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Antibiotic resistance in *Pseudomonas aeruginosa* – Mechanisms, epidemiology and evolution

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ABSTRACT

Antibiotics are powerful drugs used in the treatment of bacterial infections. The inappropriate use of these medicines has driven the dissemination of antibiotic resistance (AR) in most bacteria. *Pseudomonas aeruginosa* is an opportunistic pathogen commonly involved in environmental- and difficult-to-treat hospital-acquired infections. This species is frequently resistant to several antibiotics, being in the "critical" category of the WHO's priority pathogens list for research and development of new antibiotics. In addition to a remarkable intrinsic resistance to several antibiotics, *P. aeruginosa* can acquire resistance through chromosomal mutations and acquisition of AR genes. *P. aeruginosa* has one of the largest bacterial genomes and possesses a significant assortment of genes acquired by horizontal gene transfer (HGT), which are frequently localized within integrons and mobile genetic elements (MGEs), such as transposons, insertion sequences, genomic islands, phages, plasmids and integrative and conjugative elements (ICEs). This genomic diversity results in a non-clonal population structure, punctuated by specific clones that are associated with significant morbidity and mortality worldwide, the so-called high-risk clones. Acquisition of MGEs produces a fitness cost in the host, that can be eased over time by compensatory mutations during MGE-host coevolution. Even though plasmids and ICEs are important drivers of AR, the underlying evolutionary traits that promote this dissemination are poorly understood. In this review, we provide a comprehensive description of the main strategies involved in AR in *P. aeruginosa* and the leading drivers of HGT in this species. The most recently developed genomic tools that allowed a better understanding of the features contributing for the success of *P. aeruginosa* are discussed.

1. *Pseudomonas aeruginosa*: the making of a superbug

Pseudomonas aeruginosa is a Gram-negative rod-shaped gammaproteobacterium. It is a ubiquitous microorganism present in multiple ecological niches, thriving in aquatic and soil habitats due to its metabolic versatility. *P. aeruginosa* can colonize several living sources, such as plants, animals and humans (Gellatly and Hancock, 2013; Moradali et al., 2017; Silby et al., 2011). It is also an opportunistic human pathogen associated with an ever-widening array of life-threatening acute and chronic infections, including cystic fibrosis (CF), ventilator-associated pneumonia, urinary tract infections, otitis externa, burn and wound injuries, bone and joint infections, bacteremia and systemic infections (Shortridge et al., 2019; Bassetti et al., 2018; Burrows, 2018; Nguyen et al., 2018; Parkins et al., 2018). *P. aeruginosa* is one of the "ESKAPE" pathogens – *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *P. aeruginosa* and *Enterobacter* species –, emphasizing its impact on hospital infections and the ability of this microorganism to "escape" the activity of antibacterial drugs (Fig. 1) (Boucher et al., 2009). This species is also in the "critical" category of the World Health Organization (WHO)'s priority list of bacterial pathogens for which research and development of new antibiotics is urgently required (Fig. 1) (Tacconelli et al., 2018). Moreover, according to the International Nosocomial Infection Control Consortium report, comprising a surveillance study from January 2010 to December 2015 in 703 intensive care units (ICU) worldwide, nosocomial infections caused by *P. aeruginosa* have become a healthcare concern, mainly due to the high level of resistance to several antibiotics (Rosenthal et al., 2016). The European Centre for Disease Prevention

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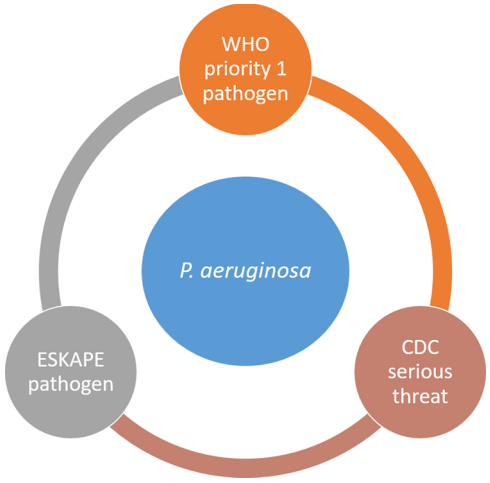

Fig. 1. Mnemonics assigned to *P. aeruginosa*. WHO classifies *P. aeruginosa* as a "critical" pathogen for which research and development of new antibiotics is urgently required, whereas the US Center for Disease Control flagged multidrug-resistant *P. aeruginosa* as a serious threat and as one of the ESKAPE pathogens causing nosocomial infections worldwide.

and Control (ECDC) Epidemiological Report 2016 on healthcare-associated infections (HAI) acquired in ICU, comprising data submitted by 15 European Union countries (plus Iceland, Liechtenstein and Norway) in 2014, revealed that *P. aeruginosa* was the most frequently isolated microorganism in ICU-acquired pneumonia episodes and one of the most prevalent in ICU-acquired urinary tract infections and ICU-acquired bloodstream infections (ECDC, 2018a). Point prevalence surveys of HAI and antimicrobial use in European long-term care facilities and acute care hospitals showed that *P. aeruginosa* was one of the microorganisms most frequently isolated from HAI (ECDC, 2013, 2014).

Metabolic versatility, the production of a myriad of virulence factors, the formation of biofilms and antibiotic resistance (AR) comprise the critical traits contributing to the pathogenicity of *P. aeruginosa* (Balasubramanian et al., 2013; Granato et al., 2018; Juan et al., 2017a). The pathogenic potential of this species is governed by the transcriptional, post-transcriptional and post-translational regulation of several systems. An intricate network of transcription factors, quorum-sensing (QS), two-component regulatory systems, non-coding RNAs and unidentified environmental signals controls the expression of these traits (Andersen et al., 2018; Balasubramanian et al., 2014; Chambonnier et al., 2016; Chen et al., 2019; Darch et al., 2018; Ferreira et al., 2019; Koeppen et al., 2016; Kollaran et al., 2019; Lee and Zhang, 2015; Mikkelsen et al., 2011; Nadal Jimenez et al., 2012; Talà et al., 2019). QS is the central regulatory mechanism, controlling biofilm formation, secretion systems, efflux pumps, motility and AR in a population density-dependent manner. The production of several virulence factors, such as alginate, toxins, proteases and siderophores is also regulated by QS. QS controls the expression of multiple virulence factors in a population-dependent manner and is activated by several regulators such as LasR, RhIR, AmpR, MvfR and VqsR (Balasubramanian et al., 2013). Additionally, *P. aeruginosa* is intrinsically resistant to a wide variety of antibiotics (Alekshun and Levy, 2007; Blair et al., 2015; Breidenstein et al., 2011; Burrows, 2018; Chevalier et al., 2017; Du et al., 2018; Hancock, 1998; Juan et al., 2017b; Li et al., 2015; Livermore, 2001; Moradali et al., 2017; Nguyen et al., 2018), and can easily switch from a planktonic to a sessile lifestyle in response to environmental changes, leading to the formation of biofilms, the hallmark of *P. aeruginosa* disease progression and chronic infections (Juan et al., 2017a; Lee and Zhang, 2015; Mikkelsen et al., 2011; Moradali et al., 2017; Schick and Kassen, 2018; Secor et al., 2018).

In this review, we explore the main strategies involved in AR in *P. aeruginosa* and the most important drivers of horizontal gene transfer (HGT) in this species, with emphasis on the barely exploited field analyzing the role of integrative and conjugative elements in the dissemination of carbapenemase-encoding genes. We also sought to provide a brief explanation on the most recently developed genomic tools that allowed a better understanding of the features contributing to the success of *P. aeruginosa*. While this review focuses on *P. aeruginosa*, the concepts discussed herein are also important to other bacteria. Novel therapeutic strategies have been extensively reviewed elsewhere (S.J. Baker et al., 2018; Bassetti et al., 2018; Burrows, 2018; Lynch et al., 2017; Nguyen et al., 2018; Pang et al., 2019).

2. Omics approaches to explore *P. aeruginosa* lifestyle

The genome of *P. aeruginosa* PAO1 reference strain, isolated more than 50 years ago from a patient wound in Melbourne, Australia, was the first to be sequenced, following a community-aided approach that was initiated on 1977 (Stover et al., 2000). The 6.3 mega base pair (Mb) genome comprising 5570 predicted open reading frames was the largest bacterial genome to be sequenced back then. Interestingly, no evidence of recent gene duplication was found, leading to the conclusion that the large genome resulted from genetic and functional diversity. Additionally, a high percentage of predicted regulatory genes (9.4%) was identified in *P. aeruginosa* PAO1 strain and directly linked to the genome size, a trend frequently encountered in bacterial species thriving to survive in several niches (Mathee et al., 2008; Stover et al., 2000). Moreover, about 150 genes identified on *P. aeruginosa* PAO1 strain are predicted to encode for outer membrane proteins involved in adhesion, motility, antibiotic and virulence factors export, an outsized number compared with other genomes (Chevalier et al., 2017). A disproportionately large number of genes (nearly 300) encoding for transport systems and enzymes involved in the uptake of nutrients was also reported (Chevalier et al., 2017; Stover et al., 2000). Genome size, predicted regulatory genes and complexity are consistent with the remarkable versatility frequently described in this species and reflects evolutionary adaptation to multiple distinct niches (Mathee et al., 2008; Stover et al., 2000). Indeed, the expansion on nutritionally restricted environments through acquisition of new metabolic functions most likely comprises a key player shaping the *P. aeruginosa* genome (Mathee et al., 2008; Schick and Kassen, 2018), an evolution pattern contrasting to the observed genome reduction upon adaptation of pathogens to a parasitic existence (Mathee et al., 2008; Moran, 2002; Moran et al., 2009; Weinert and Welch, 2017). A recent genomic and phenotypic analysis of ten different laboratory *P. aeruginosa* PAO1 isolates showed variable production of molecular products, suggesting alterations in transcriptional and translational regulation and the ongoing microevolution during culture and storage that may affect reproducibility when using the reference strain in different laboratories (Chandler et al., 2018).

