
Streptogramin	*vgb1*	42_4095	virginiamycin B lyase
Tetracycline	*tetA*	36_3666	Tetracycline resistance protein
Vancomycin	*vanW*	13_878	Vancomycin B-type resistance protein

At least 120 β-lactamases have already been confirmed in clinical isolates of *P. aeruginosa*, and the β-lactamases coding genes found in the isolate *E67* confirm the results of previous studies showing that this species consists of a natural reservoir of β-lactam resistance determinants, which compromises the use of last resource antimicrobials such as carboxypenicillins, ureidopenicillins, carbapenems and extended-spectrum cephalosporins.[14,140]. These results indicate that the isolate *E67* is considered a MDR *P. aeruginosa* isolate, since it shows resistance to more than three classes of antibiotics[119]. Alterations in bacterial enzymes DNA gyrase and DNA topoisomerase were also checked, but turned out to be inexistent.

In order to survive in an environment which in known for its high concentration of metals ions, *P. aeruginosa E67* also harbours a vast collection of metal resistance determinants consisting in a total 112 PEG (Figure 12), whereas most of them code for

components of efflux systems. This allows the isolate to tolerate the presence of the arsenic, copper, mercury, chromium, cobalt, zinc, cadmium and lead.

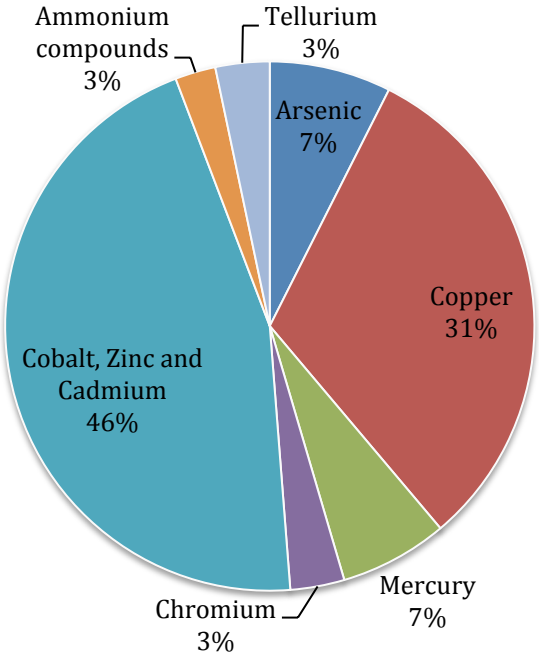

Figure 11 Distribution of the identified metal resistance PEG and other related compounds.

Amongst the resistance PEG detected, genes coding for proteins present in efflux pumps such as as the *cop* system (Figure 13B) and P-type ATPase's whose expression grants tolerance to metals like Copper, Zinc, Cobalt, Chromium and Cadmium are predominant, which confirms the importance of these systems for the survival in metal contaminated environments.[59] Two *mer* operons, one complete operon located in the PAGI-2 genomic island (Figure 13A) and an incomplete operon missing the *merA* gene were identified. These structures are responsible for the reduction of toxic Hg^{2+} to volatile Hg^{0} [141], and were detected in the isolates genome without the *merB* gene, which is a common feature in some organisms[56]. Also, mobile genetic determinants were located next to both operons. The *ars* operon (Figure 13C), which is responsible for arsenic resistance[56], was also detected. These resistance determinants have already been identified in other metal resistant isolates, mainly environmental isolates that are found in agricultural soils irrigated with wastewater and other metal contaminated sites[59,62,142]. Nonetheless, these metal resistant isolates have also been identified in clinical settings, although the degree of

resistance is considerably lower comparatively to environmental isolates[143]. Full details of AR traits are available in Table 8 and supplementary Table S1.

Regarding mobile genetic elements, we explored the RAST subsystem related transposable elements and phage elements and we detected fourteen genes coding for integrases, and twenty one for transposases, as well as eleven genes coding for site-specific recombinases, four for relaxases and five for other widespread colonization island factors.

In order to understand if detected mobile genetic elements are organized in specific chromosomal regions, we screened the E67 isolates genome for genomic islands (Figure 12).

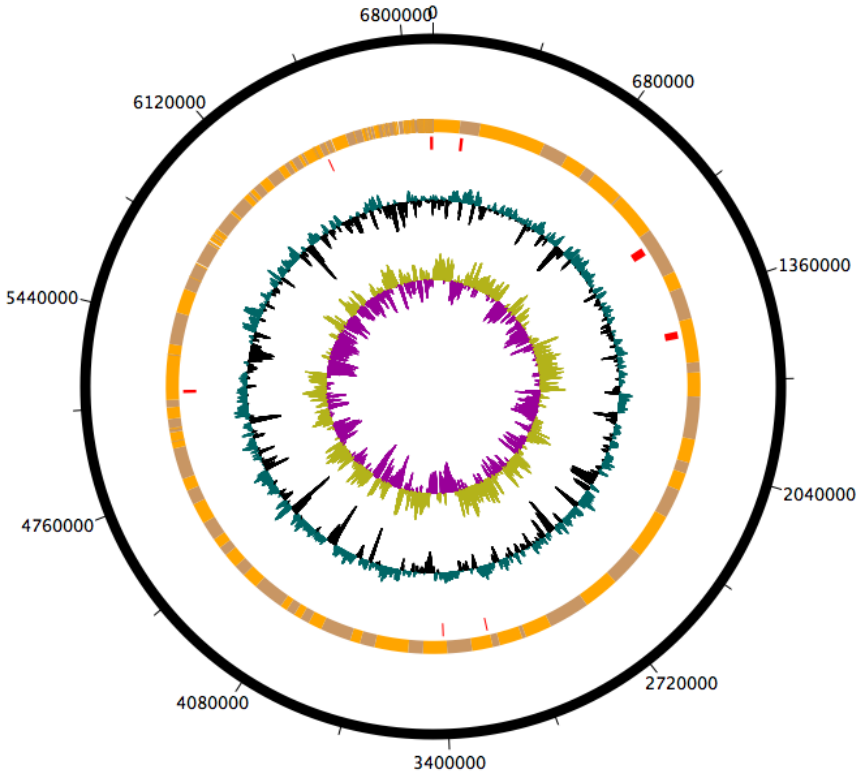

Figure 12 Graphical map of the *P. aeruginosa* E67 draft genome were genomic islands are shown in red. G+C content plot and GC skew (purple: negative values, olive: positive values) are also shown.

This analysis revealed that one of the *mer* operons (13A), a *cop* operon (13B), two chromate transport proteins and a *ars* operon (Figure 13C) are located in the mobile structure PAGI-2[78].

