U.PORTO

PHD IN PHARMACEUTICAL SCIENCES

MICROBIOLOGY SPECIALITY

Carbapenemase producers of *Pseudomonas aeruginosa*: lights on new mobile genetic elements

João Botelho

D

2018

U.PORTO

João Botelho. Carbapenemase producers of *Pseudomonas aeruginosa*: lights on new mobile genetic elements

D.FFUP **2018**

Carbapenemase producers of *Pseudomonas aeruginosa*: lights on new mobile genetic elements

João Botelho

FACULDADE DE FARMÁCIA

FCT Fundação para a Ciência e a Tecnologia
MINISTÉRIO DA EDUCAÇÃO E CIÊNCIA

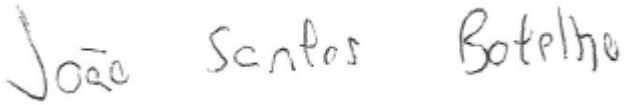
João Francisco Tomaz Santos Botelho

Carbapenemase producers of *Pseudomonas aeruginosa*: lights on new mobile genetic elements

Tese do 3.º Ciclo de Estudos Conducente ao Grau de Doutoramento em Ciências Farmacêuticas na Especialidade de Microbiologia

Trabalho realizado sob a orientação da Professora Doutora Luísa Peixe e coorientação da Doutora Filipa Grosso e Doutora Clara Sousa (Faculdade de Farmácia, Universidade do Porto, Portugal).

August 2018



Agradecimentos (Acknowledgments)

Gostaria de agradecer, em primeiro lugar, à minha orientadora Professora Doutora Luísa Peixe, e coorientadoras Doutora Filipa Grosso e Doutora Clara Sousa, por todo o auxílio e incentivo que me prestaram e que foram decisivos para a realização desta tese. Aos meus pais, irmã, namorada (e agora noiva), amigos e família mais próxima por me terem ajudado durante estes anos. À Professora Doutora São José Nascimento agradeço ter-me dado a oportunidade de realizar este trabalho no Laboratório de Microbiologia da Faculdade de Farmácia da Universidade do Porto. Queria também agradecer o apoio financeiro concedido pela Fundação para a Ciência e a Tecnologia (FCT), Ministério da Ciência, Tecnologia e Ensino Superior de Portugal. Aos restantes colegas (com destaque especial para a Joana Mourão), alunos de ERASMUS e funcionários (novo destaque para o Nuno Oliveira e Cristina Pinto da Costa) do departamento, quero agradecer toda a disponibilidade que demonstraram ao longo dos anos.

Abstract

Pseudomonas aeruginosa is an important opportunistic pathogen and a leading cause of nosocomial infections that is often difficult to treat due to its intrinsic and acquired resistance mechanisms to various antimicrobial agents, including carbapenems, the last resort group of antibiotics. The worldwide increase in carbapenem resistance is partially due to the acquisition of carbapenemase-encoding genes (CEG). Integrons and mobile genetic elements (MGE), such as transposons, insertion sequences, genomic islands (GI), plasmids and integrative and conjugative elements (ICE) are frequently involved in the spread of CEG. Those MGE contributes to the genomic diversity of *P. aeruginosa,* which present a non-clonal population structure, punctuated by specific high-risk clones that are associated with significant morbidity and mortality worldwide. Even though different carbapenemases have been described among *P. aeruginosa* isolates from Portuguese clinical settings, information regarding the clonal lineages and genetic surroundings of CEG-bearing isolates is still scarce. Also, despite the fact that in *P. aeruginosa* CEG are frequently located within the chromosome, the association of these genes with chromosome-located MGE, and particularly to ICE, has rarely been investigated in this species. Moreover, features of CEG-bearing plasmids (such as its association with virulence factors) are insufficiently described. Focusing on the main drivers promoting carbapenem resistance in *P. aeruginosa* will ultimately help to prevent and tackle the spread of antibiotic resistance (AR).

The **main goals** of this thesis were to provide an in-depth view of the main MGE (ICE and plasmids) responsible for the spread of CEG in clinical isolates of *P. aeruginosa* and to identify the major clones involved in its dissemination. This was achieved by characterizing a collection of 263 carbapenem-resistant *P. aeruginosa* isolates obtained between 1995 and 2014 at six geographically distinct hospitals from North and Centre of Portugal. Besides characterizing the AR genes among CEG-harboring MGE, we explored the presence of virulence factors. We also analyzed the different ST associated with CEG-bearing isolates. A worldwide collection of *Pseudomonas* genomes was downloaded from NCBI and used to assess the prevalence of CEG and to explore the contribution of ICE for the spread of carbapenemases among this genus.

Among the carbapenem-resistant *P. aeruginosa* isolates from our collection, 28 CEG-harboring isolates were identified, all encoding for VIM-2 with the exception of a bla_{GES-6} gene. Focusing on the CEG-harboring isolates, we identified the internationally-recognized high-risk clones, namely ST 111, 175, 235, 244 and 253. The most frequently encountered clone in our collection belonged to ST179. This ST was also detected in several patients from Spanish hospitals. These findings probably account for a clonal spread of ST179 in the Iberian Peninsula. Also, most of the isolates from this ST were identified in the same hospital throughout different years, exposing the long-term persistence of this clone. The CEG were here linked to 8 different class I integrons. In58 already described among different species was the most prevalent integron here identified. Out of the 28 CEG-bearing isolates, 9 had a plasmid and 19 a chromosomal location.

Data obtained in this project point to the presence of CEG-bearing ICE among P. *aeruginosa* clinical isolates from high-risk clones belonging to ST 111 and 235. Three complete ICE were here characterized, ranging from 80 to 90-kb long. Also, highly similar elements were identified on the genome of international P. *aeruginosa* strains deposited on NCBI. These results emphasize both the ability of these MGE to disseminate at a local and global level. Taking these observations to a wider level, we analyzed the link between CEG and ICE among all *Pseudomonas* spp. genomes available in NCBI. Out of the more than

4000 genomes, we identified CEG in less than 10% of the genomes, which is consistent with previous findings stating that chromosomal mutations are the most important drivers of resistance to carbapenems in P. *aeruginosa*. Surprisingly, we were able to identity a wide array of ICE associated with several CEG and with specific ICE families, belonging to mating-pair formation (MPF) classes G and T. Most CEG of the ICE MPF_G class were encoded within class I integrons, which co-harbor genes conferring resistance to other antibiotics. The majority of the integrons were located within Tn*3*-like and composite transposons. MPF_T class ICE carried the CEG within composite transposons which were not associated with integrons.

Plasmids also played a part in the HGT of CEG-harboring integrons. We fully sequenced the first In58-harboring plasmid, named pJB12 and isolated from a strain belonging to the high-risk clone ST175. Plasmids of the same size were identified in isolates belonging to ST179 and ST282. These c.a. 30kb-long plasmids were not self-conjugative, but may be mobilized by the presence of a helper plasmid. Interestingly, the In58 integron was also identified in a 450kb-long plasmid identified in an isolate from our collection belonging to the high-risk clone ST253, and named pJB37. As for pJB12, the acquisition of In58 by pJB37 was most likely achieved by the transposition of a new complex transposon structure. The data here presented represents the first description of a *bla*$_{VIM-2}$-harboring megaplasmid in *P. aeruginosa*. This IncP-2 plasmid was transferrable by conjugation to a spontaneous rifampicin-resistant mutant of P. *aeruginosa* PAO1 strain.

Among the CEG-bearing isolates, we described the backbone of an *exoU*-carrying GI on a ST235 isolate. We verified by *in silico* analysis that this island is frequently located in genomes belonging to this ST. ExoU is a potent cytotoxin with phospholipase activity, which is frequently related to nosocomial-acquired pneumonia, ear infections and keratitis. Expressing this toxin could participate in the poor outcome of infections due to ST235. We also explored the virulence determinants carried by the megaplasmid pJB37, such as those encoding for twitching motility proteins PilT and PilG, type IV fimbrial assembly ATPase PilB and a chemotaxis (*che*) operon. Since we verified a twitching motility reduction, we hypothesized that acquiring the megaplasmid may have comprised an adaptation to a sessile lifestyle, helping the bacteria to evade the host's hostile conditions.

The **general conclusion** of this thesis is that the spread of CEG among *P. aeruginosa* is often achieved by a wide array of MGE, comprising ICE and plasmids with different backbones that are frequently associated with internationally successful clones. Besides providing a detailed view on the backbone of different CEG-bearing plasmids, this work also shed a light on the underappreciated contribution of ICE for the spread of CEG.

Keywords: *Pseudomonas aeruginosa*, Carbapenemases, Integrative and conjugative elements, Plasmids.

Resumo

A *Pseudomonas aeruginosa* é um patogénico oportunista importante e uma das principais causas de infeções nosocomiais, frequentemente difícil de tratar devido aos seus mecanismos de resistência intrínseca e adquirida a vários agentes antimicrobianos, incluindo os carbapenemos, que são utilizados como terapêutica de última linha. O aumento a nível mundial da resistência a carbapenemos deve-se parcialmente à aquisição de genes que codificam para carbapenemases (CEG). Os integrões e elementos genéticos móveis (MGE), tais como transposões, sequências de inserção, ilhas genómicas (GI), plasmídeos e elementos integrativos e conjugativos (ICE) estão frequentemente envolvidos na disseminação dos CEG. Esses MGE contribuem para a diversidade genómica de *P. aeruginosa*, que apresenta uma estrutura populacional não-clonal, pontuada por clones específicos de alto risco que estão associados a taxas de morbidade e mortalidade significativas em todo o mundo. Apesar de terem sido descritas diferentes carbapenemases entre isolados de *P. aeruginosa* de contextos clínicos portugueses, a informação sobre as linhagens clonais e o ambiente genético dos isolados com CEG ainda é escassa. Além disso, apesar de em *P. aeruginosa* os CEG estarem habitualmente localizados no cromossoma, a associação desses genes com MGE que se integram no cromossoma, como as ICE, tem sido raramente investigada nesta espécie. De igual modo, a natureza dos plasmídeos portadores de CEG (tais como a sua associação com fatores de virulência) encontra-se insuficientemente descrita. Focando a nossa atenção nos principais fatores que promovem a resistência aos carbapenemos em *P. aeruginosa*, em última análise será possível contribuir para a prevenção e combate à disseminação da resistência aos antibióticos (AR).

Os **principais objetivos** desta tese foram facultar uma visão detalhada dos principais MGE (ICE e plasmídeos) responsáveis pela propagação dos CEG em isolados clínicos de *P. aeruginosa* e identificar os principais clones envolvidos na sua disseminação. Isto foi conseguido através da caracterização de uma coleção de 263 isolados de *P. aeruginosa* resistentes a carbapenemos, obtidos entre 1995 e 2014 em seis hospitais geograficamente distintos do Norte e Centro de Portugal. Além de caracterizar os genes AR entre MGE que transportam CEG, nós explorámos a presença de fatores de virulência. Nós também analisámos os diferentes *sequence type* (ST) associados aos isolados com CEG. Uma coleção mundial de genomas de *Pseudomonas* foi transferida do NCBI e usada para avaliar a prevalência de CEG, bem como explorar a contribuição das ICE para a disseminação de carbapenemases entre isolados deste género bacteriano.

Entre os isolados de *P. aeruginosa* resistentes a carbapenemos da nossa coleção, foram identificados 28 isolados portadores de CEG, todos codificando para VIM-2, com exceção de um gene (bla_{GES-6}). Focando a nossa atenção nos isolados que transportam CEG, identificámos os clones de alto risco reconhecidos internacionalmente, a saber, os ST 111, 175, 235, 244 e 253. O clone mais encontrado na nossa coleção pertence ao ST179. Este ST também foi detetado em vários pacientes de hospitais espanhóis. Estes resultados provavelmente são responsáveis pela disseminação clonal do ST179 na Península Ibérica. Além disso, a maioria dos isolados deste ST foi identificada no mesmo hospital em diferentes anos, expondo a persistência a longo prazo deste clone. Os CEG identificados neste trabalho estão associados a 8 integrões diferentes de classe I. O In58, já descrito em diferentes espécies, foi o integrão mais prevalente. Dos 28 isolados contendo CEG, 9 foram identificados em plasmídeos e 19 numa localização cromossómica.

Os dados obtidos neste trabalho apontam para a presença de ICE transportando CEG entre os isolados clínicos de *P. aeruginosa* pertencentes aos clones de alto risco ST 111 e 235. Três ICE completos foram caracterizados, sendo que o seu comprimento varia entre os 80 a 90 kb. Além disso, elementos altamente similares foram identificados no genoma de isolados internacionais de *P. aeruginosa* depositados no NCBI. Estes resultados enfatizam a capacidade desses MGE para disseminar a um nível tanto local como global.

A um nível mais amplo, nós analisámos a ligação entre CEG e ICE em todos os genomas de *Pseudomonas* sp*p.* disponíveis no NCBI. Dos mais de 4000 genomas, identificámos CEG em menos de 10% dos genomas, o que é consistente com estudos anteriores que atribuem às mutações cromossómicas um papel mais importante na resistência aos carbapenemos em *P. aeruginosa*. Surpreendentemente, fomos capazes de identificar uma ampla variedade de ICE associadas a vários CEG e de famílias específicas, pertencentes às classes G e T. A maioria dos CEG são codificados dentro de integrões de classe I, quando integrados em ICE da classe $MPF_{G.}$ Estes integrões incluem ainda genes que conferem resistência a outros antibióticos. A maioria dos integrões encontra-se dentro de transposões do tipo Tn*3*-like e transposões compostos. As ICEMG da classe MPF_T transportam os CEG dentro de transposões compostos, mas não associados a integrões.
Os plasmídeos também desempenham um papel na HGT dos integrões que transportam os CEG. Nós sequenciámos totalmente o primeiro plasmídeo portador de In58, denominado pJB12 e isolado de uma estirpe pertencente ao clone ST175 de alto risco. Plasmídeos do mesmo tamanho foram identificados em isolados pertencentes ao ST179 e ST282. Estes plasmídeos de tamanho aproximado de 30 kb não demonstraram ser conjugativos, mas podem ser mobilizados na presença de um plasmídeo auxiliar. Curiosamente, o integrão In58 também foi identificado num plasmídeo de tamanho aproximado de 450kb, identificado num isolado da nossa coleção pertencente ao clone de alto risco ST253, e denominado pJB37. Tanto neste como no pJB12, a aquisição do In58 foi provavelmente mediada pela inserção de novos transposões complexos. Os dados aqui apresentados representam a primeira descrição de um megaplasmídeo portador de bla_{VIM-2} em *P. aeruginosa*. A transferência por conjugação deste plasmídeo IncP-2 para um mutante espontâneo resistente à rifampicina da estirpe *P. aeruginosa* PAO1 foi bem-sucedida.
Entre os isolados portadores de CEG, também descrevemos uma GI portador de *exoU* num isolado pertencente ao ST235. Verificámos por análise *in silico* que esta ilha é frequentemente encontrada em genomas pertencentes a este ST. ExoU é uma citotoxina potente com atividade de fosfolipase e habitualmente relacionada com a pneumonia adquirida nos hospitais, a infeções nos ouvidos e ceratites. A expressão desta toxina pode contribuir para o mau prognóstico das infeções causadas por isolados do ST235. Também foram explorados os fatores de virulência do megaplasmídeo pJB37, como os que codificam para as proteínas de mobilidade de *twitching* PilT e PilG, de ATPase pilinas do tipo IV (PilB) e um operão de quimiotaxia (*che*). Uma vez que verificámos uma redução da mobilidade de *twitching*, levantámos a hipótese que a aquisição do mega-plasmídeo pode ter conduzido a uma adaptação a um estilo de vida séssil, ajudando a bactéria a escapar-se às condições hostis do hospedeiro.
A **conclusão geral** desta tese é que a disseminação dos CEG entre isolados de *P. aeruginosa* é frequentemente mediada por uma variedade de MGE, compreendendo ICE e plasmídeos com diferentes estruturas genéticas e que se encontram habitualmente associados a clones bem-sucedidos internacionalmente. Além de fornecer uma visão detalhada da composição genética de diferentes plasmídeos que transportam CEG, este trabalho também explorou o contributo subvalorizado que as ICE têm para a disseminação de CEG.

Palavras-chave: *Pseudomonas aeruginosa*, Carbapenemases, Elementos integrativos e conjugativos, Plasmídeos

Table of contents

List of abbreviations

AR – Antibiotic resistance;

CEG – Carbapenemase-encoding gene(s);

CF – Cystic fibrosis;

CRISPR-Cas – Clustered regularly interspaced short palindromic repeats-CRISPR associated proteins;

ECDC – European Center for Disease Prevention and Control;

ESPAKE – *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *P. aeruginosa* and *Enterobacter* species;

GC – Guanine-cytosine;

GI – Genomic island(s);

HAI – Healthcare-associated infection(s);

HGT – Horizontal gene transfer;

ICE – Integrative and conjugative element(s);

ICU – Intensive care unit(s);

Mb – Mega base pair(s);

MBL – Metallo-beta-lactamase(s);

MDR – Multi-drug resistance;

MGE – Mobile genetic element(s);

MPF – Mating-pair formation;

NCBI – National Center for Biotechnology Information;

PAPI – *Pseudomonas aeruginosa* pathogenicity island(s);

QS – Quorum-sensing;

ST – Sequence type(s);

T4SS – Type-IV secretion system;

WGS – Whole-genome sequencing;

WHO – World Health Organization;

‘The time may come when penicillin can be bought by anyone in the shops. Then, there is the danger that the ignorant man may easily under-dose himself and by exposing his microbes to non-lethal quantities of the drug educate them to resist penicillin’

Alexander Fleming, Nobel Lecture, December 11, 1945

Introduction

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

Pseudomonas aeruginosa is a Gram-negative rod-shaped gamma-proteobacterium. 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 [1, 2]. 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 [3–5]. *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 (**Figure 1**) [6]. 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 (**Figure 1**) [7]. 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 [8]. The European Centre for Disease Prevention 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 [9]. 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 [10, 11].

Figure 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.

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* (**Figure 2**) [12]. 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 [13–18]. 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 (**Figure 2**) [12]. Additionally, *P. aeruginosa* is intrinsically resistant to a wide variety of antibiotics [1, 19], 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 [1, 14, 15, 20].

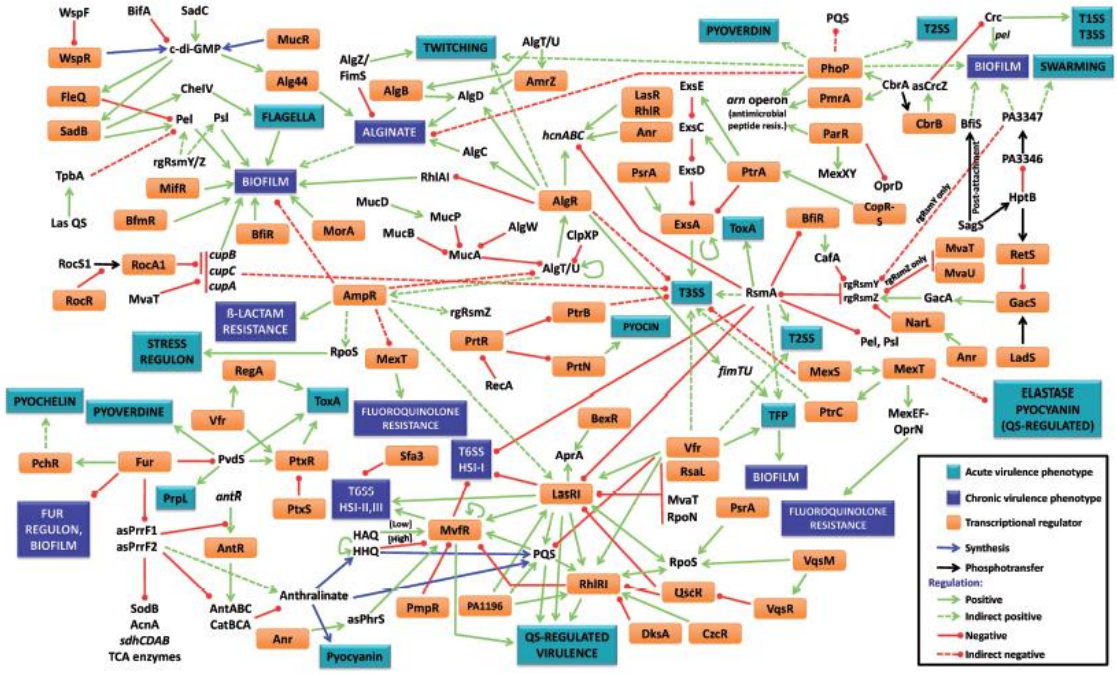


Figure 2 – The *P. aeruginosa* regulatory network. QS regulates biofilm formation, motility, AR and expression of multiple secretion systems and efflux pumps. Transition from acute to chronic *P. aeruginosa* infection can be mediated by complex regulatory pathways, such as the RetS-LadS-GacSA-RsmA and the two-component system BfiSR, MifR and BfmSR involved in biofilm formation. Iron concentration also affects the formation of biofilms, through the action of the Fur master repressor of iron uptake. Some interactions are present as indirect since the mechanisms responsible for the regulation are unknown. Adapted with permission from Balasubramanian *et al* [12].

1.2. 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 [21]. 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 on *P. aeruginosa* PAO1 strain and directly linked to the genome size, a trend frequently encountered in bacterial species thriving to survive in several niches [21]. 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. A disproportionately large number of genes (nearly 300) encoding for transport systems and enzymes involved in the uptake of nutrients was also reported [21]. Genome size, predicted regulatory genes and complexity are consistent with the remarkable versatility frequently described on this species and reflects evolutionary adaptation to multiple niches [21, 22]. Indeed, the expansion on nutritionally restricted environments through acquisition of new metabolic functions most likely comprises a key player shaping the *P. aeruginosa* genome [22], an evolution pattern contrasting to the observed genome reduction upon adaptation of pathogens to a parasitic existence [22–24].

Comparative genomic analysis performed by Mathee et al. and later revisited revealed that *P. aeruginosa* genome presents a picture of a mosaic genome, composed by a large number of core genes interspersed by strain-specific blocks of genes [22, 25–29]. The latter segments were defined as regions of genomic plasticity [22]. The sum of these unique regions comprise the accessory genome. As so, the *P. aeruginosa* pangenome is currently seen 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 [22, 25, 26, 30, 31]. Genes are classified as unique in comparative genomics when identified in a single genome. Hilker et al. performed genome sequencing analysis of representative strains of the most frequent clonal complexes in *P. aeruginosa* population and found that the respective pangenome consists of a core genome with around 4000 genes, an accessory genome of about 10000 genes and at least 30000 unique genes [29]. However, 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 [27]. 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 [27]. 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 [28]. Comparing with the genome of two major pathogens *Escherichia coli* and *Staphylococcus aureus*, *P. aeruginosa* displays a larger average size (5972 genes against 4721 and 3118 genes, respectively) [32, 33]. 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 [34]. 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 [33].

According to the EzBioCloud website (http://www.ezbiocloud.net/) [35], the mean values for size, guanine-cytosine (GC) content and number of coding sequences of the 2605 quality controlled *P. aeruginosa* genomes (of which only 117 represent complete genomes) are around 6.7 Mb, 66.2% and 6200, respectively (accessed June 13, 2018). The Pathosystems Resource Integration Center (PATRIC, https://www.patricbrc.org/) database harbors a wider collection of *P. aeruginosa* genomes, comprising 2734 whole-genome sequencing (WGS) projects and 171 complete genomes (accessed June 13, 2018) [36]. 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 [37]. 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 1000 isolates from soil, water, plants, animals and human infections. 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 [38].

Besides genomics, other omic tools yielded new insights into *P. aeruginosa*'s genome organization, biology, diversity and virulence. Koeppen et al. used RNA-seq to characterize a novel mechanism whereby *P. aruginosa* reduces the host immune response through packaged intracellular small RNAs in outer membrane vesicles [17]. 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 [39]. A recent transcriptomics study revealed that several AR genes had substantially higher expression in human infections than in laboratory conditions, most likely explaining why AR assays often underestimate resistance in patients [40]. Lassek et al. provided a quantitative proteomic 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 [41]. Recent studies explored the co-evolution of *P. aeruginosa* with *Staphylococcus aureus*, two of the most prevalent respiratory pathogens detected in CF patients, and revealed that interspecies adaptation can drive evolution of both pathogens in

the CF lung-environment [42–44]. Tognon et al. proposed that lipopolysaccharide mutations in *P. aeruginosa* are a consequence of co-evolution of *S. aureus* [42]. 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 [43].

1.3. 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 [45, 46, 55–59, 47–54]. 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 of AR [47]. 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 [60]. According to recent estimates nearly 700000 people die every year as a consequence of AR infections [45]. 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 [46].

As stated on aforementioned reports, multi-drug resistance (MDR, meaning non-susceptible to ≥1 agent in ≥3 antimicrobial categories [61]) is an increasing healthcare concern worldwide, and many of the involved mechanisms have been unveiled over the last decades. The main driver 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 [62, 63, 72, 64–71]. 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**) [73–76]. D'Costa et al. performed metagenomic analyses 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 [73]. These enzymes displayed identities between 53 and 84% with known beta-lactamases, and are frequently expressed at low levels.

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 [74]. 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 sphere 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 [73, 77–79]. 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 [63]. With this approach, it would be possible to screen the pool of resistance genes (resistome, as coined by D'Costa et al. [80]) 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 no expression [81].

Box 1. Evolution of antimicrobial resistance (AR) (cont.)

Back in 1973, the theory that environmental antibiotic-producing bacteria were the evolutionary origin of clinically relevant AR genes was starting to gain attention [82]. 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 [83]. 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 [79]. Due to the ancient origins both of natural occurring antibiotics and AR genes, it is not surprising that clinical and agricultural use of antibiotics has potentiated the dissemination of resistance [84]. Future studies should prioritize increased surveillance on hotspots for HGT of AR genes, such as the agricultural settings, hospitals and wastewater and sewage settings, through functional metagenomics, next-generation sequencing and computational analyses [77, 81].

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 [85]. *P. aeruginosa* can develop resistance to a wide range of antibiotics, mainly due to a combination of intrinsic, acquired and/or adaptive resistance [1, 3, 4, 19, 86–88]. 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* [89]. Together with the production of AmpC and MexAB-OprM, these strategies largely contribute to the basal low 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 [26]. Efflux pumps are in fact ancient elements present in bacteria long before the antibiotic era, being responsible for the extrusion of several toxic compounds [90, 91]. 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 [92]. 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 beta-lactamase and PIB-1 [93, 94].

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 [1, 19]. 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 beta-lactamase [95]. The inducible expression of MexXY confers decreased susceptibility to aminoglycosides [92]. Given the large pool of regulatory genes identified on *P. aeruginosa* genome [21], 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) [64, 88, 96]. *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* [97–99]. Also, target mutations in *ampC* were shown to increase resistance to cephalosporins [100, 101]. Regulation of AmpC (frequently designated as PDC, for *Pseudomonas*-derived cephalosporinase) comprises one of the most intricate repression-derepression systems, since the transcription of bla_{PDC} is controlled by aforementioned peptidoglycan-recycling genes [97, 100]. 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 [13, 102]. Specific mutations in DNA gyrase and topoisomerase IV-encoding genes *gyrA*, *gyrB*, *parC* and *parE* are responsible for fluoroquinolone resistance in *P. aeruginosa* [103]. 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 beta-lactams, fluoroquinolones and aminoglycosides [64, 104, 105]. 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 [106–108].

1.3.1. Carbapenemases identified on *P. aeruginosa*

Due to their high importance for human medicine, carbapenems are considered by WHO as critically-important antimicrobials that should be reserved for the treatment of infections caused by multi-drug resistant Gram-negative bacteria in humans [109]. Recent data from ECDC, presenting the latest information on European antimicrobial consumption, revealed that the population-weighted mean consumption of carbapenem did not show any

significant change during 2011-2015 (**Table 1**) [110]. According to the latest ECDC AR surveillance report, the European population-weighted mean for *P. aeruginosa* isolates with resistance to carbapenems was 17,8%, a steady-state value at least since 2012 (**Figure 2**) [46]. 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 challenges of treatment of ICU patients [9].

Table 1. Trends in the consumption of carbapenems in the hospital environment. The numbers for the EU/EEA countries represent the corresponding population-weighted mean consumption of carbapenems from 2011 to 2015, expressed as defined daily doses per 1000 inhabitants and per day. Adapted from ECDC surveillance data.

Country	2011	2012	2013	2014	2015	Trends in consumption of carbapenems, 2011–2015	Average annual change 2011–2015	Statistically significant trend
Bulgaria	0.013	0.013	0.014	0.020	0.019		0.002	>
Poland (a)				0.024	0.020			N/A
Netherlands	0.018	0.019	0.020	0.019	0.021		0.001	>
Latvia	0.029	0.019	0.022	0.027	0.033		0.002	
France	0.030	0.021	0.033	0.033	0.035		0.002	
Norway	0.044	0.045	0.046	0.047	0.039		-0.001	
Hungary	0.028	0.032	0.037	0.042	0.046		0.005	>
Lithuania (a)		0.026	0.026	0.033	0.046			N/A
Slovakia (a)		0.027	0.034	0.042	0.048			N/A
Romania	0.023*	0.024*	0.024*	0.032*	0.049*		0.006	
Estonia	0.036	0.036	0.033	0.043	0.050		0.003	
Sweden	0.052	0.053	0.056	0.053	0.050		<0.001	
EU/EEA	**0.048**	**0.053**	**0.060**	**0.058**	**0.054**		**0.002**	
Italy	0.039	0.073	0.076	0.081	0.056		0.004	
Finland (b)	0.094	0.074	0.088	0.081	0.065		-0.005	
Belgium	0.079	0.062	0.062	0.063	0.065		-0.003	
United Kingdom (a)			0.064	0.071	0.071			N/A
Slovenia	0.078	0.074	0.061	0.066	0.072		-0.002	
Croatia	0.058	0.065	0.060	0.073	0.079		0.005	>
Denmark	0.060	0.063	0.087	0.085	0.083		0.007	
Luxembourg	0.086	0.101	0.095	0.087	0.089		-0.001	
Ireland	0.057	0.061	0.088	0.109	0.091		0.011	
Malta	0.105	0.052	0.066	0.101	0.107		0.005	
Cyprus	0.087*	0.102*	0.118*	0.121*	0.132*		0.011	>
Portugal (c)	0.139	0.143	0.146	0.139	0.133		-0.002	
Greece	0.130	0.133	0.135	0.143	0.137		0.002	>

* Includes consumption in the community.
(a) These countries did not provide data for all years during 2011-2015.
(b) Includes data from consumption in remote primary healthcare centers and nursing homes.
(c) Only includes data from public hospitals.
NA, not applicable.
>, increasing trend.

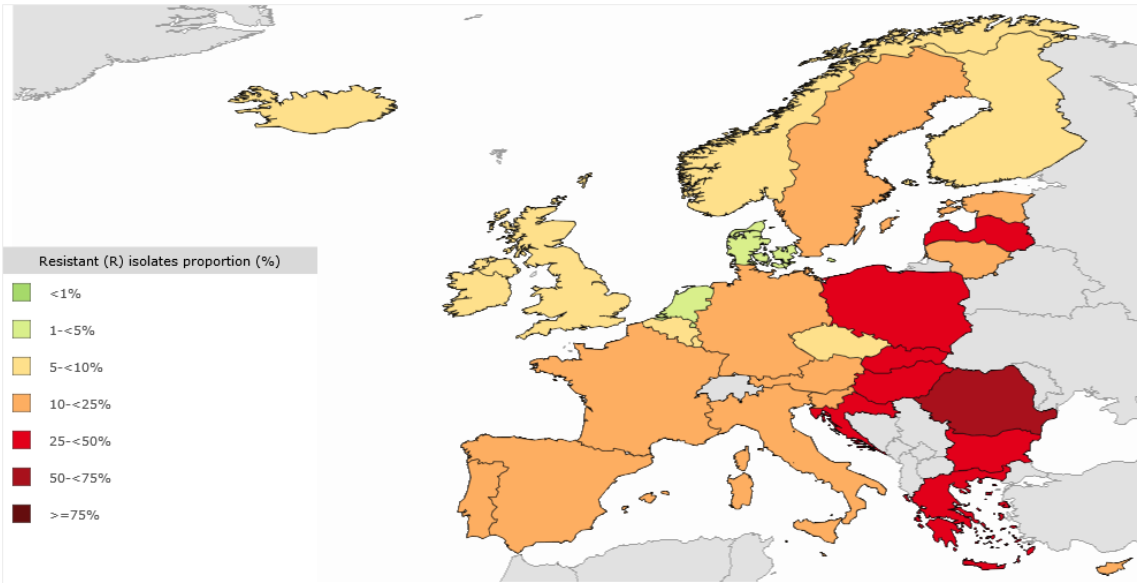


Figure 2 – Percentage of invasive *P. aeruginosa* isolates with resistance to carbapenems. This information is made available by ECDC collating data from the Member States collected through The European Surveillance System. Adapted from ECDC Surveillance Atlas of Infectious Diseases (https://ecdc.europa.eu/en/surveillance-atlas-infectious-diseases).

Carbapenem resistance in *P. aeruginosa* is often mediated by acquisition of carbapenemase-encoding genes (CEG) [1, 19, 86, 111–113]. These ß-lactamases are able to hydrolyze carbapenems and confer resistance to virtually all ß-lactam antibiotics. A recent survey conducted on 14 European countries revealed that 20% of carbapenem-resistant *P. aeruginosa* isolates were carbapenemase-producers [114]. 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* [115, 116]. 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 [117, 118]. Amid this class, several families of enzymes presenting at least 30% of amino acid similarity among them were described in *P. aeruginosa*: VIM, IMP, NDM, SPM, DIM, GIM, FIM and AIM [86, 111, 117, 119–122]. 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 [86, 117]. Even though *bla*$_{SPM-1}$ has been identified in Switzerland and in the United Kingdom [123, 124], both patients had been previously hospitalized in Brazil. The *bla*$_{VIM-2}$ is by far the most frequently reported CEG in *P. aeruginosa* [86, 117]. 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 [117].

In addition to MBL, carbapenemases belonging to Ambler class A β-lactamases, such as KPC-2 and several GES variants, have also been reported in *P. aeruginosa* [86, 111, 125, 126]. 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: OXA-40, OXA-181 and OXA-198 [127–129].

1.4. 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 (GI), prophages and phage-like elements, plasmids and integrative and conjugative elements (ICE) (**Figure 3**) [25]. 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 [62]. 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 MGE. 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. 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 [25]. 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 [26].

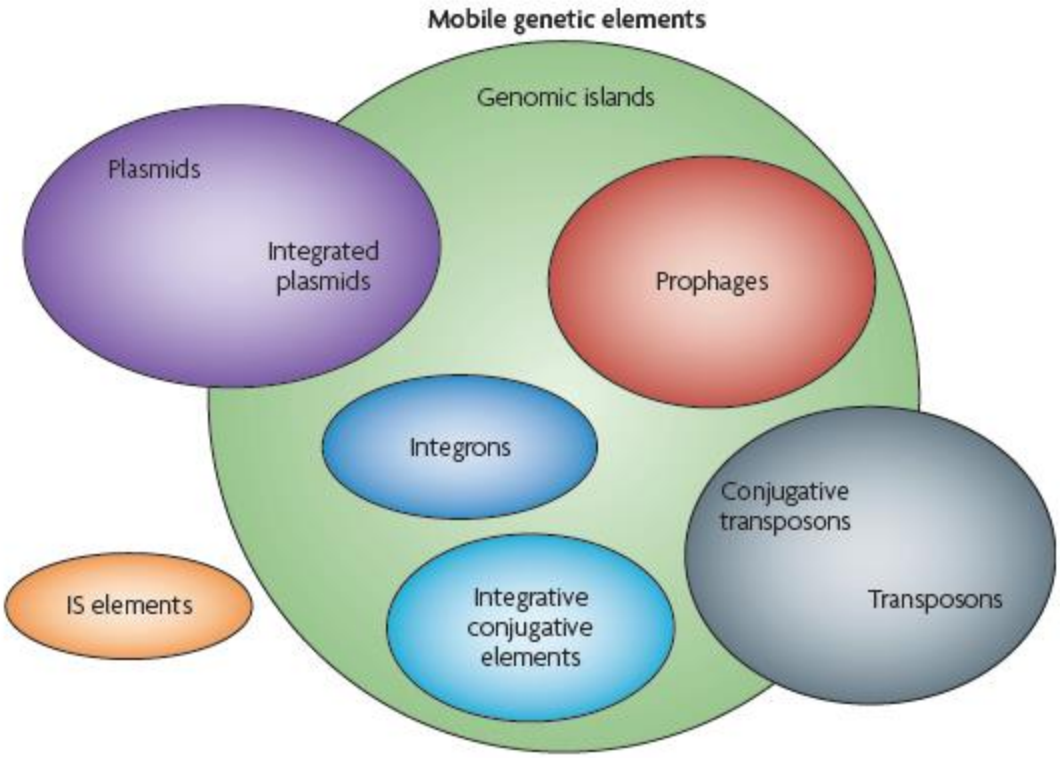


Figure 3 – A schematic representation of the different MGE. The broad definition of GI, considering all genomic regions which were likely derived from HGT, allows the clustering of several other MGE. Adapted with permission from Langille *et al* [130].

The MGE 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 MGE [22, 25, 131]. 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 [25]. However, MGE are essentially self-interested entities, promoting their self-propagation and may cause a burden to the host [132]. 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.

1.4.1. Integrons and “jumping” genes

Most CEG identified in *P. aeruginosa* are associated with class I integrons [86, 112]. Integrons are genetic elements composed by an integrase-encoding *intI* gene, an *attI* recombination site and a Pc promoter [133, 134]. 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 [112, 133, 134]. Integrons *per se* are not mobilizable, but these elements may be inserted on MGE such as transposons, plasmids and/or ICE [25]. Also, these elements may be mobilized when a complete transposition module is provided in *trans* [134]. Related integrons may be identified in different contexts, which also suggest an important contribution of homologous recombination in promoting the evolution of integrons [133, 134].

The most clinically relevant class I integrons identified are ‘sul1-type’ integrons [112, 133, 134]. 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 [133–135]. 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 IS6*100*-mediated deletions into the conserved region, were also identified [133]. Quite surprisingly, most of these integrons are associated with a Tn*402*-like transposon which lost part of its transposition module [112, 133, 134]. Transposons or “jumping genes” are MGE which integrate into and excise from the chromosome but are not capable of being horizontally transferred [136, 137]. 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 [112, 133, 138]. 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 [112, 133, 138].

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 $bla_{\text{SPM-1}}$, $bla_{\text{NDM-1}}$ and $bla_{\text{KPC-2}}$ [86, 111, 120, 125, 126, 139]. The spread of these CEG is accomplished by its association with different transposons and other MGE [112].

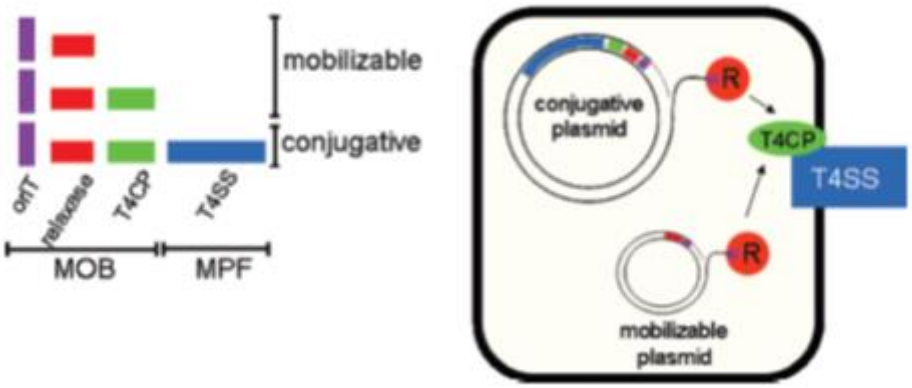
Importantly, most integrons bearing CEG co-harbor gene cassettes mediating resistance to other antibiotic classes, most frequently to aminoglycosides [86, 117]. Dissemination of these integrons may then contribute for the occurrence of multi-drug resistant phenotypes. Several carbapenemase-harboring 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*6249* and Tn*7017* transposons; a Tn*5051*-type transposon housing a *bla*$_{IMP-13}$ [140–144].