Comparative genomic analysis performed by Mathee et al. and later revisited, revealed that *P. aeruginosa* genome presents a picture of a mosaic genome, composed of a large number of core genes interspersed by strain-specific blocks of genes (Fischer et al., 2016; Freschi et al., 2018b; Hilker et al., 2015; Klockgether et al., 2011; Kung et al., 2010; Mathee et al., 2008; Valot et al., 2015). The latter segments were defined as regions of genomic plasticity (Mathee et al., 2008). The sum of these unique regions comprise the accessory genome. As such, the *P. aeruginosa* pangenome is currently envisioned as the result of the sum of the (1) core genome, comprising genes that are identified in almost all *P. aeruginosa* strains and the (2) accessory genome, encompassing strain-specific genes usually inserted *en bloc* and clustered in certain loci that may encode niche adaptation properties (Freschi et al., 2018a, 2018b; Kung et al., 2010; Mathee et al., 2008; McInerney et al., 2017;

Valot et al., 2015). Genes are classified as unique in comparative genomics when identified in a single genome. The pangenome of a given species depends on the number of sequenced genomes, and for species with an extended and diverse accessory genome such as *P. aeruginosa*, each new sequenced genome will enlarge this pool of genes and will naturally decrease those of the core genome (Freschi et al., 2018b; Klockgether et al., 2011). Klockgether et al. found that the core genome is largely collinear among *P. aeruginosa* strains and that the accessory genome is the main contributor for genome diversity (Klockgether et al., 2011). Intra-clonal genome diversity of *P. aeruginosa* major clones showed that the core genome was highly conserved, while the accessory genome was equally variable at the intra- and inter-clonal level, suggesting a stronger purifying selection in the core genome than in the accessory genome (Fischer et al., 2016). Comparing with the genome of two major pathogens *Escherichia coli* and *S. aureus*, *P. aeruginosa* displays a larger average size (Boissy et al., 2011; Touchon et al., 2009). Curiously, genome dynamics among these pathogens is remarkably different, reflecting different host-bacteria interactions. Indeed, *E. coli* displays a very large pangenome (17838 genes) and a small core genome (1976 genes), encompassing a variety of commensal and highly pathogenic strains (Touchon et al., 2009). On the other hand, the pangenome of *S. aureus* (3221 genes) is quite close to the average and core genomes size (3118 and 2245 genes, respectively), probably reflecting an optimized capacity to maintain core phenotypes and to survive the human host's conditions (Boissy et al., 2011).

According to the EzBioCloud website (http://www.ezbiocloud.net/) (Yoon et al., 2017), the mean values for size, guanine-cytosine (GC) content and number of coding sequences of the 3684 quality controlled *P. aeruginosa* genomes (of which only 165 represent complete genomes) are around 6.6 Mb, 66.2% and 6140, respectively (accessed July 30, 2019). The Pathosystems Resource Integration Center (PATRIC, https://www.patricbrc.org/) database harbors a wider collection of *P. aeruginosa* genomes, comprising 4974 whole-genome sequencing (WGS) projects and 422 complete genomes (accessed July 30, 2019) (Wattam et al., 2017). The *Pseudomonas* Genome Database (http://*Pseudomonas*.com/), besides providing significant updates to the *P. aeruginosa* PAO1 genome annotations, also allows large scale comparative curated genome analysis and visualization (Winsor et al., 2016). Another interesting repository of *P. aeruginosa* genomes, the International *Pseudomonas* Consortium Database (https://ipcd.ibis.ulaval.ca/), currently contains genomic and phenotypic data regarding more than 1700 isolates from soil, water, plants, animals and human infections (accessed July 30, 2019). The main purpose was to perform a metadata analysis linking bacterial genotype, phenotype and clinical data, as well as to build a user-friendly pipeline for genome evolution, AR and virulence (Freschi et al., 2015). BACTOME (https://bactome.helmholtz-hzi.de/cgi-bin/h-intro.cgi) is a recently developed database for the identification of genotype–phenotype correlations of *P. aeruginosa* clinical isolates, to explore sequence variation and the transcriptional landscape of this species (Hornischer et al., 2018).

Apart from genomics, other omics yielded new insights into the organization of the *P. aeruginosa* genome as well as its biology, diversity and virulence (Dötsch et al., 2015; Dunphy et al., 2019; Gao et al., 2018; Gonzalez et al., 2018; Han et al., 2019; Jensen et al., 2017; Rossi et al., 2018; Skurnik et al., 2013; Tognon et al., 2019; Wurtzel et al., 2012). Koeppen et al. used RNA-seq to characterize a novel mechanism whereby *P. aeruginosa* reduces the host immune response through packaged intracellular small RNAs in outer membrane vesicles (Koeppen et al., 2016). A recent transcriptomics study revealed that several AR genes had substantially higher expression in human infections than under laboratory conditions, most likely explaining why AR assays often underestimate resistance in patients (Cornforth et al., 2018). Turner et al. conducted RNA-seq and genome-wide insertion mutant fitness profiling (Tn-seq) analyses to investigate the genetic requirements for acute and chronic pathogenesis in *P. aeruginosa* infections, and demonstrated that the combination of these techniques is quite helpful for the study of physiology, metabolism and virulence (Turner et al., 2014). In addition, Limoli et al. highlighted the important role of alginate overproduction by mucoid strains of *P. aeruginosa* in reducing the production of anti-*S. aureus* effectors such as siderophores and rhamnolipids, promoting their co-existence in a Model of CF Respiratory Infection (Limoli et al., 2017). Lassek et al. provided a quantitative proteomics analysis from *P. aeruginosa* clinical isolates derived from several infection sites, and hypothesized that common and highly expressed proteins might be considered as promising targets for antimicrobial therapy (Lassek et al., 2016).

3. Antibiotic resistance in *P. aeruginosa*

Antibiotics are powerful medicines used in the treatment of bacterial infections. The inappropriate use of these drugs has promoted the spread of AR in most bacteria (WHO, 2015; Cassini et al., 2018; CDC, 2017; Chatterjee et al., 2018; Davies and Davies, 2010; de Kraker et al., 2016; Núñez-Núñez et al., 2018; Political Declaration, 2016; Temkin et al., 2018; Versporten et al., 2018; Waglechner and Wright, 2017; Tacconelli et al., 2018; O'Neill, 2016; Collignon et al., 2018; European Centre for Disease Prevention and Control, 2018b; ECDC, 2018b; Gillings et al., 2017; Holmes et al., 2016; Klein et al., 2018; Livermore, 2018). The WHO's report on AR provided the first ever global picture and national surveillance capacity with data provided by 114 countries, exposing high levels of AR in all regions of the world and significant gaps in tracking AR (WHO, 2015). The report also lists infection control actions to prevent a post-antibiotic era, in which common infections and minor injuries can once again kill. Maintaining the trend that has been registered throughout the years, the 12th edition of the World Economic Forum's Global Risks Report included rapid and massive spread of infectious diseases, as a result of AR, among the top societal risks confronting the world ("The Global Risks Report, 2017). According to recent estimates, nearly 700,000 people die every year as a consequence of AR infections worldwide (O'Neill, 2016). Additionally, according to the latest ECDC AR surveillance report, the European population-weighted mean for *P. aeruginosa* isolates with combined resistance (resistance to three or more antibiotic groups, including carbapenems) was 12.9%, a steady-state value at least since 2012 (ECDC, 2017). Recently, several approaches have been explored to constrain AR evolution (Durão et al., 2018), such as cellular hysteresis and antibiotic combination efficacy networks (Brochado et al., 2018; Barbosa et al., 2018, 2017; Imamovic et al., 2018; Roemhild et al., 2018), 'evolution-proof' antibiotics (Bell and MacLean, 2018; Ling et al., 2015), inactivation of evolvability factors (Ragheb et al., 2018), preventing the evolution of antibiotic tolerance (Brauner et al., 2016; Levin-Reisman et al., 2017; Meredith et al., 2018) and functional metagenomics combined with next-generation approaches (Crofts et al., 2017).

Eight categories of antimicrobial agents are frequently used to treat *P. aeruginosa* infections: aminoglycosides (gentamicin, amikacin, netilmicin, tobramycin), carbapenems (imipenem, meropenem, doripenem), cephalosporins (ceftazidime, cefepime), fluoroquinolones (ciprofloxacin, levofloxacin), penicillins with β-lactamase inhibitors (ticarcillin-clavulanic acid, piperacillin-tazobactam), monobactams (aztreonam), phosphonic acids (fosfomycin) and polymyxins (colistin and polymyxin B). Ceftazidime-avibactam and ceftolozane-tazobactam were already approved by the FDA and are available for use, while cefiderocol and imipenem-cilastatin/relebactam are currently in development (Barnes et al., 2018; Bassetti et al., 2018; Giani et al., 2018; Nguyen et al., 2018). According to Magiorakos et al., *P. aeruginosa* strains are defined as multidrug-resistant (MDR) if non-susceptible to ≥ 1 agent in ≥ 3 antimicrobial categories, extensively drug-resistant (XDR) if non-susceptible to ≥ 1 agent in all but ≤ 2 categories and pandrug-resistant (PDR) if non-susceptible to all antimicrobial agents listed (Magiorakos et al., 2012).

As stated in the aforementioned reports, MDR bacteria are an increasing healthcare concern worldwide, and many of the involved mechanisms have been unveiled over the last decades. One of the main

drivers for the emergence of MDR in the majority of Gram-negative and Gram-positive bacteria is horizontal gene transfer (HGT). An intricate network of several factors is responsible for the evolution, transmission (vertical and/or horizontal) and maintenance of AR in bacterial populations, such as i) mutation frequency, ii) the resistance level conferred by a given mechanism, iii) fitness cost associated with the burden of AR, iv) epistatic interactions, v) the development of co-, cross- or pleiotropic resistance and vi) the nature of the selective pressure (Baltrus, 2013; Bell and MacLean, 2018; Cantón and Ruiz-Garbajosa, 2011; Estrela and Brown, 2018; Frost et al., 2018; Good et al., 2017; Knopp and Andersson, 2018; Knöppel et al., 2017; Lehtinen et al., 2017; Sánchez-Diener et al., 2017; Schenk et al., 2013; Sommer et al., 2017; Yokoyama et al., 2018). Besides sub-inhibitory concentrations of antibiotics, non-antibiotic pharmaceuticals may also promote AR through HGT (Jutkina et al., 2018; Maier et al., 2018; Wang et al., 2018). Curiously, intrinsic resistance and naturally occurring tools were present in bacteria from all environments (including those without human influence) long before the appearance of the first antibiotics (Box 1) (Clemente et al., 2015; D'Costa et al., 2011; Dantas et al., 2008; Karkman et al., 2018). D'Costa et al. performed metagenomics of ancient environmental DNA from 30,000-year-old Beringian permafrost sediments and identified several genes encoding resistance to antibiotics, providing the first evidence that the presence of genes encoding for AR is indeed an ancient phenomenon that predates our use of antibiotics (D'Costa et al., 2011). These enzymes displayed 53 and 84% identity with known β-lactamases, and are frequently expressed at low levels.