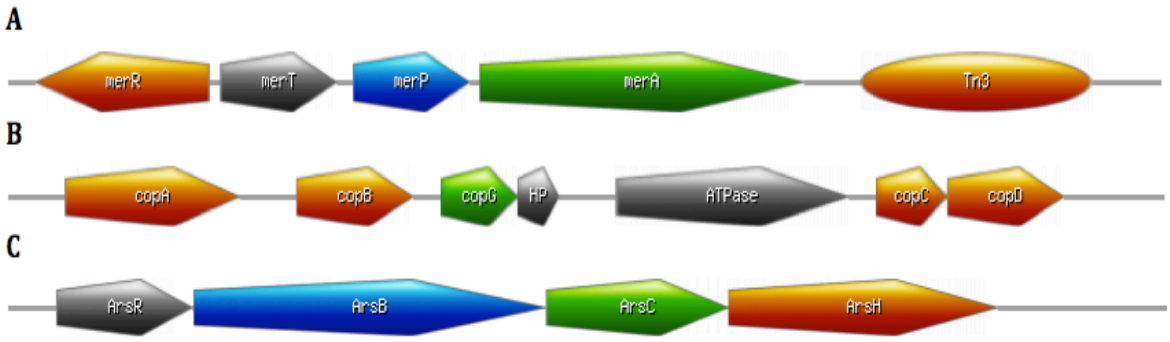

Figure 13 (A) Diagram of the *mer* operon present in the PAGI-2. Some genes which are not present in the majority of the *mer* operons, namely, *mer*C, *mer*G, *mer*B, *mer*D and *mer*E, were not detected[144]. Additionally, a gene coding a Tn3 transposase was found adjacent to the operon. **(B)** Diagram of the *cop* operon characteristic of *Pseudomonas sp*. This ATPase efflux mechanism is responsible for the periplasmic binding of Cu(II). The HP stands for hypothetical protein and the *cop*G was also found in the operon. **(C)** *Ars* operon schematic representation. It is composed by an arsenical resistance operon repressor *ArsR,* an arsenic efflux pump protein arsB coding gene, an arsenic reductase (arsC) coding gene and the arsenic resistance protein arsH coding gene.

Also, various genes coding for proteins associated with multidrug-resistance efflux pumps such as membrane fusion proteins and membrane transporters belonging to the RND family were also identified in this mobile structure.

Other full-length or nearly full-length genomic islands previously described in *P. aeruginosa* were also identified, namely PAGI-1, PAGI-3, PAGI-4, PAGI-6, PAGI-7, PAGI-8 and PAGI-9[47,78]. Most of these structures comprise genes related to virulence, metabolic and transport functions[44]. Also, PAGI-1 and PAGI-4 are amongst the better characterized *P. aeruginosa* genomic islands related to pathogenicity, having been examined in greater detail in clinical isolates, while the incidence of these islands in environmental isolates hasn't been given the same attention[145].

Various integrative conjugative elements (ICE's) resembling the ones present in the PFGI-1 genomic island described in *P. fluorescens* Pf-5 were detected mainly in a PAPI-1/PAGI-5-like genomic island and also in PAGI-2[146]. Some of these ICE's belonging to the PFGI-1 genomic island are known to contribute to the survival of *Pseudomonas* isolates by providing protection from environmental stresses[49]. A genomic island containing CRISPR repeat sequences belonging to the I-E CRISPR-Cas subtype was also detected (Figure 14). Besides their well known function as a bacterial adaptative immune

system, CRISPR systems have also been proved to play an important role in controlling horizontal gene transfer and, consequently, the dynamics of AR in *P. aeruginosa*[53].

Figure 14 Schematic representation of the CRISPR-Cas type I-E structure identified in the genome of *P.aeruginosa* E67. This structure is however incomplete, missing the flanking genes cas3 and cse1 in the downstream region and cas1 in the upstream region.

These genomic islands are known to contribute for the evolution of microbial genomes, conferring rapid changes in virulence potential and influencing traits such as AR, symbiosis, fitness and adaptation in general[46].

More information on the location and composition of the *P. aeruginosa* E67 identified genomic islands is shown in Figure 12 and Table S2.

Characterization of *C. haemolyticum* IR17: phenotype and whole genome sequence analysis

1 Phenotypic analysis

C. haemolyticum IR17 showed resistance towards several β-lactams including penicillins (amoxicillin and amoxicillin combined with the β-lactamase inhibitor clavulanic acid), extended-spectrum cephalosporins (cefotaxime and cefepime) and carbapenems (imipenem and ertapenem)[89]. On the other hand it was found to be susceptible to non β-lactams such as tetracycline, quinolones and aminoglycosides[89]. Also, when exposed to metal containing culture medium, *C. haemolyticum* IR17 showed low tolerance levels to copper, zinc, nickel and arsenic. It showed only a weak growth in medium with zinc and nickel concentration of 2mM, and no growth in medium containing the same concentration of copper and arsenic. Thus, IR17 isolate didn't possess a significant metal resistance phenotype.

Regarding the isolates optimal growth conditions, *C. haemolyticum* IR17 exhibited an optimal growth temperature of 30°C, although it also grew at 37°C but not at 25°. Also, the pH and the NaCl concentration range were 6-8 and 0.5-4.0%, respectively. The isolates phenotypic characteristics are summarized on Table 9.

Table 9 Phenotypic caracteristics of the isolate *IR17*

Characteristics*	
AR phenotype	AMX AMC CTX FEP IPM ETP
Temperature optimum	30 °C
pH range for growth	6 - 8
NaCL concentration range for growth	0.5 – 4.0 %

* AMX –amoxicillin, AMC -amoxicillin/clavulanic acid, CTX –cefotaxime, FEP –cefepime, IPM – imipenem, ETP -ertapenem

2 General genome analysis

Sequencing of *C. haemolyticum* IR17 genome yielded 3,088,021 paired-end sequence reads, which were then trimmed and assembled using CLC Genomics workbench version 6.5.1. (CLC Bio, Denmark). Results predicted that the draft genome of strain IR17 contained 5,135,899 bp assembled into 421 contigs with a GC content of 62.3 %. Also, an average 157.8–fold genome coverage was obtained. Full sequencing details are showed in Table 10.

Table 10 General genomic features of the whole genome sequence of *C. haemolyticum* IR17

Feature	
Accumulated lenght (bp)	5,135,899
Number of Q20 bases (Mbp)	832.06
Total number of reads	3,088,021
Mean read length (bp)	262,41
Number of contigs	421
N75 (bp)	28,088
N50 (bp)	53,809
N25 (bp)	80,654
Average contig length (bp)	12,199
Average GC content	62.3 %
Matched reads count	3,016,599
Number of CDS	4,797
Number of tRNA genes	71
Number of rRNA genes	6

The genomic annotation of isolate IR17 performed by RAST included 4,797 protein-coding sequences, distributed into 472 subsystems which cover 47.0 % of the genomic features, as determined by RAST (Figure 15).