1.4.2. Plasmids

Plasmids are autonomous self-replicating elements which may be transferred from cell to cell by conjugation [145–149]. Halary et al. reconstructed a network of MGE and concluded that conjugation is more frequent than transduction, a bacteriophage-mediated DNA transfer mode, emphasizing the dominant role of plasmids in HGT [150–152]. Plasmid backbone is mainly taken up by genes encoding for its own propagation and stability. The replication module defines the copy number of plasmid cells and its own survival in several hosts. Low copy-number plasmids are subjected to a higher frequency of plasmid loss, due to random assortment at cell division [145, 146]. As so, extra stability modules such as toxin-antitoxin and partition systems may be required [153, 154].

Mobility of plasmids will depend on the set of backbone genes, and so these extrachromosomal elements may be conjugative, mobilizable or non-transmissible. (**Figure 4A**). 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 [145, 146]. 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) (**Figure 4B**) [145, 146]. Interestingly, Smillie et al. found that more than half of the plasmids are non-transmissible [145].

A



B

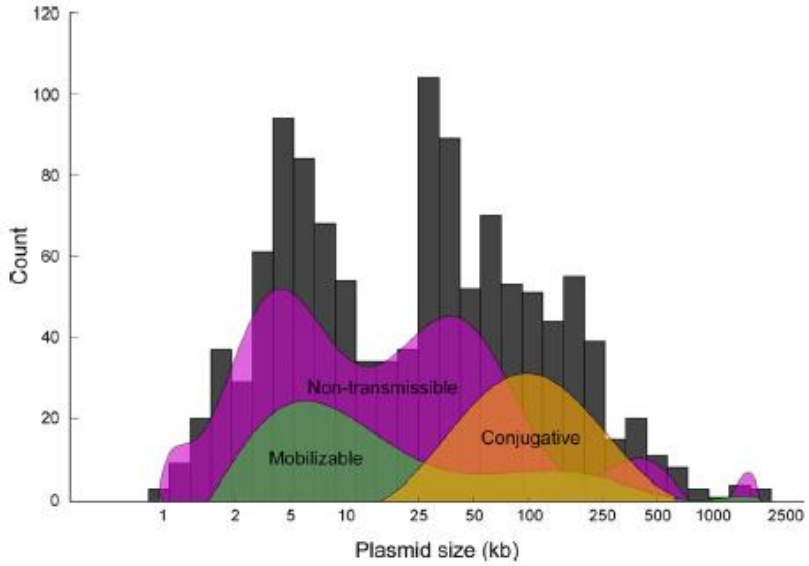


Figure 4. A – Schematic representation of conjugative and mobilizable plasmids. **B** – Distribution of conjugative, mobilizable and non-transmissible plasmids according to plasmid size. Both figures were adapted with permission from Smillie *et al* [145].

Plasmids may harbor an accessory module that confers an adaptive advantage to their host, such as virulence-encoding factors and AR genes [147, 149, 155, 156]. **Table 2** summarizes the main characteristics of the CEG-bearing plasmids that have been reported in *P. aeruginosa.*

Table 2 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	[157]
pNOR2000	VIM-2	-	22	62,8	France	Clinical	1996	[158]
pDCPR1	VIM-2	Unknown	18	58,4	Argentina	Clinical	2005 and 2012	[159]
pMRVIM0713	VIM-6	-	36	61,3	USA	Clinical	-	GenBank accession number KP975076.1
p07-406	VIM-7	-	24	63,8	USA	Clinical	2001	[160]

pP378-IMP	IMP-4	IncN	51	50,5	China	Clinical	2009-2013	[161]
pOZ176	IMP-9	IncP-2	501	57,0	China	Clinical	2000	[162]
pCOL-1 and pPA-2	KPC-2	IncP-6 and IncU	32 and 8	60,0 and 56,0	Colombia	Clinical	-	[125]
p10265-KPC	KPC-2	IncP-6	39	58,2	China	Clinical	2010	[126]
pD5170990	KPC-2	IncU	32	60,3	Brazil	-	2013	GenBank accession number KX169264.1

To date, fourteen incompatibility groups (IncP-1 to IncP-14) were characterized amongst *Pseudomonas* plasmids [112, 163]. 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 (IncP), IncP-3 (IncA/C), IncP-4 (IncQ), IncP-6 (IncG) [112, 163]. 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 phylogenetical concordance between plasmid core genes [164, 165]. 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 2**). However, plasmid reconstruction can be quite puzzling [166]. 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, a reference-dependent graph-based tool and its recently developed user-friendly web-tool (https://castillo.dicom.unican.es/upload/) [167, 168]; and the automated and reference-independent tools plasmidSPAdes and Recycler [169, 170]. 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 [165].

1.4.3. Genomic islands and integrative and conjugative elements

GI comprise a cluster of genes that had been acquired by a bacterial genome by HGT and contributed to diversification and host adaptation [25, 130, 171]. These broad definition may also comprise other MGE, such as ICE and prophages (**Figure 3**) [130]. Albeit the origin of these elements remains unknown, a growing body of evidence demonstrates that phages are one of the major sources [172]. GI frequently display a sporadic distribution along the genome, a large size, an association with phage integrase genes and a sequence composition that is significantly different from that of the host. These elements also tend to target tRNA genes and are normally flanked by direct repeats [25, 130, 171]. However, these features are not specific to these elements, which makes GI 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 GI. Also, foreign DNA tends to undergo amelioration in the host genome, limiting the detection of ancient GI and leading to false-negative predictions [130, 173]. The transfer of GI 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 GI are not inserted next to tRNA genes and not all are flanked by direct repeats [174]. As so, comparative genomic analysis and phylogeny-based methods should be engaged with sequence composition to perform a more robust prediction [130]. Comparative genomics relies on the availability of related sequenced genomes. Moreover, if the genomes are too closely related, GI 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 [130]. Currently, *in silico* analysis of GI may be accomplished by IslandViewer 4 (http://www.pathogenomics.sfu.ca/islandviewer/), an integrated interface of four different GI prediction methods [175].

In *P. aeruginosa*, the spread of AR via GI is greatly underappreciated [112, 176]. Few reports have explored the role of these elements for the transfer of CEG [142, 177–180]. Chowdhury et al. reported that GI 1 and 2 are frequently targeted by Tn*6060*-like and Tn*6163*-like carbapenemase-harboring transposons, respectively [177, 178].

P. aeruginosa genomic islands that carry genes coding for virulence factors are collectively known as pathogenicity islands (PAPI) and help to promote the pathogenic promiscuity of specific strains. For instance, several GI were reported in *P. aeruginosa* LESB58 strain from a CF patient [181, 182]. Two GI (PAPI-1 and PAPI-2) were reported on *P. aeruginosa* PA14 highly virulent strain from a burn patient [183, 184]. These elements present a mosaic structure and may contribute individually and synergistically for virulence [184]. 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 [20, 185, 186]. 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 [20, 187]. 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 GI related to PAPI-2 (ExoU islands A, B and C) [188].

ICE are self-transmissible mosaic and modular MGE that combine features of i) transposons and phages, since ICE can also integrate into and excise from the chromosome; and ii) plasmids, since ICE can also exist as circular extrachromosomal elements and can be transferred by conjugation (**Figure 5** and **Box 2**) [25, 189–196]. 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 [191, 194, 195]. ICE are a subset of GI (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 [130, 189]. Four mating-pair formation (MPF) classes cover the T4SS among Proteobacteria: MPF_T, MP_G, MPF_F and MPF_I [193]. 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 ICE. MPF_I is rarely identified [193].

Conjugative transposons are also defined as ICE. In fact, the first ICE to be discovered was Tn*916* (back then defined as a conjugative transposon) from *Enterococcus faecalis* [197]. ICE 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 [25, 189–196]. 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 ICE [25, 189–196]. 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 [198].

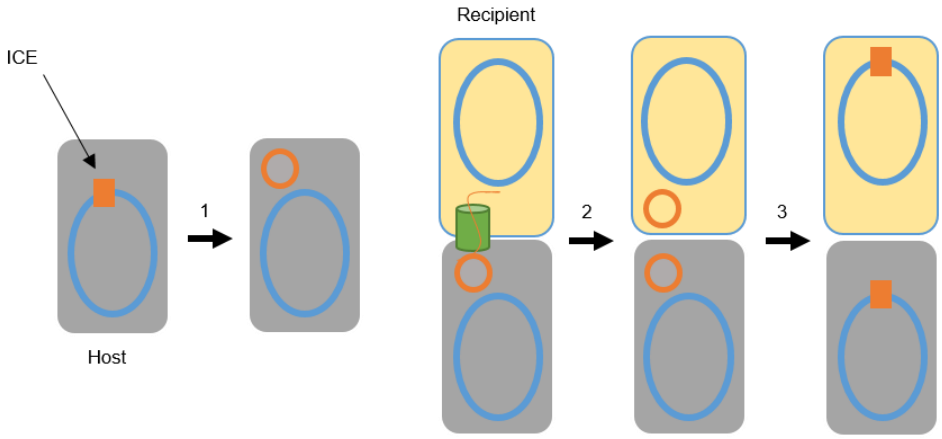


Figure 5 – The HGT of an ICE. Besides being vertically transmitted to daughter cells during cell division, ICE can be transferred to other bacteria by conjugation. ICE are normally integrated in the chromosome and under certain conditions can be excised and form a plasmid-like circular intermediate (Step 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 ICE.

Understanding the life cycles of phages, plasmids and transposons is mandatory to correctly inform our views of ICE. In fact, the regulatory and maintenance mechanisms are similar to those of phages and plasmids; integration/excision to phages and transposons; and conjugative transfer to plasmids [25, 189–196]. In fact, a growing body of evidence is challenging the line separating plasmids and ICE [190, 192, 195]. Besides the aforesaid common features, several ICE are also capable of autonomous rolling-cycle replication, which may be critical to facilitate ICE maintenance [190]. Additionally, the relaxase and the T4SS encoded by ICE resembles that of conjugative plasmids [25, 189–196]. Like mobilizable plasmids, these GI 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 GI are named integrative and mobilizable elements [199]. Guglielmini et al. constructed a phylogenetic tree of VirB4, a highly conserved ATPase from the T4SS apparatus of different conjugative plasmids and ICE, and formulated the hypothesis of interchangeable conjugation modules along their evolutionary history [192]. VirB4 proteins encoded by ICE are, however, more related to those encoded by other ICE 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 ICE and/or vice-versa. A search of more than 1,000 genomes revealed that ICE are present in most bacterial clades and may be more prevalent than conjugative plasmids in all clades [192]. These researchers also found that mobilizable plasmids and IMEs outnumber conjugative plasmids and ICE, suggesting a wide utilization of T4SS in *trans*. The majority of ICE and integrative and mobilizable elements analyzed were found in only one copy per genome [171]. Some ICE may have become fixed into the chromosome due to degeneration of its phage integrase genes and/or conjugative elements. ICE 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 [25, 196].

As most GI, ICE frequently target a single insertion site, which is often a tRNA gene [25, 189–196]. Even though multiple copies of these genes may be present among different hosts, ICE 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 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 [25, 189–196]. 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 [200, 201].

Most ICE identified among *P. aeruginosa* fall into two families: pKLC102-like and *clc*-related ICE [25], 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% [27]. Main differences between given families are related to the integration site and the type of integrase: ICE 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 [25, 189, 202]. Also, pKLC102-like ICE 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 [25]. Interestingly, Fischer et al. found that ICE 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 [28]. The ICE Tn*4371* family also represents a large group of ICE with a common backbone and which are widely distributed, such as in *P. aeruginosa* PA7 and 2192 [189, 203].

To date, only three examples of carbapenemases were associated with an ICE location in *P. aeruginosa*: a bla_{GES-5}-harboring GI2, identified in Australia, a bla_{SPM-1} inserted into ICETn*4371*6061 and reported in Brazil and a bla_{NDM-1}-harboring ICE from Singapore [139, 177, 204]. Curiously, these reports are quite recent, leading us to speculate that the advent of WGS and bioinformatics will help to accurately identify more ICE and eventually assess the real contribution for the spread of CEG.

ICEberg (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 [205]. Unfortunately, this integrated database has not been regularly updated. The CONJscan (https://research.pasteur.fr/en/software/conjscan-t4ssscan/) is a tool that scans a set of protein sequences for T4SS and relaxases of both ICE and plasmids using hidden Markov models [206, 207].

1.5. 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 [148, 208, 209]. The classic paradigm is that AR determinants endeavor a fitness cost for the bacterial host, in terms of reduced growth, competition and/or infectivity [62, 155, 208–211], even though some important exceptions were reported [212, 213]. It is also assumed that evolution of resistance occurs by only a few mutations of large effect [208, 209]. 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 [208]. 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 [214]. 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 [209, 215, 216].

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**) [208]. Indeed, Melnik et al. tracked the development of ciprofloxacin resistance in *P. aeruginosa* under constant and fluctuating antibiotic delivery, and found that high-fitness, resistant strains (broadly adapted generalists, as defined by Kassen et al. [217]) evolved quickly under fluctuating treatments and that second-site mutations were responsible for compensating the fitness cost associated with resistance [218]. As so, the authors suggested that these therapies generate persistent AR by selecting for the evolution of cost-free resistance *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 [219]. 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 [219].

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 [220]. HGT is more frequent between closely related species from the same taxonomic group, having similar genomes and an analogous GC content [220–222]. As so, the pangenome also represents the pool of genes that may be available through HGT to any close relative of the same taxon [223]. Another study found bi-directional associations among the similarity in tRNA pools of bacteria and the number of HGT events happening between them [224]. Both situations make quantification of HGT quite challenging [221, 225]. 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 [226]. 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 [227]. Also, HGT was shown to play a more important role than gene duplication for the expansion of protein families among prokaryotes [228]. HGT may also depend on the transfer mechanism [229, 230]. 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 [220, 223, 225, 228, 229, 231].

Once foreign DNA has been integrated into the host, it might need to adapt within the genome in order to resist purifying selection [23]. 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 [62]. 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 [223, 232]. 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 [223, 232].

Genetic elements such as plasmids and ICE frequently harbor AR and/or virulence genes that may provide a selective advantage for the host. Acquisition of a useful gene pool by a host genome could counterbalance the fitness cost associated with maintaining these elements [132, 233]. 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 [62].

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 maintain plasmids harboring AR genes in a given population [63, 152, 155, 234]. To accommodate this MGE, compensatory mutations may occur in the plasmid, the host or in both [148, 155, 211, 231, 235–239]. Given the abundance of non-transmissible plasmids [145], 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 [240]. Loftie-Eaton et al. exposed 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 [234]. The researchers also found that this transposition expanded plasmid-host range. Another work reported how compensatory adaptation and positive selection interact to assure persistence of non-conjugative plasmid [235]. 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 [237]. 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 [231]. However, co-evolution may not always lead to amelioration of plasmid burden [241]. 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 [63, 242, 243].

But 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, resistance mutations in essential and highly conserved genes are likely to be more costly [244]. 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 [209]. 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 [155, 209]. This host-plasmid coevolution could also help to explain why acquisition of the same plasmids by different hosts exerts a different cost [245].

ICE appear to have a bistable lifestyle that determines the change between vertical and horizontal transmission [195]. 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 ICE 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 [194, 195, 246]. 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* [246–248], 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.

1.6. 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) [249]. 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 [249]. Hospital transmission can, however, increase the prevalence of particularly adapted clones [86].

Some ST are worldwide disseminated and frequently linked to outbreaks and to the dissemination of carbapenemases. These ST have been designated as high-risk clones, of which major examples are ST235, ST111 and ST175 in *P. aeruginosa* (**Figure 6**) [86, 250–252]. The bla_{VIM-2} was the most frequently encountered CEG among ST235 and ST111 [86, 119]. Albeit being responsible for the spread of AR through HGT, these ST have also been associated with mutational resistance. In fact, the mechanisms responsible for the multi-

drug resistant phenotypes frequently observed in ST175 are mostly determined by specific mutations on *oprD*, *ampR*, *mexZ*, *gyrA* and *parC* [253–255].

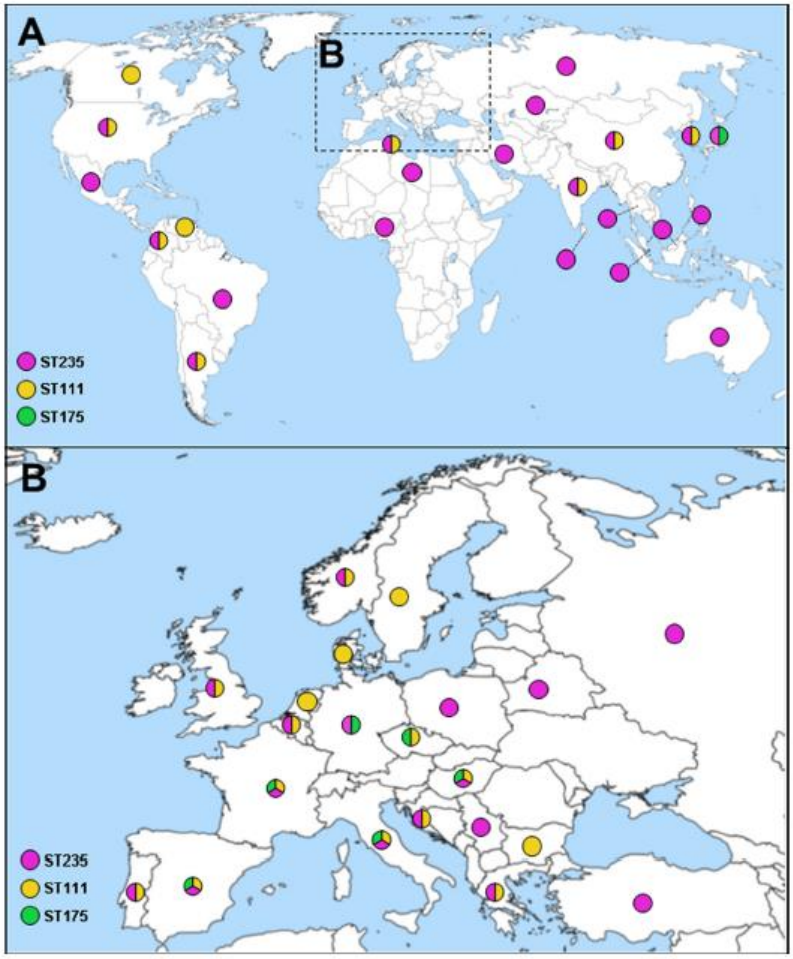


Figure 6 – Worldwide (**A**) and European (**B**) dissemination of internationally-recognized high-risk clones. Adapted with permission from Oliver *et al* [86].

Among the few major high-risk clones, ST235 is the most widely disseminated [86, 177, 178, 250, 256–259]. Nearly 100 horizontally-acquired resistance elements have been reported among ST235 isolates [86, 177, 178, 256]. The *dprA* gene was identified by Treepong et al. as a specific marker of this lineage [256]. 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 [86, 114, 119, 178, 250, 258].

The geographical distribution of the high-risk clones and the diversity of AR elements suggest that the spread of these ST is global and the acquisition of the resistance genes is mainly local [86, 119]. Previous studies suggest that environmental species may pose an important reservoir for the dissemination of clinically relevant carbapenemases, which are vertically amplified upon transfer to *P. aeruginosa* high-risk clones [260, 261]. 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 [135]. Other theories support that the high-risk clones have a naturally increased ability to acquire foreign DNA, since these ST 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 [119, 258, 262].

Mulet et al. studied the biological markers that may be responsible for the success of the *P. aeruginosa* high-risk clones [263]. The authors found that these ST were defective in motility and in 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 [263].

Treepong et al. linked the dissemination of ST235 with the selective pressure exerted by fluoroquinolones [256]. 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 [253]. Curiously, as above-mentioned, some mutations do not involve biological costs, as those targeting *gyrA* and *gyrB* genes [208]. Conferring low levels of resistance without incurring in fitness cost can help to explain the high frequency of gyrase mutations in several clinical isolates [264]. Future studies performing comparative genomic analysis of high-risk clones and non-epidemic muti-drug resistant strains will help to shed a new light on the traits responsible for the success of these ST. Also, 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 [86].

Aims and objectives

The **main aims** of this thesis were to provide an in-depth view of the main MGE (ICE and plasmids) responsible for the spread of CEG in clinical isolates of *P. aeruginosa* and to identify the clones involved in their dissemination. This was achieved by characterizing a collection of 263 carbapenem-resistant *P. aeruginosa* isolates obtained between 1995 and 2014 at six geographically distinct hospitals from North and Centre of Portugal. A worldwide collection of *Pseudomonas* genomes was also downloaded from NCBI and used to assess the prevalence of CEG and to explore the contribution of ICE for the spread of carbapenemases among this genus.

Specific objectives were the following:

- To investigate the molecular epidemiology of carbapenemase-producing *P. aeruginosa* (CPPA) isolates in Portuguese hospitals;
- To provide a detailed view of CEG-bearing MGE and to evaluate the linkage of class I integrons with different MGE, such as transposons, insertion sequences, ICE and plasmids;
- To investigate the contribution of high-risk clones and local outbreak strains in the spread of CPPA in Portugal;
- Taking into consideration that i) in pseudomonads, CEG are frequently located within the chromosome, ii) ICE are the most abundant conjugative elements in prokaryotes and iii) ICE are more frequently identified in large bacterial genomes, such as in pseudomonads, we explored the contribution of ICE to horizontal gene transfer of AR among *Pseudomonas* spp genomes publicly available;

Organization of the thesis

Chapter 1 supplies a comprehensive review of the current knowledge on topics related to AR in the opportunistic pathogen *P. aeruginosa*, focusing on the role of MGE (mainly ICE and plasmids) for the spread of CEG among CPPA isolates. This section also provides a snapshot on the population structure and the relevance of successful international clones for the spread of CEG among *P. aeruginosa* strains. It is also explored in this chapter the evolution of AR and fitness cost associated with AR in *P. aeruginosa* strains.

Chapter 3 includes the methods and results referring to this thesis's main aims and specific objectives that were published on international peer-reviewed journals.

Chapter 4 explores the main achievements of the published works, alongside future directions.

Chapter 5 provides a list of significant references used throughout chapters 1 and 4.

Chapter 6 provides the permissions to reproduce the figures and full text articles presented on this thesis.

Results and discussion

J Antimicrob Chemother
doi:10.1093/jac/dkx517

Journal of Antimicrobial Chemotherapy

Two decades of *bla*$_{VIM-2}$-producing *Pseudomonas aeruginosa* dissemination: an interplay between mobile genetic elements and successful clones

João Botelho[1], Filipa Grosso[1], Sandra Quinteira[2–4], Michael Brilhante[5], Helena Ramos[6] and Luísa Peixe[1]*

[1]*UCIBIO/REQUIMTE, Laboratório de Microbiologia, Faculdade de Farmácia, Universidade do Porto, Porto, Portugal;* [2]*CIBIO, Centro de Investigação em Biodiversidade e Recursos Genéticos, Universidade do Porto (CIBIO/UP)/InBio Laboratório Associado, Vairão, Portugal;* [3]*Faculdade de Ciências da Universidade do Porto, Departamento de Biologia, Porto, Portugal;* [4]*CESPU, Instituto de Investigação e Formação Avançada em Ciências e Tecnologias da Saúde, Gandra, PRD, Portugal;* [5]*Institute of Veterinary Bacteriology, University of Bern, Bern, Switzerland;* [6]*Serviço de Microbiologia, Centro Hospitalar do Porto, Porto, Portugal*

*Corresponding author: Laboratório de Microbiologia, Faculdade de Farmácia da Universidade do Porto, Rua Jorge Viterbo Ferreira no. 228, 4050-313 Porto, Portugal. Tel: +351 220428500; Fax: +351 226093390; E-mail: lpeixe@ff.up.pt

Received 9 August 2017; returned 6 October 2017; revised 4 December 2017; accepted 8 December 2017

Objectives: Information on clonal lineages and genetic platforms involved in the mobilization of carbapenemases between *Pseudomonas aeruginosa* strains in Portugal is scarce. Here, we outline the genetic drivers contributing to the occurrence of *bla*$_{VIM-2}$-producing *P. aeruginosa* over two decades.

Methods: A collection of carbapenem-resistant *P. aeruginosa* clinical isolates ($n = 263$, 1995–2014) was screened for carbapenemase production by Blue-Carba and PCR. Antimicrobial susceptibility testing was performed according to EUCAST and clonal analysis by MLST. Nine isolates representing different integrons and STs were selected for WGS, followed by bioinformatics.

Results: Twenty-seven *bla*$_{VIM-2}$-producing *P. aeruginosa* belonging to 10 STs were identified, with ST179 and ST111 being the most prevalent and persistent clones. *bla*$_{VIM-2}$ was associated with seven class I integrons frequently co-harbouring aminoglycoside resistance genes. In58 was commonly identified, followed by derivatives and In100. *bla*$_{VIM-2}$-harbouring transposons of the Tn*3* and Tn*402* families were linked to different plasmids or integrative conjugative elements of the *clc* family.

Conclusions: The dissemination of *bla*$_{VIM-2}$ carrying integrons is associated with a complex interplay between different mobile genetic elements, including the overlooked integrative conjugative elements, and successful spread of particular clones.

Introduction

Pseudomonas aeruginosa is a clinically important pathogen exhibiting interplay between intrinsic and acquired mechanisms of resistance to several antibiotics, including carbapenems.[1–3] The species is identified as one of the most critical Gram-negative bacteria to be included in the recent WHO list of antibiotic-resistant pathogens. It poses the greatest threat to public health, and is the focus of effort to secure and guide research and development of new antibiotics (see http://www.who.int/mediacentre/news/releases/2017/bacteria-antibiotics-needed/en/). Since 2008, *P. aeruginosa* has been reported as one of the 'ESKAPE' (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* species) pathogens, underlining its impact on nosocomial infections and the ability of the bacteria to 'escape' the activity of antimicrobial drugs.[4]

The increased spread of carbapenemase-producing *P. aeruginosa* (CPPA) isolates constitutes, at present, an important threat worldwide, with VIM (Verona imipenemase) and IMP (imipenemase) families being the most prevalent.[1–3] MBL genes are frequently embedded in class I integrons, which may also harbour other antibiotic resistance determinants. These structures can be easily mobilized by different mobile genetic elements (MGEs), such as transposons, plasmids, integrative conjugative elements (ICEs) and/or genomic islands (GIs).[1–3,5–9] Moreover, in this species, MBLs have been frequently linked to high-risk clones belonging to widespread STs 111, 175, 235, 253 and 244.[1–3,10–12]

In Portugal, different carbapenemases have been described among isolates of *P. aeruginosa*, with frequent reports of VIM-2 and occasionally IMP-5 and GES-6.[13–19] Notwithstanding this, information concerning the clonal lineages and genetic surroundings of CPPA strains in Portuguese hospitals is still scarce.[1] Here, we

Table 1. STs and genetic platforms associated with bla_{VIM-2}-producing *P. aeruginosa* isolates from Portuguese hospitals, collected from 1995 to 2014

ST[a]	Integron	Genetic location (no. isolates)	Isolate selected for WGS	Years	Hospital[b]	Sources	Reference
111	In58	ICE*6440* (~80 kb)[c] (2)	HSV3483	1995, 2013	A, C	urine, respiratory secretions	18, this study
	In100	ΔGI[d] (1)	FFUP_PS_105	2012	A	urine	17, this study
		C, non-ICE[e] (2)		2000, 2014	E, F	urine, blood	this study
175	In58	pJB12 (~30 kb) Tn*6352*[c] (4)	FFUP_PS_12	2001, 2003, 2006	A, D	urine, NA	13, this study
179	In103	pJB35 (~30 kb) (1) Tn*402*-like	FFUP_PS_35	2002	A	urine	this study
	In58	C (7)	FFUP_PS_144	2001, 2003, 2011, 2012, 2013	A, B	catheter, urine, bronchial secretions, bronchial aspirate	this study
235	In56	ICE*6441* (~90 kb)-Tn*6391* (1)	FFUP_PS_CB5	2002	E	bronchial aspirate	this study
244	In58	C-Tn*6356*-like (1)	FFUP_PS_65	2010	A	urine	this study
	In796	C (1)		2013	A	urine	this study
253	In58	pJB37 (~460 kb)-Tn*6356*[c] (3)	FFUP_PS_37	2008, 2010	A	bronchial secretions, exudate	14, this study
260	In58	C (1)		2001	D	urine	this study
282	In58	pCB58 (~30 kb) (1) Tn*402*-like	FFUP_PS_CB58	2004	F	urine	this study
815	In102	C (1)		2000	E	blood	this study
1284	In1220	C (1)		2011	A	catheter	this study

NA, not available; C, chromosome; P, plasmid.
[a]Combination of alleles (*acsA*, *aroE*, *guaA*, *mutL*, *nuoD*, *ppsA* and *trpE*) for each ST identified is as follows: ST111 (17, 5, 5, 4, 4, 4, 3), ST175 (28, 22, 5, 3, 3, 14, 19), ST179 (36, 27, 28, 3, 4, 13, 7), ST235 (38, 11, 3, 13, 1, 2, 4), ST244 (17, 5, 12, 3, 14, 4, 7), ST253 (4, 4, 16, 12, 1, 6, 3), ST260 (14, 5, 10, 7, 4, 13, 7), ST282 (6, 5, 11, 7, 3, 12, 19), ST815 (103, 11, 61, 5, 1, 6, 8) and ST1284 (32, 8, 5, 3, 5, 6, 26).
[b]Hospitals A, B and C: North; D, E and F: Centre.
[c]Structure predicted for the representative isolate submitted for WGS analysis. For related isolates, this structure was predicted by PCR mapping.
[d]Δ, truncated. This structure was related to part of the ICE*6440* and ICE*6441*.
[e]By PCR mapping. No relation to ICE*6440* or ICE*6441* was observed.

explore the drivers contributing to the occurrence of bla_{VIM-2}-producing *P. aeruginosa* in different hospitals over two decades.

Materials and methods

Bacterial isolates

A collection of carbapenem-resistant *P. aeruginosa* clinical isolates ($n = 263$, 1995–2014) was obtained from several clinical products (urine, respiratory secretions, bronchial aspirate, bronchial secretions, aspirate, blood and catheter) and distinct inpatients from six geographically distant hospitals within Portugal (Table 1).

Species identification and antimicrobial susceptibility testing

Identification was initially performed by the automated VITEK 2 system (bioMérieux, Marcy-l'Étoile, France) and afterwards confirmed by a multilocus sequence analysis (*rpoD*, *gyrB* and 16S rRNA genes) in all CPPA strains.[20]

Antimicrobial susceptibility testing of all CPPA isolates was conducted according to the EUCAST guidelines (www.eucast.org) by standard disc diffusion, except for colistin (broth microdilution method).

Carbapenemase identification and genetic location

Carbapenemase production was assessed by the Blue-Carba test.[21] PCR assays for the most prevalent carbapenemase-encoding genes were conducted as described elsewhere.[22,23]

The genetic location of carbapenemase-encoding genes was determined by I-*Ceu*I/S1 PFGE for all CPPA isolates, as previously described.[16] Hybridization was performed with specific probes for carbapenemase genes and 16S rDNA, as reported.[16]

Transfer assays of carbapenem resistance

Conjugation assays were conducted as previously described,[17] using a spontaneous rifampicin-resistant mutant of *P. aeruginosa* PAO1 as the recipient strain. A selection of transconjugants was performed in Mueller–Hinton agar plates containing rifampicin (100 μg/mL) and imipenem (4 μg/mL).

Clonal analysis

Clonal diversity analysis was assessed by MLST according to the guidelines published for *P. aeruginosa* (https://pubmlst.org/paeruginosa/).

WGS and bioinformatics

Nine CPPA isolates representing different STs, integrons and time periods were selected for WGS analysis (Table 1). Total DNA was extracted with the Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. Complete nucleotide sequencing was achieved by the Illumina HiSeq platform (100× coverage) and *de novo* assembly by Geneious assembler v 9.1.8. High-throughput sequence data quality was accessed by FastQC, while genome assembly quality was evaluated by QUAST (http://quast.bioinf.spbau.ru/). Automatic annotation of the genome was conducted by NCBI's Prokaryotic Genome Annotation Pipeline.[24] The presence of putative plasmid sequences was examined by the plasmidSPAdes tool from SPAdes 3.9.0.[25] The presence of putative GIs was inspected by the SIGI-HMM method available in IslandViewer 4 (http://www.pathogenomics.sfu.ca/islandviewer/). PHAST (http://phast.wishartlab.com/index.html) web server was used to detect the presence of prophage sequences. Antimicrobial resistance genes were identified by ResFinder (https://cge.cbs.dtu.dk/services/ResFinder/) and the RGI software from the CARD database (https://card.mcmaster.ca/home). For the ST assignment, genomes deposited in GenBank were inspected using the MLST-1.8 Server (https://cge.cbs.dtu.dk/services/MLST/).

Overlapping sequences comprising the genetic environment surrounding the different integron structures were mapped with Geneious v. 9.1.8. Primer design and PCR mapping was conducted to confirm the orientation of these regions, and the coding sequences (CDSs) were predicted using the Glimmer tool available at Geneious v. 9.1.8. Manual annotations for these regions were assessed with NCBI's BLASTp and Conserved Domain Database tools. Easyfig (http://mjsull.github.io/Easyfig/) was used to compare the backbone of different MGEs. BRIG (https://sourceforge.net/projects/brig/) software was applied to design plasmid backbones.

Nucleotide sequence accession numbers

These Whole Genome Shotgun projects have been deposited into DDBJ/ENA/GenBank under accession nos NINN00000000 (FFUP_PS_65), NINO00000000 (FFUP_PS_CB58), NINP00000000 (FFUP_PS_CB5), NINQ00000000 (FFUP_PS_12), NINR00000000 (FFUP_PS_144), NINS00000000 (FFUP_PS_105), NINT00000000 (FFUP_PS_37), NINU00000000 (FFUP_PS_35) and NINV00000000 (HSV3483). Complete nucleotide sequences of ICE*6440* and ICE*6441* were deposited into GenBank under accession nos MF168944 and MF168946, respectively. Complete nucleotide sequences of pCB58 and pJB35 were deposited into GenBank under accession nos KY630469.1 and MF168945, respectively. Nucleotide sequences of the novel integron structures were deposited into GenBank under accession nos AY775051.1 (In102), AY954726.1 (In103) and KT946596.1 (In1220).

Results and discussion

General features

Over a two-decade period, within the context of regular screening for carbapenemase producers, 27 CPPA isolates were detected (18 from hospital A, 1 from B, 1 from C, 2 from D, 3 from E and 2 from F) from a collection of 263 carbapenem-resistant *P. aeruginosa* clinical isolates.

The low prevalence of carbapenemases in our collection (10%) is similar to previous findings. In fact, the main mechanisms for carbapenem resistance in this species are OprD porin impairment, and overexpression of efflux systems.[26] The analysis revealed that all CPPA carried the bla_{VIM-2} gene, with the exception of one isolate that harboured bla_{GES-6} in a ST235 high-risk clone.[15] bla_{VIM-2} is frequently found worldwide amongst *P. aeruginosa* isolates and is commonly linked to high-risk clones that exhibit MDR or XDR phenotypes.[1–3,12] Although bla_{VIM-2} was first reported in *P. aeruginosa* in France in 1996,[27] the index case was recorded in Portugal in 1995.[18] Accordingly, VIM-2 enzyme represents the most ancient and, for almost two decades, the most frequently found MBL in CPPA Portuguese isolates.