Even though HGT plays a decisive role in the emergence of AR among several species, spontaneous mutation is the main driver in *P. aeruginosa* resistance evolution (Lister et al., 2009). *P. aeruginosa* can develop resistance to a wide range of antibiotics, mainly due to a combination of intrinsic, acquired and/or adaptive resistance (Bassetti et al., 2018; Breidenstein et al., 2011; Chevalier et al., 2017; Juan et al., 2017b; Moradali et al., 2017; Nguyen et al., 2018; Oliver et al., 2015). The main intrinsic resistance feature in *P. aeruginosa* is its low outer membrane permeability, which is ~12–100-fold lower than that reported for *E. coli* (Hancock, 1998). Together with the production of AmpC and MexAB-OprM, these strategies largely contribute to the low basal susceptibility of *P. aeruginosa* to antibiotics. Alongside the genes encoding for AmpC, GyrA/GyrB and ParC/ParE, those encoding for the four clinically relevant AR efflux pumps (MexAB-OprM, MexCD-OprJ, MexXY and MexEF-OprN) were all identified in the core genome (Valot et al., 2015). Efflux pumps are in fact ancient elements present in bacteria long before the antibiotic era, being responsible for the extrusion of several toxic compounds (Du et al., 2018; Martinez et al., 2009). The constitutive expression of MexAB-OprM is responsible for conferring a basal low susceptibility to nearly all β-lactams (with the exception of imipenem) and fluoroquinolones (Li et al., 2015). Overexpression and/or modification of this pump, alongside deletions of specific chromosomal regions and mutations leading to β-lactam target modification may promote AmpC-independent β-lactam resistance (Cabot et al., 2018). Besides AmpC, *P. aeruginosa* presents other two chromosome-encoded β-lactamase that influence the basal level of susceptibility to β-lactams: the OXA-50 class D β-lactamase and the metallo-β-lactamase PIB-1 (Fajardo et al., 2014; Girlich et al., 2004; Juan et al., 2017b). FosA homologues are present in the majority of genomes in some species, including *P. aeruginosa*, and contribute to intrinsic fosfomycin resistance (Ito et al., 2017b, 2017a).

Adaptive resistance is induced by the presence of antibiotics or other environmental factors, as biocides, pH, anaerobiosis, as well as social bacterial activities for instance swarming motility and biofilm formation (Breidenstein et al., 2011; Moradali et al., 2017; Spalding et al., 2018). These triggers may affect the basal expression level of AmpC and efflux pumps. The inducible expression of AmpC is involved in the intrinsic resistance of this species to aminopenicillins and cephalosporins and in the basal resistance level to imipenem, since these molecules prompt the expression of this β-lactamase (Livermore, 1992). The inducible expression of MexXY confers decreased susceptibility to aminoglycosides (Li et al., 2015). Given the large pool of regulatory genes identified on *P. aeruginosa* genome (Stover et al., 2000), the adaptive phenomenon is expected to play a major role in the outcome of resistance.

Mutational inactivation or loss of outer membrane protein-encoding *oprD* gene plays a key role in imipenem resistance and also reduces susceptibility to meropenem, an antibiotic of the same class (cross-resistance) (Cantón and Ruiz-Garbajosa, 2011; Chevalier et al., 2017; Skurnik et al., 2013). *P. aeruginosa* frequently develops resistance to penicillins, cephalosporins and monobactams due to AmpC overexpression by means of specific mutations in peptidoglycan-recycling genes, such as *dacB*, *ampR*, *ampD* and its homologues *ampDh2* and *ampDh3* (Cabot et al., 2011; Juan et al., 2006; Moya et al., 2009). Furthermore, target mutations in *ampC* were shown to increase resistance to cephalosporins (Berrazeg et al., 2015; Rodríguez-Martínez et al., 2009). Regulation of AmpC (frequently designated as PDC, for

Box 1
Evolution of antimicrobial resistance (AR).

A recent study conducted on the host-associated microbiome of Amerindians with no known exposure to antibiotics found multiple AR genes, revealing that these functional genes may be a natural feature of the human microbiome (Clemente et al., 2015). Additionally, environmental metagenomics studies have identified several AR genes in different niches, suggesting that besides the availability of functional AR genes, the successful transmission of these genes into the clinical setting is also related to their association with mobile genetic elements (MGE), the fitness cost of the gene and the interactions between the donor and recipient niches (D'Costa et al., 2011; Forsberg et al., 2014; Munck et al., 2015; Sommer et al., 2009). An interesting tactic to circumvent this problem was recently proposed by Sommer et al.: functional metagenomics of the human microbiome with which a specific pathogen is most likely to interact should be inspected prior to the development of a new antimicrobial drug (Sommer et al., 2017). With this approach, it would be possible to screen the pool of AR genes (resistome, as coined by D'Costa et al. (D'Costa et al., 2006)) of a given environment and to evaluate the risk assessment of a new antibiotic. Besides intrinsic and acquired resistance genes, the resistome encompasses proto-resistance genes, with the potential to evolve to a resistance function, and cryptic resistance genes, which are not obviously associated with resistance due to low or lack of expression (Crofts et al., 2017).

Back in 1973, the theory that environmental antibiotic-producing bacteria were the evolutionary origin of clinically relevant AR genes was starting to gain attention (Benveniste and Davies, 1973). A recent study by Forsberg et al. developed a functional metagenomics pipeline that unveiled the first evidence of shared multi-drug resistant genes between non-pathogenic soil bacteria and human pathogens (Forsberg et al., 2012). However, resistome composition is correlated with the phylogenetic and taxonomic structure of a microbial community, and the synteny of AR genes with MGE appears to be less frequent in environmental metagenomes than in the genome of human pathogens, suggesting that these genes may not be transferred between environmental bacteria as willingly as between clinically relevant pathogens (Forsberg et al., 2014). Due to the ancient origins of both naturally occurring antibiotics and AR genes, it is not surprising that clinical and agricultural use of antibiotics has potentiated the dissemination of resistance (Knapp et al., 2010). Future studies should prioritize increased surveillance on hotspots for HGT of AR genes, such as the agricultural settings, hospitals, wastewater and sewage settings, through functional metagenomics, next-generation sequencing and computational analyses (Pärnänen et al., 2019; Crofts et al., 2017; Munck et al., 2015).

Pseudomonas-derived cephalosporinase) comprises one of the most intricate repression-derepression systems, since the transcription of bla_{PDC} is controlled by the aforementioned peptidoglycan-recycling genes (Juan et al., 2006; Rodríguez-Martínez et al., 2009). AmpR is a major transcriptional regulator that also plays a role in switching from the acute to the chronic infection traits, by controlling the expression of virulence factors through QS, biofilm formation and the production of alginate (Balasubramanian et al., 2014; Gifford et al., 2018).

Specific mutations in DNA gyrase and topoisomerase IV-encoding genes *gyrA*, *gyrB*, *parC* and *parE* are responsible for fluoroquinolone resistance in *P. aeruginosa* (Bruchmann et al., 2013). Mutations on efflux pump regulatory genes, such as *nalC* and *nfxB*, may affect antibiotics of the same class or from different classes (pleiotropic resistance), including some β-lactams, fluoroquinolones and aminoglycosides (Cantón and Ruiz-Garbajosa, 2011; Fernández and Hancock, 2012; Livermore, 2001). Evolved colistin resistance in *P. aeruginosa* is a complex and multistep process, that likely emerges through mutation in at least five independent loci with epistatic interactions (Dößelmann et al., 2017; Jochumsen et al., 2016; Lee et al., 2016). Recently, the transferable colistin resistance gene *mcr* was described for the first time in *P. aeruginosa* within a Tn*3*-like transposon (Snesrud et al., 2018).

4. Carbapenemases identified on *P. aeruginosa*

Due to their high importance for human medicine, carbapenems are considered by the WHO as critically-important antimicrobials that should be reserved for the treatment of infections caused by multidrug resistant Gram-negative bacteria in humans (EFSA, 2013). Recent data from ECDC, presenting the latest information on European antimicrobial consumption, revealed that the population-weighted mean consumption of carbapenems did not show any significant change during 2011–2015 (ECDC, 2018c). According to the latest ECDC AR surveillance report, the European population-weighted mean for *P. aeruginosa* isolates with resistance to carbapenems was 17.4% (ECDC, 2018c). The latest ECDC epidemiological report on HAI acquired in ICU showed that the percentage of carbapenem-resistant isolates in *P. aeruginosa* was 27.7%, which represents the treatment challenges for ICU patients (European Centre for Disease Prevention and Control, 2018a).

Even though chromosomal mutations (such as those leading to the loss or inactivation of OprD porin and/or to the overexpression of efflux pumps) are still the most important promoters of carbapenem resistance in *P. aeruginosa*, the acquisition of carbapenemase-encoding genes (CEGs) is extremely relevant (Botelho et al., 2015, 2017b, 2018a, 2018b, 2018c; Breidenstein et al., 2011; Diene and Rolain, 2014; Moradali et al., 2017; Oliver et al., 2015; Partridge et al., 2018b; Queenan and Bush, 2007). These β-lactamases are able to hydrolyze carbapenems and confer resistance to virtually all β-lactam antibiotics. A recent survey conducted on 14 European countries revealed that 20% of carbapenem-resistant *P. aeruginosa* isolates were carbapenemase producers (Castanheira et al., 2014). Carbapenemases are not intrinsically expressed by *P. aeruginosa*, but rather encoded by heterologous genes acquired by HGT. Only two resident carbapenemases were identified in *Pseudomonas*: POM-1 from *Pseudomonas otitidis* and PAM-1 from *Pseudomonas alcaligenes* (Suzuki et al., 2014; Thaller et al., 2011). Similar to the majority of the carbapenemases reported among this genus, these resident carbapenemases are metallo-β-lactamases (MBL). These Ambler class B β-lactamases present a pan-β-lactam resistance phenotype with the exception of aztreonam, high carbapenemase activity and resistance to β-lactamase inhibitors (Berglund et al., 2017; Cornaglia et al., 2011; McGeary et al., 2017). Amid this class, several families of enzymes presenting at least 30% of amino acid similarity