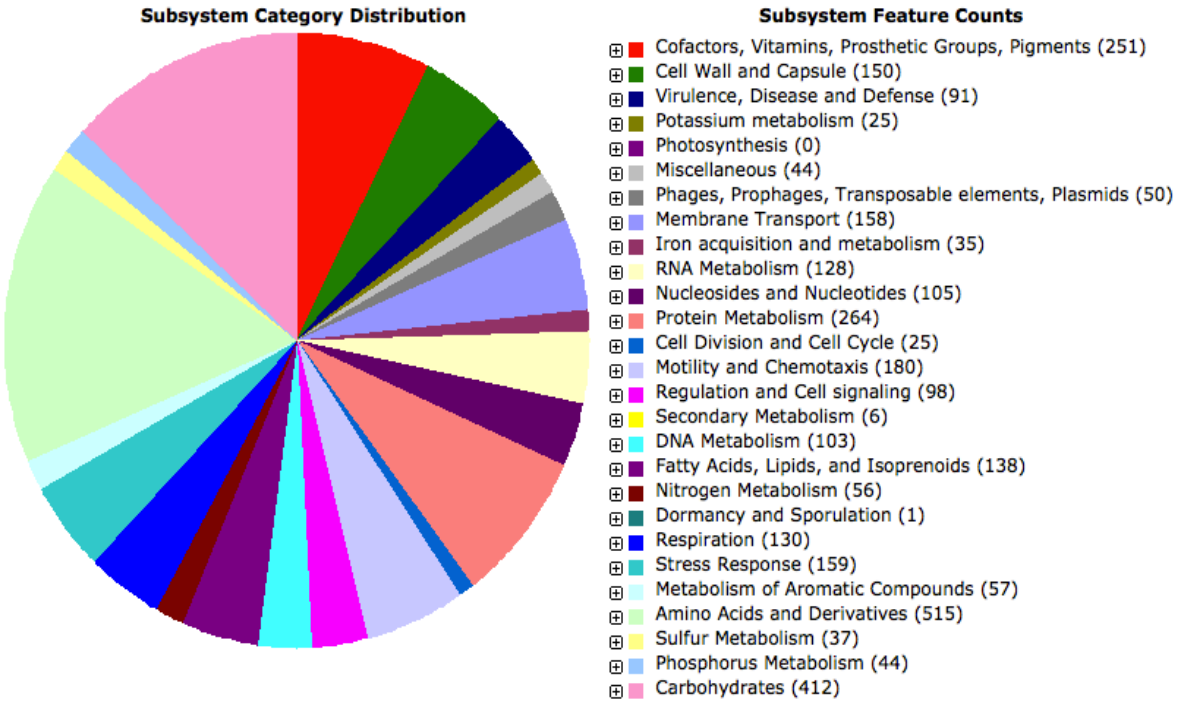

Figure 15 Main subsystem categories resulting from RAST annotation of the *C. haemolyticum* IR17 genome

Additionally, 71 tRNA genes and 6 rRNA genes were detected, according to tRNAscan-SE[123] and RNAmmer[105], respectively. Genomic data is summarized in Table 10.

The subsystems which represent a more significant part of IR17 isolates gene repertoire are amino acids and derivatives metabolism, carbohydrates and protein metabolism, as showed in Table 11.

Table 11 Functional classes of RAST predicted genes

Functional Class	Genomic features	Genes (%)
Amino acids and Derivatives	515	8.7
Carbohydrates	412	6.9
Cell Division and Cell Cycle	25	0.4
Cell Wall and Capsule	150	2.5
Cofactors, Vitamins, Prosthetic Groups, Pigments	251	4.2
DNA Metabolism	103	1.7
Dormancy and Sporulation	1	0.02
Fatty Acids, Lipids and Isoprenoids	138	2.3
Iron acquisition and metabolism	35	0.6
Metabolism of Aromatic Compounds	57	1.0
Membrane Transport	158	2.7
Miscellaneous	44	0.7
Motility and Chemotaxis	180	3.0
Nitrogen Metabolism	56	0.9
Nucleosides and Nucleotides	105	1.8
Phages, Prophages, Transposable elements, Plasmids	50	0.8
Phosphorous Metabolism	44	0.7
Potassium Metabolism	25	0.4
Protein Metabolism	264	4.4
Regulation and Cell signalling	98	1.6
Respiration	130	2.2
RNA Metabolism	128	2.2
Secondary Metabolism	6	0.1
Stress Response	159	2.7
Sulfur Metabolism	37	0.6
Virulence, Disease and Defence	91	1.5
Hypothetical	111	1.9
Unknown (conserved hypothetical)	2576	43.3
Total	5949	100.0

3 Phylogenetic analysis

The analysis of 16S rDNA gene sequence sequences and the resulting phylogenetic tree (Figure 16) confirmed the phylogenetic affiliation of strain IR17 to *C. haemolyticum* and comparison of genome sequences in the RAST server showed that the closest neighbour of *C. haemolyticum* IR17 was *C. violaceum* ATCC 12472, taking into account that this *C. violaceum* strain is the only *Chromobacterium* genus representative deposited in the SEED database.

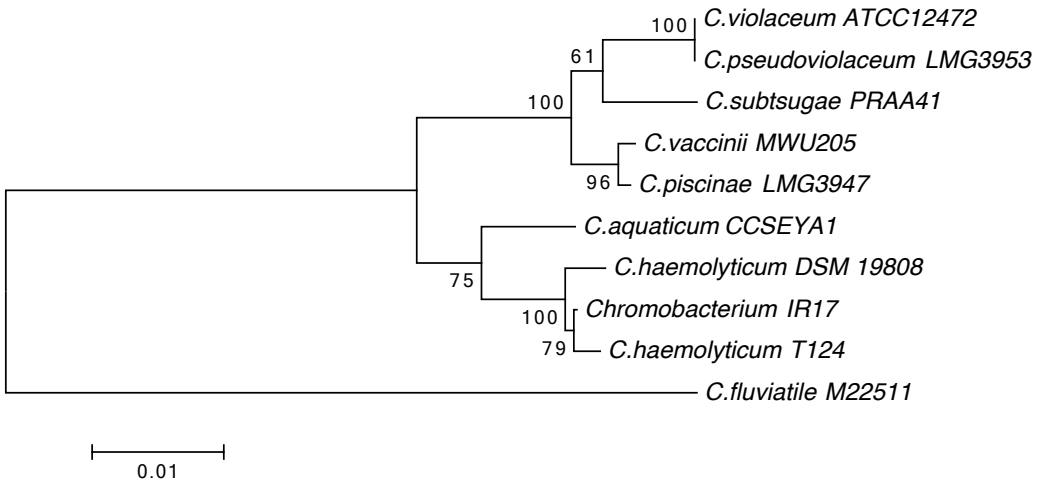

Figure 16 Phylogenetic tree based on 16S sequence of type strains of *Chromobacterium* spp. including the two *C. haemolyticum* strains full genome sequence is available and *C. haemolyticum* IR17. The numbers at the nodes represent levels (%) of bootstrap support from 1000 resampled datasets.

The resulting phylogenetic tree exhibits a significant statistical separation between *C. haemolyticum* and *C. violaceum,* which indicates that, as also proposed by Harmon *et. Al*[147], 16S rRNA sequencing is an adequate method to separate these pathogenic species in infection cases where conventional biochemical techniques may lead to their misidentification[147].

4 Antibiotic resistance profile

By analysing the RAST data regarding the virulence, disease and defence subsystem, it was possible to identify the isolates resistance arsenal and identify specific genes related to AR (Table 12).

Genetic determinants encoding resistance to β-lactams were identified as for example, class A, class C and class D β-lactamases determinants. The complete genetic determinants encoding resistance to β-lactams include, two class A, two class C and one class D β-lactamases determinants. The class D β-lactamase is a oxacillinase with 277 aminoacids (aa) that shows 42.7 % of similarity with a $bla_{\text{oxa-9}}$ found in species belonging to the *Pseudomonas, Klebsiella* and *Enterobacter* genus; the class C β-lactamases are both cephalosporinases with 388aa and 319aa that exhibit 33.6 % and 50.3 % of similarity two AmpC's characteristic of the *Aeromonas* and *Serratia* genus, respectively.