Among the isolates studied, various ST profiles ($n = 10$) were identified, including the high-risk clones ST111, ST175, ST235 and ST244 (Table 1). This reflects the high genetic diversity of carbapenemase-encoding clones, as commonly observed in *P. aeruginosa* clinical isolates from different countries.[1,2,11] ST179 was the most frequently found clone within our isolates and observed over 11 years (Table 1). VIM-2-producing ST179 was also detected in several patients with chronic respiratory infections from Spanish hospitals.[28] ST111, a recognized high-risk clone, was identified as the oldest CPPA isolate from our collection as well as in recent isolates from different hospitals (Table 1).[1–3,12] ST175 is an international clone that has been mainly associated with XDR and MDR *P. aeruginosa* isolates from Spanish hospitals.[1,2,29] ST253 is one of the five high-risk clones commonly identified in this species.[30] Besides the clinical relevance, this clone was also associated with different environmental, animal and non-clinical settings.[31,32] ST244 also represents a widely spread clone, being responsible for the propagation of VIM-2, IMP-6, VEB-1 and PER-1 enzymes in different countries.[11,33] Even though ST244 was identified in a single hospital, it was the most frequently found ST in that unit (J. Botelho, F. Grosso and L. Peixe, unpublished results). ST235, scarcely found in this study, is the most frequent high-risk clone involved in the worldwide dissemination of carbapenemases (Table 1).[1–3,12,15] STs 260, 282, 815 and 1284, each described in one isolate, were also rarely reported in other studies. Interestingly, a ST260 carrying bla_{IMP-14} and ST1284 with bla_{VIM-7} were previously reported.[34,35]

All bla_{VIM-2}-producing isolates were non-susceptible to imipenem, ceftazidime, cefepime and piperacillin/tazobactam, and presented variable susceptibility to aztreonam (52%), ciprofloxacin (22%), meropenem (7.4%), amikacin (3.7%), tobramycin (3.7%) and gentamicin (3.7%). All isolates were susceptible to colistin (MICs ranging from 0.25 to 1 mg/L). Twenty-two isolates were defined as XDR, whereas five (two isolates from ST253 and the isolates from STs 260, 282 and 815) presented a MDR phenotype.[36]

Genetic platforms associated with mobilization of the bla_{VIM-2} gene

In this study, bla_{VIM-2} was located within a wide variety of class I integrons (Figure 1 and Table 1). In58 (present in this study in 19 isolates, hospitals A, B, C, D and F) has been described in Portugal and France in association with different *Pseudomonas* species.[16,18,37] Interestingly, this class I integron was identified in the HSV3483 *P. aeruginosa* isolate, indicating its presence in Portugal at least since 1995.[18] All integron structures carried different combinations of In58 gene cassettes, with the exception of In56 and In100 (Figure 1). In796 (found in one isolate, hospital A) was previously detected in *P. aeruginosa* and *Acinetobacter bereziniae* Portuguese isolates.[38] It comprises a structure similar to In58, but lacks the *aacA4* gene. The genetic structure exhibited by In103 (one isolate, hospital A) was also similar to that of In58, except for the absence of the *aacC1* gene cassette. In1220 (one isolate, hospital A) only differed from In58 by the presence of an IS*6100* truncating the *aacA4* gene, suggesting that IS*6100*-mediated deletions into the gene cassettes might have

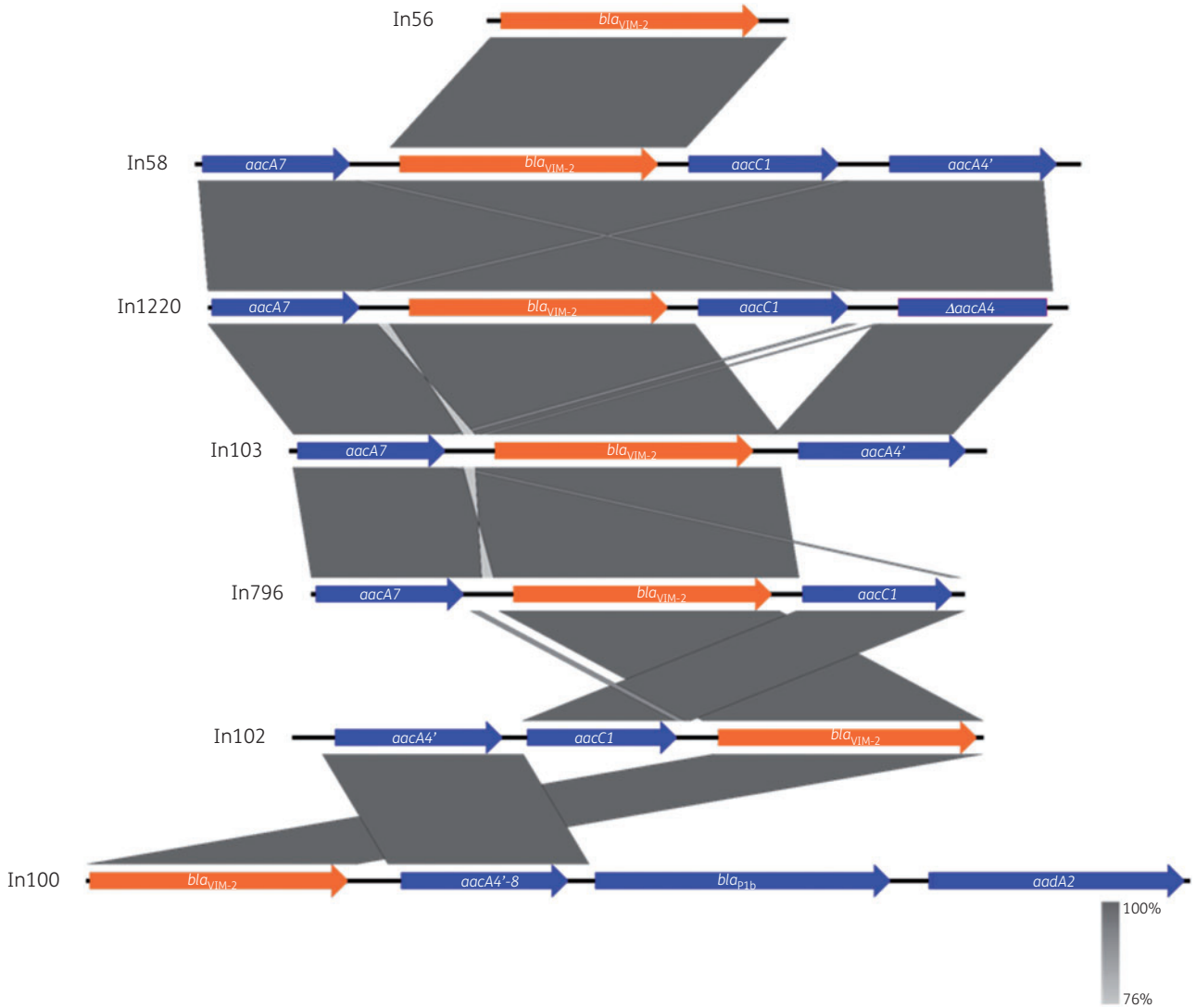


Figure 1. Schematic representation of the variable region of the class I integrons described in this study. The arrows show the orientation of the genes. bla_{VIM-2} gene is highlighted in orange. Figure was created using EasyFig. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

occurred.[39] In102 (1 isolate, hospital E) contained an *aacA4* gene followed by *aacC1* and bla_{VIM-2} gene cassettes. In56 (one isolate, hospital E), presenting the bla_{VIM-2} gene as a sole inserted gene cassette, was already reported in clinical and environmental contexts,[40] but it was only found here in one isolate (Table 1). In100 (three isolates, hospitals A, E and F), an integron with a particular gene cassette organization reflecting the antibiotic usage in therapeutics,[17] was identified in *P. aeruginosa* isolates obtained in different years from half of the sampled hospitals (Table 1). In102, In103 and In1220 represented novel integrons. Curiously, In58, In102 and In103 presented the *aacA4′* variant and In100 the *aacA4′-8* variant (Figure 1). The proteins encoded by those genes share the L83S change that typifies the AACA4′ variant, an aminoglycoside 6′-*N*-acetyltransferase type II conferring resistance to gentamicin.[41]

WGS analysis of selected isolates revealed a wide array of MGEs being responsible for the acquisition of bla_{VIM-2}-carrying integrons (Table 1). Nevertheless, in the ST179 representative isolate (FFUP_PS_144) the bla_{VIM-2}-harbouring In58 was located in the chromosome, with no evidence of its acquisition by an MGE (Table 1). A similar In58 genetic environment was observed by PCR mapping in the other ST179 isolates (Table 1). WGS analysis of a ST244 isolate (FFUP_PS_65) revealed the In58 in a transposon related to Tn*6356*, which was previously described on IncP-2 megaplasmid pJB37 from isolate FFUP_PS_37 (Table 1).[14] This transposon was inserted [direct repeats (DRs) 5′-TCATT-3′] in a backbone similar to that observed for the pJB37 megaplasmid.[14] However, no plasmid replicase nor conjugal transfer modules were here identified. These findings are also in accordance with the fact that this platform was not transferred by conjugation from FFUP_PS_65. Curiously, those

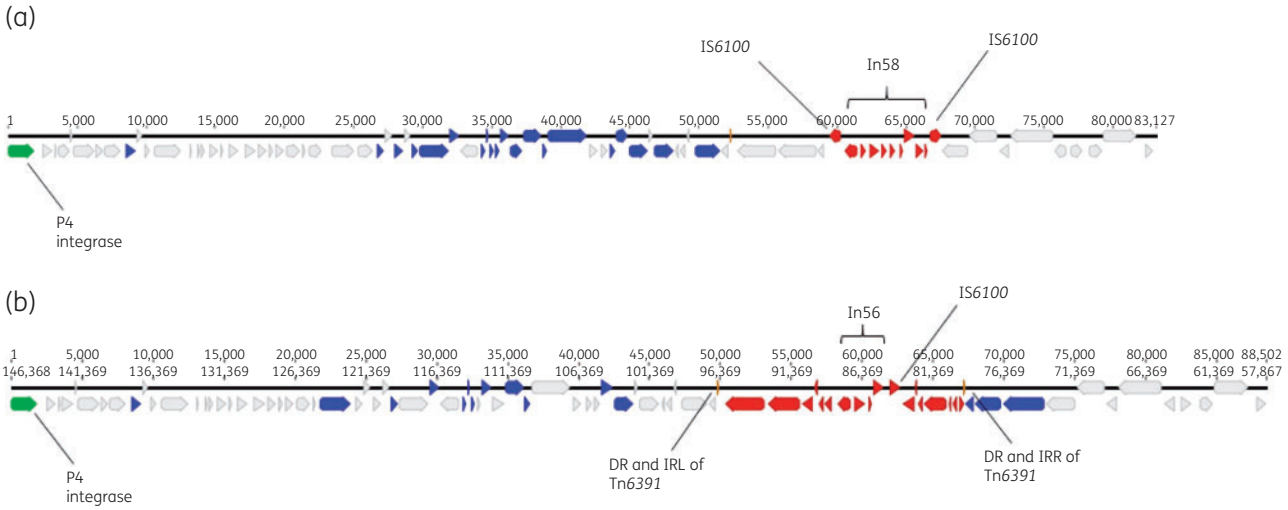


Figure 2. Schematic representation of the ICEs, ICE*6440* (a) and ICE*6441* (b). Arrows and arrowheads represent the translation orientation of the coding genes. Specific features of both ICEs are highlighted. Both figures were created using Geneious v 9.1.8. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

isolates belong to different clonal lineages, with FFUP_PS_37 belonging to ST253 (Table 1).

ICEs linked to high-risk clones ST235 and ST111

The bla_{VIM-2} gene was linked to two complete ICE sequences on two isolates (HSV3483 and FFUP_PS_CB5), associated with different integrons (In58 and In56) and with high-risk clones ST111 and ST235 (Table 1). ICEs are self-transmissible mosaics that encode their own integration/excision, conjugation, regulation and accessory modules.[6–9,42,43] These elements seem to be the most prevalent conjugative elements in all prokaryotic clades.[43] Until now, only three reports were associated with carbapenemases: a bla_{GES-5}-harbouring GI2, identified in *P. aeruginosa* draft genomes from Australia and belonging to the ST235; a bla_{GES-6}-habouring ICE associated with a *P. aeruginosa* isolate belonging to the same high-risk clone; and a bla_{SPM-1} inserted into ICETn*43716061*, frequently associated with ST277 *P. aeruginosa* isolates from Brazil.[44–47] Curiously, these reports are quite recent, which highlights the importance of WGS projects and subsequent bioinformatic analysis in the correct identification of these MGEs.

In HSV3483, the bla_{VIM-2}-carrying In58 integron was identified on an 83 339 bp ICE comprising 77 CDSs and a GC content of 62.0%, hereby named ICE*6440* (http://transposon.lstmed.ac.uk/), according to the criteria proposed by Roberts *et al.*[48] (Figure 2a and Table S1, available as Supplementary data at *JAC* Online). This ICE was highly related to bla_{GES-24}-harbouring PAGI-15 and bla_{IMP-6}-carrying PAGI-16 identified in *P. aeruginosa* clinical isolates from South Korea (accession nos KX196168.1 and KX196169.1, respectively), presenting 99% nucleotide identity and 94% of query cover.[49] Similar values (99% identity and a query cover ranging from 92% to 95%) were also found for genomic sequences of *P. aeruginosa* strains RIVM-EMC2982 and Carb01 63 from the Netherlands, E6130952 from Canada and DHS01 from France (accession nos CP016955.1, CP011317.1, CP020603.1 and CP013993.1, respectively). Comparative genome analysis revealed a wide dissemination of this backbone throughout unrelated clonal lineages: ST235 was associated with the strain E6130952 and with the isolates harbouring PAGI-15 and PAGI-16; the strains RIVM-EMC2982 and Carb01 63 belonged to ST111 and strain DHS01 to ST395. PCR mapping of another isolate belonging to ST111 and also carrying In58 revealed an identical ICE as the genetic background. The bla_{VIM-2}-harbouring In58 integron was here associated with a novel transposon hereby named Tn*6442*. This ICE was integrated next to a $tRNA^{Gly}$ gene and was inserted between a gene encoding for an α/β hydrolase and one encoding for a hypothetical protein.

FFUP_PS_CB5 isolate presented the bla_{VIM-2} gene in an 88 502 bp ICE resulting from the assembly of five contigs, hereby named ICE*6441* (Figure 2b and Table S1). This MGE comprised 84 CDSs and a GC content of 62.1%. ICE*6441* displayed high similarity values (nucleotide identity of 99% and query cover ranging from 96% to 97%) with *P. aeruginosa* strains F30658 from the USA, S04 90 from the Netherlands, NCGM257 from Japan (accession nos CP008857.1, CP011369.1 and AP014651.1, respectively) and the aforementioned strain DHS01. *In silico* analysis of these genomes revealed that strain F30658 belonged to ST111, NCGM257 to ST357 and S04 90 to ST446, also revealing a dissemination of this backbone amongst different clonal lineages. The bla_{VIM-2}-harbouring In56 integron was here associated with a novel transposon hereby named Tn*6391*. This structure was flanked by imperfect 38 bp inverted repeats typical of Tn*3*-like transposons. Tn*6391* was composed by a *tnpAR* module, followed by the In56 integron, an IS*6100* element and a mercuric resistance operon. A similar backbone (92% of query cover and 99% identity) was identified on the genome of the aforementioned strains RIVM-EMC2982 and Carb01 63. DRs (5′-CTCAA-3′) were generated upon transposition of Tn*6391* on this ICE. In fact, ICE can mount up other MGEs within their boundaries, thus also transferring these elements.[6]

In *P. aeruginosa*, most ICE fall into two large families: those linked to pKLC102 and the *clc*-like ICEs.[9] The ICEs here described are related to the ICE*clc*. The ICE_{Tn4371} family also represents a large group of ICEs with a common backbone and which are widely

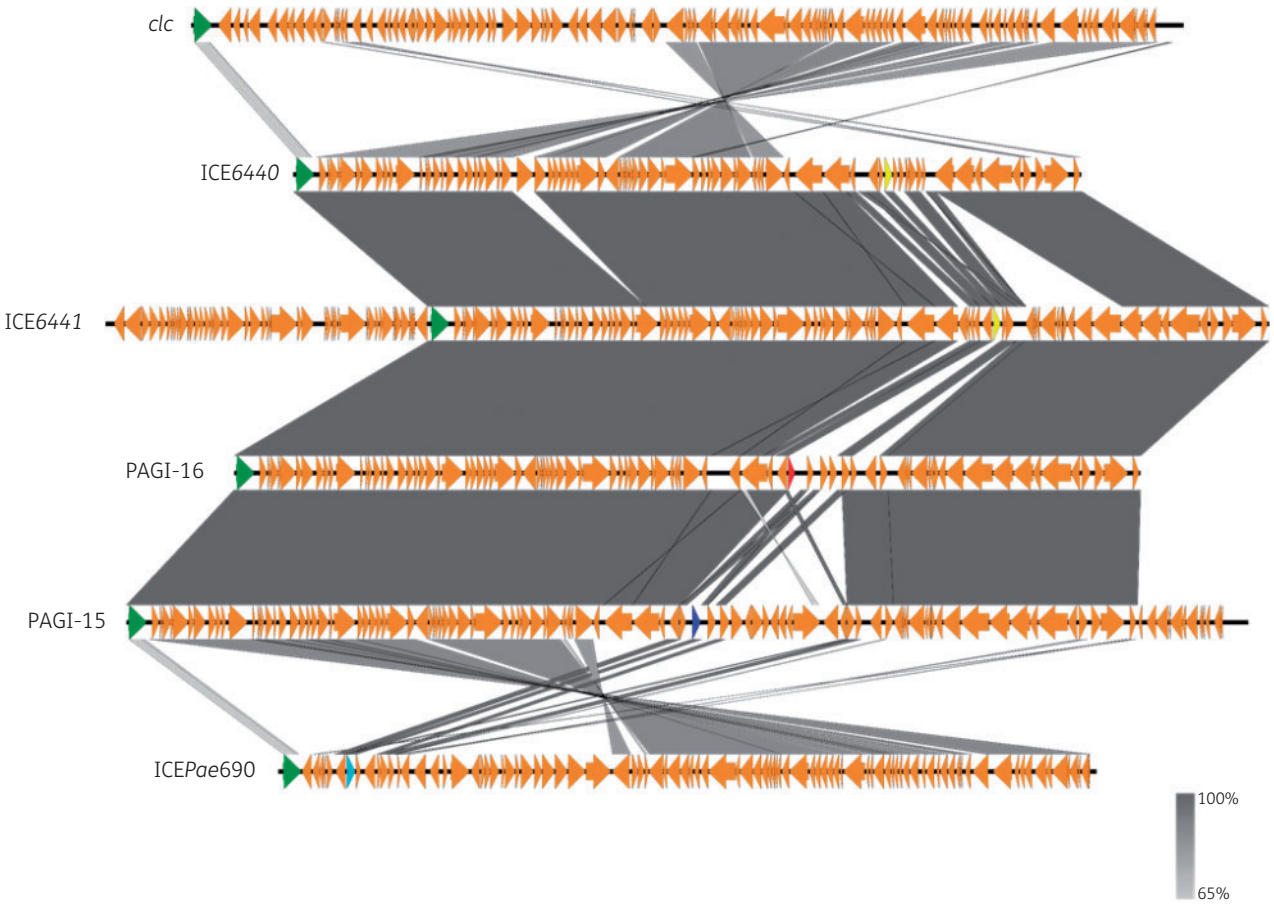


Figure 3. Genetic organization of the ICEs described in this study and related structures. Green arrowheads represent the integrase-encoding gene. Region upstream of the integrase on ICE*6441* represents the intact prophage described here. bla_{VIM-2} is highlighted in yellow. Remaining carbapenemase genes (bla_{IMP-6}, bla_{GES-6} and bla_{GES-24}) are represented by red, cyan and dark blue arrowheads, respectively. Arrows and arrowheads indicate the translation orientation of the coding genes. Figure was constructed with EasyFig. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

distributed.[45,47] When comparing the nucleotide sequence of the complete ICEs described here with the one from *clc* (accession no. AJ617740), 87% identity and a query cover ranging from 50% to 67% were identified (Figure 3). Indeed, the low query cover observed is typical of *clc*-like ICEs, as these elements display a conserved portion next to the integration site but harbour unique cargo genes.[9] These elements were also somehow similar to other previously characterized *clc*-like ICEs, such as PAGI-2 and LESGI-3 (accession nos AF440523 and FM209186, respectively).

Interestingly, the ICEs here identified shared exactly the same amino acid sequence for the integrase. Comparative genome analysis also revealed that the same integrase was found on PAGI-15, PAGI-16 and on the genome of *P. aeruginosa* strains RIVM-EMC2982, Carb01, E6130952, NCGM257 and DHS01. Site-specific recombinases of the bacteriophage P4 integrase subfamily integrases are typically found next to tRNA genes in temperate phages, integrative plasmids and GIs from different Gram-negative bacteria, emphasizing the interchangeable potential of functional modules between different MGEs along their evolutionary history.[6,7,9,42] Besides the integrase, both ICEs comprised a relaxase, type IV secretion systems (T4SS), a type IV coupling protein (T4CP) and genes encoding for maintenance functions. As mentioned above, these ICEs are highly related with PAGI-15 and PAGI-16 (Figure 3).[49] These findings help to link the ICEs here identified with the *clc* family and may contribute to the reclassification of PAGI-15 and PAGI-16 as *clc*-like ICEs. As far as we know, this is the first report of bla_{VIM-2}-harbouring ICEs. Taking into account that chromosomally encoded VIM-2 represents the most frequently identified MBL on *P. aeruginosa*,[2] and that ICEs seem to be the most prevalent conjugative elements in all prokaryotic clades,[43] we hypothesize that with the advent of WGS, the real contribution of these MGEs for the dissemination of this and other carbapenemases will be correctly assessed.

Interestingly, a 33.1 kb intact prophage region was inserted next to the $tRNA^{Gly}$ gene in ICE*6441*. ICEs possess several traits related to prophages, remaining quiescent with repressed mobility genes and being passively transmitted after cell division.[42] Genomic variation is highly influenced by the presence or absence of integrated prophages. This phage was similar to those of P2-like phages, such as the $_{\Phi}$CTX *P. aeruginosa* cytotoxin-converting phage from Japan, the $_{\Phi}$X216 *Burkholderia* bacteriophage from the USA and the Smp131 *Stenotrophomonas maltophilia* temperate phage from Taiwan (accession nos AB008550.1, JX681814.1

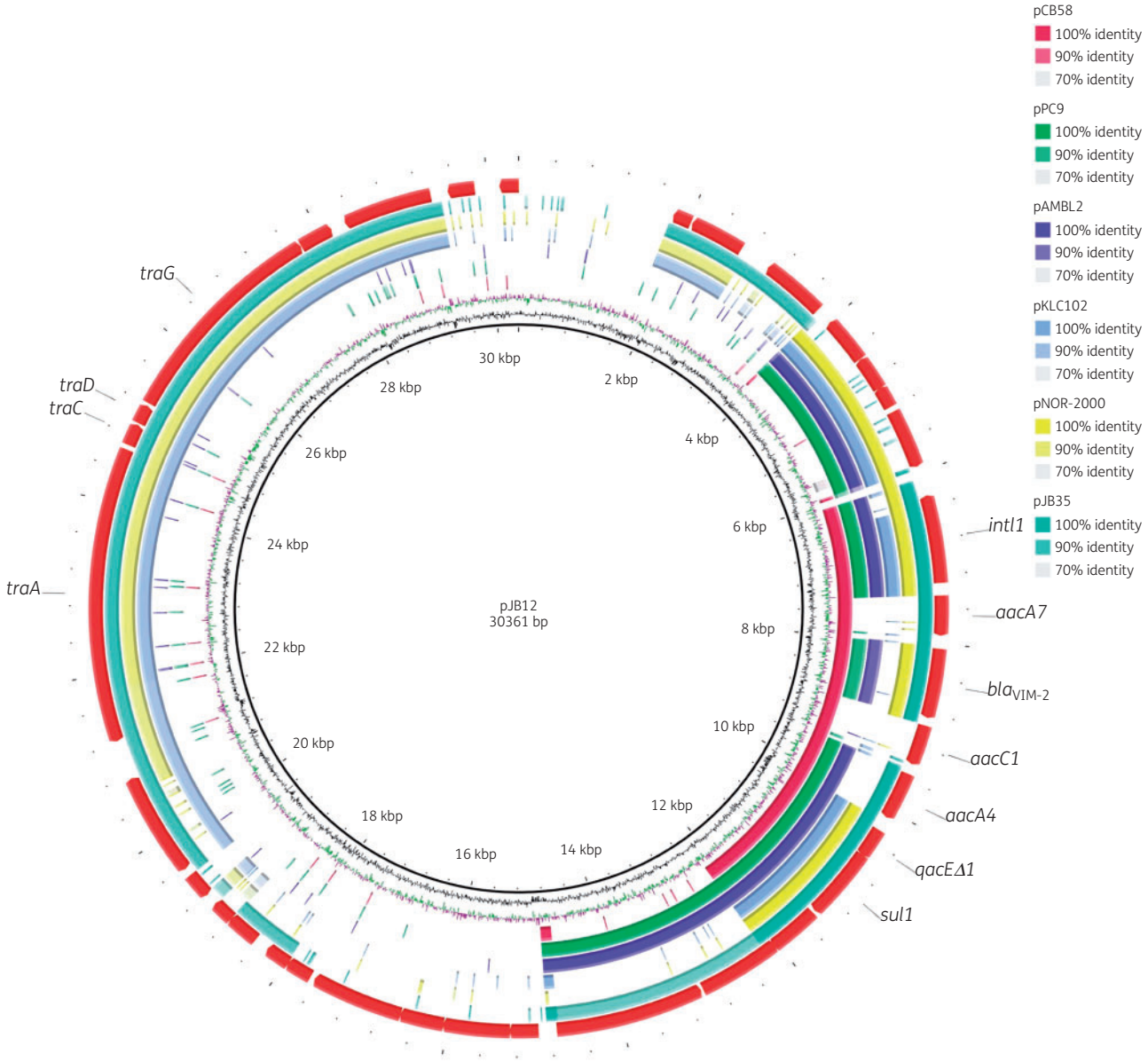


Figure 4. Comparison between the backbone structures of pJB35, pCB58 and related plasmids against pJB12. Red arrows point the translation orientation of the coding genes from pJB12. Selected features from pJB12 are annotated. The figure was created using BRIG. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

and JQ809663.1, respectively). All these phages displayed GC content values >60% and were identified on highly related species that also comprise a high GC genomic content, most likely reflecting their adaptation to these hosts. Curiously, the gene encoding for the ΦCTX phage cytotoxin was absent from the platform here reported. *In silico* analysis of this phage yielded several sequences producing significant alignments (nucleotide identity ranging from 96% to 98% and query cover from 61% to 82%) and revealed that this backbone was widely disseminated throughout different clonal lineages, such as ST175 (the FFUP_PS_12 isolate here described), ST111 (F30658 strain), ST235 (NCGM 1984), ST155 (ATCC 27853), ST357 (NCGM257) and ST395 (DHS01 strain) (accession nos CP008857.1, AP014646.1, CP015117.1, AP014651.1 and CP013993.1, respectively). These findings suggest the infection of different clonal lineages with related phages and subsequent genomic rearrangements. Even though a similar ICE and phage backbones were identified on strains NCGM257 and DHS01, these elements were not present next to each other. Upon insertion of the prophage next to the ICE, the resulting integrant exhibited three *att* sites with the same sequence (5′-GATTCCCTTCGCCCGCTCCA-3′). These elements may be transferred *en bloc* to a new host. This repeat region is similar to the *attL* and *attR* sites found for *clc*-like ICEs and was identified on ICE*6440*.[9]

Plasmids associated with different integrons and clonal lineages

The bla_{VIM-2} of FFUP_PS_35, belonging to ST179 (Table 1) was associated with a small plasmid, named pJB35. This plasmid was present in six contigs, which were finally assembled in a 31166 bp long (in agreement with the ~30 kb fragment obtained from S1 nuclease PFGE-based sizing) plasmid, comprising 39 CDSs and presenting a high GC content (62.8%), quite similar to the 62.6% of previously reported pJB12 plasmid (Table S1).[13] pJB35 was related to the latter, exhibiting 90% nucleotide identity and 68% of query cover (Figure 4). The main differences concerned: (i) the integron structure, as the bla_{VIM-2} gene is here related to a In58 variant lacking the *aacC1* gene, hereby named In103; (ii) absence of the ISPa17 element that was found upstream the integron platform on pJB12, being here replaced by a *tniABQR* module and a *merTPFADE* operon; (iii) *repA-parAB* module; and (iv) absence of the arsenic resistance (*ars*) operon (Figure 4). Plasmid pJB35 also shared a similar backbone to plasmids pKLC102 (93% identity and 57% of query cover) and pNOR2000 (95% identity and 53% of query cover), with the accession nos AY257539.1 and KC189475.1, respectively. Curiously, pKLC102 presents a dual lifestyle as it can coexist as a free plasmid and a GI in a bacterial cell.[50] DRs (5′-GCCTG-3′) were found flanking the In103 integron and the downstream found IS*6100* element, suggesting that these structures were co-mobilized.

Isolate FFUP_PS_CB58 of ST244 (Table 1) also harboured a bla_{VIM-2} plasmid of similar size as pJB12 and pJB35. However, its backbone was different (Figure 4). pCB58 plasmid was present in a single contig, was 32207 bp and displayed a 58.6% GC content, which is also in agreement with the ~30 kb fragment obtained from S1 nuclease PFGE-based sizing). pCB58 was related to bla_{VIM-1}-harbouring plasmids pPC9, from *Pseudomonas putida* HB3267 strain and isolated in France (88% query cover and 99% identity) and pAMBL2, from *P. aeruginosa* and isolated in Spain (44% query cover and 99% identity), with the accession nos CP003739.1 and KP873171.1, respectively (Figure 4). Replicase protein belongs to the Rep-3 superfamily and shared 100% homology with the ones from pPC9 and pAMBL2. Similar findings were observed for pPC9.[51] A Tn*402*-like transposon comprising the In58 integron and the IS*6100* element was here identified. A Tn*5393c*-like transposon harbouring streptomycin resistance genes was also observed. DRs (5′-ATGAA-3′), were found flanking this structure, suggesting the occurrence of a transposition event.

Curiously, pJB12, pJB35 and pCB58 were associated with different STs (Table 1). As previously reported for pJB12,[13] both plasmids lacked the complete machinery for self-conjugation, thus justifying the failure of conjugation assays. Yet, mobilization by a helper plasmid may occur, as a relaxase gene, a putative *oriT* site and a T4CP gene were identified.[52]

Conclusions

In conclusion, our results suggest that the occurrence of bla_{VIM-2} in Portuguese hospitals has been strongly influenced by the dissemination of In58 and related integrons. Although In58 has been usually chromosomally located, its prevalence and occurrence among different STs might be due to plasmid and ICE dissemination with subsequent integration into the chromosome, accompanied by recombination events. This could explain the diversity of In58-like integrons found in this study. In addition, the presence of these 'hitch-hiking' elements on several clonal lineages, including high-risk clones, justifies the high genetic plasticity and wide dissemination capacity of this carbapenemase gene.

Acknowledgements

The isolates were kindly provided by the Microbiology Departments of: Centro Hospitalar e Universitário de Coimbra, Centro Hospitalar do Porto, Instituto Português de Oncologia (Porto), Centro Hospitalar do Tâmega e Sousa, Hospital de S. Teotónio and Centro Hospitalar da Cova da Beira.

Funding

This work received financial support from the European Union (FEDER funds POCI/01/0145/FEDER/007728) and National Funds (FCT/MEC, Fundação para a Ciência e Tecnologia and Ministério da Educação e Ciência) under the Partnership Agreement PT2020 UID/MULTI/04378/2013. João Botelho and Filipa Grosso were supported by grants from Fundação para a Ciência e a Tecnologia (SFRH/BD/104095/2014 and SFRH/BPD/95556/2013, respectively).

Transparency declarations

None to declare.

Supplementary data

Table S1 appears as Supplementary data at *JAC* Online.

References

1 Castanheira M, Deshpande LM, Costello A *et al*. Epidemiology and carbapenem resistance mechanisms of carbapenem-non-susceptible *Pseudomonas aeruginosa* collected during 2009-11 in 14 European and Mediterranean countries. *J Antimicrob Chemother* 2014; **69**: 1804–14.

2 Oliver A, Mulet X, López-Causapé C *et al*. The increasing threat of *Pseudomonas aeruginosa* high-risk clones. *Drug Resist Updat* 2015; **21-22**: 41–59.

3 Wright LL, Turton JF, Livermore DM *et al*. Dominance of international 'high-risk clones' among metallo-β-lactamase-producing *Pseudomonas aeruginosa* in the UK. *J Antimicrob Chemother* 2015; **70**: 103–10.

4 Rice LB. Federal funding for the study of antimicrobial resistance in nosocomial pathogens: no ESKAPE. *J Infect Dis* 2008; **197**: 1079–81.

5 Bellanger X, Payot S, Leblond-Bourget N *et al*. Conjugative and mobilizable genomic islands in bacteria: evolution and diversity. *FEMS Microbiol Rev* 2014; **38**: 720–60.

6 Johnson CM, Grossman AD. Integrative and conjugative elements (ICEs): what they do and how they work. *Annu Rev Genet* 2015; **49**: 577–601.

7 Wozniak RA, Waldor MK. Integrative and conjugative elements: mosaic mobile genetic elements enabling dynamic lateral gene flow. *Nat Rev Microbiol* 2010; **8**: 552–63.

8 Langille MG, Hsiao WW, Brinkman FS. Detecting genomic islands using bioinformatics approaches. *Nat Rev Microbiol* 2010; **8**: 373–82.

9 Kung VL, Ozer EA, Hauser AR. The accessory genome of *Pseudomonas aeruginosa*. *Microbiol Mol Biol Rev* 2010; **74**: 621–41.

10 Woodford N, Turton JF, Livermore DM. Multiresistant Gram-negative bacteria: the role of high-risk clones in the dissemination of antibiotic resistance. *FEMS Microbiol Rev* 2011; **35**: 736–55.

11 Maatallah M, Cheriaa J, Backhrouf A *et al*. Population structure of *Pseudomonas aeruginosa* from five Mediterranean countries: evidence for frequent recombination and epidemic occurrence of CC235. *PLoS One* 2011; **6**: e25617.

12 Correa A, Del Campo R, Perenguez M *et al*. Dissemination of high-risk clones of extensively drug-resistant *Pseudomonas aeruginosa* in Colombia. *Antimicrob Agents Chemother* 2015; **59**: 2421–5.

13 Botelho J, Grosso F, Peixe L. Characterization of the plasmid pJB12 from *Pseudomonas aeruginosa* reveals Tn*6352*, a novel putative transposon associated with mobilization of *bla*$_{VIM-2}$-harboring In58 integron. *Antimicrob Agents Chemother* 2017; **61**: e02532–16.

14 Botelho J, Grosso F, Quinteira S *et al*. The complete nucleotide sequence of an IncP-2 megaplasmid unveils a mosaic architecture comprising a putative novel *bla*$_{VIM-2}$-harbouring transposon in *Pseudomonas aeruginosa*. *J Antimicrob Chemother* 2017; **72**: 2225–9.

15 Botelho J, Grosso F, Sousa C *et al*. Characterization of a new genetic environment associated with GES-6 carbapenemase from a *Pseudomonas aeruginosa* isolate belonging to the high-risk clone ST235. *J Antimicrob Chemother* 2015; **70**: 615–7.

16 Quinteira S, Peixe L. Multiniche screening reveals the clinically relevant metallo-β-lactamase VIM-2 in *Pseudomonas aeruginosa* far from the hospital setting: an ongoing dispersion process? *Appl Environ Microbiol* 2006; **72**: 3743–5.

17 Quinteira S, Sousa JC, Peixe L. Characterization of In100, a new integron carrying a metallo-β-lactamase and a carbenicillinase, from *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother* 2005; **49**: 451–3.

18 Cardoso O, Leitão R, Figueiredo A *et al*. Metallo-β-lactamase VIM-2 in clinical isolates of *Pseudomonas aeruginosa* from Portugal. *Microb Drug Resist* 2002; **8**: 93–7.

19 Brízio A, Conceição T, Pimentel M *et al*. High-level expression of IMP-5 carbapenemase owing to point mutation in the -35 promoter region of class 1 integron among *Pseudomonas aeruginosa* clinical isolates. *Int J Antimicrob Agents* 2006; **27**: 27–31.

20 Mulet M, Lalucat J, Garcia-Valdes E. DNA sequence-based analysis of the *Pseudomonas* species. *Environ Microbiol* 2010; **12**: 1513–30.

21 Pires J, Novais A, Peixe L. Blue-carba, an easy biochemical test for detection of diverse carbapenemase producers directly from bacterial cultures. *J Clin Microbiol* 2013; **51**: 4281–3.

22 Bogaerts P, Rezende de Castro R, de Mendonca R *et al*. Validation of carbapenemase and extended-spectrum β-lactamase multiplex endpoint PCR assays according to ISO 15189. *J Antimicrob Chemother* 2013; **68**: 1576–82.

23 Ellington MJ, Kistler J, Livermore DM *et al*. Multiplex PCR for rapid detection of genes encoding acquired metallo-β-lactamases. *J Antimicrob Chemother* 2007; **59**: 321–2.

24 Tatusova T, DiCuccio M, Badretdin A *et al*. NCBI prokaryotic genome annotation pipeline. *Nucleic Acids Res* 2016; **44**: 6614–24.

25 Bankevich A, Nurk S, Antipov D *et al*. SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. *J Comput Biol* 2012; **19**: 455–77.

26 Lister PD, Wolter DJ, Hanson ND. Antibacterial-resistant *Pseudomonas aeruginosa*: clinical impact and complex regulation of chromosomally encoded resistance mechanisms. *Clin Microbiol Rev* 2009; **22**: 582–610.

27 Poirel L, Lambert T, Turkoglu S *et al*. Characterization of Class 1 integrons from *Pseudomonas aeruginosa* that contain the *bla*$_{VIM-2}$ carbapenem-hydrolyzing β-lactamase gene and of two novel aminoglycoside resistance gene cassettes. *Antimicrob Agents Chemother* 2001; **45**: 546–52.

28 Juan C, Zamorano L, Mena A *et al*. Metallo-β-lactamase-producing *Pseudomonas putida* as a reservoir of multidrug resistance elements that can be transferred to successful *Pseudomonas aeruginosa* clones. *J Antimicrob Chemother* 2010; **65**: 474–8.

29 Viedma E, Juan C, Villa J *et al*. VIM-2-producing multidrug-resistant *Pseudomonas aeruginosa* ST175 clone, Spain. *Emerg Infect Dis* 2012; **18**: 1235–41.

30 Cabrolier N, Sauget M, Bertrand X *et al*. Matrix-assisted laser desorption ionization-time of flight mass spectrometry identifies *Pseudomonas aeruginosa* high-risk clones. *J Clin Microbiol* 2015; **53**: 1395–8.

31 Estepa V, Rojo-Bezares B, Torres C *et al*. Faecal carriage of *Pseudomonas aeruginosa* in healthy humans: antimicrobial susceptibility and global genetic lineages. *FEMS Microbiol Ecol* 2014; **89**: 15–9.

32 Kidd TJ, Ritchie SR, Ramsay KA *et al*. *Pseudomonas aeruginosa* exhibits frequent recombination, but only a limited association between genotype and ecological setting. *PLoS One* 2012; **7**: e44199.

33 Moyo S, Haldorsen B, Aboud S *et al*. Identification of VIM-2-producing *Pseudomonas aeruginosa* from Tanzania is associated with sequence types 244 and 640 and the location of *bla*$_{VIM-2}$ in a TniC integron. *Antimicrob Agents Chemother* 2015; **59**: 682–5.

34 Samuelsen O, Toleman MA, Sundsfjord A *et al*. Molecular epidemiology of metallo-β-lactamase-producing *Pseudomonas aeruginosa* isolates from Norway and Sweden shows import of international clones and local clonal expansion. *Antimicrob Agents Chemother* 2010; **54**: 346–52.

35 Balero de Paula S, Cayô R, Streling AP *et al*. Detection of *bla*$_{VIM-7}$ in an extensively drug-resistant *Pseudomonas aeruginosa* isolate belonging to ST1284 in Brazil. *Diagn Microbiol Infect Dis* 2017; **89**: 80–2.

36 Magiorakos AP, Srinivasan A, Carey RB *et al*. Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria—an international expert proposal for interim standard definitions for acquired resistance. *Clin Microbiol Infect* 2012; **18**: 268–81.

37 Santos C, Caetano T, Ferreira S *et al*. Tn*5090*-like class 1 integron carrying *bla*$_{VIM-2}$ in a *Pseudomonas putida* strain from Portugal. *Clin Microbiol Infect* 2010; **16**: 1558–61.

38 Domingues S, Toleman MA, Nielsen KM *et al*. Identical miniature inverted repeat transposable elements flank class 1 integrons in clinical isolates of *Acinetobacter* spp. *J Clin Microbiol* 2013; **51**: 2382–4.

39 Targant H, Doublet B, Aarestrup FM *et al*. IS*6100*-mediated genetic rearrangement within the complex class 1 integron In104 of the *Salmonella* genomic island 1. *J Antimicrob Chemother* 2010; **65**: 1543–5.

40 Quinteira S, Ferreira H, Peixe L. First isolation of *bla*$_{VIM-2}$ in an environmental isolate of *Pseudomonas pseudoalcaligenes*. *Antimicrob Agents Chemother* 2005; **49**: 2140–1.

41 Lambert T, Ploy M-C, Courvalin P. A spontaneous point mutation i the *aac(6′)-Ib′* gene results in altered substrate specificity of aminoglycoside 6′-N-acetyltransferase of a *Pseudomonas fluorescens* strain. *FEMS Microbiol Lett* 1994; **115**: 297–303.

42 Burrus V. Mechanisms of stabilization of integrative and conjugative elements. *Curr Opin Microbiol* 2017; **38**: 44–50.

43 Guglielmini J, Quintais L, Garcillán-Barcia MP *et al*. The repertoire of ICE in prokaryotes underscores the unity, diversity, and ubiquity of conjugation. *PLoS Genet* 2011; **7**: e1002222.

44 Roy Chowdhury P, Scott M, Worden P *et al*. Genomic islands 1 and 2 play key roles in the evolution of extensively drug-resistant ST235 isolates of *Pseudomonas aeruginosa*. *Open Biol* 2016; **6**: pii=150175.

45 Fonseca EL, Marin MA, Encinas F *et al*. Full characterization of the integrative and conjugative element carrying the metallo-β-lactamase *bla*$_{SPM-1}$ and bicyclomycin *bcr1* resistance genes found in the pandemic *Pseudomonas aeruginosa* clone SP/ST277. *J Antimicrob Chemother* 2015; **70**: 2547–50.

46 Botelho J, Grosso F, Peixe L. Unravelling the genome of a *Pseudomonas aeruginosa* isolate belonging to the high-risk clone ST235 reveals an integrative conjugative element housing a *bla*$_{GES-6}$ carbapenemase. *J Antimicrob Chemother* 2018; **73**: 77–83.

47 Toleman MA, Walsh TR. Combinatorial events of insertion sequences and ICE in Gram-negative bacteria. *FEMS Microbiol Rev* 2011; **35**: 912–35.

48 Roberts AP, Chandler M, Courvalin P *et al*. Revised nomenclature for transposable genetic elements. *Plasmid* 2008; **60**: 167–73.

49 Hong JS, Yoon EJ, Lee H *et al*. Clonal dissemination of *Pseudomonas aeruginosa* sequence type 235 isolates carrying bla_{IMP-6} and emergence of bla_{GES-24} and bla_{IMP-10} on novel genomic islands PAGI-15 and -16 in South Korea. *Antimicrob Agents Chemother* 2016; **60**: 7216–23.

50 Klockgether J, Reva O, Larbig K *et al*. Sequence analysis of the mobile genome island pKLC102 of *Pseudomonas aeruginosa* C. *J Bacteriol* 2003; **186**: 518–34.

51 Molina L, Udaondo Z, Duque E *et al*. Antibiotic resistance determinants in a *Pseudomonas putida* strain isolated from a hospital. *PLoS One* 2014; **9**: e81604.

52 Smillie C, Garcillán-Barcia MP, Francia MV *et al*. Mobility of plasmids. *Microbiol Mol Biol Rev* 2010; **74**: 434–52.

Table S1. Sequence of primers used to assemble the plasmids and integrative conjugative elements (ICEs) described in this study.