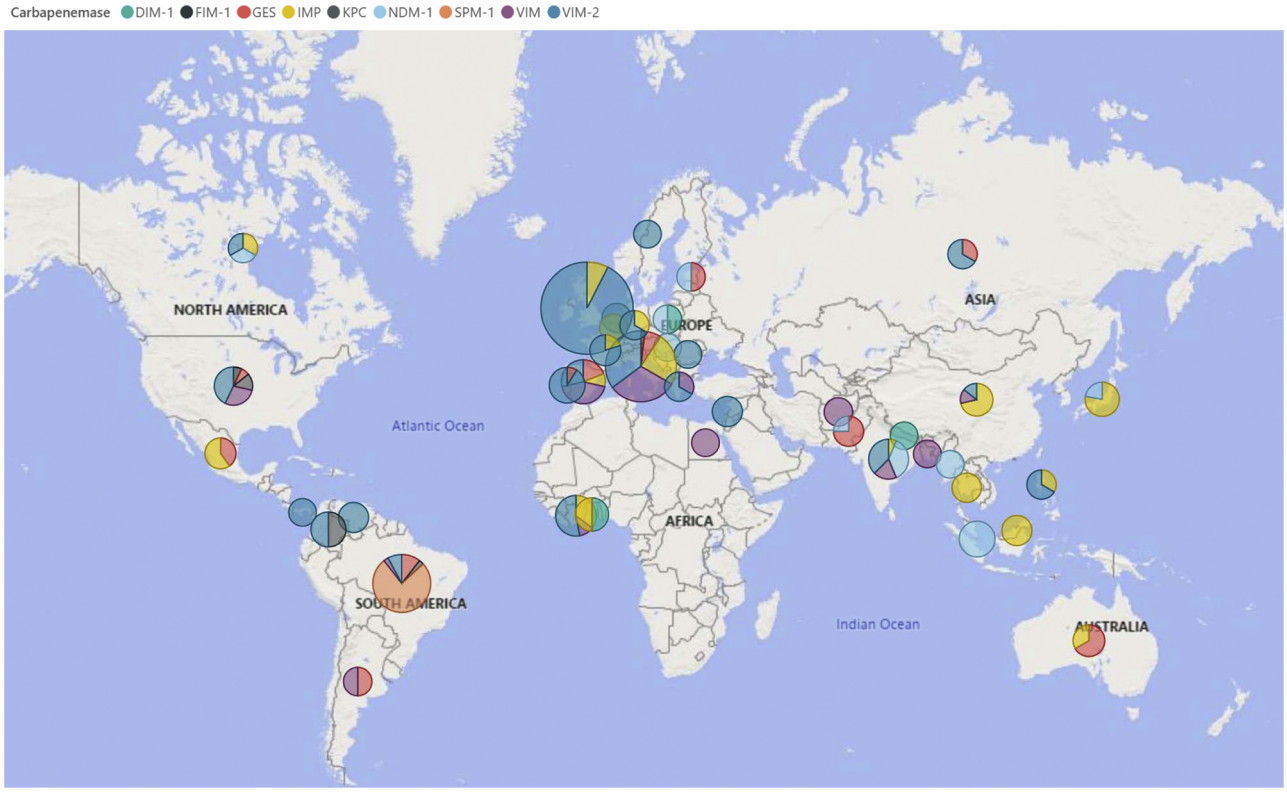

Fig. 2. Carbapenemases identified in all *P. aeruginosa* genomes available on NCBI (accessed on the 11/4/2019). 4532 genomes were downloaded and blasted against an in-house database of 688 carbapenemases (adapted from (Botelho et al., 2018c)). 397 carbapenemases were identified and country information was retrieved from the genbank files. When more than one variant was identified (such as in GES, IMP and KPC), the family name was included on the figure legend. VIM-2 was separated from the remaining VIM variants, to highlight its prevalence. The size of the circles reflects the total number of sequenced genomes with the given carbapenemase.

among them were described in *P. aeruginosa*: VIM, IMP, NDM, SPM, DIM, GIM and FIM (Fig. 2) (Berglund et al., 2017; Cornaglia et al., 2011; Nascimento et al., 2016; Oliver et al., 2015; Poirel et al., 2010; Queenan and Bush, 2007; Turton et al., 2015). The bla_{VIM}, bla_{IMP} and bla_{NDM-1} are widely disseminated, opposed to bla_{SPM-1}, bla_{GIM-1}, bla_{FIM-1} and bla_{AIM-1}, which appear to be restricted to Brazil, Germany, Italy and Australia, respectively (Cornaglia et al., 2011; Oliver et al., 2015). Even though bla_{SPM-1} has been identified in Switzerland and in the United Kingdom (Hopkins et al., 2016; Salabi et al., 2010), both patients had been previously hospitalized in Brazil. The bla_{VIM-2} is by far the most frequently reported CEG in *P. aeruginosa* (Botelho et al., 2018c; Cornaglia et al., 2011; Oliver et al., 2015). A few strains were reported to carry more than one carbapenemase (Botelho et al., 2018c). Despite the fact that the origin of these acquired MBL remains unknown, environmental bacteria sharing the same ecologic niche as clinically important pathogens are the most likely source (Cornaglia et al., 2011). Recent approaches to discover MBL inhibitors have been developed (McGeary et al., 2017).

In addition to MBL, serine carbapenemases belonging to Ambler class A β-lactamases, such as KPC-2 and several GES variants, have also been reported in *P. aeruginosa* (Fig. 2) (Botelho et al., 2015, 2018a, 2018c; Dai et al., 2016; Naas et al., 2013; Oliver et al., 2015; Queenan and Bush, 2007). Besides the intrinsic oxacilinase OXA-50, Ambler class D β-lactamases are rarely identified in this species. To date, only three class D carbapenemases have been reported in *P. aeruginosa*: OXA-40, OXA-181 and OXA-198 (El Garch et al., 2011; Evans and Amyes, 2014; Meunier et al., 2016; Sevillano et al., 2009).

5. The role of the *P. aeruginosa* accessory genome in the dissemination of resistance

As aforementioned, the accessory genome is composed of strain-specific genes that may encode niche adaptation properties. In *P. aeruginosa*, the accessory genome comprises integrons, transposons, insertion sequences, genomic islands (GIs), prophages and phage-like elements, plasmids and integrative and conjugative elements (ICEs) (Kung et al., 2010). Larger genomes are more prone to be the recipient of HGT events from divergent organisms, a correlation that might be related to the decrease effect of codon bias with total genomic size (Baltrus, 2013; diCenzo and Finan, 2017; Guglielmini et al., 2011). Since *P. aeruginosa* comprises one of the largest bacterial genomes, it is not surprising to observe the large pool of genes acquired by HGT. Most of these elements are able to mobilize, and frequently possess a mosaic structure comprising modules from different mobile genetic elements (MGEs). Since this species exhibits a high GC content, foreign DNA from other species will be frequently associated with a lower GC content. Other features such as codon and tetranucleotide usage should be analyzed in order to correctly address this distinction (Langille et al., 2010). However, once integrated into the chromosome, genes and elements acquired by HGT will experience the same selective pressure as the core genome and may lose those sequence compositional differences (Kung et al., 2010). For instance, Valot et al. found that recently acquired prophages displayed a lower GC content when compared to long-standing ones, which experienced a genetic drift and reached a similar content as that of the core genome (Valot et al., 2015).

The MGEs frequently integrate into the chromosome by targeting specific regions of genomic plasticity within the core genome. These regions act as hotspots for the insertion of MGEs (Botelho et al., 2018c; Kung et al., 2010; Mathee et al., 2008; Oliveira et al., 2017). These elements may harbor genes i) that improve bacterial persistence within different ecological niches, ii) involved in AR, iii) that encode for virulence determinants, and so their acquisition by HGT may confer advantageous traits and must be seen as a major contributor to shape genome evolution (Kung et al., 2010). However, MGE are essentially self-interested entities, promoting their self-propagation and may cause a burden to the host (Ghaly and Gillings, 2018; Rankin et al., 2011). Taking into account the significant value that mutational events already represent for AR in *P. aeruginosa*, the spread of MGE harboring AR genes illustrates the dire public health consequences of such events.

6. Integrons and "jumping" genes

Most CEGs identified in *P. aeruginosa* are associated with class I integrons (Oliver et al., 2015; Partridge et al., 2018b). Integrons are genetic elements composed of an integrase-encoding *intI* gene, an *attI* recombination site and a Pc promoter (Domingues et al., 2012; Partridge et al., 2018b). These elements present a variable region flanked by two conserved regions (5′CS and 3′CS). The integrase mediates site-specific recombination responsible for the acquisition or excision of gene cassettes harboring AR genes. Several gene cassettes may be captured by the same integron into the variable region and their expression will be further regulated by the Pc promoter (Domingues et al., 2012; Partridge, 2011; Partridge et al., 2018b). Integrons *per se* are not mobilizable, but these elements may be inserted on MGEs such as transposons, plasmids and/or ICEs (Kung et al., 2010; Partridge et al., 2018b). Also, these elements may be mobilized when a complete transposition module is provided in *trans* (Domingues et al., 2012). Related integrons may be identified in different contexts, which also suggest an important contribution of homologous recombination in promoting the evolution of integrons (Domingues et al., 2012; Partridge et al., 2018b).

The most clinically relevant class I integrons identified are 'sul1-type' integrons (Domingues et al., 2012; Partridge, 2011; Partridge et al., 2018b). These elements present a *qacEΔ1sul1* gene fusion, exposing a successful link between an antiseptic and a sulphonamide resistance gene and its further association with several AR genes, creating a so-called winner effect (Baquero, 2004; Domingues et al., 2012; Partridge, 2011). Class I integrons are derived from different lineages, comprising variable ISs. Among these, In*4*-like integrons are particularly relevant in *P. aeruginosa*. These elements present a partially duplicated IS*6100* insertion sequence downstream the 3′CS region. Variants lacking part or all of the 3′CS region, almost certainly resulting from IS*6100*-mediated deletions into the conserved region, were also identified (Partridge, 2011; Partridge et al., 2001). Surprisingly, most of these integrons are associated with a Tn*402*-like transposon which lost part of its transposition module (Domingues et al., 2012; Partridge, 2011; Partridge et al., 2018b). These defective transposons may however be mobilized in *trans* and are capable of targeting the *res* site of Tn*3*-like transposons and plasmids, establishing a Matryoshka-like arrangement that may contribute for their mobilization (Nicolas et al., 2015; Partridge, 2011; Partridge et al., 2018b; Petrovski et al., 2011). The Tn*3* family of transposons are flanked by 38-bp inverted repeats and comprise genes encoding for a transposase, resolvase and a resolution site. Tn*3*-type transposons are frequently associated with mercury resistance genes. Its dissemination throughout different bacterial species provided the bedrock for the emergence of class I integrons harboring AR genes (Nicolas et al., 2015; Partridge, 2011; Partridge et al., 2018b; Petrovski et al., 2011).

In *P. aeruginosa*, most acquired carbapenemases are present on class I integrons within Tn*402*-like transposons of chromosomal location, with the important exceptions of bla_{SPM-1}, bla_{NDM-1} and bla_{KPC-2} (Botelho et al., 2018c; Dai et al., 2016; Ding et al., 2018; Naas et al., 2013; Nascimento et al., 2016; Oliver et al., 2015; Queenan and Bush, 2007). Importantly, most integrons bearing CEGs co-harbor gene cassettes mediating resistance to other antibiotic classes (Botelho et al., 2018c; Cornaglia et al., 2011; Oliver et al., 2015; Partridge et al., 2018b). Dissemination of these integrons may then contribute to the occurrence of MDR phenotypes. Besides CEGs, integrons often harbor genes encoding extended-spectrum β-lactamases (ESBLs) mediating resistance to penicillins, third-generation cephalosporins and aztreonam (Bouheraoua et al., 2018; Garza-Ramos et al., 2015; Laudy

et al., 2017; Picao et al., 2009). Recently, aminoglycoside-modifying enzymes (AMEs, conferring selective resistance) and 16S rRNA methyltransferases (*rmt*, mediating class-wide resistance) were found to be prevalent among this species and other clinically relevant Gram-negative pathogens (Costello et al., 2018; Jackson et al., 2013). *rmt* genes were often located within class I integrons (Silveira et al., 2016; Tada et al., 2017). A quinolone resistance determinant qnrVC1 was identified within a class I integron co-harboring bla_{VIM-2} and genes conferring resistance to other antibiotics (Belotti et al., 2015). This was the first description of a *qnr* determinant in a *P. aeruginosa* strain.