Table 12 Genes conferring AR found in *C. haemolyticum* IR17

Antibiotics	Genes	Contig location	Products
Aminoglycosides	*aaadk*	131_637	Aminoglycoside 6-adenylyltransferase
	aaaCA4	44_2721	Aminoglycoside 6-N-acetyltransferase
	amrB	136_699	Multidrug resistance protein
Beta-Lactams	*ampC*	1_18-19	Beta-lactamase class C and other penicillin binding proteins
	ampC	18_1192	Class C Beta-lactamase
	ampH	18_1193	Penicillin-binding protein
	ampG	58_3372	Permease
	bla_{Oxa}	24_1644	Beta-lactamase class D
	$bla_{KPC\text{-}like}$	57_3301	Class A Beta-lactamase
	penP	73_3893	Class A Beta-lactamase
Chloramphenicol	*mdfA*	11_221	Multidrug translocase
	mdfA	61_3507	Multidrug translocase
	rarD	114_402	Chloramphenicol-sensitive protein
Fosfomycin	*fosA*	36_2308	Fosfomycin resistance protein
Lincosamide	*uup*	59_3421	ABC transporter ATP-binding protein
	ABC transporter gene	113_343	Glutathione-regulated potassium-efflux system ATP-binding protein
Polymyxin	*arnT*	126_569	Undecaprenyl phosphate-alpha-4-amino-4-deoxy-L-arabinose arabinosyl transferase
	arnD	126_570	4-deoxy-4-formamido-L-arabinose-phosphoundecaprenol deformylase
	arnA	126_571	UDP-4-amino-4-deoxy-L-arabinose formylase
	arnC	126_572	Glycosyl transferase
	eptA	126_575	Phosphoethanolamine transferase
	arnC	17_1332	Glycosyl transferase
	arnT	17_1133	Undecaprenyl phosphate-alpha-L-Ara4N transferase
Fosmidomycin	*fsR*	58_3357-3358	Fosmidomycin resistance protein
Fusaric acid		11_209	Hypothetical protein
	fusE	181_1260	Fusaric acid resistance protein
	fusB/fusC	181_1262	Fusaric acid resistance protein
	fusB/fusC	186_1962	Fusaric acid resistance protein fusB/fusC

Finally, the two class A β-lactamases are composed by 290aa and 295aa, the first one having 69.4 % of amino acid similarity with the KPC family and the second 50,4 % with the penP enzymes family.

These features are likely to have a significant role in the isolates resistance towards β-lactam antibiotics and the presence of a chromosomal gene coding for a class A carbapenemase with high resemblance to KPC justified a profounder examination through the means of a phylogenetic analysis. MEGA6 was also used to conduct a phylogenetic analysis of class-A β-lactamase coded by *C. haemolyticum* IR17 that showed a high similarity with KPC-type enzymes. To do this, the sequences of the closest KPC-2

homologues and three *Chromobacterium* β-lactamases described by Poirel *et al.* where used[33].

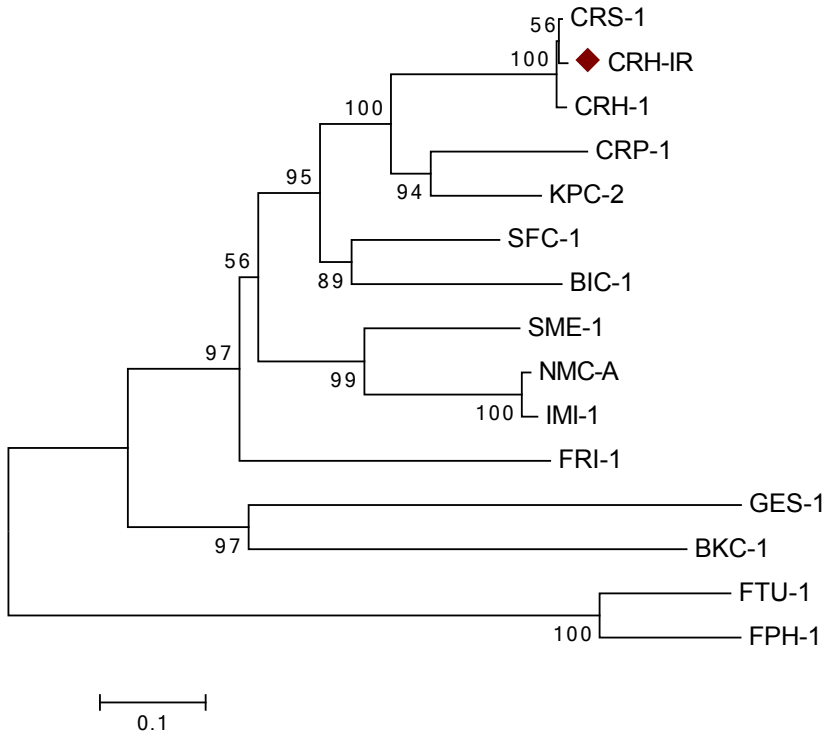

Figure 17 Phylogenetic tree based on class A β-lactamases deduced amino acid sequence detected in *C. haemolyticum* IR17 genome (marked with a shape) and closest matches previously described[33]. GenBank accession numbers: CRP-1, WP_043629745.1; CRH-1, WP_043592266.1; CRS-1, WP_039755574.1; KPC-2, AY034847; SFC-1, AY354402; BIC-1, GQ260093; NMC-A, Z21956; IMI-1, U50278; SME-1, U60295; GES-1, AAL82589; FTU-1, YP_513599.1; FPH-1, ZP_05249935.1; FRI-1, KT192551; and BKC-1, KP689347. The three was generated using the neighbor-joining method tree with 1000 bootstrap replicates. Bootstrap confidence is shown in %.

The conducted phylogenetic analysis (Figure 17) indicated that the putative class-A β-lactamase from *C. haemolyticum* (CRH-1) and from *Chromobacterium sp*. C-61 (CRS-1) were more closely related with the KPC identified in the *C. haemolyticum* IR17 genome, exhibiting 98.0 % identity in terms of amino acid sequence with CHR-1 and 99.0 % with CRS-1. Both CRS-1, CRH-1 and CRH-IR, are more closely related to KPC-2 than to other class A carbapenemases (Figure 17). As suggested by Poirel et al.[33], this suggests that bla_{KPC} genes may have their origin in *Chromobacterium* genus.

Also, chromosomal-encoded resistance to aminoglycosides was detected, namely the presence of three enzymes, one with 22.8 % similarity with an aminoglycoside O-nucleotidylyltransferase, one with 25.7 % with a phosphotransferase and the other

exhibiting 56.1 % similarity with a aminoglycoside N-acetyltransferase. Additionally, a protein was detected presenting 58.1 % amino acid similarity with the gluthanione transferase protein *Fos*a, responsible for fosfomycin resistance. Chromosomal-encoded resistance to fluoroquinolones was detected as a result of mutations identified in DNA gyrase genes (*gyrA* and *gyrB*) and topoisomerase IV subunit A and B (*parC* and *parE*) and also, genes related to AR mediated by multidrug efflux pumps were identified, as for instance components of the MATE, MFS, RND, ABC and DMT superfamilies. Some of these genes code for components of efflux systems that confer resistance to specific antibiotics. These include two MFS transporters exhibiting 68.6 % and 67.3 % similarity with components of a fosmidomycin efflux pump, four MFS transporters with 32.8 %, 34.0 %, 28.5 % and 39.7 % similarity with components of a chloramphenicol efflux pump and two RND transporters showing 60.2 % and 46.6 % similarity with components of a macrolide specific efflux system.