Primer name	Sequence	Primer name	Sequence	Size of amplicon (bp)
Plasmid identified in FFUP_PS_35 strain (pJB35)				
35_merT_Rv	CAGCAGTGTCTTCGGGTCTT	res_Rv	CTTCTCGGCAATGACCACCT	1600
tniA_Rv	GCTCGCGCCGGGCAATCCAC	VIP2	ACGACTGAGCGATTTGTGTG	3900
Vim_Fw	TGTCCGTGATGGTGATGAGT	IS6100_Rv	GGCTCTGTTGCAAAGATTGGC	4800
ICE identified in FFUP_HSV3483 strain (ICE*6440*)				
3483_hin	CGTTCTGGGTTCTGGTCGAA	IS6100_Fw	GGCTCTGTTGCAAAAATCGTG	1300
IS6100_Fw	GGCTCTGTTGCAAAAATCGTG	methylt	TGTCCAACTTCCAGAGTCGC	1300
ICE identified in FFUP_PS_CB5 strain (ICE*6441*)				
VIP2	ACGACTGAGCGATTTGTGTG	CB5_NODE11	ACAGGGTTTCGCGACTGATT	2800
VIP1	ACTCACCCCCATGGAGTTTT	CB5_NODE27	ATGGATGTGGTGGCTGAAGG	3700
VIP2	ACGACTGAGCGATTTGTGTG	CB5_tnpA_Rv	TTCAGGTACCGGTTGCAGTC	5700

AMERICAN SOCIETY FOR MICROBIOLOGY | Antimicrobial Agents and Chemotherapy®

Characterization of the pJB12 Plasmid from *Pseudomonas aeruginosa* Reveals Tn*6352*, a Novel Putative Transposon Associated with Mobilization of the bla_{VIM-2}-Harboring In58 Integron

João Botelho, Filipa Grosso, Luísa Peixe

UCIBIO/REQUIMTE, Laboratório de Microbiologia, Faculdade de Farmácia, Universidade do Porto, Porto, Portugal

ABSTRACT The bla_{VIM-2}-carrying In58 integron has been linked to a chromosomal location in different bacterial species, including *Pseudomonas aeruginosa*. This work reports the first fully sequenced In58-harboring plasmid, which is significantly different from the two previously identified bla_{VIM-2}-carrying plasmids in *P. aeruginosa*. bla_{VIM-2} might have been acquired by transposition of Tn*6352*, a novel transposon composed of the In58 and IS*Pa17* elements. The recognition of similar inverted repeat (IR) sites by IS*Pa17* reveals a common mobilization process associated with acquisition of the bla_{VIM-2} and bla_{VIM-1} genes.

KEYWORDS *Pseudomonas aeruginosa*, carbapenemase, plasmid-mediated resistance, transposon

Received 29 November 2016 **Returned for modification** 21 December 2016 **Accepted** 25 January 2017

Accepted manuscript posted online 13 February 2017

Citation Botelho J, Grosso F, Peixe L. 2017. Characterization of the pJB12 plasmid from *Pseudomonas aeruginosa* reveals Tn*6352*, a novel putative transposon associated with mobilization of the bla_{VIM-2}-harboring In58 integron. Antimicrob Agents Chemother 61:e02532-16. https://doi.org/10.1128/AAC.02532-16.

Address correspondence to Luísa Peixe, lpeixe@ff.up.pt.

Carbapenemase-producing *Pseudomonas aeruginosa* strains have been increasingly documented, a particular worrisome situation due to associated resistance to several β-lactams (1, 2). The most widespread carbapenemases produced by *P. aeruginosa* are metallo-β-lactamases (MBLs), particularly the VIM and IMP types (1, 2). VIM-2 represents the MBL most frequently found in *P. aeruginosa*, and evidence of endemic spread throughout southeast Asia and southern European countries has been documented (1–3).

In *P. aeruginosa*, bla_{VIM-2} is most often associated with class 1 integrons containing additional antibiotic resistance genes (3–11). The bla_{VIM-2} gene has been commonly associated with chromosomally located Tn*402*-like class 1 integrons (3–8). This gene also has been less frequently linked to plasmids, usually smaller than 100 kb, and cannot be transferred by conjugation, at least not to *Escherichia coli* (1, 9).

Until now, only two complete bla_{VIM-2}-carrying plasmid sequences in *P. aeruginosa*, pNOR-2000 and pDCPR1, have been described (10, 11). In order to obtain new insight about the mobile elements contributing to the dissemination of this gene, we report here the complete sequence of pJB12, a bla_{VIM-2}-carrying plasmid from a *P. aeruginosa* clinical isolate, and we explore the genetic background involved in acquisition of the bla_{VIM-2}-harboring In58 integron.

Bacterial isolate. In the context of a regular surveillance of several Portuguese hospitals conducted by our lab for the screening of MBL producers among *P. aeruginosa* strains, four isolates belonging to the high-risk sequence type 175 (ST175) clone were recovered in different years from inpatients of two geographically distinct hospitals. These isolates presented the same pulsed-field gel electrophoresis (PFGE) pattern and carried the bla_{VIM-2} gene in a ca. 30-kb plasmid (S1 nuclease PFGE-based sizing). The pJB12 plasmid was extracted from one of these isolates, FFUP_PS_12, which

was obtained in 2006 at the Centro Hospitalar do Porto (Portugal). FFUP_PS_12 was initially identified by Vitek-2 (bioMérieux), and the species was confirmed by multilocus sequence analysis (*rpoD*, *gyrB*, and 16S rRNA genes) (12). Antimicrobial susceptibility testing was conducted by standard disc diffusion, Etest (carbapenems), and broth microdilution (colistin) methods according to EUCAST guidelines (http://www.eucast.org/).

Plasmid analysis. Extraction of plasmid DNA from FFUP_PS_12 was performed with a plasmid midikit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Sequencing was accomplished with a MiSeq (Illumina) sequencer with an average median depth of coverage of 35×. The quality of the high-throughput sequence data was assessed with FastQC. *De novo* assembly of the paired-end reads was performed with SPAdes 3.9.0 (13). Evaluation of the genome assembly was performed by QUAST (http://quast.bioinf.spbau.ru/). ResFinder (https://cge.cbs.dtu.dk/services/ResFinder/) was used to identify acquired antimicrobial resistance genes. Annotation and plasmid visualization were performed by Geneious 9.1.6 (Biomatters, USA). Annotations were manually curated using BLASTn and BLASTp (https://blast.ncbi.nlm.nih.gov/Blast.cgi) similarity searches of each predicted open reading frame (ORF).

Conjugation was performed as previously described (5); briefly, a spontaneous rifampin-resistant mutant of *P. aeruginosa* PAO1 was used as the recipient strain, and transconjugant selection was performed in Mueller-Hinton (MH) agar plates containing rifampin (100 μg/ml) and imipenem (4 μg/ml). *E. coli* DH5α- and *P. aeruginosa* PAO1-competent cells were prepared and transformed by electroporation as described previously (14). Transformants were selected in MH agar plates containing imipenem (0.5 μg/ml for *E. coli* DH5α and 4 μg/ml for *P. aeruginosa* PAO1) and confirmed by PCR for bla_{VIM} and *aacA7* genes and antimicrobial susceptibility testing.

Screening and Sanger sequencing for the pJB12-like *repA* gene (with the primers rep_JB12_Fw [CTCCTTGGAGCGATACGACC] and rep_JB12_Rv [GGACTCATACAGGCTCA CGG]) were performed for the remaining isolates belonging to ST175 and with the bla_{VIM-2} gene located in a ca. 30-kb plasmid.

General features of FFUP_PS_12 and pJB12. FFUP_PS_12 displayed an extensively drug-resistant (XDR) phenotype, including resistance to imipenem (MICs, ≥32 mg/liter), meropenem (MICs, ≥32 mg/liter), ceftazidime, cefepime, piperacillin-tazobactam, gentamicin, tobramycin, amikacin, netilmicin, and ciprofloxacin and susceptibility to aztreonam and colistin (MICs, 1 mg/liter) (15). The isolate belonged to the high-risk clone ST175, which has been associated mainly with XDR and multidrug-resistant (MDR) *P. aeruginosa* isolates throughout several European countries and in Japan and was responsible for the dissemination of VIM-2, IMP-1, and IMP-22 carbapenemases (1).

The complete plasmid sequence of pJB12 was identified in a single 30,465-bp contig, which is in agreement with the result obtained from S1 nuclease PFGE-based sizing (ca. 30 kb). Since extremities overlapped, suggesting a circular sequence, a 30,361-bp plasmid was obtained. With a GC content of 62.6%, pJB12 comprised 36 ORFs, including genes associated with a transposon structure harboring the bla_{VIM-2}-carrying In58 integron (Fig. 1; see also Table S1 in the supplemental material).

***In silico* analysis and comparison of the pJB12 scaffold with related plasmids.** Plasmid pJB12 displays two plasmid stabilization systems (ParA-ParB and T/AT1-T/AT2) which ensure the maintenance and inheritance of the plasmid (Fig. 1; see also Table S1) (10). An *ars* operon, which mediates arsenic resistance, was also present (16). The identified genes (*arsR*, *arsH*, *arsC*, and *arsB*) encode for proteins that display high similarity to the ones identified in pAB3, a plasmid identified in *Acinetobacter baumannii* (GenBank accession number CP012005) (Fig. 1; Table S1). Contamination with arsenic compounds (e.g., arsenic-contaminated drinking water, arsenic compounds used in animal husbandry and as rodenticides) may lead to the selection of plasmids carrying different antimicrobial genes (16).

The putative replicase protein from pJB12 shared 83% homology with that of pMATVIM-7, a bla_{VIM-7}-carrying plasmid identified in a *P. aeruginosa* isolate recovered

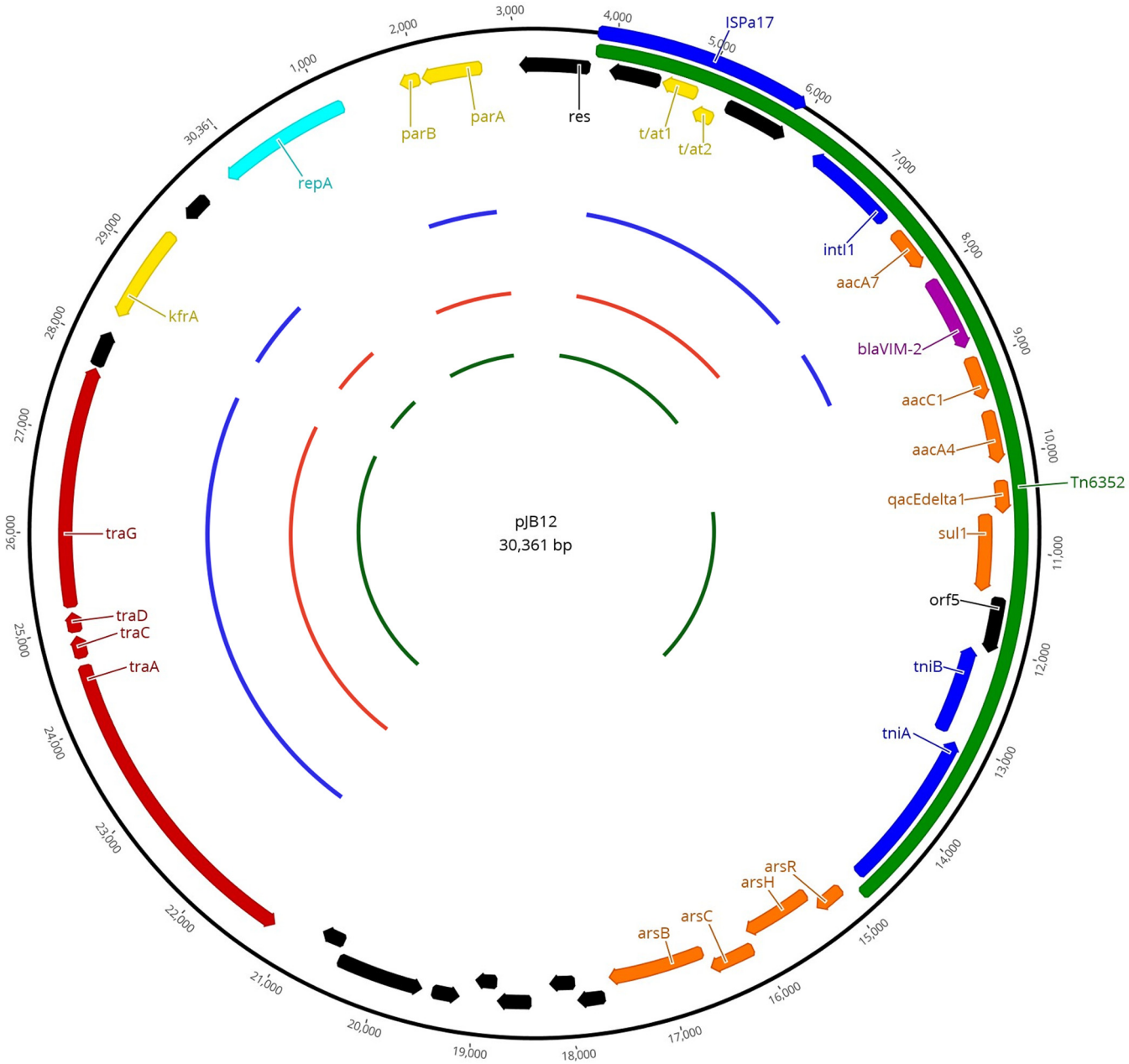


FIG 1 Schematic representation of the pJB12 plasmid. Annotated coding sequences are shown as arrows and are colored depending on gene function. Yellow arrows represent genes involved in plasmid housekeeping functions such as partition and maintenance. Red arrows point out the genes involved in conjugation transfer. Light-blue arrows indicate the replication gene. Dark-blue arrows point out genes responsible for DNA rearrangements (such as integrases and transposases). Orange arrows represent resistance genes. The purple arrow points out the bla_{VIM-2} gene. Tn*6352* is presented in dark green. Genes of unknown function are shown in black. The inner circles illustrate homology regions between plasmid pJB12 and those characterized previously. The inner dark-green circle corresponds to plasmid pAX22 from *Achromobacter xylosoxidans* (GenBank accession number NC_022242.1), and the two outer circles represent plasmids pKLC102 (red circle) and pNOR-2000 (blue circle), both from *P. aeruginosa* (GenBank accession numbers AY257538.1 and KC189475.1, respectively).

from sputum of a cancer patient diagnosed with pneumonia at a hospital in Houston, Texas (17). However, pJB12 presented a backbone different than that of pMATVIM-7 (GenBank accession number AM778842), suggesting independent evolutionary routes for the emergence of these enzymes. Interestingly, the remaining bla_{VIM-2}-carrying 30-kb plasmids in our collection presented the same nucleotide sequence for the replicase gene of plasmid pJB12. A putative origin of replication was found upstream of the *repA* gene. An AT-rich region, a 9-mer DnaA-box (5′-TTTTACACA-3′), and iterons, which are typical functional elements of plasmid replication origins, were identified (18). Considering

the replicase proteins associated with the two bla_{VIM-2}-harboring plasmids previously described in *P. aeruginosa* (pNOR-2000 and pDCPR1, GenBank accession numbers KC189475.1 and KJ577613.1, respectively) (10, 11), we found no detectable homology compared with the one found in plasmid pJB12, suggesting a separate origin for the replication regions. Nevertheless, for parts of pNOR-2000 and two other plasmids (pAX22, a bla_{VIM-1}-carrying plasmid from *Achromobacter denitrificans*, and pKLC102, an *aadB*-harboring plasmid from *P. aeruginosa*) (19, 20), the pJB12 plasmid backbone shared high nucleotide homology with the highest amino acid sequence identity being verified for plasmid pNOR-2000 (from 80% to 97% for the maintenance system and from 87% to 94% for the transfer region) (Table S1). This homology included the partitioning and stable inheritance system genes (*parA*, *parB*, and *kfrA*) and transfer genes (*traA*, *traC*, *traD*, and *traG*) (Fig. 1).

The pJB12 plasmid carried a relaxase gene (*traA*), a putative *oriT* site upstream of the *traA* gene, and a type IV coupling protein (T4CP) gene (*traG*) (10, 21). However, the machinery for self-conjugation is incomplete, since the type IV secretion system (T4SS) is absent (10, 21). Even so, pJB12 might be mobilizable in the presence of a helper plasmid (10, 21). The incomplete transfer operon we observed justifies the failure of the conjugation experiments. Electrotransformation of the pJB12 plasmid to *P. aeruginosa* PAO1 was successful, with transformants acquiring resistance to β-lactams (MICs, $\geq$32 mg/liter against imipenem) and aminoglycosides.

In58 mobilization in plasmid pJB12 resembles that observed in bla_{VIM-1}-carrying In70 from plasmid pAX22. In this study, the bla_{VIM-2} gene was associated with the In58 integron, a genetic platform that also carries aminoglycoside resistance genes (*aacA7*, *aacA4*, and *aacC1*), which was previously identified among *P. aeruginosa*, *Pseudomonas putida*, and *Citrobacter freundii* isolates in different countries (6–8). This structure was found to be chromosomally located (6, 8), and plasmid pJB12 represents the only reported plasmidic support for In58. This integron was frequently found in Portuguese *P. aeruginosa* clinical isolates carrying the bla_{VIM-2} gene associated with different high-risk clones (J. Botelho, F. Grosso, and L. Peixe, unpublished data).

In58 was associated with a defective Tn*402*-like transposon, with IS*Pa17* upstream of Tn*402* inverted repeat initial (IRi) in a head-to-tail orientation (Fig. 1). IS*Pa17* represents an atypical structure initially described in plasmid Rms149 (22). IS*Pa17* encodes a putative transposase, a toxin/antitoxin system (A/AT1 and A/AT2), and a putative resolvase (Fig. 1). IS*Pa17* may represent a role in the mobilization of defective transposons carrying class 1 integrons, as demonstrated for the bla_{VIM-1}-harboring In70 integron (19, 23). Interestingly, the IRs flanking IS*Pa17* were similar to those surrounding the Tn*402*-like transposon, suggesting that these IRs can be targeted by the transposases of both structures. Direct repeats (DRs) of the 5′-AATTG sequence were found flanking the inverted repeat left (IRL) of IS*Pa17* and the inverted repeat terminal (IRt) of the Tn*402*-like transposon, suggesting that a transposition event mediated the *en bloc* insertion of these structures (19). This new complex transposable element was inserted next to the plasmid resolvase gene and designated Tn*6352* (http://www.ucl.ac.uk/eastman/research/departments/microbial-diseases/tn) according to the criteria proposed by Roberts et al. (24). Future studies are warranted to investigate the mobility of Tn*6352* and to address if the transposition event was mediated by the IS*Pa17* transposase or by a complete *trans*-acting *tni* module of another Tn*402*-like transposon.

The 113-bp nucleotide sequence found between the inverted repeat right of IS*Pa17* and the IRi of the Tn*402*-like transposon (Fig. 2) was identical to the one found in plasmid pAX22 and the ParA-TraC-encoding region of several IncP-1ε plasmids, such as pHH128, pHH3414, and pHH3408 (25). This suggests that Tn*6352* was assembled among these plasmids after independent insertion of IS*Pa17* and the Tn*402*-like transposon (19). In fact, IncP-1ε plasmids are frequently found in environmental settings and seem to be fundamental vectors for the spread of antibiotic resistance genes (25).

In summary, this work presents the sequence of pJB12, the first complete nucleotide sequence of an In58-harboring plasmid. The bla_{VIM-2} gene was most likely acquired by

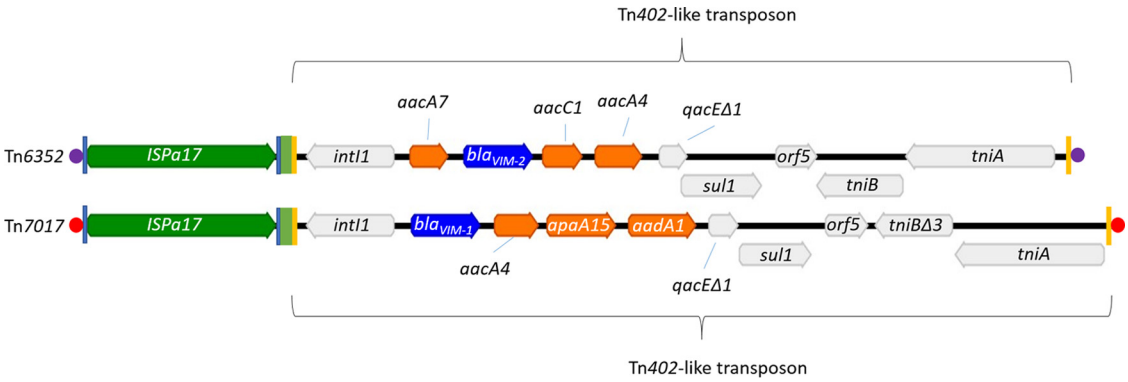


FIG 2 Comparison of putative transposons Tn*6352* and Tn*7017*. Both structures are composed of IS*Pa17*- and Tn*402*-like transposons in a head-to-tail orientation. Carbapenemase-encoding genes are represented by dark-blue arrows. Orange arrows symbolize aminoglycoside resistance genes. IS*Pa17* is represented by a green arrow. The remaining genes identified in the Tn*402*-like transposons are shown in light gray. Light-blue rectangles represent the inverted repeats (IRs) identified in IS*Pa17*. The IRs of the Tn*402*-like transposons are illustrated by yellow rectangles. The 113-bp nucleotide sequence found between the IRR of IS*Pa17* and the IRi of Tn*402*-like transposons is represented by green rectangles. Direct repeats (DRs) flanking the putative transposon Tn*6352* (5′-AATTG-3′) and Tn*7017* (5′-GTGGC-3′) are illustrated by purple and red circles, respectively.

plasmid pJB12 by transposition of Tn*6352*, a novel putative transposon comprising an In58 integron and an IS*Pa17* in a head-to-tail orientation. The recognition of DNA regions with similar IR sites by IS*Pa17* demonstrates a common mobilization process associated with acquisition of the bla_{VIM-2} and bla_{VIM-1} genes.

Accession number(s). The complete sequence of plasmid pJB12 has been submitted to GenBank under the accession number KX889311.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AAC.02532-16.

SUPPLEMENTAL FILE 1, PDF file, 0.4 MB.

ACKNOWLEDGMENTS

The isolate was kindly provided by the Microbiology Departments of Centro Hospitalar do Porto, Portugal.

This work had financial support from FCT/MEC through national funds and cofinanced by FEDER, under the Partnership Agreement PT2020 (grant no. UID/Multi/04378/2013). João Botelho and Filipa Grosso were supported by grants from Fundação para a Ciência e a Tecnologia (SFRH/BD/104095/2014 and SFRH/BPD/95556/2013, respectively).

REFERENCES

1. Oliver A, Mulet X, Lopez-Causape C, Juan C. 2015. The increasing threat of *Pseudomonas aeruginosa* high-risk clones. Drug Resist Updat 21–22:41–59. https://doi.org/10.1016/j.drup.2015.08.002.
2. Potron A, Poirel L, Nordmann P. 2015. Emerging broad-spectrum resistance in *Pseudomonas aeruginosa* and *Acinetobacter baumannii*: Mechanisms and epidemiology. Int J Antimicrob Agents 45:568–585. https://doi.org/10.1016/j.ijantimicag.2015.03.001.
3. Hong DJ, Bae IK, Jang IH, Jeong SH, Kang HK, Lee K. 2015. Epidemiology and characteristics of metallo-beta-lactamase-producing *Pseudomonas aeruginosa*. Infect Chemother 47:81–97. https://doi.org/10.3947/ic.2015.47.2.81.
4. Moyo S, Haldorsen B, Aboud S, Blomberg B, Maselle SY, Sundsfjord A, Langeland N, Samuelsen O. 2015. Identification of VIM-2-producing *Pseudomonas aeruginosa* from Tanzania is associated with sequence types 244 and 640 and the location of bla_{VIM-2} in a TniC integron. Antimicrob Agents Chemother 59:682–685. https://doi.org/10.1128/AAC.01436-13.
5. Quinteira S, Sousa JC, Peixe L. 2005. Characterization of In100, a new integron carrying a metallo-β-lactamase and a carbenicillinase, from *Pseudomonas aeruginosa*. Antimicrob Agents Chemother 49:451–453. https://doi.org/10.1128/AAC.49.1.451-453.2005.
6. Quinteira S, Peixe L. 2006. Multiniche screening reveals the clinically relevant metallo-beta-lactamase VIM-2 in *Pseudomonas aeruginosa* far from the hospital setting: an ongoing dispersion process? Appl Environ Microbiol 72:3743–3745. https://doi.org/10.1128/AEM.72.5.3743-3745.2006.
7. Santos C, Caetano T, Ferreira S, Mendo S. 2010. Tn*5090*-like class 1 integron carrying bla_{VIM-2} in a *Pseudomonas putida* strain from Portugal. Clin Microbiol Infect 16:1558–1561. https://doi.org/10.1111/j.1469-0691.2010.03165.x.
8. Poirel L, Lambert T, Turkoglu S, Ronco E, Gaillard J, Nordmann P. 2001. Characterization of class 1 integrons from *Pseudomonas aeruginosa* that contain the bla_{VIM-2} carbapenem-hydrolyzing beta-lactamase gene and of two novel aminoglycoside resistance gene cassettes. Antimicrob Agents Chemother 45:546–552. https://doi.org/10.1128/AAC.45.2.546-552.2001.
9. Dortet L, Flonta M, Boudehen YM, Creton E, Bernabeu S, Vogel A, Naas T. 2015. Dissemination of carbapenemase-producing *Enterobacteriaceae*

and *Pseudomonas aeruginosa* in Romania. Antimicrob Agents Chemother 59:7100–7103. https://doi.org/10.1128/AAC.01512-15.
10. Bonnin RA, Poirel L, Nordmann P, Eikmeyer FG, Wibberg D, Puhler A, Schluter A. 2013. Complete sequence of broad-host-range plasmid pNOR-2000 harbouring the metallo-beta-lactamase gene bla_{VIM-2} from *Pseudomonas aeruginosa*. J Antimicrob Chemother 68:1060–1065. https://doi.org/10.1093/jac/dks526.
11. Vilacoba E, Quiroga C, Pistorio M, Famiglietti A, Rodriguez H, Kovensky J, Deraspe M, Raymond F, Roy PH, Centron D. 2014. A bla_{VIM-2} plasmid disseminating in extensively drug-resistant clinical *Pseudomonas aeruginosa* and *Serratia marcescens* isolates. Antimicrob Agents Chemother 58:7017–7018. https://doi.org/10.1128/AAC.02934-14.
12. Mulet M, Lalucat J, Garcia-Valdes E. 2010. DNA sequence-based analysis of the *Pseudomonas* species. Environ Microbiol 12:1513–1530. https://doi.org/10.1111/j.1462-2920.2010.02181.x.
13. Bankevich A, Nurk S, Antipov D, Gurevich AA, Dvorkin M, Kulikov AS, Lesin VM, Nikolenko SI, Pham S, Prjibelski AD, Pyshkin AV, Sirotkin AV, Vyahhi N, Tesler G, Alekseyev MA, Pevzner PA. 2012. SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. J Comput Biol 19:455–477. https://doi.org/10.1089/cmb.2012.0021.
14. Choi KH, Kumar A, Schweizer HP. 2006. A 10-min method for preparation of highly electrocompetent *Pseudomonas aeruginosa* cells: application for DNA fragment transfer between chromosomes and plasmid transformation. J Microbiol Methods 64:391–397. https://doi.org/10.1016/j.mimet.2005.06.001.
15. Magiorakos AP, Srinivasan A, Carey RB, Carmeli Y, Falagas ME, Giske CG, Harbarth S, Hindler JF, Kahlmeter G, Olsson-Liljequist B, Paterson DL, Rice LB, Stelling J, Struelens MJ, Vatopoulos A, Weber JT, Monnet DL. 2012. Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. Clin Microbiol Infect 18:268–281. https://doi.org/10.1111/j.1469-0691.2011.03570.x.
16. Hobman JL, Crossman LC. 2015. Bacterial antimicrobial metal ion resistance. J Med Microbiol 64:471–497. https://doi.org/10.1099/jmm.0.023036-0.
17. Li H, Toleman MA, Bennett PM, Jones RN, Walsh TR. 2008. Complete sequence of p07-406, a 24,179-base-pair plasmid harboring the bla_{VIM-7} metallo-beta-lactamase gene in a *Pseudomonas aeruginosa* isolate from the United States. Antimicrob Agents Chemother 52:3099–3105. https://doi.org/10.1128/AAC.01093-07.
18. Rajewska M, Wegrzyn K, Konieczny I. 2012. AT-rich region and repeated sequences—the essential elements of replication origins of bacterial replicons. FEMS Microbiol Rev 36:408–434. https://doi.org/10.1111/j.1574-6976.2011.00300.x.
19. Di Pilato V, Pollini S, Rossolini GM. 2014. Characterization of plasmid pAX22, encoding VIM-1 metallo-β-lactamase, reveals a new putative mechanism of In70 integron mobilization. J Antimicrob Chemother 69:67–71. https://doi.org/10.1093/jac/dkt311.
20. Klockgether J, Reva O, Larbig K, Tummler B. 2004. Sequence analysis of the mobile genome island pKLC102 of *Pseudomonas aeruginos*a C. J Bacteriol 186:518–534. https://doi.org/10.1128/JB.186.2.518-534.2004.
21. Smillie C, Garcillan-Barcia MP, Francia MV, Rocha EP, de la Cruz F. 2010. Mobility of plasmids. Microbiol Mol Biol Rev 74:434–452. https://doi.org/10.1128/MMBR.00020-10.
22. Haines AS, Jones K, Cheung M, Thomas CM. 2005. The IncP-6 plasmid Rms149 consists of a small mobilizable backbone with multiple large insertions. J Bacteriol 187:4728–4738. https://doi.org/10.1128/JB.187.14.4728-4738.2005.
23. San Millan A, Toll-Riera M, Escudero JA, Cantón R, Coque TM, MacLean RC. 2015. Sequencing of plasmids pAMBL1 and pAMBL2 from *Pseudomonas aeruginosa* reveals a bla_{VIM-1} amplification causing high-level carbapenem resistance. J Antimicrob Chemother 70:3000–3003. https://doi.org/10.1093/jac/dkv222.
24. Roberts AP, Chandler M, Courvalin P, Guedon G, Mullany P, Pembroke T, Rood JI, Smith CJ, Summers AO, Tsuda M, Berg DE. 2008. Revised nomenclature for transposable genetic elements. Plasmid 60:167–173. https://doi.org/10.1016/j.plasmid.2008.08.001.
25. Heuer H, Binh CT, Jechalke S, Kopmann C, Zimmerling U, Krogerrecklenfort E, Ledger T, Gonzalez B, Top E, Smalla K. 2012. IncP-1ε plasmids are important vectors of antibiotic resistance genes in agricultural systems: diversification driven by class 1 integron gene cassettes. Front Microbiol 3:2. https://doi.org/10.3389/fmicb.2012.00002.

Table S1. ORFs identified in plasmid pJB12.

Gene name	Position	Length	Direction	Function	Amino acid Identity (GenBank accession number)
repA	30141 - 1198	1419	Reverse	Putative plasmid replicase	88% RepA pMATVIM-7 (CAO91756.1)
parB	1812 - 2027	216	Reverse	Plasmid stabilization and partitioting system	80% ParB pNOR-2000 (AGG19226.1)
parA	2048 - 2680	633	Reverse	Plasmid stabilization and partitioting system	97% ParA pNOR-2000 (AGG19225.1)
res	3052 - 3798	747	Reverse	Putatitve resolvase	74% Res pMATVIM-7 (CAO91761.1)
pJB12.5	3998 - 4540	543	Reverse	Putatitve transposase	100% TnpA pAX22 (YP_008508469.1)
t/at1	4546 - 4941	396	Reverse	Toxin/antitoxin system	100% T/AT2 pAX22 (YP_008508471.1)
t/at2	4938 - 5189	252	Reverse	Toxin/antitoxin system	100% T/AT1 pAX22 (YP_008508470.1)
pJB12.8	5254 - 5937	684	Forward	Putative invertase	100% Putative recombinase pAX22 (YP_008508472.1)
intI1	6284 - 7297	1014	Reverse	Class 1 integrase	100% IntI1 (AAK19119.1)
aacA7	7444 - 7902	459	Forward	Aminoglycoside N(6')-acetyltransferase type 1	100% AAC(6')-Il (AAK19120.1)
bla$_{VIM-2}$	8055 - 8855	801	Forward	Metallo-β-lactamase	100% VIM-2 (AAK19121.1)
aacC1	8949 - 9413	465	Forward	3-N-aminoglycoside acetyltransferase	100% AAC(3')-I (AAK19122.1)
aacA4	9534 - 10088	555	Forward	Aminoglycoside N(6')-acetyltransferase type 2	100% AAC(6')-Ib' (AAK19123.1)
qacEΔ1	10257 - 10604	348	Forward	SMR family efflux pump	100% QACEΔ1 (AAK19124.1)
sul1	10598 - 11437	840	Forward	Sulfonamide resistance	100% SUL-1 (AAK19125.1)
orf5	11469 - 12065	597	Forward	Hypothetical protein	100% hypothetical protein (AAK19126.1)

tniB	12034 - 13002	969	Reverse	ATP-binding	95% TniBΔ3 pAX22 (YP_008508481.1)
tniA	13029 - 14744	1716	Reverse	Putative transposase	100% TniA pAX22 (YP_008508482.1)
arsR	14955 - 15287	333	Forward	Metalloregulatory repressor	91% ArsR pAB3 (AKQ28764.1)
arsH	15284 - 16051	768	Forward	Oxidoreductase	95% ArsH pAB3 (AKQ28783.1)
arsC	16048 - 16554	507	Forward	Arsenate reductase	85% Arsenic reductase pAB3 (AKQ28782.1)
arsB	16551 - 17612	1062	Forward	Arsenic pump membrane	95% Arsenic transporter pAB3 (AKQ28781.1)
pJB12.23	17667 – 17969	303	Forward	Putative plasmid stabilization protein	84% Putatitve plasmid stabilization protein pRWC72a (AFV47236.1)
pJB12.24	17966 - 18253	288	Forward	Putatitve plasmid stabilization protein	86% Putatitve plasmid stabilization protein pRWC72a (AFV47237.1)
pJB12.25	18425 - 18796	372	Forward	Hypothetical protein	76% hypothetical protein pMRVIM0713 (AKJ19124.1)
pJB12.26	18793 - 19044	252	Forward	Hypothetical protein	100% Growth regulator HB13 (ERF04107.1)
pJB12.27	19177 - 19473	297	Reverse	Hypothetical protein	100% hypothetical protein HB13 (ERF04108.1)
pJB12.28	19564 - 20493	930	Reverse	Putative serine protease	94% TNCP23 pKLC102 (AAP22630.1)
pJB12.29	20492 - 20770	279	Forward	Hypothetical protein	92% TNCP22b pKLC102 (AAP22629.1)
traA	21227 - 24631	3405	Reverse	Conjugal transfer	94% TraA pNOR-2000 (AGG19209.1)
traC	24690 - 24941	252	Forward	Conjugal transfer	87% TraC pNOR-2000 (AGG19208.1)
traD	24964 - 25185	222	Forward	Conjugal transfer	93% TraD pNOR-2000 (AGG19207.1)
traG	25220 - 27742	2523	Forward	Conjugal transfer	93% TraG pNOR-2000 (AGG19206.1)
pJB12.34	27752 - 28144	393	Forward	Hypothetical protein	89% hypothetical protein pAMBL1 (AKH45393.1)
kfrA	28316 - 29344	1029	Reverse	Transcriptional regulation	91% KfrA pNOR-2000 (AGG19205.1)

pJB12.36	29547 - 29855	309	Reverse	Hypothetical protein	96% hypothetical protein pMRVIM0713 (AKJ19104.1)

J Antimicrob Chemother 2017; **72**: 2225–2229
doi:10.1093/jac/dkx143 Advance Access publication 12 May 2017

Journal of Antimicrobial Chemotherapy

The complete nucleotide sequence of an IncP-2 megaplasmid unveils a mosaic architecture comprising a putative novel *bla*$_{VIM-2}$-harbouring transposon in *Pseudomonas aeruginosa*

João Botelho[1], Filipa Grosso[1], Sandra Quinteira[2–4], Aymen Mabrouk[5,6] and Luísa Peixe[1]*

[1]*UCIBIO/REQUIMTE, Laboratório de Microbiologia, Faculdade de Farmácia, Universidade do Porto, Porto, Portugal;* [2]*CIBIO, Centro de Investigação em Biodiversidade e Recursos Genéticos, Universidade do Porto (CIBIO/UP)/InBio Laboratório Associado, Vairão, Portugal;* [3]*Faculdade de Ciências da Universidade do Porto, Departamento de Biologia, Porto, Portugal;* [4]*CESPU, Instituto de Investigação e Formação Avançada em Ciências e Tecnologias da Saúde, Gandra PRD, Portugal;* [5]*Laboratories UR12ES02 – The National Bone Marrow Transplant Centre, Tunis, Tunisia;* [6]*University of Carthage, Faculty of Sciences of Bizerte, Tunis, Tunisia*

*Corresponding author. Tel: +351-220428580; Fax: +351-226093390; E-mail: lpeixe@ff.up.pt

Received 8 February 2017; returned 15 March 2017; revised 6 April 2017; accepted 19 April 2017

Objectives: In *Pseudomonas aeruginosa*, *bla*$_{VIM-2}$ has been mostly associated with a chromosomal location and rarely with a plasmid backbone. Until now, only three complete *bla*$_{VIM-2}$-carrying plasmid sequences have been described in this species. Here we explore the modular structure of pJB37, the first *bla*$_{VIM-2}$-carrying megaplasmid described in *P. aeruginosa*.

Methods: The complete nucleotide sequence of plasmid pJB37 was determined with an Illumina HiSeq, with *de novo* assembly by SPAdes, annotation by RAST and searching for antimicrobial resistance genes and virulence factors. Conjugation assays were conducted.

Results: Megaplasmid pJB37 (464 804 bp long and GC content of 57.2%) comprised: an IncP-2 *repA-oriV-parAB* region; a conjugative transfer region (*traF*, *traG*, *virD2* and *trbBCDEJLFGI* genes); Tn*6356*, a new putative *bla*$_{VIM-2}$-carrying transposon; heavy metal (mercury and tellurite) resistance operons; and an arsenal of virulence genes. Plasmid pJB37 was transferable by conjugation to a spontaneous rifampicin-resistant mutant of *P. aeruginosa* PAO1. Here, a *bla*$_{VIM-2}$-harbouring In58 integron was associated with a new complex transposable structure, herein named Tn*6356*, suggesting that In58 was most likely acquired by insertion of this element.

Conclusions: The mosaic arrangement exhibited by the pJB37 IncP-2 megaplasmid, which highlights the vast assembly potential of distinct genetic elements in a *Pseudomonas* widespread plasmid platform, gives new insights into bacterial adaptation and evolution.

Introduction

Pseudomonas aeruginosa is a clinically important pathogen and a leading cause of nosocomial infections that are often difficult to treat due to its intrinsic and acquired resistance mechanisms to various antimicrobial agents, including carbapenems.[1–3] The carbapenem resistance observed in this species is frequently mediated by the production of carbapenemases, mainly MBLs, with VIM and IMP families being the most widespread.[1–3] The VIM-2 enzyme shows an endemic spread throughout Southern European and Southeast Asian countries and represents the most frequently reported MBL in *P. aeruginosa*.[1–3] In this species, *bla*$_{VIM-2}$ is commonly linked to a chromosomal location and to class 1 integrons containing additional gene cassettes.[4–7] This gene has rarely been associated with a plasmid location.[1,8]

Until now, only three complete *bla*$_{VIM-2}$-carrying plasmid sequences have been described in *P. aeruginosa*: pNOR-2000, pDCPR1 and pJB12.[9,10,11] These plasmids presented a small size (≤30 kb) and were unable to be transferred by conjugation. The majority of transmissible resistance plasmids identified in this species belong to the incompatibility group P-2.[12,13] IncP-2 plasmids are usually very large and single copy, have a limited host range and are among the most common plasmids encountered in clinical isolates of *P. aeruginosa*.[12,13] Although these megaplasmids are ubiquitous in the environment,[12,13] the only complete sequence reported so far in *P. aeruginosa* corresponds to pOZ176, a *bla*$_{IMP-9}$-carrying plasmid from China.[13] Interestingly, a *bla*$_{DIM-2}$-harbouring megaplasmid of unknown incompatibility group has also been reported in China in a *Pseudomonas putida* clinical

isolate,[14] suggesting an important role of megaplasmids in the acquisition of carbapenemase genes.