Several carbapenemase-harboring transposons have been identified in *P. aeruginosa*, such as KPC-2-encoding Tn*4401*, a Tn*3*-like transposon assuring high-frequency transposition; bla_{VIM}-harboring Tn*6001*, Tn*6162*, Tn*6249*, Tn*6352* and Tn*6356* transposons; a Tn*5051*-type transposon housing a bla_{IMP-13} (Botelho et al., 2017a, 2017b; Chowdhury et al., 2017; Cuzon et al., 2011; Di Pilato et al., 2015; Naas et al., 2013; Papagiannitsis et al., 2017; Pfennigwerth et al., 2017; Roy Chowdhury et al., 2009; Samuelsen et al., 2009; Shimizu et al., 2015; Toleman et al., 2003; Tseng et al., 2009; van der Zee et al., 2018; Zhan et al., 2018). Besides CEGs, transposons may carry genes conferring resistance to other antibiotics (Dubois et al., 2002; Feng et al., 2016; Subedi et al., 2018; Uemura et al., 2010). A transferable colistin resistance gene (*mcr-5*, and reclassified as *mcr-5.1* according to the nomenclature proposed by Partridge et al. (Partridge et al., 2018a)) was recently reported within a Tn*3*-like transposon in a *P. aeruginosa* clinical isolate (Snesrud et al., 2018). This was the first report of *mcr* in colistin-unsusceptible *P. aeruginosa*.

Recently, a transposable element typing pipeline named TETyper (https://github.com/aesheppard/TETyper) was developed (Sheppard et al., 2018). This tool classifies variation and genetic contexts of transposable elements from short-read WGS data. Accurate annotation of integrons and transposable elements may be achieved by Galileo™ AMR (https://galileoamr.arcbio.com/mara/) (Arc Bio, Cambridge, MA) (Partridge and Tsafnat, 2018). The INTEGRALL database (http://integrall.bio.ua.pt/) provides an easy access to the integron DNA sequences and genetic arrangements (Moura et al., 2009). IntegronFinder (https://github.com/gem-pasteur/Integron_Finder) is a bioinformatics tool to find integrons in bacterial genomes (Cury et al., 2016).

7. Plasmids

Transposons or "jumping genes" are MGEs which integrate into and excise from the chromosome but are not capable of being horizontally transferred (Roberts et al., 2008; Roberts and Mullany, 2011). To move between cells, transposons are frequently encountered within plasmids. These autonomous self-replicating elements may be transferred from cell to cell by conjugation (Garcillán-Barcia et al., 2011; Rozwandowicz et al., 2018; San Millan, 2018; San Millan and MacLean, 2017; Smillie et al., 2010). Halary et al. reconstructed a network of MGEs and concluded that conjugation is more frequent than transduction, a bacteriophage-mediated DNA transfer mode, emphasizing the dominant role of plasmids in HGT (Halary et al., 2010; Lopatkin et al., 2017; Ramsay and Firth, 2017). The plasmid backbone is mainly taken up by genes encoding for its own propagation and stability (Hülter et al., 2017). The replication module defines the copy number of plasmid cells and its own survival in several hosts (Ilhan et al., 2018). Low copy-number plasmids are subjected to a higher frequency of plasmid loss, due to random assortment at cell division (Garcillán-Barcia et al., 2011; Smillie et al., 2010). As so, extra stability modules such as toxin-antitoxin and partition systems may be required (Baxter and Funnell, 2014; Díaz-Orejas et al., 2017).

Mobility of plasmids will depend on the set of backbone genes, and so these extrachromosomal elements may be conjugative, mobilizable or non-transmissible. Conjugative plasmids carry all the machinery necessary for self-propagation: (i) a relaxase, a key protein in conjugation; (ii) an origin of transfer site, a small DNA sequence that is recognized by the relaxase and that is required in *cis* for a plasmid to be properly transferred; (iii) a set of genes encoding for the type-IV secretion system (T4SS), which provides a membrane-spanning secretion channel connecting the donor to the recipient strain; and (iv) a type-IV coupling protein-encoding gene, associated with the link between the relaxosome and the mating channel (Garcillán-Barcia et al., 2011; Smillie et al., 2010). Mobilizable plasmids can spare the complete set of genes encoding for the T4SS, and may use those of a helper plasmid present in the cell to be successfully transferred. Conjugative plasmids tend to be low copy number and large-sized, while mobilizable plasmids are frequently high copy number and low-sized (< 30 kb) (Garcillán-Barcia et al., 2011; Smillie et al., 2010). Interestingly, Smillie et al. found that more than half of the plasmids are non-transmissible (Smillie et al., 2010), and these elements may in fact use diverse strategies to facilitate their mobilization (Peña-Miller et al., 2015; Ramsay and Firth, 2017; Wein et al., 2019).

Plasmids may harbor an accessory module (comprising for example virulence-encoding factors and/or AR genes) that confers an adaptive advantage upon their host (Loftie-Eaton et al., 2017; Rozwandowicz et al., 2018; San Millan, 2018; Svara and Rankin, 2011). Table 1 summarizes the main characteristics of the CEG-bearing plasmids that have been reported in *P. aeruginosa.* Besides CEGs, plasmids may carry genes conferring resistance to other antibiotics (Chávez-Jacobo et al., 2018; Jeannot et al., 2018).

To date, fourteen incompatibility groups (IncP-1 to IncP-14) were characterized amongst *Pseudomonas* plasmids (Partridge et al., 2018b; Thomas and Haines, 2004). Narrow host range plasmids comprise IncP types 2, 5, 7, 10, 12 and 13 and cannot be transferred onto *E. coli*. On the other hand, other groups seem to display a broad host range, being also included in the plasmid type scheme of *Enterobacteriaceae*: IncP-1

Table 1
List of relevant CEG-harboring plasmids in *P. aeruginosa* deposited on the National Center for Biotechnology Information (NCBI).

Plasmid	Carbapenemase	Inc type(s)	Size(s) (kb)	GC content (%)	Country	Origin	Year	References
pAMBL1 and pAMBL2	VIM-1	–	26 and 24	63.5 and 60.4	Spain	Clinical	2006–07	(San Millan et al., 2015a)
pNOR2000	VIM-2	–	22	62.8	France	Clinical	1996	(Bonnin et al., 2013)
pDCPR1	VIM-2	Unknown	18	58.4	Argentina	Clinical	2005	(Vilacoba et al., 2014)
pJB12	VIM-2	Unknown	30	62.6	Portugal	Clinical	2006	(Botelho et al., 2017a)
pJB37	VIM-2	IncP-2	465	57.2	Portugal	Clinical	2008	(Botelho et al., 2017b)
pS04 90	VIM-2	Unknown	159	57.7	Netherlands	Clinical	–	(van der Zee et al., 2018)
p07-406	VIM-7	–	24	63.8	USA	Clinical	2001	(Li et al., 2008)
pP378-IMP	IMP-4	IncN	51	50.5	China	Clinical	2009–2013	(Feng et al., 2016)
pOZ176	IMP-9	IncP-2	501	57.0	China	Clinical	2000	(Xiong et al., 2013)
pBM413	IMP-45	Unknown	423	56.4	China	Clinical	2012	(J. Liu et al., 2018)
pHN39-SIM	SIM-2	Unknown	282	56.9	China	Clinical	2012	(Sun et al., 2016)
pCOL-1 and pPA-2	KPC-2	IncP-6 and IncU	32 and 8	60.0 and 56.0	Colombia	Clinical	–	(Naas et al., 2013)
p10265-KPC	KPC-2	IncP-6	39	58.2	China	Clinical	2010	(Dai et al., 2016)
pOXA-198	OXA-198	IncP-11	49	60.5	Belgium	Clinical	2010 and 2013	(Bonnin et al., 2018)

(IncP), IncP-3 (IncA/C), IncP-4 (IncQ), IncP-6 (IncG) (Partridge et al., 2018b; Thomas and Haines, 2004). Contrarily to what occurred with *Enterobacteriaceae*, a replicon-based PCR for *P. aeruginosa* plasmids has not been created yet. Moreover, plasmid typing among *P. aeruginosa* is particularly challenging due to insertion, deletion, co-integration and exchange events that will determine low phylogenetic concordance between plasmid core genes (Orlek et al., 2017; Tazzyman and Bonhoeffer, 2014). The advent of WGS enabled the *in silico* analyses of a wide array of plasmids, most of them provided by assembly of short-read sequencing data (see References on Table 1). However, plasmid reconstruction can be quite puzzling (Arredondo-Alonso et al., 2017). Performing plasmid isolation before sequencing may optimize this task, but is laborious. The most appropriate solution is to undergo long-read sequencing (such as Pacific Biosciences or Oxford Nanopore MinION), which allows a more accurate reconstruction. Several *in silico* platforms are available to analyze plasmids: PLACNET (https://sourceforge.net/projects/placnet/), a reference-dependent graph-based tool and its recently developed user-friendly web-tool (https://castillo.dicom.unican.es/upload/) (Lanza et al., 2014; Vielva et al., 2017); the automated and reference-independent tools plasmidSPAdes (http://cab.spbu.ru/software/spades/) and Recycler (https://github.com/Shamir-Lab/Recycler) (Antipov et al., 2016; Rozov et al., 2016); PlasFlow (https://github.com/smaegol/PlasFlow), that employs a neural network approach to identify plasmid sequences in metagenomics data (Krawczyk et al., 2018); PLSDB (https://ccb-microbe.cs.uni-saarland.de/plsdb/), a complete bacterial plasmids database (Galata et al., 2018), Plasmid Atlas (https://github.com/tiagofilipe12/pATLAS), a web interface to browse for plasmids and their associated genes (Jesus et al., 2019) and PlasmidSeeker (https://github.com/bioinfo-ut/PlasmidSeeker), a k-mer based tool for the identification of known plasmids from sequencing reads (Roosaare et al., 2018). Future advances in plasmid metagenomics and optical mapping of intact plasmids combined with sequencing-based analysis will enhance our knowledge of the plasmidome present in different environments and to improve plasmid assembly, respectively (Orlek et al., 2017; Bikkarolla et al., 2019).

Reducing antibiotic use alone is insufficient to tackle AR since plasmids are often transferred at high rates in the absence of antibiotic selection (Lopatkin et al., 2017). Recently, novel strategies combining conjugation inhibition and promoting plasmid loss to reduce the global burden of AR were proposed (Buckner et al., 2018).

8. Genomic islands and integrative and conjugative elements

GIs comprise a cluster of genes that had been acquired by a bacterial genome by HGT and contributed to diversification and host adaptation (Bellanger et al., 2014; Kung et al., 2010; Langille et al., 2010). These broad definition may also comprise other MGEs, such as ICEs and prophages (Langille et al., 2010). GIs frequently display a sporadic distribution along the genome, a large size, an association with phage integrase genes and a sequence composition that is significantly distinct from that of the host. These elements also tend to target tRNA genes and are normally flanked by direct repeats (Bellanger et al., 2014; Kung et al., 2010; Langille et al., 2010). However, these features are not specific to these elements, which makes GIs prediction a difficult task. In fact, highly expressed genes frequently present a different sequence composition than the rest of the genome, yielding false-positive predictions of GIs. Also, foreign DNA tends to undergo amelioration in the host genome, limiting the detection of ancient GIs and leading to false-negative predictions (Karlin, 2001; Langille et al., 2010). The transfer of GIs from a donor to the recipient species with a similar sequence composition will also result in false-negatives. An additional difficulty is the fact that several GIs are not inserted next to tRNA genes and not all are flanked by direct repeats (Vernikos and Parkhill, 2008). As such, comparative genomic analysis and phylogeny-based methods should be engaged with sequence composition to perform a more robust prediction (Langille et al., 2010). Comparative genomics relies on the availability of related sequenced genomes. Moreover, if the genomes are too closely related, GIs that were inserted before the genomes diverged may not be predicted. Using at least one genome that has recently diverged, will lead to more accurate predictions (Langille et al., 2010).