Through the means of a BLAST search in the NCBI database, we found all of these elements possess high similarity with others that were already described in other *Chromobacterium* genomes. Full details of AR traits are available in Table 12 and supplementary Table S3.

Moreover, genome analysis revealed that the genome of *C. haemolyticulm* IR17 presented several gene clusters related to metals resistance, namely to copper, zinc, arsenic, chromium, cobalt and cadmium. However, taking into account the results revealing a low tolerance level to metals, these genes expression doesn't seem to have a relevant role in the isolates metal resistance phenotype.

It is important to notice that, when performing a WGS analysis, the presence of a determinate set of resistance genes doesn't mean that they are necessarily expressed phenotypically. However, there is the potential that these genes may function as vectors by being transferred to another bacterial host, which may be pathogenic and may, this way, express the ARG and develop resistance.

When screening the RAST subsystems related to mobile genetic elements, we detected a significant arsenal composed by thirteen transposases, five integrases, one putative resolvase and seven recombinases present in the *C. haemolyticum's* IR17 genome. Also, we searched the IR17 isolates genome for genomic islands and found the presence of genes coding for multidrug resistance efflux pumps proteins and also genes coding for an

arsenate reductase and a aminoglycoside 6-adenyltransferase which confer resistance to arsenic and aminoglycosides, respectively, in these structures.

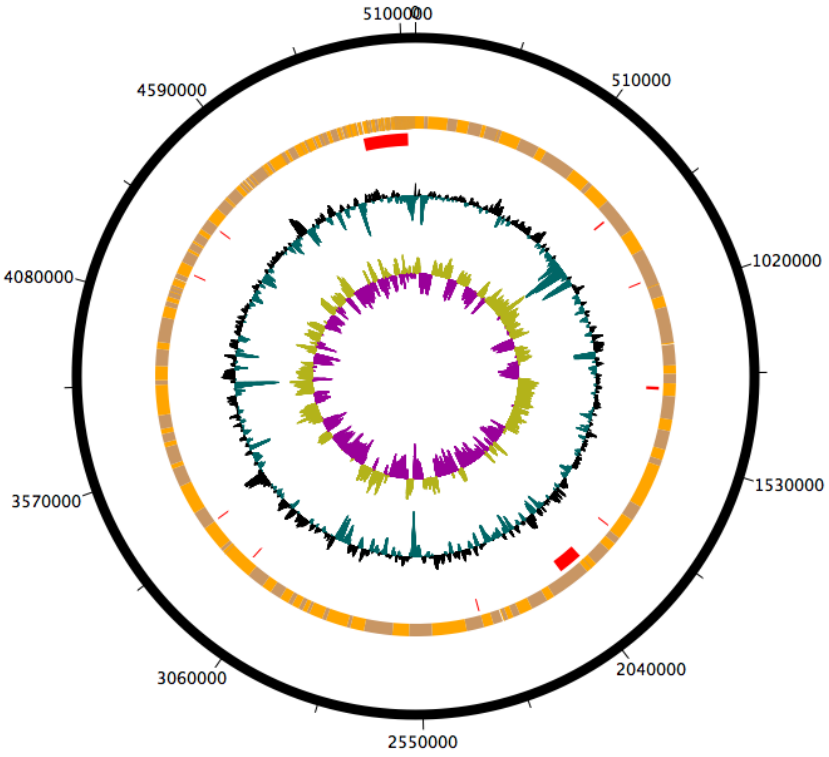

Figure 18 Graphical map of the *C. haemolyticum IR17* draft genome, were genomic islands are shown in red. G+C content plot and GC skew (purple: negative values, olive: positive values) are also show

Furthermore, similar to the *C. violaceum* ATCC 12472 and *C. haemolyticum* T124 previously reported genomes, *C. haemolyticum* IR17 also contains the virulence-associated type III secretion genes cluster.

More information on the location and composition of the *C. haemolyticum* identified genomic islands is shown in Figure 18 and Table S4.

CONCLUSIONS

The aim of this study was to analyse the resistome and mobilome of *P. aeruginosa* and *C. haemolyticum* environmental isolates using a WGS approach, making also a comparative study with the results obtained from phenotypic tests. *P. aeruginosa* E67 isolate was obtained from a polluted salt marsh known to be heavily contaminated with metals[95], while the *C. haemolyticum* IR17 was obtained from non polluted river water[89]. Thus, this study also aimed to find if there is a relation between the isolates resistance arsenal and the possible influence of the polluted environments in which these isolates exist.

Regarding *P. aeruginosa* E67, this study led to the following conclusions:

- MDR *P. aeruginosa* strains are present in metal-contaminated environments.
- *P. aeruginosa* E67 owns a rich arsenal of resistance determinants.
- MLST analysis of *P. aeruginosa E67* showed that it fits the profile of ST395, a ST that is represented by high-risk clones often associated to MDR phenotypes.
- Multidrug resistance efflux pumps represent the majority of the isolates resistance arsenal and are responsible for its resistance to several metals and antibiotics.
- There is a predominance of cross-resistance mechanisms associated to the overexpression of multidrug efflux pumps.
- Several genetic determinants contributing to motility were identified.

Concerning *C. haemolyticum* IR17, the following conclusions were drawn:

- *C. haemolyticum* IR17 is sensible to all the metals tested, namely copper, zinc, nickel and arsenic.
- *C. haemolyticum* IR17 is sensible to most of the tested antibiotic classes.
- Overall, metal and antibiotic resistance profile may be related to an absence of selective pressures in unpolluted river water.
- Isolate IR17 is resistant to some last resource β-lactam antibiotics with great clinical relevance such as carbapenems.
- 16S rRNA sequencing is an efficient method in distinguishing C. *haemolyticum* and *C. violaceum* isolates. Thus, in cases of infection, it can be used when conventional biochemical methods are inconclusive.

- The *in silico* genome analysis, revealed the presence of several β-lactamase coding genes in *C. haemolyticum's* IR17 genome and also several efflux pumps.
- The presence of a gene encoding a new class A β-lactamase with possible carbapenemase activity in the isolates genome was detected. It most likely contributes to the isolates resistance towards β-lactam antibiotics, namely carbapenems. Also, the sequence of this β-lactamase presents maximum homology with KPC-type carbapenemases.
- The presence of a $bla_{\text{KPC-like}}$ in the IR17's genome supports the recent argument stating that the *Chromobacterium* genus is involved in the evolution of KPC encoding genes[33].

Globally, the current study contributes to an expansion in the comprehension of the resistome owned by *P. aeruginosa* and *C. haemolyticum*. By adopting a WGS approach, we are also increasing the availability of genome sequences that can be used for the performance of comparative genomic analysis. In the case of *P. aeruginosa*, there is still few data regarding environmental isolates and concerning *C. haemolyticum*, there is a general lack of information, since only two genomes have been sequenced and are currently available. Also, the interest in analysing environmental isolates is reinforced with this study, since these microorganisms can contain resistance mechanisms with clinical relevance that can be transferred to pathogenic bacteria.