Here we report the complete nucleotide sequence and the characterization of plasmid pJB37, which is, as far as we know, the first bla_{VIM-2}-carrying megaplasmid to be described in *P. aeruginosa*, and we characterize the different genetic elements that constitute its backbone.

Materials and methods

Bacterial isolate

Following a regular surveillance led by our laboratory for the screening of MBL producers among clinical isolates, three *P. aeruginosa* belonging to the high-risk clone ST253, presenting the same PFGE pattern and carrying the bla_{VIM-2} gene in an ~450 kb plasmid (S1 nuclease PFGE-based sizing), were isolated in different years (2008 and 2010) from distinct inpatients attending the same hospital. WGS of one of these isolates (FFUP_PS_37) was performed. This carbapenem-resistant *P. aeruginosa* isolate was recovered in 2008 from bronchial secretions of a patient with a respiratory infection admitted to the General Internal Medicine unit of a Portuguese hospital. FFUP_PS_37 was initially identified using the VITEK-2 system (bioMérieux) and species was confirmed by multilocus sequence analysis (*rpoD*, *gyrB* and 16S rRNA genes).[15] Antimicrobial susceptibility testing was conducted by standard disc diffusion, Etest (carbapenems) and broth microdilution (colistin) methods, according to the EUCAST guidelines (http://www.eucast.org/).

Genome sequencing and plasmid analysis

Total DNA from the FFUP_PS_37 *P. aeruginosa* isolate was extracted using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Sequencing was accomplished with a HiSeq (Illumina) sequencer, with coverage of ×100. The quality of the high-throughput sequence data was assessed by FastQC. *De novo* assembly of the paired-end reads was performed using SPAdes v. 3.9.0.[16] The quality of genome assembly was evaluated with QUAST (http://quast.bioinf.spbau.ru/) and ResFinder (https://cge.cbs.dtu.dk/services/ResFinder/) was applied for identification of acquired antimicrobial resistance genes. Virulence genes were inspected using the Virulence Factor Database (http://www.mgc.ac.cn/VFs/main.htm) and the VRprofile web-based tool (http://bioinfo-mml.sjtu.edu.cn/VRprofile/index.php). Putative plasmid contigs were analysed and re-assembled to form a single megaplasmid using Geneious v. 9.1.6. Primer design and PCR mapping were conducted to confirm that the contigs formed a single megaplasmid. Automatic annotation was conducted using the RAST server (http://rast.nmpdr.org/). Annotations were manually curated using BLASTn and BLASTp searches.

Conjugation assays were conducted as previously described,[5] using a spontaneous rifampicin-resistant mutant of *P. aeruginosa* PAO1 as recipient strain. Transconjugant selection was performed using Mueller–Hinton agar plates containing rifampicin (100 mg/L) and imipenem (4 mg/L). Twitching motility assays were performed for the donor, recipient and transconjugant strains as previously described.[17]

PCR mapping, screening for the pJB37-like *repA* gene (rep_JB37_Fw: CGTTCTTCTCGCTTGCCAAG; rep_ JB37_Rv: TACAGCCAGCGTTCGATACC) and Sanger sequencing were performed for the remaining isolates that belonged to ST253 and presented the bla_{VIM-2} gene in an ~450 kb plasmid.

Nucleotide sequence accession number

The complete sequence of plasmid pJB37 has been submitted to GenBank under the accession number KY494864.

Results and discussion

Main features of the FFUP_PS_37 isolate and the pJB37 plasmid

The FFUP_PS_37 *P. aeruginosa* clinical isolate showed resistance to imipenem, meropenem, ceftazidime, cefepime, aztreonam, piperacillin / tazobactam, gentamicin, tobramycin, amikacin and netilmicin and remained susceptible to ciprofloxacin and colistin (MIC 0.25 mg/L), which is consistent with an XDR phenotype.[18] FFUP_PS_37 belonged to ST253, one of the five high-risk clones frequently identified in *P. aeruginosa*.[19] Besides its clinical relevance, ST253 was also linked to different environmental, animal and non-clinical settings.[20,21] Consequently, this wide dispersion throughout intimately connected ecological niches certainly potentiates the risk of causing human infections.

The complete plasmid sequence of pJB37 was 464 804 bp long, containing 588 ORFs and presenting a GC content of 57.2% (Figure 1). The modular structure of plasmid pJB37 comprises: (i) the IncP-2 *repA-oriV-parAB* region; (ii) the conjugative transfer region; (iii) Tn*6356*, a new putative bla_{VIM-2}-carrying transposon; (iv) heavy metal (mercury and tellurite) resistance operons; and (v) virulence genes (Figures 1 and 2).

Scaffold of plasmid pJB37 and its basic features

Plasmid pJB37 was highly related to bla_{IMP-9}-harbouring IncP-2 plasmid pOZ176 isolated in China (85% query cover and 99% nucleotide similarity) (accession number KC543497.1) (Figure 1).[13] Also, 77% and 78% query cover and 99% and 98% nucleotide similarities were observed when comparing with megaplasmid pRBL16 from an oestrogen-degrading *Pseudomonas citronellolis* isolate and bla_{IMP-45}-carrying megaplasmid pBM413 from *P. aeruginosa* (accession numbers NZ_CP015879.1 and CP016215.1, respectively), both from China (Figure 1).

The putative replicase protein shared 91% homology with the one from pOZ176 (accession number AGL46263.1). Interestingly, PCR mapping and Sanger sequencing unveiled the same replicase gene and the same backbone of plasmid pJB37 among the remaining 450 kb bla_{VIM-2}-carrying plasmids from our *P. aeruginosa* collection. The 64 bp origin of replication (bp 391 538–391 590), comprising three copies of iterons (TCGTGCTATCAGGAGTA), was similar to the one found in pOZ176.[13]

Comparative analysis of the transfer region of pJB37 (*traF*, *traG*, *virD2* and *trbBCDEJLFGI* genes) revealed that it was quite similar to the one found in pOZ176 (encoded proteins exhibit a similarity ranging from 76% for TraF to 95% for TrbF). Conjugal transfer of plasmid pJB37 to a spontaneous rifampicin-resistant mutant of *P. aeruginosa* PAO1 was successful. Screening for bla_{VIM} by PCR gave positive results for the transconjugant, here named *P. aeruginosa* PAO1(pJB37), which exhibited an MIC value of 16 mg/L for both imipenem and meropenem. The MIC values for the donor strain were > 32 mg/L for both imipenem and meropenem, while for the receptor strain the value dropped to 1 and 0.5 mg/L for imipenem and meropenem, respectively. Conjugal transfer of pOZ176 was successful when using *P. aeruginosa* NCTC 50814 as recipient, but not when *Escherichia coli* UB1637/R was used.[13] Altogether, these findings may reveal adaptation of IncP-2 plasmids to *P. aeruginosa*, confirming its narrow host range nature.

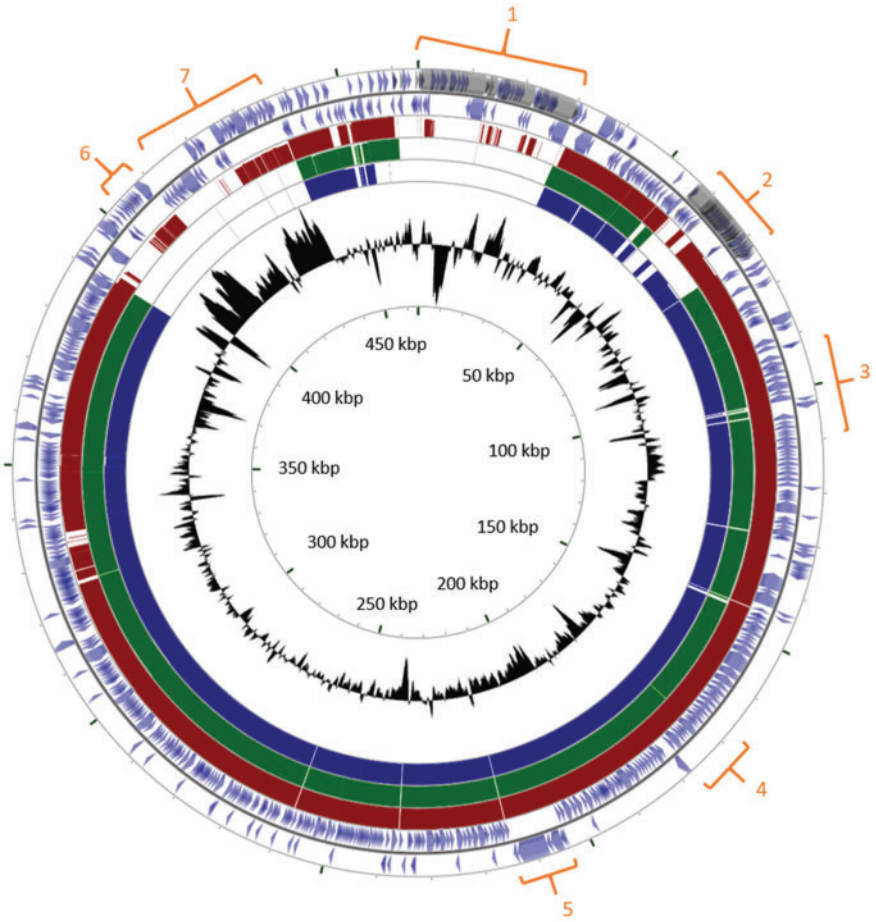


Figure 1. Genetic organization of the pJB37 megaplasmid and comparison with similar plasmid backbones. Coding sequences from pJB37 are represented by blue arrowheads in the outermost circles, comprising both the forward and reverse frames. Homology regions are represented in red for the bla_{IMP-9}-harbouring pOZ176 plasmid from *P. aeruginosa*, green for the pRBL16 plasmid from an oestrogen-degrading *P. citronellolis* isolate and blue for bla_{IMP-45}-carrying megaplasmid pBM413 from *P. aeruginosa*. The black circle illustrates G + C skew. The innermost circle indicates the scale. Labels 1–7 highlight the modular structure of pJB37: 1, Tn*5041*-like mercury resistance transposon, disrupted by the insertion of a Tn*4661*-like transposon and a *mer* operon; 2, Tn*6356*, a novel bla_{VIM-2}-harbouring transposon; 3, *ter* (tellurite resistance) operon; 4, *pil* (pili) operon; 5, *che* (chemotaxis) operon; 6, IncP-2 *tra-parBA-oriV-repA* region; and 7, conjugative transfer region. Labels 1, 2, 6 and 7 represent regions of pJB37 with weak or no similarity with the remaining plasmids. The genome map was created using CGview software. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

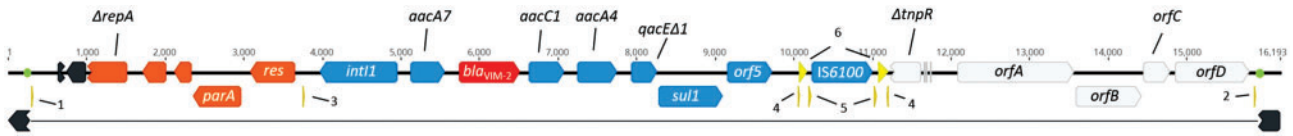


Figure 2. Schematic representation of the putative novel transposon Tn*6356*, disrupting the gene coding for a regulator of protease activity, HflC. Light blue arrows represent the genes associated with the In4-like lineage. The bla_{VIM-2} gene is illustrated in red. Orange arrows highlight the region similar to plasmids pAB5S9 and pAB5S9b from *A. salmonicida* subsp. *salmonicida*. Δ stands for truncated genes. Light grey arrows represent the Δ*tnpR-orfABCD* module similar to the one identified in the Tn*1403* transposon. Labels 1–6 highlight specific repeat regions: 1, 38 bp IR left (5′-GGGGAGCCCGCAGAATTCGGAAAAAATCGTACGCTAAG-3′) of Tn*6356*; 2, 38 bp IR right (5′-GTTAGCGTACGATTTTTTCCGAATTCTGCGGGCTCCCC-3′) of Tn*6356*; 3, IR initial of Tn*402*-like transposon; 4, IRs terminal of Tn*402*-like transposon; 5, IR left and right of IS*6100*; and 6, 123 and 152 bp short segments in inverse orientation that typically flank IS*6100* in In4-like structures. DRs (5′-TTCATT-3′) flanking the 38 bp IRs are illustrated by green circles. Geneious software v. 9.1.6 was used to construct the transposon. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

Plasmid maintenance regions of IncP-2 plasmids are frequently found in the genome of several clinical and environmental species.[12] In fact, two portions of the pJB37 plasmid scaffold (the IncP-2 *repA-oriV-parAB* region and the conjugative transfer region) were encountered in the chromosome of *Delftia tsuruhatensis* strain CM13, *P. putida* strain IEC33019 and *P. aeruginosa* strain Carb0163, and in the backbone of *Klebsiella pneumoniae* strain Kp29642964TF and *Enterobacter cloacae* complex sp. 35734 p35734-141.404 kb plasmids (accession numbers CP017420.1, CP016634.1, CP011317.1, KT935446.1 and CP010360.2, respectively). The GC content of these portions (67.1% and 63.2%, respectively) (Figure 1) was different from that observed for the megaplasmid (57.2%) and similar to the 65.2% GC content of the FFUP_PS_37 host genome. *In silico* analysis revealed that these portions shared a similar GC content to pOZ176 (66.9% and 66.7%, respectively). These findings may reveal an important contribution of different plasmid backbones for the assembly process of IncP-2 plasmids.[12]

Plasmid pJB37 also carried an arsenal of virulence factors, such as twitching motility proteins PilT and PilG, type IV fimbrial assembly ATPase PilB and a chemotaxis (*che*) operon (Figure 1). *pilB*, *pilG* and *pilT* genes are involved in several traits, such as the biogenesis and mechanical function of type IV pili (T4P), twitching motility and biofilm formation.[22] The *che* operon is required for flagella-mediated chemotaxis in *P. aeruginosa* and is similar to the Pil-Chp system.[13,22] This system is also involved in the regulation of intracellular levels of cyclic AMP (cAMP), a messenger that activates Vfr.[22] Vfr is a cAMP-binding protein that plays a vital role in several virulence traits, such as the expression of T4P.[22] This regulator was identified on pJB37 and the chromosome of FFUP_PS_37. The Vfr copy from the chromosome presented 100% amino acid identity with the one from PAO1, the reference genome. Interestingly, the amino acid sequence of the regulator copy identified on the megaplasmid was not the same. Non-silent mutations of the *vfr* gene have previously been associated with impairment in pili biogenesis or function.[21] Comparative analysis of the conserved domains of both Vfr copies revealed different ligand-binding sites and different flexible hinge regions.

In this study, motility assays using *P. aeruginosa* PAO1 as control revealed that the FFUP_PS_37 strain did not demonstrate T4P-mediated twitching motility. The twitch zone observed on *P. aeruginosa* PAO1(pJB37) was smaller than the one for the PAO1 strain. This suggests that the acquisition by PAO1 of the mutant *vfr* encoded by pJB37 resulted in a twitching motility reduction, probably due to a higher affinity of the mutant for the ligand-binding sites. Motile *P. aeruginosa* exhibit a more virulent phenotype and lead to the activation of the host immune system responses. In fact, T4P is relevant in the acute phase of infection but is frequently down-regulated in chronic infections.[22] Consequently, acquisition of the megaplasmid may comprise an adaptation to a sessile lifestyle, helping bacteria to evade hostile conditions.[22]

The peculiar structure of Tn6356 harbouring the bla$_{VIM-2}$ gene

In the novel pJB37 plasmid, described here, the *bla*$_{VIM-2}$ gene was associated with an In58 class 1 integron, a genetic platform that carries three aminoglycoside resistance genes (*aacA7*, *aacA4* and *aacC1*) and which was previously identified among *P. aeruginosa*, *P. putida* and *Citrobacter freundii* isolates in different countries.[6,7,23]

The In58 integron structure was derived from the In4 lineage, which typically comprises *orf5* and/or *orf6* genes downstream from the Tn*402*-like transposon, as well as a partial copy of IS*6100* attached to a full copy of the same IS.[24] However, in the present study the In4-like integron lacked the partial copy of the insertion sequence (Figure 2). This observation has been reported elsewhere.[13,25,26] Due to the known genetic rearrangement ability of this IS,[26,27] an additional recombination potential can be expected for an In58 integron.

Here, In58 was associated with a novel transposon structure, flanked by 38 bp IRs of Tn*3*-like transposons. This atypical element was composed of three blocks: (i) a Δ*repA-parA-resA* segment; (ii) the In4-like structure; and (iii) a Δ*tnpR-orfABCD* module similar to the one identified in the Tn*1403* transposon (Figure 2).[28] The Δ*repA-parA-resA* segment was 99% similar to the one identified in plasmids pAB5S9 and pAB5S9b from *Aeromonas salmonicida* subsp. *salmonicida* (accession numbers ABQ41438.1 and AIM49713.1, respectively). The replicase protein, truncated due to the insertion of an ancestral mobile element, exhibited 100% homology with the replicases of unknown incompatibility group found in these plasmids.[29] The putative transposon displayed a hybrid construction, which might have resulted from site-specific and/or homologous recombination events that took place before and/or after insertion. The insertion of this putative transposon in plasmid pJB37 disrupted a gene coding for a regulator of protease activity, HflC (Figure 2). Here, a 6 bp target site duplication (5′-TTCATT-3′) was found surrounding the IRs of the transposon, suggesting that an insertion event mediated the *en bloc* insertion of the regions comprising this atypical element (Figure 2). This novel and complex transposable element was designated here as Tn*6356* (http://www.ucl.ac.uk/eastman/research/departments/microbial-diseases/tn), according to the criteria proposed by Roberts *et al.*[30] Future studies are warranted to investigate the events that mediated the acquisition of Tn*6356*.

Conclusions

In this work we present the complete sequence of pJB37, an IncP-2 megaplasmid associated with a *bla*$_{VIM-2}$-harbouring In58 integron from a *P. aeruginosa* clinical isolate belonging to the well-recognized ST253 high-risk clone. In58 was most likely acquired by insertion of Tn*6356*, the putative hybrid transposon now described. The mosaic structure exhibited by the pJB37 megaplasmid, which highlights the vast assembly potential of distinct genetic elements in a *Pseudomonas* widespread plasmid platform, helps to shed new light on bacterial adaptation and evolution.

Acknowledgements

Isolates were kindly provided by the Microbiology Department of Centro Hospitalar do Porto, Portugal.

Funding

This work received financial support from the European Union (FEDER funds POCI/01/0145/FEDER/007728) and National Funds (FCT/MEC, Fundação para a Ciência e Tecnologia and Ministério da Educação e

Ciência) under the Partnership Agreement PT2020UID/MULTI/04378/2013. João Botelho and Filipa Grosso were supported by grants from Fundação para a Ciência e a Tecnologia (SFRH/BD/104095/2014 and SFRH/BPD/95556/2013, respectively).

Transparency declarations

None to declare.

References

1 Oliver A, Mulet X, Lopez-Causape C *et al*. The increasing threat of *Pseudomonas aeruginosa* high-risk clones. *Drug Resist Updat* 2015; **21–22**: 41–59.

2 Potron A, Poirel L, Nordmann P. Emerging broad-spectrum resistance in *Pseudomonas aeruginosa* and *Acinetobacter baumannii*: mechanisms and epidemiology. *Int J Antimicrob Agents* 2015; **45**: 568–85.

3 Hong DJ, Bae IK, Jang IH *et al*. Epidemiology and characteristics of metallo-β-lactamase-producing *Pseudomonas aeruginosa*. *Infect Chemother* 2015; **47**: 81–97.

4 Moyo S, Haldorsen B, Aboud S *et al*. Identification of VIM-2-producing *Pseudomonas aeruginosa* from Tanzania is associated with sequence types 244 and 640 and the location of bla_{VIM-2} in a TniC integron. *Antimicrob Agents Chemother* 2015; **59**: 682–5.

5 Quinteira S, Sousa JC, Peixe L. Characterization of In100, a new integron carrying a metallo-β-lactamase and a carbenicillinase, from *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother* 2005; **49**: 451–3.

6 Quinteira S, Peixe L. Multiniche screening reveals the clinically relevant metallo-β-lactamase VIM-2 in *Pseudomonas aeruginosa* far from the hospital setting: an ongoing dispersion process? *Appl Environ Microbiol* 2006; **72**: 3743–5.

7 Poirel L, Lambert T, Turkoglu S *et al*. Characterization of class 1 integrons from *Pseudomonas aeruginosa* that contain the bla_{VIM-2} carbapenem-hydrolyzing β-lactamase gene and of two novel aminoglycoside resistance gene cassettes. *Antimicrob Agents Chemother* 2001; **45**: 546–52.

8 Dortet L, Flonta M, Boudehen YM *et al*. Dissemination of carbapenemase-producing Enterobacteriaceae and *Pseudomonas aeruginosa* in Romania. *Antimicrob Agents Chemother* 2015; **59**: 7100–3.

9 Bonnin RA, Poirel L, Nordmann P *et al*. Complete sequence of broad-host-range plasmid pNOR-2000 harbouring the metallo-β-lactamase gene bla_{VIM-2} from *Pseudomonas aeruginosa*. *J Antimicrob Chemother* 2013; **68**: 1060–5.

10 Vilacoba E, Quiroga C, Pistorio M *et al*. A bla_{VIM-2} plasmid disseminating in extensively drug-resistant clinical *Pseudomonas aeruginosa* and *Serratia marcescens* isolates. *Antimicrob Agents Chemother* 2014; **58**: 7017–8.

11 Botelho J, Grosso F, Peixe L. Characterization of the plasmid pJB12 from *Pseudomonas aeruginosa* reveals Tn*6352*, a novel putative transposon associated with mobilization of bla_{VIM-2}-harboring In58 integron. *Antimicrob Agents Chemother* 2017; **61**: e02532–16.

12 Jacoby GA, Sutton L, Knobel L *et al*. Properties of IncP-2 plasmids of *Pseudomonas* spp. *Antimicrob Agents Chemother* 1983; **24**: 168–75.

13 Xiong J, Alexander DC, Ma JH *et al*. Complete sequence of pOZ176, a 500-kilobase IncP-2 plasmid encoding IMP-9-mediated carbapenem resistance, from outbreak isolate *Pseudomonas aeruginosa* 96. *Antimicrob Agents Chemother* 2013; **57**: 3775–82.

14 Sun F, Zhou D, Wang Q *et al*. Genetic characterization of a novel bla_{DIM-2}-carrying megaplasmid p12969-DIM from clinical *Pseudomonas putida*. *J Antimicrob Chemother* 2016; **71**: 909–12.

15 Mulet M, Lalucat J, Garcia-Valdes E. DNA sequence-based analysis of the *Pseudomonas* species. *Environ Microbiol* 2010; **12**: 1513–30.

16 Bankevich A, Nurk S, Antipov D *et al*. SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. *J Comput Biol* 2012; **19**: 455–77.

17 Beatson SA, Whitchurch CB, Semmler ABT *et al*. Quorum sensing is not required for twitching motility in *Pseudomonas aeruginosa*. *J Bacteriol* 2002; **184**: 3598–604.

18 Magiorakos AP, Srinivasan A, Carey RB *et al*. Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. *Clin Microbiol Infect* 2012; **18**: 268–81.

19 Cabrolier N, Sauget M, Bertrand X *et al*. Matrix-assisted laser desorption ionization-time of flight mass spectrometry identifies *Pseudomonas aeruginosa* high-risk clones. *J Clin Microbiol* 2015; **53**: 1395–8.

20 Estepa V, Rojo-Bezares B, Torres C *et al*. Faecal carriage of *Pseudomonas aeruginosa* in healthy humans: antimicrobial susceptibility and global genetic lineages. *FEMS Microbiol Ecol* 2014; **89**: 15–9.

21 Kidd TJ, Ritchie SR, Ramsay KA *et al*. *Pseudomonas aeruginosa* exhibits frequent recombination, but only a limited association between genotype and ecological setting. *PLoS One* 2012; **7**: e44199.

22 Burrows LL. *Pseudomonas aeruginosa* twitching motility: type IV pili in action. *Annu Rev Microbiol* 2012; **66**: 493–520.

23 Santos C, Caetano T, Ferreira S *et al*. Tn*5090*-like class 1 integron carrying bla_{VIM-2} in a *Pseudomonas putida* strain from Portugal. *Clin Microbiol Infect* 2010; **16**: 1558–61.

24 Partridge SR, Brown HJ, Stokes HW *et al*. Transposons Tn*1696* and Tn*21* and their integrons In4 and In2 have independent origins. *Antimicrob Agents Chemother* 2001; **45**: 1263–70.

25 Boyd D, Cloeckaert A, Chaslus-Dancla E *et al*. Characterization of variant *Salmonella* genomic island 1 multidrug resistance regions from serovars Typhimurium DT104 and Agona. *Antimicrob Agents Chemother* 2002; **46**: 1714–22.

26 Targant H, Doublet B, Aarestrup FM *et al*. IS*6100*-mediated genetic rearrangement within the complex class 1 integron In104 of the *Salmonella* genomic island 1. *J Antimicrob Chemother* 2010; **65**: 1543–5.

27 Coyne S, Courvalin P, Galimand M. Acquisition of multidrug resistance transposon Tn*6061* and IS*6100*-mediated large chromosomal inversions in *Pseudomonas aeruginosa* clinical isolates. *Microbiology* 2010; **156**: 1448–58.

28 Stokes HW, Elbourne LD, Hall RM. Tn*1403*, a multiple-antibiotic resistance transposon made up of three distinct transposons. *Antimicrob Agents Chemother* 2007; **51**: 1827–9.

29 Vincent AT, Trudel MV, Paquet VE *et al*. Detection of variants of the pRAS3, pAB5S9, and pSN254 plasmids in *Aeromonas salmonicida* subsp. *salmonicida*: multidrug resistance, interspecies exchanges, and plasmid reshaping. *Antimicrob Agents Chemother* 2014; **58**: 7367–74.

30 Roberts AP, Chandler M, Courvalin P *et al*. Revised nomenclature for transposable genetic elements. *Plasmid* 2008; **60**: 167–73.

the intact type VII MDRGI was obtained from both C179b and its transformant C179b_11168, but not from C179b_81-176. Considering the high level of similarity between the genes in the type VII MDRGI and the pTet plasmid in 81-176, there is a strong possibility that the MDRGI inserted into pTet instead of the chromosome (Figure 1). We performed several analyses to locate the exact insertion site of the MDRGI in 81-176. S1-nuclease PFGE and Southern blotting indicated that *erm*(B) and the tetracycline resistance gene *tet*(O) were located on a ~50 kb plasmid (Figure S1). It should be noted that this original *tet*(O) gene in pTet showed 94.6% nucleotide identity to that in the type VII MDRGI. A modified random primer-walking strategy revealed that the MDRGI was linked to the genes on the pTet plasmid. Further sequence analysis indicated that the pTet plasmid kept its original features [*tet*(O), *cpp2*, *cpp3*, *cpp4* and *repa*] and 8 out of 13 ORFs from the MDRGI were incorporated between *cpp2* and *cpp3* (Figure 1). Finally, long-range PCR using the primers cpp50-F (5′-CCACTTGGCTTTATCTTTTGC-3′) and cpp7-R (5′-GCAATGTCTTTCTCATATTCTGC-3′) (Figure 1), which are located in *cpp50* and *cpp7* on pTet, confirmed the presence of the MDRGI in C179b_81-176. These results showed that type VII MDRGI could be transferred amongst different *C. jejuni* strains and could be integrated into both plasmid and chromosome. Based on these and previous findings,[5,6] we concluded that the characterized MDRGIs could be integrated into at least four regions in *Campylobacter* and the plasticity of the insertion regions enhances the possible dissemination of MDRGIs among *Campylobacter*.

To determine the stability of *erm*(B) and its associated type VII MDRGI in transformants, two transformants (C179b_11168 and C179b_81-176) were subcultured daily in antibiotic-free Mueller–Hinton (MH) broth for 14 days. Cultures were diluted and then plated onto MH agar plates to distinguish single colonies. PCR screening of 20 colonies using the primers described above revealed that all of the colonies maintained the acquired MDRGI, confirming that the two transformants remained stable in the absence of antibiotics for ≥14 passages (Table S1). These findings suggest that natural transformation might contribute to the dissemination of high-level resistance to macrolides amongst *Campylobacter* species.

In conclusion, this study characterized the *erm*(B)-associated MDRGI in *C. jejuni*. Together with our previous study, these findings indicated that MDRGIs carrying *erm*(B) could be disseminated amongst *C. jejuni* isolates and be transferred from *C. coli* to *C. jejuni* in multiple regions. Even though the isolation rate of *C. jejuni* carrying *erm*(B) is relatively low at this stage, *C. jejuni* accounts for ~90% of campylobacteriosis in humans. Thus, it is worthwhile to monitor the emergence and spread of *erm*(B) and its associated MDRGI in *C. jejuni*.

Funding

The study was funded by grants from the National Basic Research Program of China and the National Natural Science Foundation of China (2013CB127200 and U1031004).

Transparency declarations

None to declare.

Supplementary data

Table S1 and Figure S1 are available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/).

References

1 Ruiz-Palacios GM. The health burden of *Campylobacter* infection and the impact of antimicrobial resistance: playing chicken. *Clin Infect Dis* 2007; **44**: 701–3.

2 Luangtongkum T, Jeon B, Han J *et al*. Antibiotic resistance in *Campylobacter*: emergence, transmission and persistence. *Future Microbiol* 2009; **4**: 189–200.

3 Gupta A, Nelson JM, Barrett TJ *et al*. Antimicrobial resistance among *Campylobacter* strains, United States, 1997–2001. *Emerg Infect Dis* 2004; **10**: 1102–9.

4 Luangtongkum T, Shen Z, Seng VW *et al*. Impaired fitness and transmission of macrolide-resistant *Campylobacter jejuni* in its natural host. *Antimicrob Agents Chemother* 2012; **56**: 1300–8.

5 Qin S, Wang Y, Zhang Q *et al*. Report of ribosomal RNA methylase gene *erm*(B) in multidrug-resistant *Campylobacter coli*. *J Antimicrob Chemother* 2014; **69**: 964–8.

6 Wang Y, Zhang M, Deng F *et al*. Emergence of multidrug-resistant *Campylobacter* species isolates with a horizontally acquired rRNA methylase. *Antimicrob Agents Chemother* 2014; **58**: 5405–12.

7 Bacon DJ, Alm RA, Burr DH *et al*. Involvement of a plasmid in virulence of *Campylobacter jejuni* 81-176. *Infect Immun* 2000; **68**: 4384–90.

8 Wang Y, Taylor DE. Natural transformation in *Campylobacter* species. *J Bacteriol* 1990; **172**: 949–55.

J Antimicrob Chemother 2015
doi:10.1093/jac/dku391
Advance Access publication 25 September 2014

Characterization of a new genetic environment associated with GES-6 carbapenemase from a *Pseudomonas aeruginosa* isolate belonging to the high-risk clone ST235

João Botelho[1], Filipa Grosso[1], Clara Sousa[2] and Luísa Peixe[1]*

[1]*REQUIMTE, Laboratório de Microbiologia, Faculdade de Farmácia, Universidade do Porto, Porto, Portugal;* [2]*CEB, Centro de Engenharia Biológica, Universidade do Minho, Braga, Portugal*

*Corresponding author. Tel: +351-22-042-85-80; Fax: +351-22-042-85-90; E-mail: lpeixe@ff.up.pt

Keywords: carbapenem resistance, class I integrons, IS*Pa55*, IS*110* family, site-specific recombination

Sir,

Pseudomonas aeruginosa is an opportunistic pathogen commonly associated with nosocomial infections.[1] The spread of *P. aeruginosa*

strains resistant to carbapenems constitutes an emerging threat worldwide.[1] The GES family of enzymes has been identified in several Gram-negative bacteria, such as *P. aeruginosa*, *Klebsiella pneumoniae* and *Escherichia coli*, being responsible for an extended resistance spectrum to several β-lactams.[2,3] To date, according to the Lahey Clinic web site (http://www.lahey.org/Studies/), 24 variants of GES have been identified with some presenting carbapenemase activity, which is a matter of great concern. Few GES variants have been identified in Portugal, all of them found in Enterobacteriaceae.[3] In Portugal, carbapenemases associated with *P. aeruginosa* include VIM-2 (the most frequent) and also IMP-5.[4] Here, we report the first known worldwide description of the bla_{GES-6} gene in *P. aeruginosa* strains associated with a new genetic environment and also the first description of GES-producing *P. aeruginosa* in Portugal.

In June 2012, a carbapenem-resistant *P. aeruginosa* isolate was recovered from bronchial secretions of a patient with pneumonia admitted to the intensive care unit of a Portuguese hospital. Antibiotic susceptibility assessed by standard disc diffusion, agar dilution and Etest methods[5] demonstrated that the isolate was XDR,[6] being non-susceptible to imipenem (MIC >32 mg/L), meropenem (MIC >32 mg/L), ceftazidime, cefepime, aztreonam, piperacillin/tazobactam, gentamicin, netilmicin, amikacin, tobramycin and ciprofloxacin, but susceptible to colistin (MIC = 2 mg/L). Carbapenemase production was confirmed by UV spectrophotometric assay and Blue-Carba.[7] Screening of *bla* genes encoding metallo-β-lactamases (MBLs)[8] gave negative results. The occurrence of ESBLs (bla_{BEL}, bla_{VEB}, bla_{PER} and bla_{GES}) was searched by PCR,[8] revealing the presence of bla_{GES}, which by sequencing was identified as bla_{GES-6}. The nucleotide sequence differed from bla_{GES-6} (GenBank accession no. AY494718) by only one nucleotide (G instead of A at position 54), corresponding to a silent mutation. GES-6 is a class A β-lactamase with carbapenemase activity, differing from GES-1 by two amino acid substitutions (E104K and G170S), that was only described previously in Greece in a *K. pneumoniae* clinical isolate with reduced susceptibility to carbapenems.[9] Association of bla_{GES-6} with class I integrons was performed by PCR and sequencing.[4] A new class I integron structure of ~5000 bp named In1076 by INTEGRALL (http://integrall.bio.ua.pt/) revealed an array of two gene cassettes attached to an IS (Figure 1; GenBank accession no. KM210290), with the bla_{GES-6} gene in the first position. The start codon (ATG) of bla_{GES-6} was preceded by two putative promoter regions [with a strong promoter, Pc; −35 (TTGACA) and −10 (TAAACT)] and a P2 promoter in its inactive form.[10] An *aacA7* gene encoding a type I aminoglycoside acetyltransferase [AAC(6′)-II], which confers resistance to amikacin, netilmicin and tobramycin,[1] was found immediately downstream of the bla_{GES-6} gene. The 59 base element of the *aacA7* gene cassette was interrupted at position 13 by a novel IS of 1543 bp, belonging to the IS*110* family, IS*1111* group, named IS*Pa55* and deposited in the IS finder database (https://www-is.biotoul.fr/) (Figure 1). Members of this group do not originate direct flanking target repeats and frequently encode a single ORF flanked by relatively long non-coding sequences (IS*Pa55* presented 78 and 330 bp at the left and right ends, respectively).[11] The subterminal inverted repeats (IRs) (typical of the IS*1111* group) were 5′-GAGTAAAAAGGAGACTTCCCG-3′ (left IR) and 5′-CGGGAAGCTCCTTATG-3′ (right IR). IS*Pa55* encodes a transposase related to the IS*110* family, sharing 64% amino acid identity with that of IS*Pa34* identified in *P. aeruginosa* (GenBank accession no. ADC38937). The N-terminal regions of transposases of this family and the PIV/Moov family of DNA recombinases share a similar DEDD motif ($D_{11}E_{55}D_{94}D_{97}$ in IS*Pa55*), suggesting they may use similar catalytic mechanisms.[12] The junction promoter (P_{junc}) originated by circularization of IS*Pa55* [TTGACG (17 bp) TAAAAA] is similar to the consensus promoters for representative IS*1111* family members and is expected to increase the expression of the transposase transcript.[12] IS*Pa55* disrupts the *attC* site of the *aacA7* gene, as occurs with other IS*110* family members that can insert themselves by site-specific recombination,[12] which may compromise the excision of the corresponding gene cassettes. The genetic location of the bla_{GES-6} gene was determined by I-CeuI-PFGE followed by hybridization with probes specific for bla_{GES-6} and 16S rRNA genes.[4] Both probes hybridized in the same band, revealing that the genetic platform bearing bla_{GES-6} was chromosomally located, although bla_{GES} genes have been more frequently associated with plasmids.[2,9] MLST was performed according to the *P. aeruginosa* database (http://pubmlst.org/paeruginosa/), revealing that the isolate belonged to the worldwide-disseminated high-risk ST235 clone associated with multidrug resistance and disseminated in Portuguese hospitals (J. Botelho, F. Grosso, C. Sousa and L. Peixe, unpublished results). Interestingly, ST235 has been previously associated with GES-1 and GES-5 in Spain.[13]

In summary, we present the first known worldwide report of the bla_{GES-6} carbapenemase gene in *P. aeruginosa* belonging to the high-risk ST235 clone and inserted in a new class I integron that is chromosomally located. Moreover, this is also the first known report of a GES-producing *P. aeruginosa* in Portugal. This study highlights the risk of further dissemination of the XDR ST235 *P. aeruginosa* clone and of genetic backgrounds containing class A carbapenemase genes in our country, which deserves future monitoring.

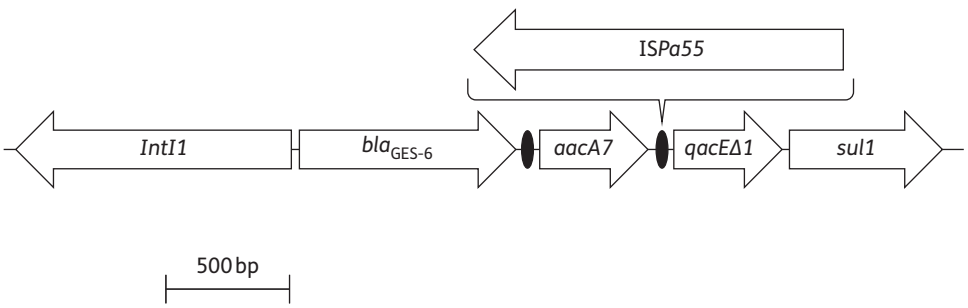


Figure 1. Schematic representation of the genetic environment of the *P. aeruginosa* CB1 integron (GenBank accession no. KM210290). The arrows indicate the translation orientation of the coding genes. The 59 base elements are indicated by black circles.

Funding

This work was funded by FEDER funds through the Operational Programme for Competitiveness Factors – COMPETE and by National Funds through FCT – Foundation for Science and Technology under the project Pest-C/EQB/LA0006/2013. C. S. and F. G. were supported by post-doctoral grants from Fundação para a Ciência e a Tecnologia (SFRH/BPD/70548/2010 and SFRH/BPD/95556/2013, respectively).

Transparency declarations

None to declare.

References

1 Dubois V, Arpin C, Dupart V *et al*. β-Lactam and aminoglycoside resistance rates and mechanisms among *Pseudomonas aeruginosa* in French general practice (community and private healthcare centres). *J Antimicrob Chemother* 2008; **62**: 316–23.

2 Poirel L, Bonnin RA, Nordmann P. Genetic support and diversity of acquired extended-spectrum β-lactamases in Gram-negative rods. *Infect Genet Evol* 2012; **12**: 883–93.

3 Jones-Dias D, Manageiro V, Ferreira E *et al*. Diversity of extended-spectrum and plasmid-mediated AmpC β-lactamases in Enterobacteriaceae isolates from Portuguese health care facilities. *J Microbiol* 2014; **52**: 496–503.

4 Quinteira S, Peixe L. Multiniche screening reveals the clinically relevant metallo-β-lactamase VIM-2 in *Pseudomonas aeruginosa* far from the hospital setting: an ongoing dispersion process? *Appl Environ Microbiol* 2006; **72**: 3743–5.