In *P. aeruginosa*, the spread of AR via GIs is greatly underappreciated (Partridge et al., 2018b; Stokes et al., 2012). Few reports have explored the role of these elements for the transfer of CEGs (Botelho et al., 2018c; Chowdhury et al., 2017; Di Pilato et al., 2015; Hong et al., 2016; Martinez et al., 2012; Roy Chowdhury et al., 2016). Chowdhury et al. reported that GIs 1 and 2 are frequently targeted by Tn*6060*-like and Tn*6163*-like carbapenemase-harboring transposons, respectively (Chowdhury et al., 2017; Roy Chowdhury et al., 2016).

P. aeruginosa genomic islands that carry genes encoding for virulence factors are collectively known as pathogenicity islands (PAPI) and help to promote the pathogenic promiscuity of specific strains. For instance, several GIs were reported in *P. aeruginosa* LESB58 strain from a CF patient (Jani et al., 2016; Winstanley et al., 2009). Two GIs (PAPI-1 and PAPI-2) were reported in a *P. aeruginosa* PA14 highly virulent strain from a burn patient (Harrison et al., 2010; He et al., 2004). These elements present a mosaic structure and may contribute individually and synergistically to virulence (He et al., 2004). PAPI-2 harbors the *exoU* gene and its chaperone, *spcU*. ExoU is a phospholipase effector cytotoxin that, alongside ExoY, ExoT and ExoS, is secreted by the *P. aeruginosa* type-III secretion system (Dortet et al., 2018; Juan et al., 2017a; Sawa et al., 2016). ExoY and ExoT are identified in the majority of strains, while ExoS and ExoU are mutually exclusive. In fact, *exoS+/exoU-* genotype is typically associated with an invasive phenotype, while the *exoS-/exoU+* genotype is related to highly cytotoxic strains such as PA14 and frequently linked to chronic infections (Diaz and Hauser, 2010; Juan et al., 2017a). ExoU is a potent virulence determinant in animal models and is associated with poor clinical outcomes in human patients. The *exoU* gene was also identified on GIs related to PAPI-2 (ExoU islands A, B and C) (Botelho et al., 2018a; Kulasekara et al., 2006).

Currently, *in silico* analysis of GIs may be accomplished by IslandViewer 4 (http://www.pathogenomics.sfu.ca/islandviewer/), an integrated interface of four different GI prediction methods: IslandPick, IslandPath-DIMOB, SIGI-HMM, and Islander (Bertelli et al., 2017).

ICEs are self-transmissible mosaic and modular MGEs that combine features of i) transposons and phages, since ICEs can also integrate into and excise from the chromosome; and ii) plasmids, since ICEs can also exist as circular extrachromosomal elements and can be transferred by

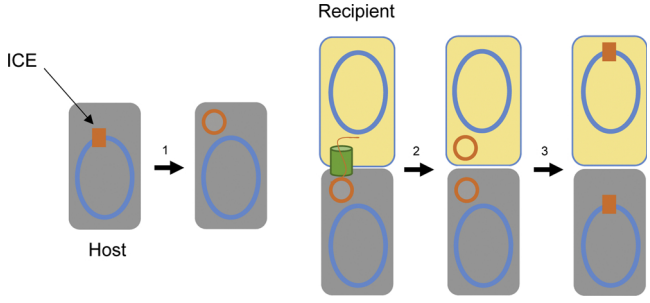

Fig. 3. The HGT of an ICE. Besides being vertically transmitted to daughter cells during cell division, ICEs can be transferred to other bacteria by conjugation. ICEs are normally integrated in the chromosome and under certain conditions can be excised and form a plasmid-like circular intermediate (Step 1). In the presence of recipient cells, donor cells can undergo a conjugative transfer event of a single DNA strand by an ICE-encoded T4SS channel (green cylinder) through rolling circle replication (Step 2). DNA replication in the recipient bacterium generates the complementary strand to synthesize the double stranded, circular form of the ICE. Recombination events result in integration of the ICE into the chromosome of each cell (Step 3).

Box 2
The mosaic structure of ICEs.

Understanding the life cycles of phages, plasmids and transposons is mandatory to correctly inform our views on ICEs. The regulatory and maintenance mechanisms are similar to those of phages and plasmids; integration/excision to phages and transposons; and conjugative transfer to plasmids (Burrus, 2017; Carraro et al., 2015; Cury et al., 2017; Delavat et al., 2017; Guglielmini et al., 2011; Johnson and Grossman, 2015; Kung et al., 2010; Toleman and Walsh, 2011; Wozniak and Waldor, 2010). In fact, a growing body of evidence is challenging the line separating plasmids and ICEs (Delavat et al., 2017; Guglielmini et al., 2011; Johnson and Grossman, 2015). Besides the aforementioned common features, several ICEs are also capable of autonomous rolling-cycle replication, which may be critical to facilitate ICE maintenance (Johnson and Grossman, 2015). Additionally, the relaxase and the T4SS encoded by ICEs resembles that of conjugative plasmids (Burrus, 2017; Carraro et al., 2015; Cury et al., 2017; Delavat et al., 2017; Guglielmini et al., 2011; Johnson and Grossman, 2015; Kung et al., 2010; Toleman and Walsh, 2011; Wozniak and Waldor, 2010). Like mobilizable plasmids, these GIs may not harbor the complete machinery for self-conjugation and may use the T4SS apparatus from a co-resident ICE or from a conjugative plasmid to be successfully transferred in *trans*. In this case, these GIs are named integrative and mobilizable elements (Guédon et al., 2017). Guglielmini et al. constructed a phylogenetic tree of VirB4, a highly conserved ATPase from the T4SS apparatus of different conjugative plasmids and ICEs, and formulated the hypothesis of interchangeable conjugation modules along the evolutionary history of these elements (Guglielmini et al., 2011). VirB4 proteins encoded by ICEs are, however, more related to those encoded by other ICEs and the same happened for plasmids. A close interplay between these elements in the deepest clades of the phylogenetic tree was observed, suggesting that plasmids may behave like ICEs and/or *vice versa*. A search of more than 1000 genomes revealed that ICEs are present in most bacterial clades and may be more prevalent than conjugative plasmids in all clades (Guglielmini et al., 2011). These researchers also found that mobilizable plasmids and integrative and mobilizable elements outnumber conjugative plasmids and ICEs, suggesting a wide utilization of T4SS in *trans*. The majority of ICEs and integrative and mobilizable elements analyzed were found in only one copy per genome (Bellanger et al., 2014). Some ICEs may have become fixed into the chromosome due to degeneration of its phage integrase genes and/or conjugative elements. ICEs are unlikely to share just one common ancestor. Since a wide variety of functional modules has been identified, the hypothesis that different ICE groups arose independently and evolved through multiple recombination events (with specific modules being acquired at different times and from different sources), seems more robust (Kung et al., 2010; Wozniak and Waldor, 2010).

conjugation (Fig. 3 and Box 2) (Burrus, 2017; Carraro et al., 2015; Cury et al., 2017; Delavat et al., 2017; Guglielmini et al., 2011; Johnson and Grossman, 2015; Kung et al., 2010; Toleman and Walsh, 2011; Wozniak and Waldor, 2010). These elements replicate as part of the host genome and are vertically inherited with the chromosome, remaining quiescent and with most of their mobility genes repressed (Burrus, 2017; Carraro et al., 2015; Carraro and Burrus, 2015; Delavat et al., 2017). ICEs are a subset of GIs (since they may also present a different GC content than that of the host chromosome, may harbor phage-related genes and may be flanked by direct and/or inverted repeats) which encode a self-conjugative transfer and integration modules (Langille et al., 2010; Toleman and Walsh, 2011). Four mating-pair formation (MPF) classes cover the T4SS among Proteobacteria: MPF_T, MPF_G, MPF_F and MPF_I (Guglielmini et al., 2013). The first is widely disseminated among conjugative plasmids and ICEs, while MPF_F is more prevalent in plasmids of γ-Proteobacteria and MPF_G is found essentially on ICEs. MPF_I is rarely identified (Guglielmini et al., 2013).

Conjugative transposons are also defined as ICEs. In fact, the first ICE to be discovered was Tn*916* (back then defined as a conjugative transposon) from *Enterococcus faecalis* (Flannagan and Clewell, 1991). Since Tn*916*-like ICEs are major drivers of HGT of AR genes (Roberts and Mullany, 2011), strategies to block their transposition by interfering with the transposase-DNA complex architecture were recently proposed as new possibilities to overcome AR (Rubio-Cosials et al., 2018).

ICEs are encompassed by integration/excision, conjugation, maintenance/regulation and accessory modules, the latter being associated with virulence, catabolic functions and/or AR and displaying little relationship between them (Burrus, 2017; Carraro et al., 2015; Cury et al., 2017; Delavat et al., 2017; Guglielmini et al., 2011; Johnson and Grossman, 2015; Kung et al., 2010; Toleman and Walsh, 2011; Wozniak and Waldor, 2010). These accessory or cargo genes are inserted in hotspot regions without disrupting ICE function. Interchangeable functional modules play a major role in shaping the evolution of ICEs (Burrus, 2017; Carraro et al., 2015; Cury et al., 2017; Delavat et al., 2017; Guglielmini et al., 2011; Johnson and Grossman, 2015; Kung et al., 2010; Toleman and Walsh, 2011; Wozniak and Waldor, 2010). Frequently, genes encoding for similar functions are clustered together. Interestingly, a recent study reported that separate chromosomal clusters may be regrouped as 'tripartite' ICE and be later transferred (Haskett et al., 2016).

As most GIs, ICEs frequently target a single insertion site, which is often a tRNA gene (Botelho et al., 2018c; Burrus, 2017; Carraro et al., 2015; Cury et al., 2017; Delavat et al., 2017; Guglielmini et al., 2011; Johnson and Grossman, 2015; Kung et al., 2010; Toleman and Walsh, 2011; Wozniak and Waldor, 2010). Even though multiple copies of these genes may be present among different hosts, ICEs tend to target only one of these loci, most likely due to specific interactions between the attachments sites of the ICE (*attP*, based on the phage nomenclature) and of the corresponding bacterial host (*attB*). However, promiscuous ICE can insert in different sites and so can be found in several locations of some bacterial genomes. This insertion is mediated by site-specific recombination promoted by a phage-like integrase, most frequently belonging to the tyrosine recombinase family. These enzymes may also be involved in the excision process (Burrus, 2017; Carraro et al., 2015; Cury et al., 2017; Delavat et al., 2017; Guglielmini et al., 2011; Johnson and Grossman, 2015; Kung et al., 2010; Toleman and Walsh, 2011; Wozniak and Waldor, 2010). The IntB13 tyrosine recombinase from ICE*clc* is an example of a P4-family integrase with unusual long length that mediates both integration and excision of the ICE (Gaillard et al., 2006; Ravatn et al., 1998).