Studies that apply whole-genome analysis are already proven to be an excellent means for deciphering the AR dynamics in environmental compartments and in the future, as this technology becomes integrated into the clinical setting, in conjunction with established susceptibility tests, antibiotic treatment will be enhanced in a more rigorous, rapid and appropriate manner.

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APPENDICE

Table S1 Drug transporter and efflux pumps found in *P. aeruginosa* E67

Drug transporters	Genes	Contig Location	Gene products
Multiple Antibiotic Resistance MAR Locus	marC	19_1680	Multiple antibiotic inner membrane resistance protein
	marC	75_5951	Membrane protein
RND family transporter	opmQ	27_3489	Outer membrane pyoverdine eflux protein
	tolC	33_3489	Outer membrane efflux protein
	hlyD	27_2677	HlyD family membrane fusion protein
	bepF	99_6672	Transporter, periplasmic adaptor subunit
	bepE	99_6673	Membrane transporter protein
MexCD-OprJ	nfxB	15_1179	Transcriptional regulator
	mexC	15_1180	Membrane fusion protein MexC
	mexD	15_1181	Transporter permease subunit
	oprJ	15_1182	Outer membrane lipoprotein from NodT family
	nfxB	15_1183	Transcriptional regulator
MexXY-OprM	oprM	2_1718-1719	Outer membrane protein
	mexY	2_1720-1722	Inner membrane trasnporter
	mexX	2_1723-1724	Membrane fusion protein
	mexZ	2_1725	TetR family transcriptional regulator
MexCD-OprJ	nfxB	15_1179	Transcriptional regulator
	mexC	15_1180	Membrane fusion protein
	mexD	15_1181	RND transporter
	oprJ	215_1182	Outer membrane lipoprotein
	nfxB	15_1183	Transcriptional regulator
MexJK-OprM	mexL	10_147	Transcriptional regulator
	mexJ	10_146	Membrane fusion protein
	mexK	10_145	Multidrug efflux transporter
	oprM	10_143	Outer membrane efflux protein
MexHI-OpmD	mexG	67_5565	Transmembrane protein
	mexH	67_5566	Membrane fusion protein
	mexI	67_5567	Transporter Protein
	OpmD	67_5569	Outer membrane protein
MexEF-oprN	mexT	16_1283	LysR-type transcriptional regulator
	mexE	16_1284	Membrane fusion protein
	mexF	16_1285	Transporter Protein
	oprN	16_1286	Outer membrane protein
	mexE	122_797	Periplasmic adaptor subunit
	mexF	122_798	Transporter protein
	oprN	122_799	Outer membrane protein
MexAB-oprM	mexR	42_4044	Transcriptional regulator MexR
	mexA	42_4045	MexAB-oprM family transporter periplasmic adaptor subunit
	mexB	42_4046	MexAB-oprM family transporter periplasmic adaptor subunit
	oprM	42_4047	Outer membrane efflux protein
	mexB	48_4426-4427	Multidrug efflux transporter
	mexA	48_4428	Efflux membrane fusion protein

	oprM	48_4429	Outer membrane lipoprotein
	MexB	61_6714	Inner membrane transporter
	MexA	61_6715-6716	Membrane fusion protein
	nalD	74_5928	Transcription repressor
ABC family transporter	ABC permease	15_1186	ABC transporter, permease protein
	ABC permease	15_1187	ABC transporter, permease protein
	tolC	15_1188	Outer membrane protein
	HlyD	15_1189	HlyD family secretion protein
	ATPase	22_2118	ATPase components of ABC transporters with duplicated ATPase domains
	ATPase	10_141	ABC-type multidrug transport system, ATPase component
	ATPase	10_188	ABC-type multidrug transport system, ATPase and permease component
	ABC permease	19_1653	ABC-type multidrug transport system, permease component
	ABC permease	34_3542	ABC-type multidrug transport system, permease component
	ABC transporter	20_1849	ATP-binding protein
	ABC transporter	22_2073	ATP-binding componente
	ABC transporter	249_2536	Substrate-binding periplasmic component
	ABC permease	3_2983	Arabinose ABC transporter permease
	ABC transporter	295_2865	Substrate-binding periplasmic component of uncharacterized ABC transporter
	ABC trasnporter	28_2756	ATP-binding/permease fusion ABC transporter
	ABC transporter	36_3685	ATP-binding/permease fusion ABC transporter
	ccsA	10_223	CcsA related protein
	ABC permease	63_5240	Permease componente of ABC-type dipeptide/oligopeptide/nickel transport systems
	ABC transporter	65_5305	Periplasmic substrate binding protein
		3_3065	Hypothetical protein
	ABC transporter	67_5496	ABC-type bacteriocin/lantibiotic exporters
	ABC transporter	67_5553	Binding protein component
	ABC transporter	30_3133-3134	ABC transporter
		49_4500-4501	Hypothetical protein
	ABC transporter	84_6032	ATP-binding component
	ABC transporter	68_6507-5610	ABC transporter
	ABC transporter	68_5621-5623	ABC-type protéase exporter
	ABC transporter	9_6222-6223	ABC transporter
	ABC transporter	61_6736-6737	ABC transporter
	ABC transporter	17_1497-1499	ABC transporter
	ABC permease	84_6033	ABC-type antimicrobial peptide transport system component
	ABC transporter	49_4556-4558	ABC transporter
	ABC transporter	146_1132	Periplasmic component
		63_5084	Hypothetical protein
	ABC transporter	17_1537-1539	ABC transporter
	ABC transporter	13_994	ATP-binding protein

	ABC transporter	15_1185	ATP-binding protein
	ABC transporter	17_1512	ATP binding protein
		63_5094	Hypothetical protein
	ABC transporter	30_3135	ATP-binding protein
	yvcR	49_4499	ABC transporterATP-bindin componente
		24_2429	Hypothetical protein
	ABC permease	34_3543	ABC permease
	ABC transporter	84_6005	ATPase component
	ABC transporter	3_2882-2883	ABC transporter
	ABC permease	19_1654	ABC permease
	ABC transporter	1_83-84	ABC transporter
	ABC transporter	22_2119	ATPase component
	msbA	33_3462	Lipid A export ATP-binding/permease protein
	msrA	33_3434	Peptide methionine sulfoxide reductase
Macrolide resistance	macB	27_2678	Macrolide transport system ATP-binding/permease protein
	macA	27_2679	Pyoverdine-specific efflux macA-like protein
MATE family trasnporter	pmpM	24_2429	MATE family efflux transporter
	norM	4_3952	MATE family efflux transporter
	MATE family	43_4223	multidrug resistance protein
Membrane components of tripartite multidrug resistance system	dsbA	100_329	Disulfide bond formation protein
	Tripartite multidrug resistance system protein	3_3191	Outer membrane protein
	Tripartite multidrug resistance system protein	40_3998	Transporter protein
	Tripartite multidrug resistance system protein	50_4679	Outer membrane transporter protein
	Tripartite multidrug resistance system protein	50_4680-4681	Membrane fusion component
	Tripartite multidrug resistance system protein	86_6156	Outer membrane protein
	Tripartite multidrug resistance system protein	211_2059	Outer membrane component of tripartite multidrug resistance system
	hlyD	86_6155	Hemolysin secretion protein D
	tolC	15_1188	Outer membrane protein
	hlyD	15_1189	HlyD family membrane fusion protein
	hlyD	52_4784	putative secretion protein
	hlyD	60_5035	Multidrug resistance efflux pump
	romA	111_537	Outer membrane protein romA
	romA	21_1889	Outer membrane protein romA
	romA	21_1922	Outer membrane protein romA
Drug/Metabolite transporter superfamily	DMT permease	22_2099	Permease of the drug/metabolite transporter (DMT) superfamily
	DMT protein	24_2428	Putative DMT superfamily metabolite efflux protein percursor