5 Clinical and Laboratory Standards Institute. *Performance Standards for Antimicrobial Susceptibility Testing: Twenty-third Informational Supplement M100-S23*. CLSI, Wayne, PA, USA, 2013.

6 Magiorakos AP, Srinivasan A, Carey RB *et al*. Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria—an international expert proposal for interim standard definitions for acquired resistance. *Clin Microbiol Infect* 2012; **18**: 268–81.

7 Pires J, Novais A, Peixe L. Blue-carba, an easy biochemical test for detection of diverse carbapenemase producers directly from bacterial cultures. *J Clin Microbiol* 2013; **51**: 4281–3.

8 Bogaerts P, Rezende de Castro R, de Mendonca R *et al*. Validation of carbapenemase and extended-spectrum β-lactamase multiplex endpoint PCR assays according to ISO 15189. *J Antimicrob Chemother* 2013; **68**: 1576–82.

9 Vourli S, Giakkoupi P, Miriagou V *et al*. Novel GES/IBC extended-spectrum β-lactamase variants with carbapenemase activity in clinical enterobacteria. *FEMS Microbiol Lett* 2004; **234**: 209–13.

10 Fluit AC, FJ S. Class 1 integrons, gene cassettes, mobility, and epidemiology. *Eur J Clin Microbiol Infect Dis* 1999; **18**: 761–70.

11 Poirel L, Brinas L, Fortineau N *et al*. Integron-encoded GES-type extended-spectrum β-lactamase with increased activity toward aztreonam in *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother* 2005; **49**: 3593–7.

12 Post V, Hall RM. Insertion sequences in the IS*1111* family that target the *attC* recombination sites of integron-associated gene cassettes. *FEMS Microbiol Lett* 2009; **290**: 182–7.

13 Viedma E, Juan C, Acosta J *et al*. Nosocomial spread of colistin-only-sensitive sequence type 235 *Pseudomonas aeruginosa* isolates producing the extended-spectrum β-lactamases GES-1 and GES-5 in Spain. *Antimicrob Agents Chemother* 2009; **53**: 4930–3.

J Antimicrob Chemother 2015
doi:10.1093/jac/dku407
Advance Access publication 14 October 2014

The complete sequence of *Salmonella* genomic island SGI2

Mohammad Hamidian[1], Kathryn E. Holt[2] and Ruth M. Hall[1]*

[1]School of Molecular Bioscience, The University of Sydney, NSW 2006, Australia; [2]Department of Biochemistry and Molecular Biology and Bio21 Molecular Science and Biotechnology Institute, The University of Melbourne, Melbourne, Victoria, Australia

*Corresponding author. Tel: +61-2-9351-3465; Fax: +61-2-9351-5858; E-mail: ruth.hall@sydney.edu.au

Keywords: multiple antibiotic resistance, class 1 integrons, *S. enterica*

Sir,

In *Salmonella enterica*, resistance to several different antibiotics is often caused by the presence of a class 1 integron or a complex class 1 integron located inside a genomic island that integrates precisely into the chromosome at the end of the 3′-end of the *thdF* gene (recently renamed *trmE*).[1] All of the antibiotic resistance genes lie within the boundaries of the complex class 1 integron and many variants that can be derived from SGI1 via the loss, gain or replacement of antibiotic resistance genes within this integron have been identified using a combination of PCR and hybridization.[1] Two island forms, SGI1 and SGI2, have arisen independently via the incorporation of a class 1 integron at different positions into backbones that are closely related, but not identical.[1–3] While the location of the integron in SGI1 (vertical arrow in Figure 1) is upstream of a resolvase-encoding gene (*resG*) as expected for this group of mobile elements, the integron in SGI2 is found 6889 bp away within an ORF designated S023 (Figure 1).

SGI2 was first found in the *S. enterica* serovar Emek strain SRC19, which was recovered from sewage effluent in September 1999 in Australia and was resistant to chloramphenicol, sulfamethoxazole, tetracycline and trimethoprim as well as nalidixic acid.[4] We originally reported SGI2 as a variant of SGI1 (SGI1-J).[2] However, further analysis revealed that it could not have arisen from SGI1 and hence was not an SGI1 variant.[4] We therefore renamed this island SGI2.[2] The structure of the complex class 1 integron was later revised to include an additional partial *tni* module and part of the backbone was also sequenced, revealing a significant number of single nucleotide differences between the SGI1 and SGI2 backbones.[3] SGI2 was also found in serovar Emek isolates from the UK (1999) and a traveller returning to Australia from Thailand in 2002.[4] SGI2-A, a deletion derivative that has lost the *cmlA9* gene (chloramphenicol and florfenicol resistance[4]) and *tet*(G) determinant (tetracycline resistance), was also found in a returning traveller.[4]

In order to facilitate future work on SGI2 and to compare the complete SGI2 backbone with that of SGI1, here we have completed the sequence of SGI2 by sequencing the genome of SRC19 on an Illumina HiSeq at the Australian Genomic Research

J Antimicrob Chemother
doi:10.1093/jac/dkx337

Journal of Antimicrobial Chemotherapy

Unravelling the genome of a *Pseudomonas aeruginosa* isolate belonging to the high-risk clone ST235 reveals an integrative conjugative element housing a bla_{GES-6} carbapenemase

João Botelho, Filipa Grosso and Luísa Peixe*

UCIBIO/REQUIMTE, Laboratório de Microbiologia, Faculdade de Farmácia, Universidade do Porto, Porto, Portugal

*Corresponding author. Laboratório de Microbiologia, Faculdade de Farmácia da Universidade do Porto, Rua Jorge Viterbo Ferreira 228, 4050-313 Porto, Portugal. Tel: +351220428500; Fax +351226093390; E-mail: lpeixe@ff.up.pt

Received 5 May 2017; returned 20 June 2017; revised 2 August 2017; accepted 19 August 2017

Objectives: In *Pseudomonas aeruginosa*, the bla_{GES-6} carbapenemase gene was previously associated with an In1076 class I integron. Here, we conducted a genome-based analysis and explored the genetic platform associated with the mobility of this gene.

Methods: WGS of a bla_{GES-6}-harbouring *P. aeruginosa* isolate (FFUP_PS_690) was performed with Illumina HiSeq, *de novo* assembly was performed using SPAdes and subsequent bioinformatic analysis was performed concerning antibiotic resistance genes, virulence features and mobile genetic elements.

Results: The FFUP_PS_690 isolate belongs to the ST235 high-risk clone and houses a novel integrative conjugative element (ICE), hereby named ICE*Pae690*. This *clc*-like ICE comprises the bla_{GES-6}-harbouring In1076 integron and specific modules. An ExoU island A variant was also identified.

Conclusions: The presence of a 'hitch-hiking' bla_{GES-6}-harbouring In1076 integron in an ICE and an *exoU*-carrying genomic island highlight the potential spread of these elements through conjugation and/or clonal expansion of the ST235 lineage.

Introduction

Pseudomonas aeruginosa is an opportunistic pathogen frequently implicated in nosocomial infections.[1,2] Developing antibiotic therapies to eradicate this bacterium remains challenging, mainly due to its intrinsic and acquired resistance mechanisms to several antimicrobial agents, including carbapenems.[1,2] The carbapenem resistance phenotype observed in *P. aeruginosa* is often mediated by the expression of carbapenemases.[1,2] Carbapenemase genes are frequently linked to integron structures, which may also harbour other antibiotic resistance determinants.[1,2] Class I integrons can be mobilized due to their association with different mobile genetic elements (MGEs), such as transposons, plasmids, integrative conjugative elements (ICEs) and/or genomic islands (GIs).[1–6] GIs typically harbour genes responsible for integration, maintenance and transfer events.[1–6] Additionally, GIs may carry genes involved in antibiotic and heavy metal resistance, as well as virulence determinants, conferring an adaptive advantage to the host in different environments.[1–6]

In Portugal, carbapenemases associated with *P. aeruginosa* include VIM-2, IMP-5 and GES-6.[7–9] The bla_{VIM-2} gene has been frequently identified in several *P. aeruginosa* isolates from Portuguese hospitals, associated with MDR and with high-risk clones (J. Botelho, F. Grosso, S. Quinteira, M. Brilhante, H. Ramos and L. Peixe, unpublished results). Previous studies conducted by our lab contributed to the characterization of different bla_{VIM-2}-carrying plasmids, highlighting the propagation of the In58 integron throughout distinct backbones.[7,8]

The GES family of enzymes has been identified in several Gram-negative species, such as *P. aeruginosa*, *Klebsiella pneumoniae* and *Escherichia coli*, and is responsible for an extended resistance spectrum to several β-lactams.[10] To date, 27 GES variants have been identified according to the Lahey Clinic website (http://www.lahey.org/Studies/). GES-6 is a class A β-lactamase with carbapenemase activity, which differs from GES-1 by two amino acid substitutions (E104K and G170S). The bla_{GES-6} gene was only previously described in Greece in a *K. pneumoniae* clinical isolate and in an 80 kb IncM1 plasmid from an *Enterobacter cloacae* clinical isolate recovered in Belgium.[11,12] In *P. aeruginosa*, this carbapenemase was first identified in a clinical isolate belonging to ST235.[9] The bla_{GES-6} gene was associated with In1076, a class 1 integron co-harbouring the aminoglycoside resistance *aacA7* gene and IS*Pa55*.[9] However, the genetic surroundings of this integron were barely inspected. Here, we explore the MGEs mediating the acquisition of the bla_{GES-6} gene in a recently detected *P. aeruginosa* ST235 isolate and analyse its genome-wide repertoire.

Materials and methods

Species identification, antimicrobial susceptibility testing and clonal analysis

The FFUP_PS_690 isolate was collected in February 2015 from the urine of a 90-year-old outpatient. The strain was initially identified by the automated VITEK 2 system (bioMérieux) and the species was confirmed by multilocus sequence analysis (*rpoD*, *gyrB* and 16S rRNA genes).[13] Antimicrobial susceptibility testing was conducted according to the EUCAST guidelines (http://www.eucast.org/) by the automated VITEK 2 system, except for colistin (broth microdilution method). MLST was performed according to the *P. aeruginosa* database (http://pubmlst.org/paeruginosa/).

WGS and bioinformatics

Total DNA from the FFUP_PS_690 isolate was extracted by the Wizard Genomic DNA Purification Kit (Promega) according to the manufacturer's instructions. The complete nucleotide sequence of FFUP_PS_690 was obtained with the Illumina HiSeq sequencing platform and *de novo* assembly by SPAdes 3.9.0.[14] The quality of the high-throughput sequence data was assessed using FastQC, and the quality of the genome assembly was evaluated by QUAST (http://quast.bioinf.spbau.ru/). SNP-based analysis using the *P. aeruginosa* PAO1 reference genome and closely related strains of the same MLST type was conducted by Geneious version 9.1.8 with default parameters. Automatic annotation of the genome was conducted by NCBI's Prokaryotic Genome Annotation Pipeline.[15] The presence of putative plasmid sequences was inspected by plasmidSPAdes tool from SPAdes 3.9.0. The presence of putative GIs was inspected by the SIGI-HMM method available in IslandViewer 4 (http://www.pathogenomics.sfu.ca/islandviewer/) and the VRprofile web-based tool (http://bioinfo-mml.sjtu.edu.cn/VRprofile/index.php). Antimicrobial resistance genes were identified by ResFinder (https://cge.cbs.dtu.dk/services/ResFinder/) and the RGI software from the CARD database (https://card.mcmaster.ca/home). Individual contigs were manually inspected for the presence of the In1076 integron using BLASTn. Overlapping sequences comprising the genetic environment surrounding the integron were mapped with Geneious version 9.1.8 and Bandage (https://rrwick.github.io/Bandage/). Primer design and PCR mapping was conducted to confirm the orientation of this region, and the coding sequences (CDSs) were predicted using the Glimmer tool available in Geneious version 9.1.8. Manual annotations for this region were assessed with NCBI's BLASTp and Conserved Domain Database (CDD) tools. Easyfig (http://mjsull.github.io/Easyfig/) was used to compare the backbone of different MGEs. Comparative genome analysis was achieved with the CGView Comparison Tool (http://stothard.afns.ualberta.ca/downloads/CCT/), encompassing BLAST-based searches.

Transfer assay of ICE

The conjugation assay was performed as previously described,[16] with minor modifications. Briefly, a spontaneous rifampicin-resistant mutant of *P. aeruginosa* PAO1 was used as recipient strain. Cultures of the donor (FFUP_PS_690) and recipient strains were grown overnight in brain heart infusion (BHI) broth and subsequently mixed in a 1:1 ratio. Cell mixtures were centrifuged for 15 min at 4500 **g**, resuspended in 60 μL of BHI broth and spotted on Mueller–Hinton (MH) agar plates. After a 6 h period of incubation at 37 °C, cells were collected in 1 mL of BHI broth and 100 μL was plated onto selective medium. Transconjugant selection was performed using MH agar plates containing rifampicin (100 mg/L) and imipenem (2 mg/L). MLST analysis of the transconjugant was conducted to confirm the success of the transfer assay.

Nucleotide sequence accession number

This Whole Genome Shotgun project has been deposited at DDBJ/ENA/GenBank under accession number NBEZ00000000. The version described in this paper is version NBEZ01000000. The complete nucleotide sequence of the bla_{GES-6}-harbouring ICE has been submitted to GenBank under accession number KY852375.

Results and discussion

General features and genome-wide analysis of antibiotic resistance and virulence determinants

P. aeruginosa isolate FFUP_PS_690 was non-susceptible to ticarcillin/clavulanic acid (MIC ≥128 mg/L), piperacillin/tazobactam (≥128 mg/L), ceftazidime (≥64 mg/L), cefepime (16 mg/L), aztreonam (16 mg/L), imipenem (≥16 mg/L), meropenem (≥16 mg/L), amikacin (16 mg/L), gentamicin (≥16 mg/L), tobramycin (≥16 mg/L) and ciprofloxacin (≥4 mg/L) and only susceptible to colistin (0.5 mg/L), which is consistent with an XDR phenotype.[17] FFUP_PS_690 belonged to ST235, the high-risk clone with the widest distribution throughout different continents and responsible for the dissemination of horizontally acquired carbapenemases.[1,2]

The draft genome sequence of FFUP_PS_690, composed of 111 contigs, was 6.8 Mb in size, and had a 66.1% GC content and 6314 ORFs. Comparative genome analysis using the CGView Comparison Tool (http://stothard.afns.ualberta.ca/downloads/CCT/) showed that the FFUP_PS_690 genome was highly related to a set of ST235 genomes, namely the complete genome sequences of NCGM 1984, NCGM 1900 and NCGM2.S1 strains (accession numbers AP014646.1, AP014622.1, AP012280.1, respectively), isolated in Japan, the draft genome sequences of RNS_PA1, RNS_PA46 and RNS_PAE05 strains (accession numbers NZ_LSZV00000000.1, NZ_LSZU00000000.1 and NZ_LSZT00000000.1, respectively), isolated in Australia, and the draft genome sequence of the S-Pae strain (accession number NZ_LYLN00000000.1), recovered in France (Figure 1). A bidirectional analysis conducted on the RAST server between this genome and aforementioned strains revealed that around 5700 ORFs shared 100% protein identity. The main differences were observed among phage-associated proteins and other horizontally acquired regions. In fact, phage populations are frequently observed in the ST235 lineage. These findings help to support the hypothesis of geographic-specific infection of this lineage with different phages and subsequent genomic rearrangements.[5]

SNP-based analysis comparing FFUP_PS_690 with the *P. aeruginosa* PAO1 reference genome (accession number NC_002516) revealed a total of 48879 polymorphisms within ORFs, of which 9899 comprised non-synonymous mutations. When comparing FFUP_PS_690 with the aforementioned closely related genomes, these values dropped to 5696, 5772 and 6413 (all variations within ORFs) and 1179, 1356 and 1400 (non-silent mutations within ORFs) for complete genomes of *P. aeruginosa* strains NCGM 1900, NCGM 1984 and NCGM2.S1, respectively. Specific SNPs lead to amino acid changes in antibiotic resistance determinants (Table 1). Sequence analysis of the *oprD* gene revealed the occurrence of a 10 bp deletion when compared with WT *oprD* from PAO1, causing a frameshift mutation that led to a premature stop codon and which most likely contributed to the high imipenem MIC (≥16 mg/L) observed.[18] Missense mutations in peptidoglycan-recycling genes (*dacB*, *ampR*, *ampD* and its homologues *ampDh2* and *ampDh3*) were also inspected. The double mutant *ampD ampDh3* (PAΔDDh3) was previously linked to an increase in basal and induced AmpC levels.[19] These mutations may lead to the

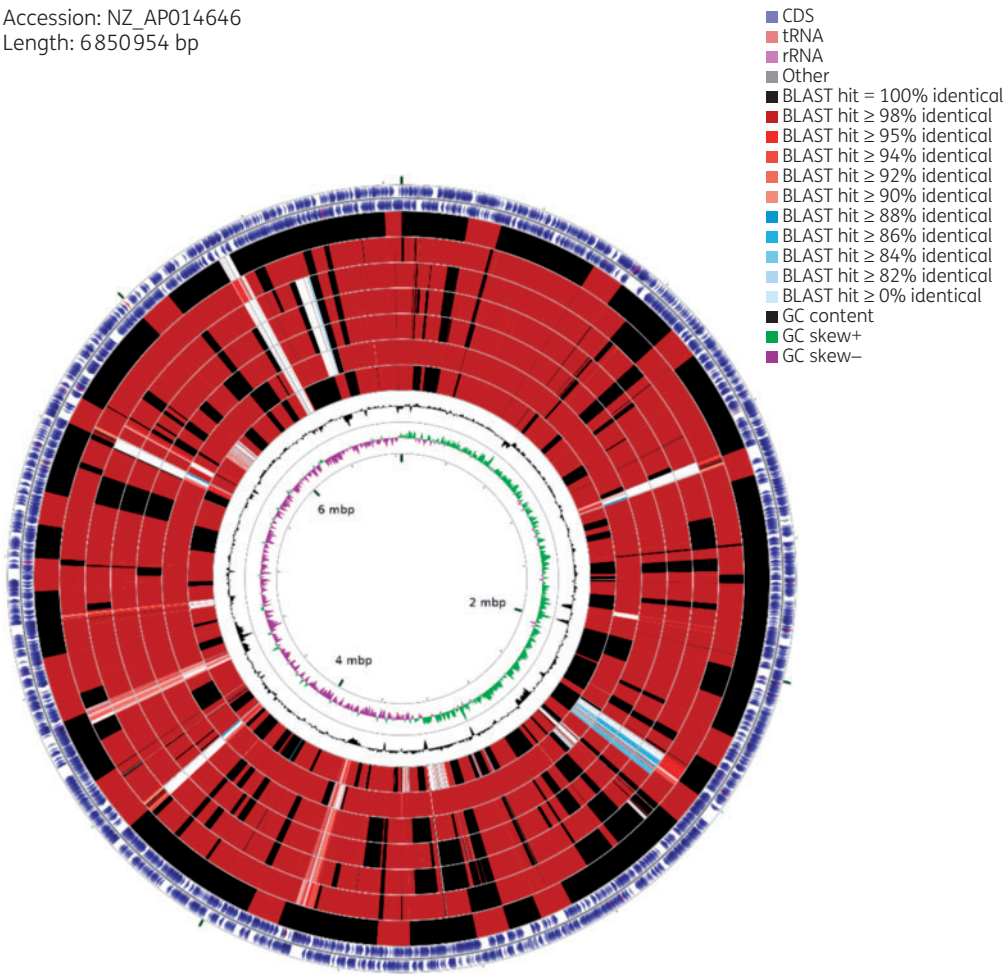


Figure 1. Sequence-based alignment of *P. aeruginosa* ST235 genomes, using the NCGM_1984 strain (accession number NZ_AP014646) as mentioned in the top left corner as the reference genome and NCGM 1900, FFUP_PS_690, RNS_PA1, RNS_PA46, RNS_PAE05, NCGM2.S1 and S-Pae (from the outermost to the innermost circle, respectively) strains as comparison organisms. Different colours are attributed to a range of nucleotide BLAST hits, as specified in the figure key. The outermost arrows and arrowheads represent specific features of the reference genome, as specified in the figure key. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

overproduction of the AmpC variant reported here (PDC-35) (Table 1), helping to decrease the susceptibility of this isolate to cephalosporins.[20] No missense mutations were observed in AmpDh2 and DacB. The mutations reported for AmpR (Table 1) were previously described,[18] but not related to enhanced resistance. T83I and D87Y mutations in the QRDR of GyrA plus an S87L alteration in ParC were identified (Table 1) and may help to explain the MIC of ciprofloxacin (≥4 mg/L) observed here.[18]

Twenty-three GI-like regions were identified in FFUP_PS_690. Of these, two presented important features related to antibiotic resistance and virulence, which will be described below.

An ICE location for the bla$_{GES-6}$ *carbapenemase gene*

The *bla*$_{GES-6}$ gene was located in an In1076 integron, as previously described (Figures 2 and 3 and Table S1, available as Supplementary data at *JAC* Online),[9] and was associated with a Tn*402*-like structure. The typical 25 bp initial IR of this transposon was found downstream of the *intI1* gene. However, the terminal IR of Tn*402* was not identified in the 3′ CS region, suggesting the occurrence of genetic rearrangements. The In1076 integron was here associated with a putative GI, being 86203 bp in size and comprising 91 coding sequences with a 63.1% GC content (Figure 2). This GC value is slightly lower than the one observed for the genome of FFUP_PS_690 (66.1%), suggesting a different origin for this MGE. PCR mapping revealed that the same genetic structure was present in the previously described *P. aeruginosa* CB1 clinical isolate, which was the first report of *bla*$_{GES-6}$ in this species.[9]

Since the definition of a GI embraces any cluster of genes that has been horizontally acquired, other MGEs such as conjugative transposons, prophages and ICEs are included in this definition.[21] ICEs are self-transmissible mosaics that encode their own integration/excision, conjugation, regulation and accessory modules.[3,4,6,22,23] These elements seem to be the most prevalent conjugative elements in all prokaryotic clades.[23]

Table 1. Non-silent SNPs related to antimicrobial resistance in the genome sequence of *P. aeruginosa* FFUP_PS_690

PAO1 locus	Gene	CDS annotation	CDS position	PAO1 nt	Isolate 690 SNP	AA position	PAO1 AA	FFUP_PS_690 AA	NCGM 1900 AA	RNS_PA46 AA	S-Pae AA
PA4110	*ampC*	β-lactamase	290	C	T	97	A	V	V	V	V
			313	A	G	105	T	A	A	A	A
			613	G	C	205	V	L	L	L	L
			1172	G	C	391	G	A	A	A	A
PA4522	*ampD*	*N*-acetyl-anhydromuramyl-L-alanine amidase	29	A	C	10	V	G	V	V	V
			443	C	G	148	G	A	A	A	A
PA0807	*ampDh3*	protein AmpDh3	623	C	T	208	A	V	V	V	V
PA4109	*ampR*	transcriptional regulator AmpR	848	C	T	283	G	E	E	E	E
			863	A	C	288	M	R	R	R	R
PA3168	*gyrA*	DNA gyrase subunit A	248	G	A	83	T	I	I	I	I
			259	C	A	87	D	Y	D	D	D
PA4964	*parC*	DNA topoisomerase IV subunit A	260	G	A	87	S	L	L	L	L
PA5514	bla_{OXA-50}	β-lactamase	46	A	G	16	T	A	A	A	A
			74	A	G	25	Q	R	R	R	R

AA, amino acid.

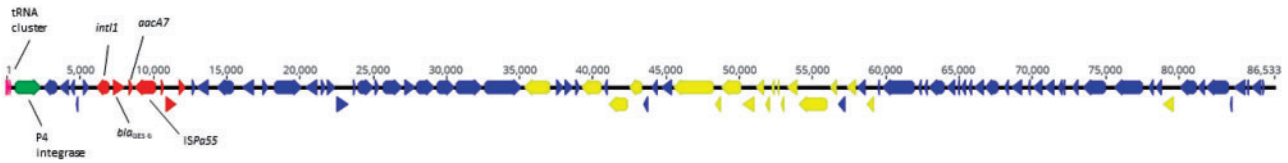


Figure 2. Schematic representation of ICE*Pae690*. Pink arrowheads highlight the tRNA cluster. The gene encoding the P4 integrase is represented by a green arrow. The different ORFs that comprise the In1076 integron are presented in red. Yellow arrows and arrowheads are related to the conjugative transfer module. The remaining ORFs of the ICE are represented by blue arrows and arrowheads. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

The MGE identified here is an ICE, hereby named ICE*Pae690*. A keyword search for 'integrative conjugative elements AND beta-lactamase' produced 21 hits in PubMed (latest search performed on 5 May 2017). Of these, only two reports were associated with carbapenemases: a bla_{GES-5}-harbouring GI2, identified in *P. aeruginosa* draft genomes from Australia and also belonging to ST235;[5] and an ICE_{Tn4371}6061 with bla_{SPM-1}, frequently observed in ST277 *P. aeruginosa* isolates from Brazil.[24] Curiously, these reports are quite recent, which highlights the importance of WGS projects and subsequent bioinformatic analysis in the correct identification of these MGEs. The ICE*Pae690* backbone was unrelated to the one from GI2 and ICE_{Tn4371}6061, supporting the high genome plasticity frequently observed in ICEs.[6]

ICE*Pae690* was very similar to a fragment of the 197350 bp PAGI-13 sequence from *P. aeruginosa* strain CCBH4851 identified in Brazil (accession number KT454971.1),[25] exhibiting 93% query cover and 99% nucleotide identity (Figure 3). PAGI-13 harbours a 16S rRNA methylase (*rmtD*) and the In163 integron (carrying *aacA4*, bla_{OXA-56} and *aadA7*).[25] A pairwise comparison revealed that 77 out of the 91 CDSs from ICE*Pae690* shared 100% protein identity with the ones from PAGI-13, comprising ICE proteins and DNA repair proteins. The main differences reside in the integron structure and ORFs encoding hypothetical proteins. Integrases were highly related, comprising a single L430P shift in ICE*Pae690*. Interestingly, similar query cover and identity values were observed for several ST277 *P. aeruginosa* isolates from Brazil: PA7790, PA1088, PA11803 and PA8281 (accession numbers CP014999.1, CP015001.1, CP015003.1 and CP015002.1, respectively).[26] It was also somehow related to previously reported GIs from this species: 87% identity and 57%, 54% and 53% query cover for PAGI-2(C) from Germany, and PAGI-15 and PAGI-16 from South Korea (accession numbers AF440523, KX196168.1 and KX196169.1, respectively). Future studies will help to evaluate if the aforementioned elements are in fact ICEs and to assess the real contribution of these MGEs to the dissemination of carbapenemases.

In *P. aeruginosa*, most ICEs fall into two large families: those linked to pKLC102 and the *clc*-like ICEs.[6] ICE*Pae690* is related to the second, exhibiting 88% identity and 61% query cover when compared with ICE*clc* (accession number AJ617740). It was also similar to other *clc*-like ICEs, such as PAGI-2(C) and PAGI-3(SG) (accession numbers AF440523 and AF440524, respectively) (Figure 3).[27] The low query cover observed is typical of the *clc*-like ICEs, since these elements display a conserved portion next to the integration site but harbour unique cargo genes (Figure 3).[6] The 105 bp ICE*clc* element was first reported in *Pseudomonas knackmussii* strain B13 and harbours cargo

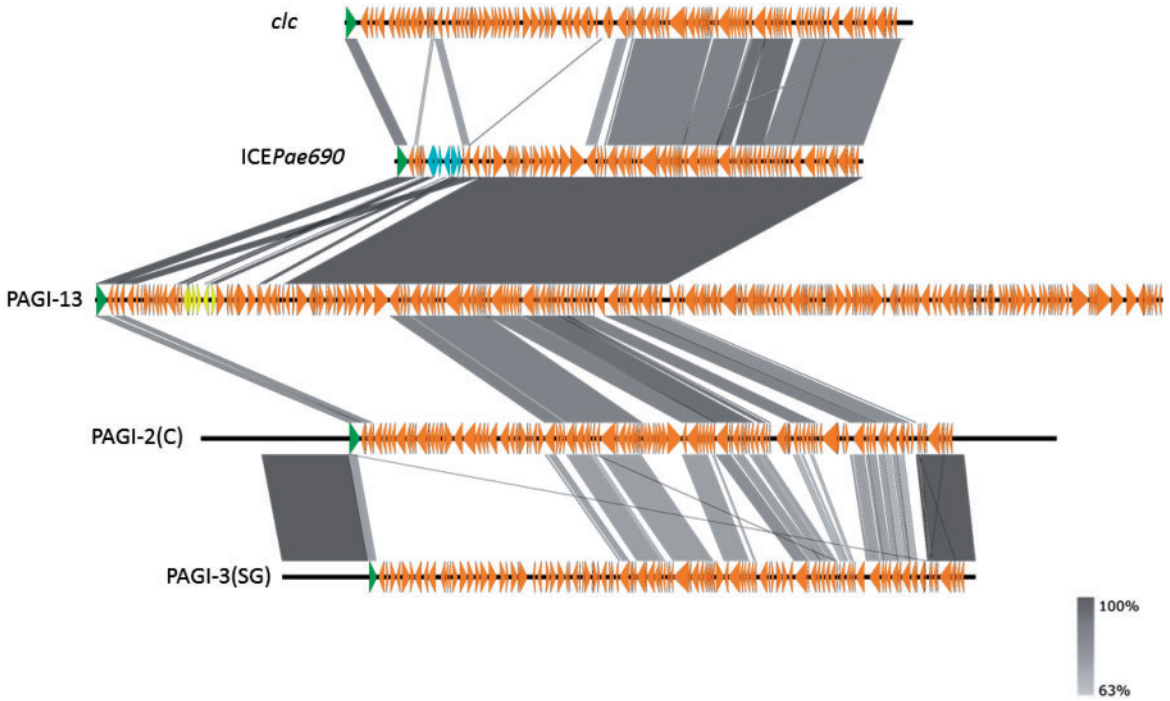


Figure 3. Linear comparison of different MGE backbones related to ICE*Pae690*. CDSs are illustrated by orange arrowheads. The integrase is symbolized by a green arrowhead. The In1076 integron from ICE*Pae690* is highlighted in blue. The In163 integron from PAGI-13 is represented by yellow arrowheads. Different shades of grey point to the nucleotide sequence identity between the different backbones. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

genes responsible for catabolic activities. This element was successfully transferred into γ- and β-proteobacteria.[6]

Besides the In1076 integron, ICE*Pae690* carried genes encoding an integrase, conjugative elements including a relaxase, type IV secretion system (T4SS) proteins, a type IV coupling protein (T4CP) and genes encoding maintenance functions. The relaxase belongs to the TraI-2 superfamily, although unrelated to the TraI nickase/helicase from IncF plasmids.[28] The T4CP is a TraG/TraD family ATPase and is part of the TrwB-AAD-bind superfamily, essential for macromolecular transport and export.[28] The T4SS proteins reported here are related to the TraU, TraG-N, TraC_F_IV and TraK superfamilies and are associated with conjugal DNA transfer and assembly of the pilus filament.[28] A putative *oriT* site was bioinformatically identified upstream of the relaxase gene.[21] The complete machinery for self-conjugation is in accordance with the finding that ICE*Pae690* was successfully transferred to a spontaneous rifampicin-resistant mutant of *P. aeruginosa* PAO1. Screening for bla_{GES} and its genetic environment by PCR gave positive results for the transconjugant, here named *P. aeruginosa* PAO1 (ICE*Pae690*), which exhibited an MIC value of imipenem of 12 mg/L. The MIC values of imipenem for the donor strain were >32 mg/L, while for the recipient strain the value dropped to 1 mg/L. MLST analysis confirmed the success of the transfer to *P. aeruginosa* PAO1.

ICE*Pae690* was integrated next to a $tRNA^{Gly}$ gene. This gene is part of a cluster comprising one $tRNA^{Glu}$ gene and two tandem $tRNA^{Gly}$ genes.[27] Several 16 bp DRs (5′-CTCGTTTCCCGCTCCA), similar to the *attL* and *attR* sites found for *clc*-like ICEs, were reported flanking ICE*Pae690*.[27] These two *att* sites result from site-specific recombination between the attachment sites on the ICE (*attP*) and in the bacterial host (*attB*).[3,4,22] Next to the $tRNA^{Gly}$ gene, a site-specific recombinase of the bacteriophage P4 integrase subfamily was identified. P4-like integrases are typically found next to tRNA genes in temperate phages, integrative plasmids and GIs from different Gram-negative bacteria, emphasizing the interchangeable potential of functional modules between different MGEs along their evolutionary history.[3,4,6,22,23] This integrase is essential for both integration and excision of the MGEs,[3,4,6,22,23] and was related to the integrase from the ICE*clc*-element, exhibiting 87% amino acid identity.

A GI location for the exoU gene

The genome of FFUP_PS_690 encodes a variety of toxins of the type III secretion system (T3SS). The T3SS is a needle-like structure that injects effectors directly into the host cell.[29,30] Most *P. aeruginosa* strains harbour a combination of three toxins, among which the *exoT* and *exoY* genes are the most conserved.[29,30] The third effector is either *exoS* or *exoU*, which are almost mutually exclusive.[25,30] The reason for this incompatibility within the same genome remains unknown. The *exoS*+/*exoU*− genotype is frequently related to an invasive phenotype, while *exoS*−/*exoU*+ is linked to a highly cytotoxic scenario.[30]

A pathogenicity GI was here identified and exhibited 99% nucleotide similarity and 100% query cover with the ExoU island A from *P. aeruginosa* strain 6077.[29] The *exoU* gene was flanked by *spcU*, encoding its chaperone, and a putative transposase. ExoU is a potent cytotoxin with phospholipase activity, which is frequently related to nosocomial-acquired pneumonia, ear infections and

keratitis.[5,29–31] During acute pneumonia, ExoU is injected into and impairs alveolar macrophages and phagocytes, compromising the host defence mechanisms and helping bacteria to survive in this environment.[31] The *exoS*−/*exoU*+ genotype is related to specific clonal lineages, such as ST235.[30] Interestingly, *in silico* analysis revealed the presence of ExoU island A at the same chromosomal location on the genome of the aforementioned ST235 strains NCGM 1900, NCGM 1984 and NCGM2.S1, reflecting a preference of these islands for the typical *attB* sequence of the $tRNA^{Lys}$ gene from the host. The *exoU* gene is less prevalent than other T3SS effector genes in the *P. aeruginosa* genome, which may be explained by its recent acquisition via horizontal transmission into particular lineages.[29,32]

Conclusions

WGS projects and bioinformatic analysis have helped to shed new light on ICE-mediated genome plasticity. The presence of 'hitchhiking' genetic elements, such as the bla_{GES-6}-harbouring In1076 integron in a putative ICE, signals its potential spread through conjugation and/or clonal expansion of the ST235 high-risk clone. Since ICEs are part of most clades of bacteria and represent the most abundant conjugative element in prokaryotes, more studies are mandatory to access the real contribution of these MGEs to the horizontal transfer of antibiotic resistance determinants.

Acknowledgements

The isolate was kindly provided by Dr Carlos da Silva Torres (Medicina Laboratorial Porto, Portugal).

Funding

This work received financial support from the European Union (FEDER funds POCI/01/0145/FEDER/007728) and National Funds (FCT/MEC, Fundação para a Ciência e Tecnologia and Ministério da Educação e Ciência) under the Partnership Agreement PT2020 UID/MULTI/04378/2013. J. B. and F. G. were supported by grants from Fundação para a Ciência e a Tecnologia (SFRH/BD/104095/2014 and SFRH/BPD/95556/2013, respectively).

Transparency declarations

None to declare.

Supplementary data

Table S1 appears as Supplementary data at *JAC* Online.

References

1 Potron A, Poirel L, Nordmann P. Emerging broad-spectrum resistance in *Pseudomonas aeruginosa* and *Acinetobacter baumannii*: mechanisms and epidemiology. *Int J Antimicrob Agents* 2015; **45**: 568–85.

2 Oliver A, Mulet X, Lopez-Causape C *et al*. The increasing threat of *Pseudomonas aeruginosa* high-risk clones. *Drug Resist Updat* 2015; **21-22**: 41–59.

3 Johnson CM, Grossman AD. Integrative and conjugative elements (ICEs): what they do and how they work. *Annu Rev Genet* 2015; **49**: 577–601.

4 Wozniak RA, Waldor MK. Integrative and conjugative elements: mosaic mobile genetic elements enabling dynamic lateral gene flow. *Nat Rev Microbiol* 2010; **8**: 552–63.

5 Roy Chowdhury P, Scott M, Worden P *et al*. Genomic islands 1 and 2 play key roles in the evolution of extensively drug-resistant ST235 isolates of *Pseudomonas aeruginosa*. *Open Biol* 2016; **6**: 150175.

6 Kung VL, Ozer EA, Hauser AR. The accessory genome of *Pseudomonas aeruginosa*. *Microbiol Mol Biol Rev* 2010; **74**: 621–41.

7 Botelho J, Grosso F, Peixe L. Characterization of the plasmid pJB12 from *Pseudomonas aeruginosa* reveals Tn*6352*, a novel putative transposon associated with mobilization of bla_{VIM-2}-harboring In58 integron. *Antimicrob Agents Chemother* 2017; **61**: e02532-16.

8 Botelho J, Grosso F, Quinteira S *et al*. The complete nucleotide sequence of an IncP-2 megaplasmid unveils a mosaic architecture comprising a putative novel bla_{VIM-2}-harbouring transposon in *Pseudomonas aeruginosa*. *J Antimicrob Chemother* 2017; **72**: 2225–9.

9 Botelho J, Grosso F, Sousa C *et al*. Characterization of a new genetic environment associated with GES-6 carbapenemase from a *Pseudomonas aeruginosa* isolate belonging to the high-risk clone ST235. *J Antimicrob Chemother* 2015; **70**: 615–7.

10 Poirel L, Bonnin RA, Nordmann P. Genetic support and diversity of acquired extended-spectrum β-lactamases in Gram-negative rods. *Infect Genet Evol* 2012; **12**: 883–93.

11 Cuzon G, Bogaerts P, Bauraing C *et al*. Spread of plasmids carrying multiple GES variants. *Antimicrob Agents Chemother* 2016; **60**: 5040–3.

12 Vourli S, Giakkoupi P, Miriagou V *et al*. Novel GES/IBC extended-spectrum β-lactamase variants with carbapenemase activity in clinical enterobacteria. *FEMS Microbiol Lett* 2004; **234**: 209–13.

13 Mulet M, Lalucat J, Garcia-Valdes E. DNA sequence-based analysis of the *Pseudomonas* species. *Environ Microbiol* 2010; **12**: 1513–30.

14 Bankevich A, Nurk S, Antipov D *et al*. SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. *J Comput Biol* 2012; **19**: 455–77.

15 Tatusova T, DiCuccio M, Badretdin A *et al*. NCBI prokaryotic genome annotation pipeline. *Nucleic Acids Res* 2016; **44**: 6614–24.

16 Michael GB, Kadlec K, Sweeney MT *et al*. ICE*Pmu1*, an integrative conjugative element (ICE) of *Pasteurella multocida*: structure and transfer. *J Antimicrob Chemother* 2012; **67**: 91–100.

17 Magiorakos AP, Srinivasan A, Carey RB *et al*. Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria—an international expert proposal for interim standard definitions for acquired resistance. *Clin Microbiol Infect* 2012; **18**: 268–81.

18 Cabot G, Ocampo-Sosa AA, Dominguez MA *et al*. Genetic markers of widespread extensively drug-resistant *Pseudomonas aeruginosa* high-risk clones. *Antimicrob Agents Chemother* 2012; **56**: 6349–57.

19 Juan C, Moya B, Perez JL *et al*. Stepwise upregulation of the *Pseudomonas aeruginosa* chromosomal cephalosporinase conferring high-level β-lactam resistance involves three AmpD homologues. *Antimicrob Agents Chemother* 2006; **50**: 1780–7.

20 Rodriguez-Martinez JM, Poirel L, Nordmann P. Extended-spectrum cephalosporinases in *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother* 2009; **53**: 1766–71.