Most ICEs identified among *P. aeruginosa* fall into two families: pKLC102-like and *clc*-related ICEs (Kung et al., 2010), a distinction that is not always consensual. For example, Klockgether et al. preferred to consider these elements as members of the same family with a common ancestry, taking into account the conserved function and synteny of the backbone genes, sharing an amino acid sequence identity of at least 20% (Klockgether et al., 2011). Main differences between these families are related to the integration site and the type of integrase: ICEs related to pKLC102 (such as PAGI-4, PAGI-5 and aforementioned ExoU island A and pathogenicity islands PAPI-1 and PAPI-2) frequently integrate into the 3′ end of $tRNA^{Lys}$ genes and present XerC/XerD-like integrases, while ICE*clc*-like elements (such as PAGI-2, PAGI-3 and LESGI-3) are more prone to target the 3′ end of a cluster of $tRNA^{Gly}$ genes and frequently harbor a bacteriophage P4-integrase (Botelho et al., 2018c; Kung et al., 2010; Larbig et al., 2002; Toleman and Walsh, 2011). Also, pKLC102-like ICEs typically share a set of syntenic conserved core genes, while ICE*clc*-like elements tend to exhibit a bipartite structure, with a conserved portion next to the integration site and unique cargo genes in the remaining ICE sequence (Kung et al., 2010). Interestingly,

Fischer et al. found that ICEs from the pKLC102 and ICE*clc* families were frequently identified among several representatives of the major *P. aeruginosa* clones C and PA14 and were the main contributors for genomic diversity (Fischer et al., 2016). The ICE Tn*4371* family also represents a large group of ICEs with a common backbone and which are widely distributed, such as in *P. aeruginosa* PA7 and 2192 (Ryan et al., 2009; Toleman and Walsh, 2011).

To date, few reports of carbapenemases were associated with an ICE location in *P. aeruginosa*, such as a bla_{GES-5}-harboring GI2 identified in Australia, a bla_{GES-6}-bearing ICE retrieved from a representative of the high-risk clone ST235, a bla_{SPM-1} inserted into ICE_{Tn4371}6061 and reported in Brazil and a bla_{NDM-1}-harboring ICE from Singapore and Poland (Botelho et al., 2018a; Chowdhury et al., 2017; Ding et al., 2018; Fonseca et al., 2015; Urbanowicz et al., 2019). Curiously, these reports are quite recent, leading us to speculate that the advent of WGS and bioinformatics will help to accurately identify more ICEs and eventually assess the real contribution for the spread of CEGs. In fact, a recent *in silico* analysis revealed that ICEs may play a vital role in disseminating CEGs among pseudomonads (Botelho et al., 2018c).

ICEberg 2.0 (http://db-mml.sjtu.edu.cn/ICEberg/) is a web-based resource that provides significant information about ICE identified in Gram-negative and Gram-positive bacteria (M. Liu et al., 2018). ICEberg also provides a tool to predict ICEs in bacterial genome sequences, named ICEfinder. The CONJscan (https://github.com/gem-pasteur/Macsyfinder_models) is a tool that scans a set of protein sequences for T4SS and relaxases of both ICE and plasmids using hidden Markov models (Abby et al., 2016; Guglielmini et al., 2014).

9. To be or not to be: the fitness cost and maintenance of antibiotic resistance

Fitness is the capacity of a genotype or individual to survive and reproduce (Andersson and Hughes, 2010; San Millan and MacLean, 2017; Vogwill and MacLean, 2015). The classic paradigm is that AR determinants endeavor a fitness cost for the bacterial host, in terms of reduced growth, competition and/or infectivity (Andersson and Hughes, 2010; Baltrus, 2013; Hall et al., 2017; Loftie-Eaton et al., 2017; Melnyk et al., 2015; Rodriguez-Beltran et al., 2018; San Millan et al., 2018; Vogwill and MacLean, 2015), even though some important exceptions were reported (Böttger et al., 1998; Sander et al., 2002). It is also assumed that evolution of resistance occurs by only a few mutations of large effect (Andersson and Hughes, 2010; Vogwill and MacLean, 2015). Despite conferring high levels of resistance, mutants with a high fitness cost are less likely to outcompete susceptible strains in a given population. On the contrary, low-level mutations can be fixed if associated with a low fitness cost (Andersson and Hughes, 2010). Paradoxically, the weaker the selection for resistance (for example, by non-lethal antibiotic concentrations) the higher the likelihood for persistence of mutants with a low fitness cost and mutator phenotypes (Andersson and Hughes, 2014). Actually, a growing body of evidence suggests that high levels of AR evolve due to combinations of multiple mutations that exert a minimal epistatic cost to the host (Trindade et al., 2009; Vogwill and MacLean, 2015; Ward et al., 2009; Wistrand-Yuen et al., 2018).

The long-term fate of resistant mutants is largely influenced by the evolution of compensatory mechanisms, which may restore fitness without compromising resistance to antibiotics (Box 3) (Andersson and Hughes, 2010). Indeed, Melnik et al. tracked the development of ciprofloxacin resistance in *P. aeruginosa* under constant and fluctuating antibiotic administration, and found that high-fitness, resistant strains (broadly adapted generalists, as defined by Kassen et al. (Kassen, 2002)) evolved quickly under fluctuating treatments and that second-site mutations were responsible for compensating the fitness cost associated with AR (Melnyk et al., 2017). As such, the authors suggested that these therapies generate persistent AR by selecting for the evolution of cost-free resistance of *P. aeruginosa* strains. Pacheco et al. studied the independent overexpression of the four most important efflux pumps involved in AR (MexAB-OprM, MexCD-OprJ, MexEF-OprN and MexXY) by *P. aeruginosa* mutants (Olivares Pacheco et al., 2017). These researchers verified that the lack of fitness cost observed among these mutants was associated with a metabolic rewiring as a compensation derived from overexpressing these pumps. This non-mutational mechanism includes an increased expression of the nitrate respiratory chain under aerobic conditions (Olivares Pacheco et al., 2017).

Genetic elements such as plasmids and ICE frequently harbor AR and/or virulence genes that may provide a selective advantage for the

Box 3
Evolution and fitness cost of HGT.

HGT plays a decisive role in AR spreading among different Gram-negative and Gram-positive bacteria. A survey conducted by Kloesges et al. suggests that nearly 75% of the genes from a given genome have been horizontally transferred at least once during evolution (Kloesges et al., 2011). HGT is more frequent between closely related species from the same taxonomic group, having similar genomes and an analogous GC content (Andam and Gogarten, 2011; Hooper et al., 2009; Kloesges et al., 2011). As so, the pangenome also represents the pool of genes that may be available through HGT to any close relative of the same taxon (Soucy et al., 2015). Another study found bi-directional associations among the similarity in tRNA pools of bacteria and the number of HGT events happening between them (Tuller et al., 2011). Both situations make quantification of HGT quite challenging (Andam and Gogarten, 2011; Polz et al., 2013). Although HGT among distantly related species can still occur, there seems to exist a biological barrier for gene acquisition from donors with different genomic GC content. Species such as *Aeromonas salmonicida* act as a natural mixing pot which harbors *Pseudomonas* and enteric types of plasmids and thus allow the exchange of genetic material (Olsen and Wright, 1976). Most genes subjected to HGT are responsible for metabolic functions, while the transfer of genes related to information processing (such as replication, transcription and translation) is rare (Cohen et al., 2011). Also, HGT was shown to play a more important role than gene duplication for the expansion of protein families among prokaryotes (Treangen and Rocha, 2011). HGT may also depend on the transfer mechanism (Popa et al., 2011; Popa and Dagan, 2011). As a consequence of HGT, prokaryotic genome evolution models should be viewed less as a bifurcated tree, and more as network models, which allow the depiction of several events such as recombination, gene fusion and HGT (Harrison and Brockhurst, 2012; Kloesges et al., 2011; Polz et al., 2013; Popa et al., 2011; Soucy et al., 2015; Treangen and Rocha, 2011).

Once foreign DNA has been integrated into the host, it might need to adapt within the genome in order to resist purifying selection (Moran et al., 2009). In order to survive, the genes usually need to incur in a low fitness cost to the host and/or to provide a selective advantage to themselves or to the recipient strain (Baltrus, 2013). Their expression will largely be governed by the presence of promoters and/or regulatory mechanisms on the vicinity of its insertion loci. Nonetheless, comparative genomic analysis unveiled that many genes acquired by HGT appear to have neutral effects for the host (Gogarten and Townsend, 2005; Soucy et al., 2015). For example, introns are self-splicing elements that provide a nearly neutral effect, and can persist since the success in invading hosts compensates for the fitness cost to the host associated with the additional DNA, RNA and protein synthesis. Neutral acquisitions can later be subjected to novel combinations, allowing its domestication over time if a beneficial phenotype is garanteed. If imported genes remain neutral, puryfing selection will most likely lead to its lose (Gogarten and Townsend, 2005; Soucy et al., 2015).

host. Acquisition of a useful gene pool by a host genome could counterbalance the fitness cost associated with maintaining these elements (Broaders et al., 2013; Rankin et al., 2011). Even though every HGT may exert a fitness cost, and even if these costs are offset in cases of benefits for the host, these events leave lasting genomic signatures that shape evolution of the MGE to fit within the host genome and/or the host to accommodate the new guest (Baltrus, 2013).

Besides selection on beneficial traits, efficient replication, segregation, re-infection by conjugation, growth inhibition or killing of plasmid-free cells by toxin-antitoxin systems and a low cost have an important role in maintaining plasmids harboring AR genes in a given population (Loftie-Eaton et al., 2017, 2016; Lopatkin et al., 2017; Sommer et al., 2017). To accommodate this MGE, compensatory mutations may occur in the plasmid, the host or in both (Harrison et al., 2015; Harrison and Brockhurst, 2012; Kottara et al., 2018; Loftie-Eaton et al., 2017; San Millan et al., 2014b; Rodriguez-Beltran et al., 2018; San Millan et al., 2016, 2015b; San Millan and MacLean, 2017; Stevenson et al., 2018; Zwanzig et al., 2019). Given the abundance of non-transmissible plasmids (Smillie et al., 2010), San Millan et al. showed that epistatic interactions between co-resident plasmids unable to be transferred by conjugation, decreases the cost associated with harboring several plasmids in *P. aeruginosa* and promotes persistence (San Millan et al., 2014a). Loftie-Eaton et al. uncovered a plasmid-host epistasis mechanism promoting plasmid stability in the coevolved host through acquisition of a transposon-encoded putative toxin-antitoxin and cointegrate resolution system, alongside host mutations (Loftie-Eaton et al., 2016). The researchers also found that this transposition expanded plasmid-host range. Another study reported how compensatory adaptation and positive selection interact to assure persistence of non-conjugative plasmid (San Millan et al., 2014b). Harrison et al. explored the compensatory evolution mechanisms responsible for ameliorating the cost of maintaining the plasmid, in both parasitic (cost prevail over benefits, a situation where plasmids are frequently lost due to purifying selection) and mutualistic (benefits take the lead and selection favors the capture of beneficial genes into the chromosome and subsequent loss of the plasmid backbone) treatments (Harrison et al., 2015). Such plasmid-plasmid and plasmid-host interactions expose the multiple solutions for co-evolution, and so to better understand HGT one should use co-evolutionary instead of a simple evolutionary approach (Harrison and Brockhurst, 2012). However, co-evolution may not always lead to amelioration of plasmid burden (Smith, 2011). Most plasmids carry several AR genes, increasing the probability of co-selection and thereby decreasing the effect of purifying selection on a high fitness cost resistance gene. Worryingly, this effect could dissociate the evolution of resistance to a given antibiotic from the use of the same agent (Sommer et al., 2017; Sundqvist et al., 2010; Yen and Papin, 2017).