	DMT permease	29_2840	Permease of the drug/metabolite transporter (DMT) superfamily
	DMT permease	30_3177	Permease of the drug/metabolite transporter (DMT) superfamily
	DMT permease	30_3194	Permease of the drug/metabolite transporter (DMT) superfamily
	DMT permease	31_3228	Permease of the drug/metabolite transporter (DMT) superfamily
	DMT permease	39_3853	Permease of the drug/metabolite transporter (DMT) superfamily
	DMT permease	39_3857	Permease of the drug/metabolite transporter (DMT) superfamily
	DMT permease	43_4207	Permease of the drug/metabolite transporter (DMT) superfamily
	DMT permease	46_4365	Permease of the drug/metabolite transporter (DMT) superfamily
	DMT permease	52_4786	Permease of the drug/metabolite transporter (DMT) superfamily
	DMT permease	7_5690	Permease of the drug/metabolite transporter (DMT) superfamily
	DMT permease	72_5857	Permease of the drug/metabolite transporter (DMT) superfamily
	DMT permease	9_6275	Permease of the drug/metabolite transporter (DMT) superfamily
	DMT permease	91_6474	Permease of the drug/metabolite transporter (DMT) superfamily
Major facilitator superfamily	MFS transporter	36_3666	Tetracycline resistance protein
	MFS transporter	86_6154	MFS transporter
	MFS permease	92_6511	MFS permease
	MFS transporter	24_2380	MFS transporter
	MFS transporter	22_2100	MFS transporter
	MFS transporter	22_2101	MFS transporter
	MFS permease	10_181	MFS permease
	MFS protein	10_191	MFS protein
	MFS permease	10_225	MFS permease
	MFS permease	10_261	MFS permease
	MFS permease	10_284	MFS permease
	MFS transporter	74_5929	MFS transporter
	MFS permease	92_4511	MFS permease
	araC	86_6155	Transcriptional regulator
	emrB	50_4682	Multidrug resistance protein B
	MFS	9_6402	MFS protein
	MFS	9_6403	MFS protein
	MFS permease	108_390	MFS permease
	MFS permease	128_842	MFS permease
	MFS transporter	13_944	MFS transporter
	MFS	14_1086	MFS protein
	MFS permease	24_2369	MFS permease
	MFS permease	24_2419	MFS permease

	MFS permease	24_2529	MFS permease
	MFS permease	42_4082	MFS permease
	MFS transporter	25_2540	MFS transporter
	MFS transporter	25_2540	MFS transporter
	MFS permease	3_3060	MFS permease
	MFS permease	33_3420	MFS permease
	MFS permease	33_3420	MFS permease
	MFS permease	42_4119	MFS permease
	MFS permease	48_4460	MFS permease
	MFS permease	68_5579	MFS permease
	MFS permease	68_5784	MFS permease
	MFS permease	72_5847	MFS permease
	MFS permease	84_6014	MFS permease
	MFS permease	9_6262	MFS permease
	MFS permease	9_6263	MFS permease
	MFS permease	67_5545	MFS permease
	MFS transporter	21_2043	MFS transporter
	MFS permease	29_2798	MFS permease
	MFS transporter	4_3936	MFS transporter
	MFS transporter	84_6065	MFS transporter
	MFS transporter	9_6264	MFS transporter
	MFF transporter	52_4789	MFS transporter
	MFS	21_1995	MFS protein
	MFS transporter	63_5109	MFS transporter
	MFS transporter	68_5605	MFS transporter
	MFS transporter	7_5677	MFS transporter
	MFS transporter	25_2609	MFS transporter
	MFS transporter	3_3002	MFS transporter
	MFS transporter	4_3967	MFS transporter
	MFS transporter	15_1154	MFS transporter
	MFS transporter	19_1665	MFS transporter
	MFS transporter	5_4664	MFS transporter
	MFS transporter	112_574	MFS transporter
	MFS transporter	112_575	MFS transporter
	EmrB/QacA subfamily	2_1765	Drug resistance transporter
	EmrB/QacA subfamily	18_1564	Drug resistance transporter
	EmrB/QacA subfamily	24_2377	Drug resistance transporter
	EmrB/QacA subfamily	24_2380	Drug resistance transporter
	DHA2 family	68_5584	Multidrug resistance permease
Small Multidrug Resistance (SMR) family	emrE	33_3470	Small multidrug resistance pump
	SMR efflux pumps	52_4884	Small multidrug resistance family-3 protein

Table S2 Predicted genomic islands in the genome of *Pseudomonas aeruginosa E67*

E67 genomic Island annotation				
Predicted Island	Start position	End position	Size (bp)	Annotation
1	118,872	132,170	13,298	Taurine dioxygenase, NADP transhydrogenase, Ferric sidephore transport system, proton channel, Biopolymer transport, Malonate descarboxylase, Triphosphoribosyl-dephospho-CoA synthetas and transferase;
3	1,080,969	1,172,023	91,054	PAGI-5/PAPI-1(Contains genes with putative metabolic functions and a PFGI-1-like cluster with hypothetical regulatory proteins)

4	1,480,484	1,512,877	32,393	MFS permease, Transcriptional regulators, Hydrolases, Gluthanione peroxidase, Ribonuclease D, Thiosulfate sulfurtransferase, Hypothetical proteins
5	3,484,262	3,589,262	105,000	PAGI-2 (Transport of heavy metals and ions, cytochrome c biogenesis, mercury resistance, metabolic capacities)
	4,616,249	4,623,604	7,355	PAGI-9 (hypothetical proteins)
6	4,752,201	4,774,680	22,479	PAGI-7 (contains transcriptional regulator and is integrated in a ATP-dependent helicase)
7	5,145,307	5,159,050	13,743	Isochrorismate synthase siderophore, Pyochelin biosynthetic protein PchC, 2,3-dihydroxybenzoate-AMP ligase siderophore, Hypothetical proteins
8	5,504,879	5,524,775	19,896	PAGI-4 (Contains genes with putative metabolic functions)
9	6,234,299	6,253,186	18,887	PAGI-8 (Predicted to encode an ATPase, a Zn-dependent transcriptional regulator, and a DotA/TraY-like protein)
10	6,269,282	6,313,853	44,571	PAGI-6 (phage related genomic Island)
11	6,884,676	6,908,671	23,995	PAGI-1 (contains genes potentially involved in oxidative stress) and CRISPR-associated proteins)
12	7,757,760	8,787,760	103,000	PAGI-3 (Cargo genes thought to confer metabolic, transport, and resistance capacities)