21 Langille MG, Hsiao WW, Brinkman FS. Detecting genomic islands using bioinformatics approaches. *Nat Rev Microbiol* 2010; **8**: 373–82.

22 Bellanger X, Payot S, Leblond-Bourget N *et al*. Conjugative and mobilizable genomic islands in bacteria: evolution and diversity. *FEMS Microbiol Rev* 2014; **38**: 720–60.

23 Guglielmini J, Quintais L, Garcillan-Barcia MP *et al*. The repertoire of ICE in prokaryotes underscores the unity, diversity, and ubiquity of conjugation. *PLoS Genet* 2011; **7**: e1002222.

24 Fonseca EL, Marin MA, Encinas F *et al*. Full characterization of the integrative and conjugative element carrying the metallo-β-lactamase *bla*$_{SPM-1}$ and bicyclomycin *bcr1* resistance genes found in the pandemic *Pseudomonas aeruginosa* clone SP/ST277. *J Antimicrob Chemother* 2015; **70**: 2547–50.

25 Silveira MC, Albano RM, Asensi MD *et al*. Description of genomic islands associated to the multidrug-resistant *Pseudomonas aeruginosa* clone ST277. *Infect Genet Evol* 2016; **42**: 60–5.

26 Nascimento AP, Ortiz MF, Martins WM *et al*. Intraclonal genome stability of the metallo-β-lactamase SPM-1-producing *Pseudomonas aeruginosa* ST277, an endemic clone disseminated in Brazilian hospitals. *Front Microbiol* 2016; **7**: 1946.

27 Larbig KD, Christmann A, Johann A *et al*. Gene islands integrated into tRNA^{Gly} genes confer genome diversity on a *Pseudomonas aeruginosa* clone. *J Bacteriol* 2002; **184**: 6665–80.

28 Smillie C, Garcillan-Barcia MP, Francia MV *et al*. Mobility of plasmids. *Microbiol Mol Biol Rev* 2010; **74**: 434–52.

29 Kulasekara BR, Kulasekara HD, Wolfgang MC *et al*. Acquisition and evolution of the *exoU* locus in *Pseudomonas aeruginosa*. *J Bacteriol* 2006; **188**: 4037–50.

30 Juan C, Pena C, Oliver A. Host and pathogen biomarkers for severe *Pseudomonas aeruginosa* infections. *J Infect Dis* 2017; **215** Suppl 1: S44–51.

31 Diaz MH, Hauser AR. *Pseudomonas aeruginosa* cytotoxin ExoU is injected into phagocytic cells during acute pneumonia. *Infect Immun* 2010; **78**: 1447–56.

32 Petit SM, Lavenir R, Colinon-Dupuich C *et al*. Lagooning of wastewaters favors dissemination of clinically relevant *Pseudomonas aeruginosa*. *Res Microbiol* 2013; **164**: 856–66.

Table S1 - ORFs identified in ICE*Pae*690 and respective predictive annotated functions.

ORF	Start	End	Length	Direction	product
ICEPae690.1	541	2484	1944	Forward	Integrase
ICEPae690.2	2522	3490	969	Reverse	Putative peroxidase
ICEPae690.3	3609	4346	738	Reverse	LysR family transcriptional regulator
ICEPae690.4	4358	4753	396	Reverse	DNA-binding protein
ICEPae690.5	4750	5001	252	Reverse	toxin-antitoxin system antitoxin component AbrB family
ICEPae690.6	5183	5749	567	Forward	Invertase
intI1	6097	7110	1014	Reverse	IntI1 class I integrase
bla$_{GES-6}$	7293	8156	864	Forward	Carbapenem-hydrolyzing class A beta-lactamase GES-6
aacA7	8280	8738	459	Forward	Aminoglycoside N-acetyltransferase AAC(6')-Il
ICEPae690.10	9113	10210	1098	Reverse	Transposase of ISPa55
qacEΔ1	10512	10859	348	Forward	QacEdelta1 multidrug efflux protein
sul1	10853	11692	840	Forward	Sul1 sulfonamide-resistant dihydropteroate synthase
orf5	11724	12320	597	Forward	Orf5 acetyltransferase
ICEPae690.14	12656	12949	294	Forward	Transcriptional regulator
ICEPae690.15	12972	13862	891	Reverse	LysR family transcriptional regulator
ICEPae690.16	14366	15580	1215	Reverse	Aldehyde dismutase
ICEPae690.17	16047	16988	942	Reverse	LysR family transcriptional regulator
ICEPae690.18	17481	17912	432	Forward	Hypothetical protein
ICEPae690.19	18287	20263	1977	Forward	Excinuclease ABC subunit B
ICEPae690.20	20414	21319	906	Reverse	LysR family transcriptional regulator
ICEPae690.21	21474	21881	408	Forward	Isopropylmalate/homocitrate/citramalate synthase
ICEPae690.22	21883	22491	609	Forward	Hypothetical protein
ICEPae690.23	22488	23378	891	Forward	Nitrilase
ICEPae690.24	23661	23936	276	Forward	Regulator
ICEPae690.25	23996	25105	1110	Forward	S-(hydroxymethyl)glutathione dehydrogenase/classIII alcohol dehydrogenase
ICEPae690.26	25149	25547	399	Forward	Glyoxalase
ICEPae690.27	25730	27064	1335	Forward	Hypothetical protein
ICEPae690.28	27123	27953	831	Forward	S-formylglutathione hydrolase
ICEPae690.29	27972	29279	1308	Forward	NADH dehydrogenase
ICEPae690.30	29331	30644	1314	Forward	Recombination factor protein RarA
ICEPae690.31	30705	32570	1866	Forward	Sodium:proton antiporter
ICEPae690.32	32596	35247	2652	Forward	Excinuclease ABC subunit A
ICEPae690.33	35328	37166	1839	Reverse	Integrating conjugative element relaxase
ICEPae690.34	37484	38110	627	Forward	Hypothetical protein
ICEPae690.35	38123	38755	633	Forward	Hypothetical protein
ICEPae690.36	38840	39205	366	Forward	Hypothetical protein

ICEPae690.37	39217	40737	1521	Reverse	TraG-like protein
ICEPae690.38	40753	41109	357	Reverse	Hypothetical protein
ICEPae690.39	41106	42500	1395	Reverse	Integrating conjugative element protein
ICEPae690.40	42510	43457	948	Reverse	TraU protein
ICEPae690.41	43454	43900	447	Reverse	Hypothetical protein
ICEPae690.42	44062	44556	495	Reverse	DNA repair protein RadC
ICEPae690.43	44732	45496	765	Reverse	Disulfide bond formation protein DsbA
ICEPae690.44	45510	48377	2868	Reverse	Conjugative transfer ATPase
ICEPae690.45	48377	48817	441	Reverse	Conjugal transfer protein
ICEPae690.46	48798	50216	1419	Reverse	Integrating conjugative element protein
ICEPae690.47	50206	51111	906	Reverse	Integrating conjugative element protein
ICEPae690.48	51108	51797	690	Reverse	Integrating conjugative element protein
ICEPae690.49	51794	52192	399	Reverse	Conjugative transfer region protein
ICEPae690.50	52204	52563	360	Reverse	Integrating conjugative element membrane protein
ICEPae690.51	52580	52813	234	Reverse	Integrating conjugative element protein
ICEPae690.52	52810	53193	384	Reverse	Integrative conjugative element protein
ICEPae690.53	53292	54041	750	Reverse	Integrating conjugative element membrane protein
ICEPae690.54	54038	56101	2064	Reverse	Conjugative coupling factor TraD PFGI-1 class
ICEPae690.55	56232	56771	540	Reverse	Integrating conjugative element protein
ICEPae690.56	56768	57373	606	Reverse	Lytic transglycosylase
ICEPae690.57	57355	58080	726	Reverse	Integrating conjugative element protein
ICEPae690.58	58095	58736	642	Reverse	Chromosome segregation protein SMC
ICEPae690.59	58733	59332	600	Reverse	Integrating conjugative element protein pill pfgi-1
ICEPae690.60	59480	59686	207	Reverse	Hypothetical protein
ICEPae690.61	59802	62081	2280	Reverse	DEAD/DEAH box helicase
ICEPae690.62	62217	62522	306	Reverse	Hypothetical protein
ICEPae690.63	62613	62933	321	Reverse	Hypothetical protein
ICEPae690.64	62985	64094	1110	Reverse	SAM-dependent methyltransferase
ICEPae690.65	64159	64806	648	Reverse	Hypothetical protein
ICEPae690.66	64883	65143	261	Reverse	Hypothetical protein
ICEPae690.67	65160	65567	408	Reverse	Hypothetical protein
ICEPae690.68	65670	65993	324	Reverse	Hypothetical protein
ICEPae690.69	66088	66777	690	Reverse	Hypothetical protein
ICEPae690.70	66835	67752	918	Reverse	Hypothetical protein
ICEPae690.71	68121	68474	354	Reverse	Hypothetical protein
ICEPae690.72	68599	69411	813	Reverse	Hypothetical protein
ICEPae690.73	69700	69978	279	Reverse	Hypothetical protein
ICEPae690.74	70076	70813	738	Reverse	Hypothetical protein
ICEPae690.75	70894	71580	687	Reverse	Hypothetical protein
ICEPae690.76	71754	72146	393	Reverse	Hypothetical protein
ICEPae690.77	72168	72380	213	Reverse	Hypothetical protein
ICEPae690.78	72712	73269	558	Reverse	Hypothetical protein

ICEPae690.79	73512	75119	1608	Reverse	DNA methyltransferase
ICEPae690.80	75639	77651	2013	Reverse	DNA topoisomerase III
ICEPae690.81	77930	78370	441	Reverse	Single-stranded DNA-binding protein
ICEPae690.82	78443	78970	528	Reverse	Hypothetical protein
ICEPae690.83	78967	79746	780	Reverse	Integrating conjugative element protein
ICEPae690.84	80065	81291	1227	Reverse	Hypothetical protein
ICEPae690.85	81295	81855	561	Reverse	Hypothetical protein
ICEPae690.86	81871	83520	1650	Reverse	Hypothetical protein
ICEPae690.87	83513	83761	249	Reverse	Hypothetical protein
ICEPae690.88	83745	84620	876	Reverse	Cobyrinic acid ac-diamide synthase
ICEPae690.89	84663	84875	213	Reverse	Phage transcriptional regulator AlpA
ICEPae690.90	84994	85746	753	Reverse	Hypothetical protein
ICEPae690.91	85776	85931	156	Forward	Hypothetical protein

Carbapenemases on the move: it's good to be on ICE

João Botelho[1*], Adam P. Roberts[2,3], Ricardo León-Sampedro[4,5], Filipa Grosso[1], Luísa Peixe[1*]

Running Title: Integrative conjugative elements in *Pseudomonas* spp.

[1]UCIBIO/REQUIMTE, Laboratório de Microbiologia, Faculdade de Farmácia, Universidade do Porto, Porto, Portugal.

[2]Department of Parasitology, Liverpool School of Tropical Medicine, Liverpool, United Kingdom.

[3]Research Centre for Drugs and Diagnostics, Liverpool School of Tropical Medicine, Liverpool, United Kingdom.

[4]Department of Microbiology, University Hospital Ramón y Cajal, Ramón y Cajal Health Research Institute (IRYCIS), Madrid, Spain.

[5]Biomedical Research Networking Center for Epidemiology and Public Health (CIBER-ESP), Madrid, Spain.

*Addresses for correspondence: João Botelho, Laboratório de Microbiologia. Faculdade de Farmácia da Universidade do Porto, Rua Jorge Viterbo Ferreira nº 228, 4050-313 Porto, Portugal; phone: +351 220428500; e-mail: joaobotelho9@hotmail.com; Luísa Peixe, Laboratório de Microbiologia. Faculdade de Farmácia da Universidade do Porto, Rua Jorge Viterbo Ferreira nº 228, 4050-313 Porto, Portugal; phone: +351 220428500; e-mail: lpeixe@ff.up.pt.

Abstract

The evolution and spread of antibiotic resistance is often mediated by mobile genetic elements. Integrative and conjugative elements (ICEs) are the most abundant conjugative elements among prokaryotes. However, the contribution of ICEs to horizontal gene transfer of antibiotic resistance has been largely unexplored. Here we report that ICEs belonging to mating-pair formation (MPF) classes G and T are highly prevalent among the opportunistic pathogen *Pseudomonas aeruginosa*, contributing to the spread of carbapenemase-encoding genes (CEGs). Most CEGs of the MPF_G class were encoded within class I integrons, which co-harbour genes conferring resistance to other antibiotics. The majority of the integrons were located within Tn*3*-like and composite transposons. A conserved attachment site could be predicted for the MPF_G class ICEs. MPF_T class ICEs carried the CEGs within composite transposons which were not associated with integrons. The data presented here provides a global snapshot of the different CEG-harbouring ICEs and sheds light on the underappreciated contribution of these elements for the evolution and dissemination of antibiotic resistance on *P. aeruginosa*.

Introduction

Among the non-fermenting Gram-negative bacteria, the *Pseudomonas* genus is the one with the highest number of species [1, 2]. *Pseudomonas aeruginosa*, an opportunistic human pathogen associated with an ever-widening array of life-threatening acute and chronic infections, is the most clinically relevant species within this genus [3–5]. *P. aeruginosa* is one of the CDC "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 [6]. *P. aeruginosa* can develop resistance to a wide range of antibiotics due to a combination of intrinsic, adaptive, and acquired resistance mechanisms, such as the reduction of its outer membrane permeability, over-expression of constitutive or inducible efflux pumps, overproduction of AmpC cephalosporinase, and the acquisition of antibiotic resistance genes (ARGs) through horizontal gene transfer (HGT) [4, 7, 8]. *P. aeruginosa* has a non-clonal population structure, punctuated by specific sequence types (STs) that are globally disseminated and frequently linked to the dissemination of ARGs [4, 9]. These STs have been designated as high-risk clones, of which major examples are ST111, ST175, ST235 and ST244.

Due to its high importance for human medicine, carbapenems are considered by the World Health Organization (WHO) as Critically-Important Antimicrobials that should be reserved for the treatment of human infections caused by MDR Gram-negative bacteria [10], such as *P. aeruginosa*. Carbapenem-resistant *P. aeruginosa* is in the "critical" category of the WHO's priority list of bacterial pathogens for which research and development into new antibiotics is urgently required [11]. Besides *P. aeruginosa*, carbapenem resistance has been reported in

other *Pseudomonas* spp. and is often mediated by the acquisition of carbapenemase-encoding genes (CEGs) [12–14]. Carbapenemases are able to hydrolyse carbapenems and confer resistance to virtually all ß-lactam antibiotics [15]. When it comes to the *Pseudomonas* genus, CEGs are mostly present on class I integrons within the chromosome [4]. Class I integrons are genetic elements that carry ARGs and an integrase gene, which controls integration and excision of genes [16–18] . Mobile genetic elements (MGEs) such as transposons, plasmids and integrative and conjugative elements (ICEs), are responsible for the spread of ARGs [19–23]. The phage-inducible chromosomal islands are a recently reported family of MGEs, but unrelated to the carriage of ARGs [24].

Usually, the genes acquired by HGT are integrated in common hotspots in the host's chromosome, comprising a cluster of genes designated by genomic islands (GIs) [19, 25, 26]. This broad definition may also encompass other MGEs, such as ICEs and prophages. Although the exact origin of these elements remains unknown, a growing body of evidence shows that phages are one of the likely major ancestors of ICEs [27] [28]. ICEs are self-transmissible mosaic and modular MGEs that combine features of transposons and phages (ICEs can integrate into and excise from the chromosome), and plasmids (ICEs can also exist as circular extrachromosomal elements, replicate autonomously and be transferred by conjugation) [21, 25, 29–31]. Integrative and mobilizable elements (IMEs) encode their own integration and excision systems, but take advantage of the conjugation machinery of co-resident conjugative elements to be successfully transferred [32]. ICEs usually replicate as part of the host genome and are vertically inherited, remaining quiescent, and with most mobility genes repressed [33, 34]. These elements also encode recombinases related to those in phages and other transposable elements. Conjugation involves three mandatory components: a relaxase (MOB), a T4SS and a type-IV coupling protein (T4CP) [35, 36]. Four mating-pair formation

(MPF) classes cover the T4SS among Proteobacteria: MPF_T, MPF_G, MPF_F and MPF_I [37]. 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*. 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 their evolutionary history [38]. A close interplay between these elements in the ancient clades of the phylogenetic tree was observed, suggesting that plasmids may behave like ICEs and vice-versa, reinforcing the common assumption that the line separating ICEs and conjugative plasmids is blurring [30, 39]. These authors also searched more than 1000 genomes and found that ICEs are present in most bacterial clades and are more prevalent than conjugative plasmids [38]. It was also observed that the larger the genome, the higher the likelihood to harbour a conjugative element at a given moment, which supports the common assumption that bacteria with large genomes are more prone to acquire genes by HGT [40, 41].

Delimiting ICEs in genomic data remains particularly challenging [26]. Some signatures features are frequently observed, such as a sporadic distribution, sequence composition bias, insertion next to or within a tRNA gene, bordering attachment (*att*) sites and over-representation of mobility genes of the type-IV secretion system (T4SS). However, some ICEs present atypical features and may not be detected by these approaches [26, 40]. In *P. aeruginosa*, most ICEs fall into three large families: the ICE*clc*, pKLC102 and Tn*4371*. The PAGI2(C), PAGI3(SG), PAGI-13, PAGI-15 and PAGI-16 were previously described as members of the ICE*clc* family, while the PAPI-1, PAPI-2, PAGI-4 and PAGI-5 were linked to the pKLC102 family [19]. The ICE_{Tn4371} family also represents a large group of ICEs with a common backbone

and which are widely distributed, such as in *P. aeruginosa* UCBPP-PA14, PA7 and PACS171b strains [21]. These ICEs have been frequently implicated in virulence [42, 43].

Previous reports characterized the complete nucleotide sequence of extra-chromosomal genetic elements housing different CEGs in pseudomonads [20, 44–47]; however, the association of CEGs with chromosome-located MGEs has rarely been investigated [48–50]. Taking into consideration that i) in pseudomonads, CEGs are frequently located within the chromosome, ii) ICEs are the most abundant conjugative elements in prokaryotes and iii) ICEs are more frequently identified in large bacterial genomes, such as in pseudomonads, we hypothesize that ICEs may play a key role in the horizontal spread of CEGs. To investigate this hypothesis, we developed an *in silico* approach to explore the association between ICEs and CEGs in pseudomonads.

Methods

Carbapenemases database

Antimicrobial resistance translated sequences were retrieved from the Bacterial Antimicrobial Resistance Reference Gene Database available on NCBI (ftp://ftp.ncbi.nlm.nih.gov/pathogen/Antimicrobial_resistance/AMRFinder/data/2018-04-16.1/). The resulting 4250 proteins were narrowed down to 695 different carbapenemases to create a binary DIAMOND (v. 0.9.21, https://github.com/bbuchfink/diamond) database [51]. Only the sequences presenting ‘carbapenem-hydrolyzing’ or ‘metallo-beta-lactamase’ on fasta-headers were used to build this local database.

137 **Genome collection and blast search**

138 A total of 4565 *Pseudomonas* genomes was downloaded from NCBI (accessed on the 24th of
139 April, 2018). These genomes were blasted against the local carbapenemase database using
140 the following command: 'diamond blastx –d DB.dmnd –o hits.txt --id 100 --subject-cover 100
141 -f 6 --sensitive'.

142

143 **Bioinformatic prediction of ICEs and genetic environment analyses**

144 The CEG-harbouring *Pseudomonas* genomes were annotated through Prokka v. 1.12
145 (https://github.com/tseemann/prokka) [52]. The translated coding sequences were analysed
146 in TXSScan/CONJscan platform to inspect the presence of ICEs
147 (https://galaxy.pasteur.fr/root?tool_id=toolshed.pasteur.fr%2Frepos%2Fodoppelt%2Fconjsc
148 an%2FConjScan%2F1.0.2) [37]. All ICEs harbouring CEGs predicted by TXSScan/CONJscan
149 were inspected for direct repeats that define the boundaries of the element. The complete
150 nucleotide sequence in Genbank format of corresponding records was imported into
151 Geneious v. 9.1.8 to help delimiting genomic regions flanking the ICEs [53]. Complete ICE
152 sequences were aligned with EasyFig v. 2.2.2 (http://mjsull.github.io/Easyfig/files.html) [54].
153 Screening of complete ICEs for ARGs was achieved by ABRicate v. 0.8
154 (https://github.com/tseemann/abricate). Phage and insertion sequences were inspected
155 through PHASTER (http://phaster.ca/) and ISfinder (https://www-is.biotoul.fr/), respectively
156 [55, 56]. Multiple Antibiotic Resistance Annotator (MARA, http://mara.spokade.com) was
157 used to explore the genetic background of the CEGs [57]. Orthologous assignment and
158 functional annotation of integrase sequences was achieved through EggNOG v. 4.5.1

159 (http://eggnogdb.embl.de/#/app/home) and InterProScan 5
160 (https://www.ebi.ac.uk/interpro/search/sequence-search) [58, 59].

161

162 **Phylogenomics**

163 All CEG-harbouring *P. aeruginosa* genomes were mapped against the *P. aeruginosa* PAO1
164 reference strain (accession number NC_002516.2), to infer a phylogeny based on the
165 concatenated alignment of high quality single nucleotide polymorphisms (SNP) using CSI
166 Phylogeny and standard settings [60]. The phylogenetic tree was plotted using the iTOL
167 platform (https://itol.embl.de/).

168

169 **MLST and taxonomic assignment of unidentified species**

170 To predict the sequence type (ST) of the strains harbouring ICEs, the *P. aeruginosa* MLST
171 website (https://pubmlst.org/paeruginosa/) developed by Keith Jolley and hosted at the
172 University of Oxford was used [61]. Taxonomic assignment of unidentified species carrying
173 ICEs was achieved by JSpeciesWS v. 3.0.17 (http://jspecies.ribohost.com/jspeciesws/#home)
174 [62].

175

176

177

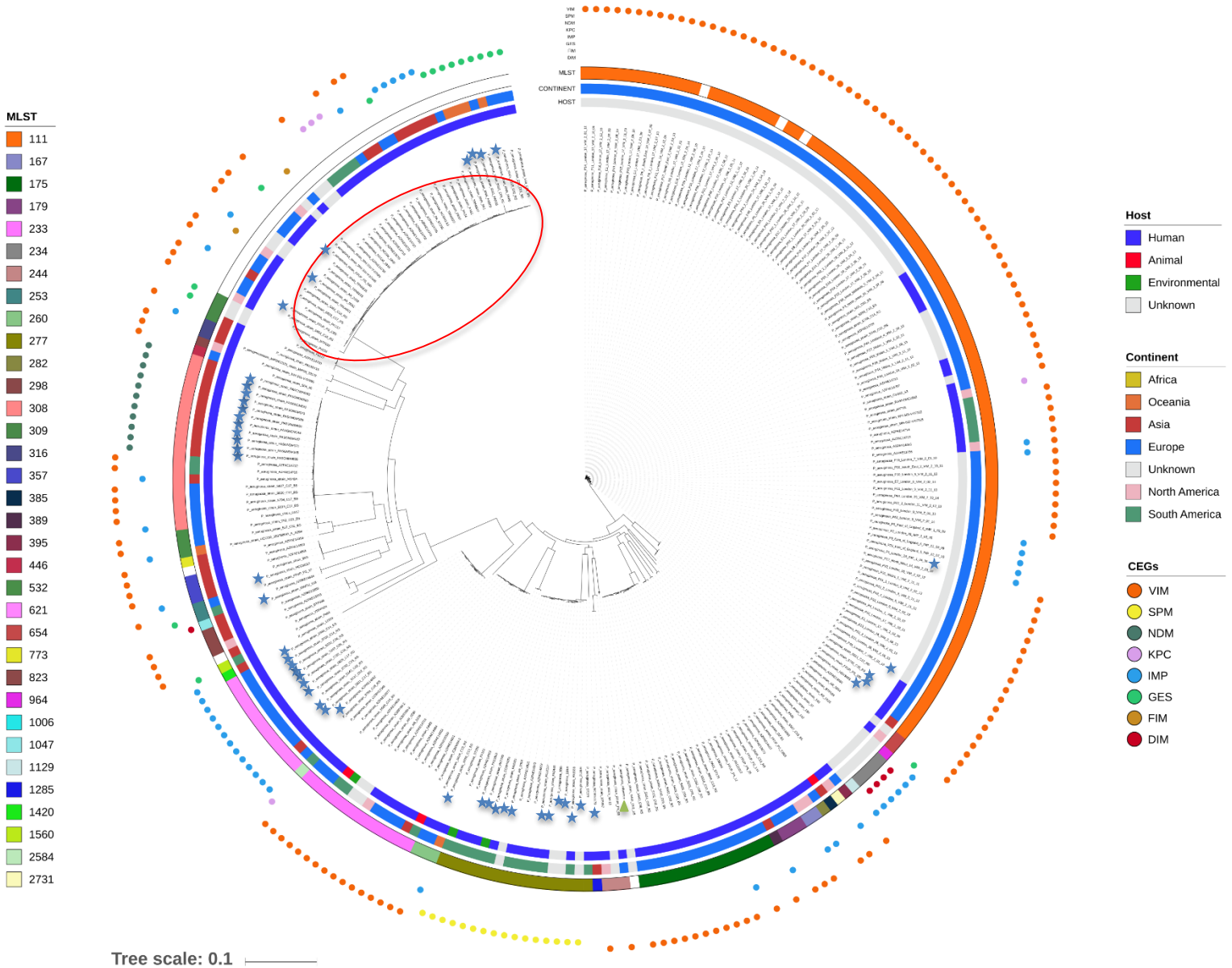
178

179

Results

A plethora of carbapenemase-encoding genes was identified in a subset of *Pseudomonas* species

From the total *Pseudomonas* genomes analysed (n=4565), 313 CEGs were identified in 297 genomes (**Figure 1** and **Table S1**). As expected, $bla_{\text{VIM-2}}$ represents the majority of the CEGs found among *Pseudomonas* spp., being detected mainly in *P. aeruginosa*, followed by *P. plecoglocissida*, *P. guariconensis*, *P. putida*, *P. stutzeri* and 16 genomes corresponding to unidentified species (**Table S1**). Curiously, some strains presented two CEGs, either presenting a duplication of the same gene, such as $bla_{\text{IMP-34}}$ from NCGM 1900 and NCGM 1984 Japanese isolates, or harbouring different CEGs, such as $bla_{\text{IMP-1}}$ and $bla_{\text{DIM-1}}$ in isolates 97, 130 and 142 recovered in Ghana (**Table S1**, highlighted in red). A wide variety of STs was also observed, including the high-risk clones ST111, ST175 and ST244.


MLST
111
167
175
179
233
234
244
253
260
277
282
298
308
309
316
357
385
389
395
446
532
621
654
773
823
964
1006
1047
1129
1285
1420
1560
2584
2731
VIM
SPM
NDM
KPC
IMP
GES
FIM
DIM
MLST
CONTINENT
HOST
Host
Human
Animal
Environmental
Unknown
Continent
Africa
Oceania
Asia
Europe
Unknown
North America
South America
CEGs
VIM
SPM
NDM
KPC
IMP
GES
FIM
DIM
Tree scale: 0.1

192

Figure 1 – Whole-genome phylogeny of the CEG-carrying *P. aeruginosa* isolates. The maximum-likelihood phylogenetic tree was constructed using 146,106 SNPs spanning the whole genome and using the *P. aeruginosa* PAO1 genome (highlighted by a green triangle) as a reference. Multilocus sequence typing (MLST), continent and host data are reported on the outer-most, middle and inner-most circles, respectively. The strains belonging to a double ST profile (ST235/ST2613) are included within the red ellipse. Blue stars point out *P. aeruginosa* strains for which a CEG-harbouring ICE was predicted. The *P. aeruginosa* AR_0356 genome (accession number CP027169.1) was removed from the tree since it corresponds to a strain of which host and origin are unknown. The phylogenetic distance from the tree root to this genome is 1 (calculated with the tree scale). The Newick format file for the original tree is included in the Supplementary information.

Detection of ICEs encoding carbapenemases in *Pseudomonas* spp.

65.5% (205/313, **Table S1**) of the CEG hits are located within small contigs, with a sequence smaller than 20kb in length. The presence of repeated regions, such as those encoding for transposases, tend to split the genome when second-generation sequencing approaches are used. Based on information retrieved from NCBI (accessed on the 24th of May, 2018), the total number of bacterial genomes sequenced at the chromosome/complete genome level is 12,077, while the number of genomes sequenced at the scaffold/contig is much larger (127,231). With this sequencing limitation, we were still able to identify 49 ICEs associated with CEGs (n=20 with complete sequence) among all pseudomonads genomes (**Table 1**, **Table S1 and Figure 1**). When an ICE location was attributed to a CEG located on a small contig, the assumption was based on previously published data, as pointed out on **Table 1**. Besides the

aforementioned ICEs, we also identified a putative MGE within *Pseudomonas* sp. NBRC 111143 strain (**Table S1**). The T4CP-encoding gene was absent from this *bla*$_{IMP-10}$-carrying element, which could be due to contig fragmentation or gene absence. In case the gene is actually missing, this element could still be mobilized by the conjugation machinery of an ICE or conjugative plasmid(s) present in the host, and should be classified as an IME.

The ICEs identified here were all integrated within *P. aeruginosa* genomes (with the exception of the one element identified in *Pseudomonas* sp. PONIH3 genome) and AT-rich when compared to their host's chromosome; the mean GC value for this species is 66.2% according to EZBioCloud (https://www.ezbiocloud.net/taxon?tn=Pseudomonas%20aeruginosa) (**Table 1**).

226 **Table 1.** Main characteristics of CEG-carrying ICEs described in this study.

ICE family	Type of integrase[1]	CEG	Nº strains	ST[2]	Country	Isolation source[3]	CONJscan T4SS type[4]	Size range (if complete, kb)[5]	GC range (if complete, %)[6]	CEG within a class I integron	CEG within a transposon	Other ARGs[7]	References
Tn*4371*	Shufflon-specific DNA recombinase Rci and Bacteriophage Hp1-like	*bla*$_{NDM-1}$	11	308	Singapore	Urine, foot wound swab, endotracheal tube aspirate	T	73.7	64.7	No	Yes (IS*CR24*-composite)	Δ*ble*, Δ*bla*$_{PME-1}$	[63], this study
		bla$_{SPM-1}$ (as single or double copy	11	277	Brazil	Urine, bloodstream, tracheal aspirate, catheter tip, NA	T	43.8 – 57.7	64.9 – 65.6	No	Yes (IS*CR4* composite)	None	[64, 65], this study
		bla$_{KPC-2}$ (double copy)	1	NA	USA	Wastewater	T	61.2	59.2	No	Yes (complex transposon)	*bla*$_{SHV-12}$, *qnrB19*	[66], this study

ICE*clc*	Bacteriophage P4	*bla*$_{IMP-13}$	10	621	Italy, India	Urinary tract infection, respiratory sample, blood	G	NA	NA	Yes	Yes (Tn*3*-like)	*aacA4-C329*, *sul1*	[67], this study
		bla$_{GES-5}$	4	235	Australia	Rectal swab, blood culture, hospital ward, hospital gel hand wash	G	92.8	61.9	Yes	Yes (Tn*3*-like)	*aacA4r15*, *gcuE15*, *aphA15*, *sul1*	[48], this study
		bla$_{VIM-2}$	4	111, 235	Portugal, UK	Urine, bronchial aspirate, NA	G	83.4 – 88.9	62.0	Yes	Yes (Tn*3*-like)	*aacC2b*, *aacA7*, *aacC1*, *aacA4-C329*, *sul1*	[50, 68], this study
		bla$_{IMP-1}$	3	111, 357, 1285	Japan, UK	Midstream urine, NA	G	76.2 – 96.4	61.9 – 62.3	Yes	Yes (Tn*3*-like)	Δ*aacA4-C329*, *aadB*, *aacA28*, *aadA1a*, *cmlA9*, *tet(G)*, *sul1*	[68, 69], This study

		bla$_{DIM-1}$	1	1047	Nepal	Urinary catheter	G	88.7	62.8	Yes	Yes (IS*6100* composite)	*dfrB5*, Δ*aacA4-C329*, *rmtF*, *catB12*	This study
		bla$_{GES-6}$	1	235	Portugal	Urine	G	86.6	63.0	Yes	Yes (defective Tn*402*-like)	*aacA7*, *sul1*	[49]
		bla$_{IMP-14}$	1	2613	NA	NA	NA	NA	NA	Yes	Yes (IS*6100* composite within a Tn*3*-like)	*aadB*, *bla*$_{OXA-10-A}$, *aacA4-T329*, *sul1*	This study
		bla$_{VIM-1}$	1	111	Italy	Blood	G	NA	NA	Yes	Yes (Tn*3*-like)	aacA4-C329, *bla*$_{OXA-2}$, *gcu10*, *aadA13*, *sul1*	[67], this study

ARGs, antibiotic resistance genes; ICE, integrative and conjugative element; NA, Not available; ST, sequence type;

[1]NA is shown when no integrase was identified;

[2]NA is shown when the ICE was identified on a species for which no MLST scheme has been developed;

[3]NA is shown when the isolation source was not provided by sequence authors;

[4]NA is shown when no output was obtained by the platform or the conjugative module system was incomplete due to contig fragmentation;

[5, 6]NA is shown when the ICE sequence was incomplete due to contig fragmentation or delimitation of the entire element was not successful;

[7]Representation of total ARGs associated with the same CEG; a given strain harbouring the referred CEG may not present all ARGs here reported; Δ represents incomplete genes.

All ICEs identified here possessed only one tyrosine integrase (**Figure 2**). ICEs belonging to the ICE*clc* family (MPF_G class) carried an integrase belonging to the bacteriophage P4-like family, while ICEs belonging to the ICE_{Tn4371} family (MPF_T class) carried an integrase belonging to shufflon-specific DNA recombinase Rci and Bacteriophage Hp1-like family (**Table 1**).[31] Rci and Hp1-like were only distantly related (13% amino acid identity) to P4-like integrases. Orthologous assignment of these integrases revealed that the former and the later integrases identified were present in more than 100 and 400 proteobacteria species, respectively. While P4-like integrases were more prevalent on γ-proteobacteria, half of the strains carrying Rci and Hp1-like integrases belong to the α-proteobacteria.

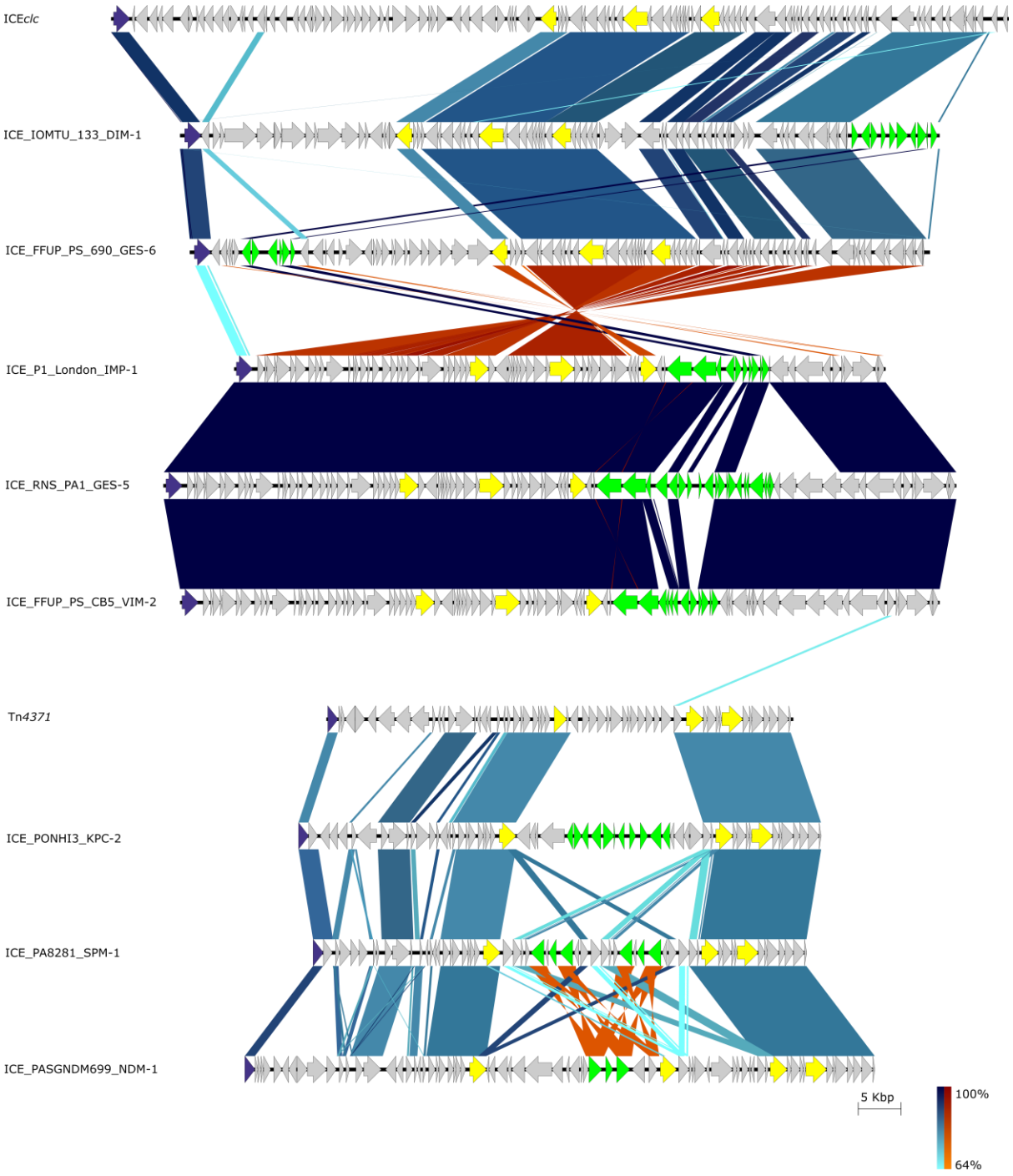


Figure 2 – Blastn comparison among multiple ICEs described in this study. A gradient of blue and red colours is observed for normal and inverted BLAST matches, respectively. Model elements (ICE*clc* for the MPF^{G} and Tn*4371* for the MPF^{T} classes, respectively) were also included for comparison. The arrows and arrowheads point the orientation of the translated coding sequences. In purple are highlighted the integrases, in yellow the mandatory features

of a conjugative system according to Cury *et al*. [40] and in green the transposons harbouring the CEGs. A more detailed view of some of these transposons is displayed in **Figure 3**.

We observed that MPF_G class ICEs tend to integrate next to a single copy of $tRNA^{Gly}$ or a cluster of two $tRNA^{Glu}$ and one $tRNA^{Gly}$ genes, which is in agreement with previous findings [26, 40]. A conserved 8-bp *att* site (5´-CCGCTCCA) flanked all complete ICEs of the MPF_G class identified here (**Table 1**). Notably, most ICEs of this class were adjacent to phages (either at the 5'- or the 3'-end) targeting the same *att* site as the neighbour ICE. No *att* site could be identified for the integration of MPF_T class ICEs. A gene encoding for a catechol 1,2-dioxygenase and a gene encoding for a protein with no described conserved domain were found flanking the bla_{SPM-1}-harbouring ICEs. Regarding the elements carrying bla_{NDM-1}, a gene encoding for a different protein also with no conserved domain identified and a gene encoding for the type III secretion system adenylate cyclase effector ExoY were separated upon insertion of these ICEs. Integration next to hypothetical proteins or tRNA genes was commonly observed.

Carbapenemases are frequently encoded within transposons

CEGs were associated with class I integrons frequently co-harbouring aminoglycoside resistance genes when associated with MPF class G ICEs (**Table 1**). Class I integrons were often associated with a wide array of transposons, such as the Tn*3* superfamily transposons and the IS*6100* composite elements (**Table 1**). MPF_T class ICEs were targeted by more complex elements, such as the composite transposons carrying bla_{SPM-1} and bla_{NDM-1} (**Table 1**). The bla_{NDM-1} gene was identified in Singapore in ICE_{Tn4371}6385 and associated with ST308, as recently reported [63]. The bla_{NDM-1} was flanked by two IS*CR24*-like transposases. bla_{SPM-1} was

linked to ICE$_{Tn4371}$6061, a recently described ICE [64]. Again, the CEG was located within an IS*CR4*-like composite transposon. IS*CR* elements are atypical elements of the IS*91* family which represent a well-recognized system of gene capture and mobilization by a rolling-circle transposition process [21, 70].