However, evolving resistance by plasmid acquisition exerts a higher cost than resistance by chromosomal mutations? The answer is not simple. Apparently, the acquisition of a MGE by the host frequently imposes a fitness, with a high cost. However, AR mutations in essential and highly conserved genes are likely to be more costly (Alekshun and Levy, 2007). A recent meta-analysis approach revealed that evolving resistance by acquisition of plasmids was associated with a smaller cost, which could help to explain why these elements are so widely disseminated (Vogwill and MacLean, 2015). Also, the host may re-acquire a plasmid that was carried during its evolutionary past, and so compensatory mutations (within the host's chromosome or the plasmid) can offset the cost of the recently acquired plasmid (Loftie-Eaton et al., 2017; Vogwill and MacLean, 2015). This host-plasmid coevolution could also help to explain why acquisition of the same plasmids by different hosts exerts a different cost (Starikova et al., 2013).

ICEs appear to have a bistable lifestyle that determines the change between vertical and horizontal transmission (Delavat et al., 2017). In the ICE integrated state, core functions are downregulated in order to exert a low fitness cost on the host and to ensure the likelihood of vertical transmission, while beneficial genes carried by the ICEs may pose a selective advantage to the host; the excision and subsequent horizontal transfer state can be activated at low frequencies upon external triggers in specific growth conditions (Burrus, 2017; Delavat et al., 2017, 2016). Activation occurs at a low level most likely to avoid harming the host and to guarantee sufficient HGT and population survival. So far, this theory has only been observed for ICE*clc* (Delavat et al., 2016; Gaillard et al., 2008; Miyazaki et al., 2018), and single cell observations should be extended to other ICE systems. Future studies should also address the mechanisms producing and maintaining this dual lifestyle and the contribution of selective forces driving ICE-host co-evolution.

10. Population structure of *P. aeruginosa* and the impact of high-risk clones

P. aeruginosa has a non-clonal epidemic population structure, punctuated by specific sequence types (ST) (Jolley et al., 2018; Pirnay et al., 2009). Its population structure is comparable to that from *Neisseria meningitidis*, a superficially clonal structure with high recombination frequency, in which rare successful epidemic lineages tend to arise. There seems to be a consensus that clinical and environmental isolates are indistinguishable and that there is no association between a given clone and a specific habitat (Pirnay et al., 2009). Hospital transmission can, however, increase the prevalence of particularly adapted clones (Oliver et al., 2015).

Some STs are worldwide disseminated and frequently linked to outbreaks and to the dissemination of carbapenemases. These STs have been designated high-risk clones, of which major examples are ST235, ST111 and ST175 in *P. aeruginosa* (Kos et al., 2015; Oliver et al., 2015; Sánchez-Diener et al., 2017; Woodford et al., 2011). The $bla_{\text{VIM-2}}$ was the most frequently encountered CEG among ST235 and ST111 (Oliver et al., 2015; Turton et al., 2015). Albeit being responsible for the spread of AR through HGT, these STs have also been associated with mutational resistance. In fact, the mechanisms responsible for the multidrug resistant phenotypes frequently observed in ST175 are mostly determined by specific mutations on *oprD*, *ampR*, *mexZ*, *gyrA* and *parC* (Cabot et al., 2016, 2012; López-Causapé et al., 2018).

Among the few major high-risk clones, ST235 is the most widely disseminated (Chowdhury et al., 2017; Kos et al., 2015; Maatallah et al., 2011; Miyoshi-Akiyama et al., 2017; Oliver et al., 2015; Roy Chowdhury et al., 2016; Treepong et al., 2018; van Belkum et al., 2015). Nearly 100 horizontally-acquired resistance elements have been reported among ST235 isolates (Chowdhury et al., 2017; Oliver et al., 2015; Roy Chowdhury et al., 2016; Treepong et al., 2018). The *dprA* gene was identified by Treepong et al. as a specific marker of this lineage (Treepong et al., 2018). Due to the reported contribution of DprA in homologous recombination and acquisition of foreign DNA, its presence in ST235 lineage may help to explain the increased ability to acquire AR elements. Class A and B carbapenemases were also frequently associated with ST111 isolates (Castanheira et al., 2014; Chowdhury et al., 2017; Kos et al., 2015; Oliver et al., 2015; Turton et al., 2015; van Belkum et al., 2015).

The geographical distribution of the high-risk clones and the diversity of AR elements suggest that the spread of these STs is global and the acquisition of the resistance genes is mainly local (Oliver et al., 2015; Turton et al., 2015). Previous studies suggest that environmental species may constitute an important reservoir for the dissemination of clinically relevant carbapenemases, which are vertically amplified upon transfer to *P. aeruginosa* high-risk clones (Jiang et al., 2017; Juan et al., 2010; Scotta et al., 2011). The high prevalence of these elements among high-risk clones may be partially explained by the genetic capitalism theory, given that a widely disseminated ST should have a greater probability of acquiring new AR genes and to be further selected and amplified due to the high antibiotic pressure in the hospital environment (Baquero, 2004). Other theories support that the high-risk clones

have a naturally increased ability to acquire foreign DNA, since these STs appear to have lost the CRISPR (clustered regularly interspaced short palindromic repeats)-Cas (CRISPR associated proteins) system, which act as an adaptive immune system in prokaryotic cells and protects them from invasion by bacteriophages and plasmids (Bondy-Denomy and Davidson, 2014; Chen et al., 2018; England et al., 2018; Li et al., 2016; Turton et al., 2015; van Belkum et al., 2015).

Mulet et al. studied the biological markers that may be responsible for the success of *P. aeruginosa* high-risk clones (Mulet et al., 2013). The authors found that these STs were defective in motility and pigment production, presented a reduced *in vitro* fitness in competition experiments and showed increased levels of biofilm formation and spontaneous mutant frequencies. Interestingly, these traits resemble those resulting from adaptation to chronic infections (Mulet et al., 2013).

Treepong et al. linked the dissemination of ST235 with the selective pressure exerted by fluoroquinolones (Treepong et al., 2018). A similar observation for ST175 was previously reported by Cabot et al., since all strains tested presented the same set of quinolone resistance-determining region mutations (Cabot et al., 2012). Curiously, as above-mentioned, some mutations did not involve biological costs, as those targeting *gyrA* and *gyrB* genes (Andersson and Hughes, 2010). Conferring low levels of AR without incurring in fitness cost can help to explain the high frequency of gyrase mutations in several clinical isolates (Wong and Kassen, 2011). Future studies performing comparative genomic analysis of high-risk clones and non-epidemic multi-drug resistant strains will help to shed a new light on the traits responsible for the success of these STs. Moreover, the existence of specific features that promote the development of *de novo* mutations and/or the acquisition of AR genes by HGT still needs to be further explored (Oliver et al., 2015).

11. Conclusions and future directions

An intriguing question is why the class I integrons tend to be integrated into the chromosome rather than inserted in plasmids. Conducting an *in silico* analysis of CEGs deposited in public databases and analyzing specific traits may help to explain this preference. It would also be interesting to explore the molecular basis for why chromosomal mutations are more important for resistance to carbapenems than acquisition of MGEs harboring AR genes. One possible explanation may be the burden of HGT to the host, and combination of multiple mutations that exert a minimal epistatic cost to the host may yield high levels of AR *per se* (Kassen, 2002; Trindade et al., 2009; Vogwill and MacLean, 2015; Ward et al., 2009).

High-risk clones appear to be more capable of acquiring and/or maintaining AR genes when comparing with other clones. However, the underlying basis for this remains a mystery. Previous reports suggested that the absence, modification or downregulation of proper defense systems against foreign DNA (such as CRISPR-Cas, toxin-antitoxin and phage-exclusion) in high-risk clones would lead to its increased invasion by MGEs (Bondy-Denomy and Davidson, 2014; Kos et al., 2015; Turton et al., 2015; van Belkum et al., 2015). These observations should be extended to a wider pool of clones in order to address if these systems are indeed absent from all representatives of this subset of *P. aeruginosa* lineages. Another possible explanation for this riddle could be that high-risk clones may share stable lineage-specific GIs that are highly capable of acquiring AR gene cassettes by the class I integrons presented in these platforms. Performing comparative genomic studies to identity the presence/absence of specific genes in high-risk clones and in other *P. aeruginosa* strains may help to address the reason why these clones are so successful. It would also be interesting to expand the work conducted by Khaledi *et al.* and perform a RNA-seq analysis to determine whether high-risk clones exhibit a different gene expression profile (Bruchmann et al., 2015; Khaledi et al., 2016). Another question that needs to be addressed is the nature of high-risk clones (Woodford et al., 2011): are these clones a minor pre-existing variant that recently succeeded, with resistance as a key driver, and/or a previously successful antibiotic-susceptible variant that underwent clonal expansion due to the acquisition of AR genes?

In the future, the approach taken to study HGT and the spread of AR genes in *P. aeruginosa* needs to be rethought, and we believe a greater emphasis should be placed on the contribution of GIs and more specifically ICEs. With the advent of WGS, the real contribution of these MGEs for the dissemination of CEGs will be correctly addressed. WGS analysis, together with epidemiological data, may also help to give further insights into the differences between members of high-risk clones and may be able to identify the routes of transmission of *P. aeruginosa* between patients and different sources. More work is needed to identify the main hospital reservoirs of MDR *P. aeruginosa*, which may include plumbing systems, ventilators and/or long-term colonization of patients (Livingston et al., 2018). It is vital to have an in-depth understanding of these MDR organisms in order to prevent infections and design control strategies to limit their future spread. The genomic era and integrated molecular epidemiology approaches may help outbreak investigations, genome-based prediction of AR and long-term persistence surveillance of MDR *P. aeruginosa* in real-time (Boolchandani et al., 2019; S. Baker et al., 2018; Besser et al., 2018; Davis et al., 2015; Gardy and Loman, 2017; Quainoo et al., 2017; Quick et al., 2014; Rossen et al., 2018; Su et al., 2018; Turton et al., 2015).

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