Table S3 Drug transporter and efflux pumps found in C. Haemolyticum IR17

Drug transporters	**Genes**	**Contig Location**	**Gene products**
Multiple Antibiotic Resistance MAR Locus	marC	28_1780	Multiple antibiotic resistance protein
	marC	3_1900	Multiple antibiotic resistance protein
	marC	57_3310	Probable multiple antibiotic resistance protein
RND family transporter		136_700	Membrane fusion protein
	cmeC	147_899	Outer membrane lipoprotein
	cmeC	150_977	Outer membrane lipoprotein
	cmeA	156_1019	Membrane fusion protein
	cmeB	156_1020	Inner membrane trasnporter
		186_1268	Membrane fusion protein
		72_2787	Membrane fusion protein
		73_3910	HlyD family secretion protein
		24_1627	Probable membrane fusion protein
	RND transporter	49_2899	RND transporter
	cmeC	55_3184	Outer membrane lipoprotein
	nodT	57_3302	Outer membrane lipoprotein
		57_3305	Membrane fusion protein
		24_1626	Acriflavin resistance protein

		417_2619	Acriflavin resistance protein
ABC family transporter	ABC permease	156_1016	ABC permease component
	ABC permease	108_168	ABC transporter permease protein
	ABC permease	11_187	Probable permease of ABC transporter
	ABC transporter	15_932	Probable ABC transporter ATP binding protein
	ABC transporter	156_1017	Probable ABC transporter ATP binding protein
	ABC permease	186_1272	Probable permease of ABC transporter
	ABC transporter	24_1567	ABC-transporter protein
	ABC transporter	29_1837	ABC-transporter protein
	ABC transporter	29_1841	ABC-transporter protein
	ABC transporter	34_2136-2137	Permease of ABC transporter
	ABC permease	41_2605	Permease of ABC transporter
	ABC permease	44_2658	Permease of ABC transporter
	ABC transporter	44_2697	ATPase componente of ABC transporter
	ABC transporter	55_3112	ABC-transporter protein
	ABC trasnporter	61_3537-3538	ABC-transporter protein
	ABC transporter	62_3549	ABC-transporter protein
	ABC transporter	62_3553	ABC-transporter protein
	ABC transporter	73_3898-3899	ABC-transporter protein
	ABC transporter	73_3909	ATP-binding protein
	ABC transporter	8_4152	ABC-transporter protein
	ABC transporter	87_4472	Hypothetical protein
Macrolide resistance	macB	150_978	Macrolide export ATP-binding/permease protein
	macA	150_979	Macrolide-specific efflux protein
MATE family trasnporter	MATE family	93_4740	Multi antimicrobial extrusiion protein
	MATE family	29_1817	multidrug resistance protein
Membrane components of tripartite multidrug resistance system	Tripartite multidrug resistance system protein	181_1264	Outer membrane protein
	Tripartite multidrug resistance system protein	58_3390	Outer membrane protein
	Tripartite multidrug resistance system protein	58__3391	Outer membrane transporter protein
Drug/Metabolite transporter superfamily	DMT permease	126_574	Permease of the drug/metabolite transporter (DMT) superfamily
	DMT permease	14_813	Permease of the drug/metabolite transporter (DMT) superfamily
	DMT permease	14_851	Permease of the drug/metabolite transporter (DMT) superfamily
	DMT permease	18_1213	Permease of the drug/metabolite transporter (DMT) superfamily
	DMT permease	20_1372	Permease of the drug/metabolite transporter (DMT) superfamily
	DMT permease	26_1714	Permease of the drug/metabolite transporter (DMT) superfamily

	DMT permease	30_1957	Permease of the drug/metabolite transporter (DMT) superfamily
	DMT permease	31_2026	Permease of the drug/metabolite transporter (DMT) superfamily
	DMT permease	44_2737	Permease of the drug/metabolite transporter (DMT) superfamily
	DMT permease	46_2817	Permease of the drug/metabolite transporter (DMT) superfamily
	DMT permease	52_2960	Permease of the drug/metabolite transporter (DMT) superfamily
	DMT permease	73_3870	Permease of the drug/metabolite transporter (DMT) superfamily
	DMT permease	92_4675-4676	Permease of the drug/metabolite transporter (DMT) superfamily
Major facilitator superfamily	MFS permease	141_870	MFS permease
	MFS transporter	20_1367	Probable MFS transporter
	MFS transporter	77_4001	MFS transporter
	MFS transporter	58_3387	MFS transporter
		80_4189	Multidrug resistance protein
	MFS transporter	80_4211	Probable MFS transporter
	MFS transporter	82_4306	Probable MFS transporter percursor
	MFS transporter	92_4669	MFS transporter
		35_2298	Multidrug resistance protein D
		80_4201	Multidrug resistance protein B
		40_2287	Probable transport transmembrane protein
		35_2226	Probable multidrug resistance protein
	yceL	3_1857	MFS superfamily export protein
		27_1753	Multidrug resistance protein B
Other multidrug resistance efflux pumps	tetR	156_1018	Transcription repressor of multidrug efflux pump acrAB operon
		17_1147	Probable multidrug resistance protein
	tolC	49_2900	Type I secretion outer membrane protein

Table S4 Predicted genomic islands in the genome of *C. Haemolyticum IR17*

IR17 genomic Island annotation				
Predicted Island	Start position	End position	Size (bp)	Annotation
1	719,580	725,603	6,023	Arsenate reductase; Components of tripartite multidrug resistance system; Tyrosine-protein kinase Wzc; Capsule polysaccharide export protein; Putative outer membrane lipoprotein;
2	961,884	967,309	5,425	Hypothetical proteins;

3	1,319,436	1,329,560	10,124	Site-specific recombinase XerC; Hypothetical proteins;
4	1,822,827	1,827,030	4,370	Molybdenum ABC transporter ; Hypothetical proteins;
5-8	1,962,339	2,045,042	82,703	Exoenzymes regulatory protein AepA percursor; Transcriptional regulator; Prolidase; Outer membrane usher protein; Sensory box histidine kinase; NERD domain protein Hypothetical proteins; Permease of the DMT superfamily;
9	2,352,725	2,356,925	4,200	Tyrosine recombinase XerC; Hypothetical proteins
10	3,165,069	3,170,367	5,298	Collagen triple helix repeat domain; Sulfate and thiosulfate binding protein CysP; Hypothetical protein;
11	3,341,226	3,345,656	4,430	Probable transmembrane sensor; Probable ABC transporter; Transcriptional regulator; Formiminoglutamase; Hypothetical protein;
12	4,201,558	4,206,900	5,342	Lipid carrier; UDP-N-acetylglucosamine 4,6.dehydratase; UDP-glucose-4-epimerase; UDP-N-acetylglucosamine 2-epimerase
13	4,373,434	4,378,281	4,847	Glycosyltransferase; dTDP-glucose 4,6-dehydratase; Hypothetical protein
14-18	4,959,190	5,109,946	150,756	Hypothetical proteins; Putrescine transport system permease; Putrescine transport ATP-binding protein PotG; Regulator of nucleoside diphosphate kinase; Probable dipeptyl aminopeptidase; Retron-type RNA-directed DNA polymerase; Aminoglycoside 6-adenylyltransferase