Besides previously described *bla*$_{NDM-1}$ and *bla*$_{SPM-1}$ harbouring ICEs, we characterize here new ICE elements of MPF$_G$ and MPF$_T$ classes (**Table 1** and **Figure 3**). The *bla*$_{DIM-1}$-harbouring ICE from IOMTU 133 strain was integrated between the 3'-end of a tRNA^{Gly} gene (IOMTU133_RS11660) and a gene encoding for the R body protein RebB (IOMTU133_RS12085). *bla*$_{DIM-1}$ was first described as a single gene cassette located within a class I integron associated with a 70-kb *Pseudomonas stutzeri* plasmid recovered in the Netherlands [13]. However, the integron carrying *bla*$_{DIM-1}$ in strain IOMTU 133 was unrelated to the one from the *P. stutzeri* plasmid, harbouring genes encoding for aminoglycoside (*aacA4-C329* and *rmtf*), trimethoprim (*dfrB5*) and chloramphenicol (*catB12*) resistance (**Figure 3A**). Direct repeats (DRs) were found flanking the entire IS*6100* composite transposon (5'-TTCGAGTC), indicating the transposition of this element into the ICE element. Besides being identified as a composite transposon, IS*6100* was frequently observed as a single copy at the 3'end of the class I integron (**Figures 3B and 3C**), suggesting that these elements were derived from the In*4* lineage [71]. The *bla*$_{IMP-1}$ from the NCGM257 strain identified in Japan belonged to a different ST (ST357) than the frequently identified ST235 associated with the spread of this CEG in this country [72]. The CEG was also shown to be associated with a novel complex class I integron, co-harbouring *aadB*, *cmlA9* and *tet(G)* genes encoding resistance to aminoglycosides, chloramphenicol and tetracyclines, respectively (**Figure 3B**). This integron was inserted (DRs 5'- GAGTC) within a mercury resistance transposon. This genetic organization was frequently recovered among other ICE-harbouring strains, such as the ones

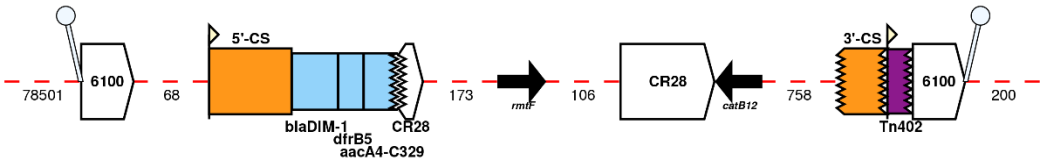
298 associated with *bla*$_{GES-5}$, *bla*$_{IMP-13}$ and *bla*$_{IMP-14}$ (**Table 1**). The entire ICE was integrated into the
299 chromosome of NCGM257 strain between the 3'-end of a tRNA^{Gly} gene (PA257_RS24790) and
300 the aforementioned *Pseudomonas* phage Pf1-like element. The new ICE identified on the
301 P1_London_28_IMP_1_04_05 strain presented *bla*$_{IMP-1}$ in a different In*4*-like integron than
302 that observed for the NCGM257 strain, even though both elements were associated with a
303 Tn*3*-like transposon (**Figure 3C**). Unlike most ICEs of the MPF$_G$ class, its integration occurred
304 between a gene encoding for a LysR family transcriptional regulator (AFJ02_RS19410) and a
305 gene encoding for a hypothetical protein (AFJ02_RS19770). Regarding the *bla*$_{KPC-2}$-harbouring
306 *Pseudomonas* sp. PONHI3 strain, a tetra correlation search revealed that this strain was highly
307 similar (Z-score above the 0.999 cut-off) to *Pseudomonas mosselii* SJ10 (accession number
308 NZ_CP009365.1). Average nucleotide identity based on BLAST (ANIb) analysis of these
309 genomes revealed that both strains belong to the same species, since the ANIb value was
310 above the 95% cut-off for species delineation [73]. However, the ANIb value for both strains
311 was below the cut-off when compared with the *P. mosselii* DSM 17497 type train (accession
312 number NZ_JHYW00000000.1), suggesting that both strains may comprise novel species
313 within the *Pseudomonas putida* phylogenetic group [2]. The PONHI3 strain carried a double
314 copy of *bla*$_{KPC-2}$ within an ICE from MPF$_T$ class. A complex genetic environment was found
315 surrounding these genes (**Figure 3D**). This ICE was integrated between a gene encoding for a
316 biopolymer transport protein ExbD/TolR (C3F42_RS18665) and a gene encoding for an
317 alpha/beta hydrolase (C3F42_RS18995).

318

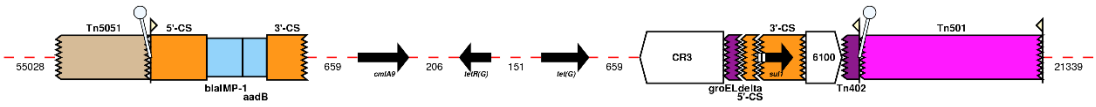
319

320

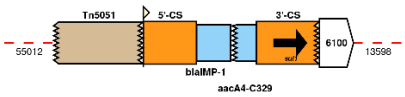
A



B



C



D

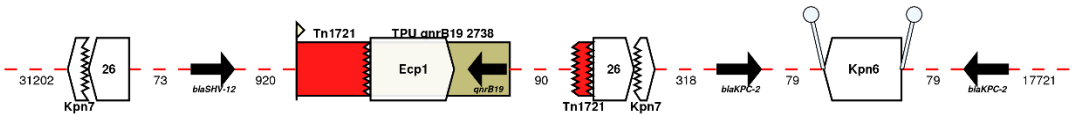


Figure 3. Genetic environment of novel ICEs harbouring *bla*$_{\text{DIM-1}}$ (A), *bla*$_{\text{IMP-1}}$ (B and C) and a double copy of *bla*$_{\text{KPC-2}}$ (D). Arrows indicate the direction of transcription for genes. The dashed part of the arrow indicates which end is missing, for other features the missing end is shown by a zig-zag line. Gene cassettes are shown by pale blue boxes, the conserved sequences (5' and 3'-CS) of integrons as orange boxes and insertion sequences as white block arrows labelled with the IS number/name, with the pointed end indicating the inverted right

repeat (IRR). Gaps >50 bp are indicated by dashed red lines and the length in bp given. Unit transposons are shown as boxes of different colors and their IRs are shown as flags, with the flat side at the outer boundary of the transposon. Direct repeats are shown as 'lollipops' of the same color.

An atypical GI encoding carbapenemases

Besides ICEs, we also identified an atypical 19.8-kb long GI harbouring *bla*$_{VIM-2}$ in *P. aeruginosa* AZPAE13853 and AZPAE13858 strains from India (**Figure S1**). A similar element was also observed in *P. aeruginosa* BTP038 strain from the USA, with the exception that the Tn*402*-like transposon harbouring *bla*$_{VIM-2}$ was orientated in an inverted position. Five base-pair DRs (5'-CTCTG in AZPAE13853 and AZPAE13858 and 5'-CTGAG in BTP038 strains) were found flanking this transposon structure. Importantly, in these strains the GIs were flanked by identical signal recognition particle RNAs (srpRNAs), indicating a strong site preference for these elements.

Discussion

Our results show that *bla*$_{VIM}$ and *bla*$_{IMP}$ are widely disseminated, both geographically and phylogenetically (across *Pseudomonas* spp.). Moreover, and as previously described, *bla*$_{VIM-2}$ was the most frequently reported CEG (**Figure 1** and **Table S1**) [4]. On the other hand, *bla*$_{SPM-1}$ is still restricted to *P. aeruginosa* and Brazil (or patients who had been previously hospitalized in Brazil) [65]. Even though ST235 has been frequently linked to the dissemination of ARGs, no CEG-harbouring strains belonging to this ST were identified. Curiously, some strains (highlighted on **Figure 1**) belong to a double ST profile

(ST235/ST2613), since the strains carry a double copy with different allele sequences of the house-keeping gene *acsA*, encoding for an acetyl-coenzyme A synthetase. These genes only display 80,3% nucleotide identity. We plan to conduct comparative genomic studies to explore the idiosyncrasies of these double ST profile strains.

Not all CEGs are likely to be geographically and phylogenetically disseminated, but those that are more promiscuous present a serious threat. The geographical distribution of the high-risk clones and the diversity of CEGs propose that the spread of these STs is global and the acquisition of the resistance genes is mainly local [4, 68]. Previous studies suggest that environmental species may pose an important reservoir for the dissemination of clinically relevant carbapenemases, which are vertically amplified upon transfer to *P. aeruginosa* high-risk clones [12, 14]. 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 CEGs and to be further selected and amplified due to the high antibiotic pressure in the hospital environment [74]. 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 [75–77].

This study underestimates the extent of host range because only ICEs in sequenced genomes were detected. Also, identification of new ICEs could only be achieved in complete genomes or contigs with a sequence length large enough to include the full (nor near complete) sequence of the ICE. As so, it is important to highlight the need to perform third generation

sequencing on CEG-harbouring genomes to avoid fragmentation of the genetic environment surrounding the gene and to provide a wider view of complete supporting ICEs and other MGEs. All ICE elements here identified fulfilled the criteria to be considered conjugative as proposed by Cury *et al*.: a relaxase, a VirB4/TraU, a T4CP and minimum set of MPF type-specific genes [40]. ICEs tend to integrate within the host's chromosome by the action of a tyrosine recombinase, even though some elements may use serine or DDE recombinases instead [30]. Though rare, some elements encode for more than one integrase, most likely resulting from independent integration of different MGEs [40]. Conserved sites are hotspots for ICE integration due to their high conservation among closely related bacteria, and so expanding the host range and be stably maintained after conjugative transfer [78, 79]. ICEs were often integrated next to phages highly similar to the *Pseudomonas* phage Pf1 (NC_001331.1), a class II filamentous bacteriophage belonging to the *Inoviridae* family [77]. Pf1-like phages are widely disseminated among *P. aeruginosa* strains and may have a role in bacterial evolution and virulence [80–82]. Interestingly, no representative of the pKLC102 family was linked to the dissemination of CEGs. This may be explained due to a higher affinity of the transposons carrying the CEGs for hotspots located within representatives of the other two families.

MGEs specifically targeting conserved regions of the genome such as tRNAs are common and this specificity represents an evolutionary strategy whereby the target site of an element is almost guaranteed to be present, due to its essentiality, and very unlikely to change due to biochemical constraints of the gene product. We think a similar situation exists for the elements found between the small srpRNAs described on the atypical GI element here identified and is in contrast to the more permissive nature of target site selection shown for example, by elements of the Tn*916*/Tn*1545* family [83].

Here, we revealed that different Tn*3*-like and composite transposons harbouring a wide array of CEGs were transposed into MPF G and T ICE classes, which were most likely responsible for the dissemination of these genes through HGT and/or clonal expansion of successful *Pseudomonas* clones. This study sheds light on the underappreciated contribution of ICEs for the spread of CEGs among pseudomonads (and potentially further afield). With the ever-growing number of third-generation sequenced genomes and the development of more sophisticated bioinformatics, the real contribution of these ICEs will likely rapidly emerge.

Recently, it was shown that interfering with the transposase-DNA complex architecture of a Tn*916*-like conjugative transposon (also known as ICE) lead to transposition inhibition to a new host [84]. In the future, it would be interesting to determine if the same mechanism is observed for tyrosine recombinases present in ICE*clc* and Tn*4371* derivatives, as well as in other MPF ICE classes, as a potential approach to interfere with the spread of antimicrobial resistance.

Acknowledgments

This study received financial support from the European Union (FEDER funds POCI/01/0145/FEDER/007728) and National Funds (FCT/MEC, Fundação para a Ciência e Tecnologia and Ministério da Educação e Ciência) under the Partnership Agreement PT2020 UID/MULTI/04378/2013. J. B. and F. G. were supported by grants from Fundação para a Ciência e a Tecnologia (SFRH/BD/104095/2014 and SFRH/BPD/95556/2013, respectively). We thank Álvaro San Millan for helpful discussions. We also thank Benjamin Buchfink (DIAMOND), Jean Cury (TXSScan/CONJscan) and Sally Partridge (MARA) for their valuable assistance.

Author contributions

JB, APR, FG and LP designed the study; JB and RLS performed the *in silico* analysis; JB wrote the manuscript. All the authors approved the final manuscript.

Competing interests

None to declare.

References

1. Parte AC. LPSN—list of prokaryotic names with standing in nomenclature. *Nucleic Acids Res* 2014; **42**: D613–D616.

2. Gomila M, Peña A, Mulet M, Lalucat J, García-Valdés E. Phylogenomics and systematics in Pseudomonas. *Front Microbiol* 2015; **6**: 214.

3. Moradali MF, Ghods S, Rehm BHA. Pseudomonas aeruginosa Lifestyle: A Paradigm for Adaptation, Survival, and Persistence. *Front Cell Infect Microbiol* 2017; **7**: 39.

4. Oliver A, Mulet X, López-Causapé C, Juan C. The increasing threat of Pseudomonas aeruginosa high-risk clones. *Drug Resist Updat* 2015; **21**–**22**: 41–59.

5. Juan C, Peña C, Oliver A. Host and Pathogen Biomarkers for Severe Pseudomonas aeruginosa Infections. *J Infect Dis* 2017; **215**: S44–S51.

6. Boucher HW, Talbot GH, Bradley JS, Edwards JE, Gilbert D, Rice LB, et al. Bad Bugs, No Drugs: No ESKAPE! An Update from the Infectious Diseases Society of America. *Clin Infect Dis* 2009; **48**: 1–12.

7. Breidenstein EBM, de la Fuente-Núñez C, Hancock REW. Pseudomonas aeruginosa: all roads lead to resistance. *Trends Microbiol* 2011; **19**: 419–26.

8. Cornaglia G, Giamarellou H, Rossolini GM. Metallo-β-lactamases: a last frontier for β-lactams? *Lancet*

Infect Dis 2011; **11**: 381–93.

9. Kidd TJ, Ritchie SR, Ramsay KA, Grimwood K, Bell SC, Rainey PB. Pseudomonas aeruginosa Exhibits Frequent Recombination, but Only a Limited Association between Genotype and Ecological Setting. *PLoS One* 2012; **7**: e44199.

10. Scientific Opinion on Carbapenem resistance in food animal ecosystems. *EFSA J* 2013; **11**.

11. World Health Organization. GLOBAL PRIORITY LIST OF ANTIBIOTIC-RESISTANT BACTERIA TO GUIDE RESEARCH, DISCOVERY, AND DEVELOPMENT OF NEW ANTIBIOTICS. http://www.who.int/news-room/detail/27-02-2017-who-publishes-list-of-bacteria-for-which-new-antibiotics-are-urgently-needed. Accessed 11 Aug 2018.

12. Scotta C, Juan C, Cabot G, Oliver A, Lalucat J, Bennasar A, et al. Environmental microbiota represents a natural reservoir for dissemination of clinically relevant metallo-beta-lactamases. *Antimicrob Agents Chemother* 2011; **55**: 5376–9.

13. Poirel L, Rodríguez-Martínez J-M, Al Naiemi N, Debets-Ossenkopp YJ, Nordmann P. Characterization of DIM-1, an integron-encoded metallo-beta-lactamase from a Pseudomonas stutzeri clinical isolate in the Netherlands. *Antimicrob Agents Chemother* 2010; **54**: 2420–4.

14. Juan C, Zamorano L, Mena A, Alberti S, Perez JL, Oliver A. Metallo- -lactamase-producing Pseudomonas putida as a reservoir of multidrug resistance elements that can be transferred to successful Pseudomonas aeruginosa clones. *J Antimicrob Chemother* 2010; **65**: 474–478.

15. Queenan AM, Bush K. Carbapenemases: the versatile beta-lactamases. *Clin Microbiol Rev* 2007; **20**: 440–58, table of contents.

16. Amos GCA, Ploumakis S, Zhang L, Hawkey PM, Gaze WH, Wellington EMH. The widespread dissemination of integrons throughout bacterial communities in a riverine system. *ISME J* 2018; **12**: 681–691.

17. Mazel D. Integrons: agents of bacterial evolution. *Nat Rev Microbiol* 2006; **4**: 608–620.

18. Gaze WH, Zhang L, Abdouslam NA, Hawkey PM, Calvo-Bado L, Royle J, et al. Impacts of anthropogenic

activity on the ecology of class 1 integrons and integron-associated genes in the environment. *ISME J* 2011; **5**: 1253–1261.

19. Kung VL, Ozer EA, Hauser AR. The accessory genome of Pseudomonas aeruginosa. *Microbiol Mol Biol Rev* 2010; **74**: 621–41.

20. Partridge SR, Kwong SM, Firth N, Jensen SO. Mobile Genetic Elements Associated with Antimicrobial Resistance. *Clin Microbiol Rev* 2018; **31**: e00088-17.

21. Toleman MA, Walsh TR. Combinatorial events of insertion sequences and ICE in Gram-negative bacteria. *FEMS Microbiol Rev* 2011; **35**: 912–935.

22. Enault F, Briet A, Bouteille L, Roux S, Sullivan MB, Petit M-A. Phages rarely encode antibiotic resistance genes: a cautionary tale for virome analyses. *ISME J* 2017; **11**: 237–247.

23. San Millan A. Evolution of Plasmid-Mediated Antibiotic Resistance in the Clinical Context. *Trends Microbiol* 2018; **0**.

24. Fillol-Salom A, Martínez-Rubio R, Abdulrahman RF, Chen J, Davies R, Penadés JR. Phage-inducible chromosomal islands are ubiquitous within the bacterial universe. *ISME J* 2018; 1.

25. Bellanger X, Payot S, Leblond-Bourget N, Guédon G. Conjugative and mobilizable genomic islands in bacteria: evolution and diversity. *FEMS Microbiol Rev* 2014; **38**: 720–760.

26. Langille MGI, Hsiao WWL, Brinkman FSL. Detecting genomic islands using bioinformatics approaches. *Nat Rev Microbiol* 2010; **8**: 373–382.

27. Chen J, Novick RP. Phage-mediated intergeneric transfer of toxin genes. *Science* 2009; **323**: 139–41.

28. Tazzyman SJ, Hall AR. Lytic phages obscure the cost of antibiotic resistance in Escherichia coli. *ISME J* 2015; **9**: 809–820.

29. Wozniak RAF, Waldor MK. Integrative and conjugative elements: mosaic mobile genetic elements enabling dynamic lateral gene flow. *Nat Rev Microbiol* 2010; **8**: 552–563.

30. Johnson CM, Grossman AD. Integrative and Conjugative Elements (ICEs): What They Do and How They Study. *Annu Rev Genet* 2015; **49**: 577–601.

31. Delavat F, Miyazaki R, Carraro N, Pradervand N, van der Meer JR. The hidden life of integrative and conjugative elements. *FEMS Microbiol Rev* 2017; **41**: 512–537.

32. Guédon G, Libante V, Coluzzi C, Payot S, Leblond-Bourget N. The Obscure World of Integrative and Mobilizable Elements, Highly Widespread Elements that Pirate Bacterial Conjugative Systems. *Genes (Basel)* 2017; **8**: 337.

33. Delavat F, Mitri S, Pelet S, van der Meer JR. Highly variable individual donor cell fates characterize robust horizontal gene transfer of an integrative and conjugative element. *Proc Natl Acad Sci U S A* 2016; **113**: E3375-83.

34. Burrus V. Mechanisms of stabilization of integrative and conjugative elements. *Curr Opin Microbiol* 2017; **38**: 44–50.

35. Smillie C, Garcillán-Barcia MP, Francia MV, Rocha EPC, de la Cruz F. Mobility of plasmids. *Microbiol Mol Biol Rev* 2010; **74**: 434–52.

36. Garcillán-Barcia MP, Alvarado A, de la Cruz F. Identification of bacterial plasmids based on mobility and plasmid population biology. *FEMS Microbiol Rev* 2011; **35**: 936–956.

37. Guglielmini J, Néron B, Abby SS, Garcillán-Barcia MP, de la Cruz F, Rocha EPC. Key components of the eight classes of type IV secretion systems involved in bacterial conjugation or protein secretion. *Nucleic Acids Res* 2014; **42**: 5715–27.

38. Guglielmini J, Quintais L, Garcillán-Barcia MP, de la Cruz F, Rocha EPC. The Repertoire of ICE in Prokaryotes Underscores the Unity, Diversity, and Ubiquity of Conjugation. *PLoS Genet* 2011; **7**: e1002222.

39. Carraro N, Poulin D, Burrus V. Replication and Active Partition of Integrative and Conjugative Elements (ICEs) of the SXT/R391 Family: The Line between ICEs and Conjugative Plasmids Is Getting Thinner. *PLOS Genet* 2015; **11**: e1005298.

40. Cury J, Touchon M, Rocha EPC. Integrative and conjugative elements and their hosts: composition, distribution and organization. *Nucleic Acids Res* 2017; **45**: 8943–8956.

525 41. Baltrus DA. Exploring the costs of horizontal gene transfer. *Trends Ecol Evol* 2013; **28**: 489–495.

526 42. Harrison EM, Carter MEK, Luck S, Ou H-Y, He X, Deng Z, et al. Pathogenicity islands PAPI-1 and PAPI-2
527 contribute individually and synergistically to the virulence of Pseudomonas aeruginosa strain PA14.
528 *Infect Immun* 2010; **78**: 1437–46.

529 43. He J, Baldini RL, Déziel E, Saucier M, Zhang Q, Liberati NT, et al. The broad host range pathogen
530 Pseudomonas aeruginosa strain PA14 carries two pathogenicity islands harboring plant and animal
531 virulence genes. *Proc Natl Acad Sci U S A* 2004; **101**: 2530–5.

532 44. Botelho J, Grosso F, Peixe L. Characterization of the pJB12 Plasmid from Pseudomonas aeruginosa
533 Reveals Tn6352, a Novel Putative Transposon Associated with Mobilization of the blaVIM-2-Harboring
534 In58 Integron. *Antimicrob Agents Chemother* 2017; **61**: e02532-16.

535 45. Botelho J, Grosso F, Quinteira S, Mabrouk A, Peixe L. The complete nucleotide sequence of an IncP-2
536 megaplasmid unveils a mosaic architecture comprising a putative novel blaVIM-2-harbouring
537 transposon in Pseudomonas aeruginosa. *J Antimicrob Chemother* 2017; **72**: 2225–2229.

538 46. San Millan A, Toll-Riera M, Escudero JA, Cantón R, Coque TM, MacLean RC. Sequencing of plasmids
539 pAMBL1 and pAMBL2 from *Pseudomonas aeruginosa* reveals a *bla* $_{VIM-1}$ amplification causing high-level
540 carbapenem resistance. *J Antimicrob Chemother* 2015; **70**: 3000–3003.

541 47. Sun F, Zhou D, Wang Q, Feng J, Feng W, Luo W, et al. Genetic characterization of a novel *bla* $_{DIM-2}$ -
542 carrying megaplasmid p12969-DIM from clinical *Pseudomonas putida*. *J Antimicrob Chemother* 2016;
543 **71**: 909–912.

544 48. Roy Chowdhury P, Scott M, Worden P, Huntington P, Hudson B, Karagiannis T, et al. Genomic islands 1
545 and 2 play key roles in the evolution of extensively drug-resistant ST235 isolates of Pseudomonas
546 aeruginosa. *Open Biol* 2016; **6**.

547 49. Botelho J, Grosso F, Peixe L. Unravelling the genome of a Pseudomonas aeruginosa isolate belonging to
548 the high-risk clone ST235 reveals an integrative conjugative element housing a blaGES-6
549 carbapenemase. *J Antimicrob Chemother* 2018; **73**: 77–83.

550 50. Botelho J, Grosso F, Quinteira S, Brilhante M, Ramos H, Peixe L. Two decades of blaVIM-2-producing

Pseudomonas aeruginosa dissemination: an interplay between mobile genetic elements and successful clones. *J Antimicrob Chemother* 2018; **73**: 873–882.

51. Buchfink B, Xie C, Huson DH. Fast and sensitive protein alignment using DIAMOND. *Nat Methods* 2015; **12**: 59–60.

52. Seemann T. Prokka: rapid prokaryotic genome annotation. *Bioinformatics* 2014; **30**: 2068–2069.

53. Kearse M, Moir R, Wilson A, Stones-Havas S, Cheung M, Sturrock S, et al. Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. *Bioinformatics* 2012; **28**: 1647–9.

54. Sullivan MJ, Petty NK, Beatson SA. Easyfig: a genome comparison visualizer. *Bioinformatics* 2011; **27**: 1009–10.

55. Arndt D, Grant JR, Marcu A, Sajed T, Pon A, Liang Y, et al. PHASTER: a better, faster version of the PHAST phage search tool. *Nucleic Acids Res* 2016; **44**: W16–W21.

56. Siguier P, Perochon J, Lestrade L, Mahillon J, Chandler M. ISfinder: the reference centre for bacterial insertion sequences. *Nucleic Acids Res* 2006; **34**: D32–D36.

57. Partridge SR, Tsafnat G. Automated annotation of mobile antibiotic resistance in Gram-negative bacteria: the Multiple Antibiotic Resistance Annotator (MARA) and database. *J Antimicrob Chemother* 2018; **73**: 883–890.

58. Huerta-Cepas J, Forslund K, Coelho LP, Szklarczyk D, Jensen LJ, von Mering C, et al. Fast Genome-Wide Functional Annotation through Orthology Assignment by eggNOG-Mapper. *Mol Biol Evol* 2017; **34**: 2115–2122.

59. Jones P, Binns D, Chang H-Y, Fraser M, Li W, McAnulla C, et al. InterProScan 5: genome-scale protein function classification. *Bioinformatics* 2014; **30**: 1236–40.

60. Kaas RS, Leekitcharoenphon P, Aarestrup FM, Lund O. Solving the Problem of Comparing Whole Bacterial Genomes across Different Sequencing Platforms. *PLoS One* 2014; **9**: e104984.

61. Jolley KA, Maiden MCJ. BIGSdb: Scalable analysis of bacterial genome variation at the population level.

BMC Bioinformatics 2010; **11**: 595.

62. Richter M, Rosselló-Móra R, Oliver Glöckner F, Peplies J. JSpeciesWS: a web server for prokaryotic species circumscription based on pairwise genome comparison. *Bioinformatics* 2016; **32**: 929–931.

63. Ding Y, Teo JWP, Drautz-Moses DI, Schuster SC, Givskov M, Yang L. Acquisition of resistance to carbapenem and macrolide-mediated quorum sensing inhibition by Pseudomonas aeruginosa via ICETn43716385. *Commun Biol* 2018; **1**: 57.

64. Fonseca EL, Marin MA, Encinas F, Vicente ACP. Full characterization of the integrative and conjugative element carrying the metallo-β-lactamase *bla* $_{SPM-1}$ and bicyclomycin *bcr1* resistance genes found in the pandemic *Pseudomonas aeruginosa* clone SP/ST277 : Figure 1. *J Antimicrob Chemother* 2015; **70**: 2547–2550.

65. Nascimento APB, Ortiz MF, Martins WMBS, Morais GL, Fehlberg LCC, Almeida LGP, et al. Intraclonal Genome Stability of the Metallo-β-lactamase SPM-1-producing Pseudomonas aeruginosa ST277, an Endemic Clone Disseminated in Brazilian Hospitals. *Front Microbiol* 2016; **7**: 1946.

66. Weingarten RA, Johnson RC, Conlan S, Ramsburg AM, Dekker JP, Lau AF, et al. Genomic Analysis of Hospital Plumbing Reveals Diverse Reservoir of Bacterial Plasmids Conferring Carbapenem Resistance. *MBio* 2018; **9**: e02011-17.

67. Giani T, Arena F, Pollini S, Di Pilato V, D'Andrea MM, Henrici De Angelis L, et al. Italian nationwide survey on Pseudomonas aeruginosa from invasive infections: activity of ceftolozane/tazobactam and comparators, and molecular epidemiology of carbapenemase producers. *J Antimicrob Chemother* 2018; **73**: 664–671.

68. Turton JF, Wright L, Underwood A, Witney AA, Chan Y-T, Al-Shahib A, et al. High-Resolution Analysis by Whole-Genome Sequencing of an International Lineage (Sequence Type 111) of Pseudomonas aeruginosa Associated with Metallo-Carbapenemases in the United Kingdom. *J Clin Microbiol* 2015; **53**: 2622–31.

69. Tada T, Kitao T, Miyoshi-Akiyama T, Kirikae T. Genome sequence of multidrug-resistant Pseudomonas aeruginosa NCGM1179. *J Bacteriol* 2011; **193**: 6397.

602 70. Toleman MA, Bennett PM, Walsh TR. ISCR elements: novel gene-capturing systems of the 21st
603 century? *Microbiol Mol Biol Rev* 2006; **70**: 296–316.

604 71. Partridge SR. Analysis of antibiotic resistance regions in Gram-negative bacteria. *FEMS Microbiol Rev*
605 2011; **35**: 820–855.

606 72. Shimizu W, Kayama S, Kouda S, Ogura Y, Kobayashi K, Shigemoto N, et al. Persistence and epidemic
607 propagation of a Pseudomonas aeruginosa sequence type 235 clone harboring an IS26 composite
608 transposon carrying the blaIMP-1 integron in Hiroshima, Japan, 2005 to 2012. *Antimicrob Agents*
609 *Chemother* 2015; **59**: 2678–87.

610 73. Varghese NJ, Mukherjee S, Ivanova N, Konstantinidis KT, Mavrommatis K, Kyrpides NC, et al. Microbial
611 species delineation using whole genome sequences. *Nucleic Acids Res* 2015; **43**: 6761–71.

612 74. Baquero F. From pieces to patterns: evolutionary engineering in bacterial pathogens. *Nat Rev*
613 *Microbiol* 2004; **2**: 510–518.

614 75. Miyoshi-Akiyama T, Tada T, Ohmagari N, Viet Hung N, Tharavichitkul P, Pokhrel BM, et al. Emergence
615 and Spread of Epidemic Multidrug-Resistant Pseudomonas aeruginosa. *Genome Biol Evol* 2017; **9**:
616 3238–3245.

617 76. Bondy-Denomy J, Davidson AR. To acquire or resist: the complex biological effects of CRISPR-Cas
618 systems. *Trends Microbiol* 2014; **22**: 218–25.

619 77. van Belkum A, Soriaga LB, LaFave MC, Akella S, Veyrieras J-B, Barbu EM, et al. Phylogenetic Distribution
620 of CRISPR-Cas Systems in Antibiotic-Resistant Pseudomonas aeruginosa. *MBio* 2015; **6**: e01796-15.

621 78. Touchon M, Hoede C, Tenaillon O, Barbe V, Baeriswyl S, Bidet P, et al. Organised Genome Dynamics in
622 the Escherichia coli Species Results in Highly Diverse Adaptive Paths. *PLoS Genet* 2009; **5**: e1000344.

623 79. Rocha EPC. The replication-related organization of bacterial genomes. *Microbiology* 2004; **150**: 1609–
624 1627.

625 80. Knezevic P, Voet M, Lavigne R. Prevalence of Pf1-like (pro)phage genetic elements among
626 Pseudomonas aeruginosa isolates. *Virology* 2015; **483**: 64–71.

627 81. Whiteley M, Bangera MG, Bumgarner RE, Parsek MR, Teitzel GM, Lory S, et al. Gene expression in
628 Pseudomonas aeruginosa biofilms. *Nature* 2001; **413**: 860–864.

629 82. Secor PR, Michaels LA, Smigiel KS, Rohani MG, Jennings LK, Hisert KB, et al. Filamentous Bacteriophage
630 Produced by Pseudomonas aeruginosa Alters the Inflammatory Response and Promotes Noninvasive
631 Infection In Vivo. *Infect Immun* 2017; **85**: e00648-16.

632 83. Roberts AP, Mullany P. Tn *916* -like genetic elements: a diverse group of modular mobile elements
633 conferring antibiotic resistance. *FEMS Microbiol Rev* 2011; **35**: 856–871.

634 84. Rubio-Cosials A, Schulz EC, Lambertsen L, Smyshlyaev G, Rojas-Cordova C, Forslund K, et al.
635 Transposase-DNA Complex Structures Reveal Mechanisms for Conjugative Transposition of Antibiotic
636 Resistance. *Cell* 2018; **173**: 208–220.e20.

637

638

Table S1 – General features of the hits. Hits associated with ICEs are highlighted in blue. Strains for which more than one CEG was identified are represented in red.

CEG	Accession number	Evalue	Bitscore	Organism	Strain	Sequence Length	Collection Date	Country	Isolation Source
DIM-1	NZ_NXHR01000090.1	5.3e-147	513.5	*Pseudomonas aeruginosa*	97	3093	2015	Ghana: Kumasi	Urine
DIM-1	NZ_NXHP01000163.1	5.3e-147	513.5	*Pseudomonas aeruginosa*	130	3093	2015	Ghana: Kumasi	Wound swab
DIM-1	NZ_NXHO01000169.1	5.3e-147	513.5	*Pseudomonas aeruginosa*	140	3093	2015	Ghana: Kumasi	Wound swab
DIM-1	NZ_NXHN01000226.1	5.3e-147	513.5	*Pseudomonas aeruginosa*	142	3093	2015	Ghana: Kumasi	Pleural fluid
DIM-1	NZ_AP017302.1	2.6e-143	512.3	*Pseudomonas aeruginosa*	IOMTU 133	6897018	2012	Nepal: Kathmandu	Urinary catheter
FIM-1	NZ_JTWC01000189.1	1.1e-154	538.5	*Pseudomonas aeruginosa*	AZPAE14730	2154	2012	Italy	Respiratory tract infection
FIM-1	NZ_LYTT01000036.1	3.0e-154	538.5	*Pseudomonas aeruginosa*	TRN6601	5964	2012	USA	NA
GES-20	NZ_JNHD01000009.1	7.8e-161	565.5	*Pseudomonas aeruginosa*	PA_ST235	206205	2008	Spain: Madrid	Blood
GES-20	NZ_CP021774.1	2.7e-159	565.5	*Pseudomonas aeruginosa*	Pa124	7008516	2006	Mexico: Mexico city	Bronchial washing
GES-20	NZ_CP022000.1	2.7e-159	565.5	*Pseudomonas aeruginosa*	Pa127	7148302	2006	Mexico: Mexico city	Bronchial washing
GES-5	NZ_JTQT01000068.1	1.8e-161	567.0	*Pseudomonas aeruginosa*	AZPAE14948	138245	2009	Argentina: Victoria	Intra-abdominal tract infection
GES-5	NZ_LFMR01000003.1	6.9e-161	567.0	*Pseudomonas aeruginosa*	BTP034	527128	2014	USA: Rochester, Minnesota	Associated Infection
GES-5	NZ_PQGF01000119.1	2.1e-163	567.0	*Pseudomonas aeruginosa*	Pa64	1603	2009	Brazil	Soft tissue
GES-5	NZ_LSZV01000002.1	2.9e-161	567.0	*Pseudomonas aeruginosa*	RNS_PA1	223457	2006	Australia: Sydney	Rectal swab
GES-5	NZ_LVED01000149.1	6.1e-163	567.0	*Pseudomonas aeruginosa*	RNS_PA69	4698	2007	Australia: Sydney	Blood culture
GES-5	NZ_LSZT01000004.1	9.1e-163	567.0	*Pseudomonas aeruginosa*	RNS_PAE05	7001	2007	Australia: Sydney	Hospital ward
GES-5	NZ_LVEF01000109.1	6.1e-163	567.0	*Pseudomonas aeruginosa*	RNS_PAE08	4698	2007	Australia: Sydney	Hospital gel hand wash

GES-5	NZ_NFGS01000071.1	6.4e-163	567.0	*Pseudomonas aeruginosa*	S122_C02_RS	4907	2013/2014	Italy: Lecco	Respiratory Sample
GES-5	NZ_NFGX01000057.1	6.4e-163	567.0	*Pseudomonas aeruginosa*	S39_C01_BS	4907	2013/2014	Italy: Milano	Blood
GES-5	NZ_NFGL01000183.1	5.8e-163	567.0	*Pseudomonas aeruginosa*	S402_C09_RS	4454	2013/2014	Italy: Udine	Respiratory Sample
GES-5	NZ_NFGW01000066.1	5.8e-163	567.0	*Pseudomonas aeruginosa*	S49_C01_BS	4454	2013/2014	Italy: Milano	Blood
GES-5	NZ_LYTY01000075.1	3.6e-163	567.0	*Pseudomonas aeruginosa*	TRN6637	2779	2013	Russia	NA
GES-6	NZ_NBEZ01000053.1	4.4e-163	567.0	*Pseudomonas aeruginosa*	FFUP_PS_690	3393	2015	Portugal: Porto	Urine
IMP-1	NZ_NXHR01000094.1	4.2e-145	506.9	*Pseudomonas aeruginosa*	97	2659	2015	Ghana: Kumasi	Urine
IMP-1	NZ_NXHP01000170.1	4.2e-145	506.9	*Pseudomonas aeruginosa*	130	2659	2015	Ghana: Kumasi	Wound swab
IMP-1	NZ_NXHO01000173.1	4.2e-145	506.9	*Pseudomonas aeruginosa*	140	2659	2015	Ghana: Kumasi	Wound swab
IMP-1	NZ_NXHN01000235.1	4.2e-145	506.9	*Pseudomonas aeruginosa*	142	2659	2015	Ghana: Kumasi	Pleural fluid
IMP-1	NZ_CP024477.1	1.1e-141	506.9	*Pseudomonas aeruginosa*	12939	6621378	2013	China	Medium
IMP-1	NZ_MPBP01000001.1	1.6e-141	506.1	*Pseudomonas aeruginosa*	AR_0103	5781139	NA	NA	NA
IMP-1	NZ_MPBP01000001.1	1.6e-141	506.1	*Pseudomonas aeruginosa*	AR_0103	5781139	NA	NA	NA
IMP-1	NZ_BCAQ01000182.1	5.2e-145	506.5	*Pseudomonas sp.*	GTC 16473	2488	NA	NA	NA
IMP-1	NZ_BCAS01000276.1	3.1e-145	505.8	*Pseudomonas sp.*	GTC 16482	875	NA	NA	NA
IMP-1	NZ_BCAV01000087.1	1.5e-144	506.1	*Pseudomonas sp.*	NBRC 111119	5517	NA	NA	NA
IMP-1	NZ_BCAX01000074.1	4.7e-144	506.1	*Pseudomonas sp.*	NBRC 111121	17324	NA	NA	NA
IMP-1	NZ_BCBC01000204.1	3.1e-145	505.8	*Pseudomonas sp.*	NBRC 111126	874	NA	NA	NA
IMP-1	NZ_BCBF01000218.1	1.9e-145	506.5	*Pseudomonas sp.*	NBRC 111129	888	NA	NA	NA
IMP-1	NZ_BCBU01000102.1	5.0e-144	506.1	*Pseudomonas sp.*	NBRC 111144	18180	NA	NA	NA
IMP-1	NZ_DF126593.1	1.9e-141	506.1	*Pseudomonas aeruginosa*	NCGM1179	6910294	NA	NA	NA

IMP-1	NC_017549.1	1.8e-141	506.1	*Pseudomonas aeruginosa*	NCGM2.S1	6764661	NA	NA	NA
IMP-1	NZ_AP014651.1	1.9e-141	506.1	*Pseudomonas aeruginosa*	NCGM257	7090694	2014	NA	Midstream urine
IMP-1	NZ_CVUY01000246.1	3.3e-143	506.5	*Pseudomonas aeruginosa*	P1_London_28_IMP_1_04_05	158152	NA	NA	NA
IMP-1	NZ_CVWC01000363.1	7.9e-144	506.5	*Pseudomonas aeruginosa*	P2_London_28_IMP_1_06_05	37866	NA	NA	NA
IMP-1	NZ_CWFP01000391.1	5.3e-144	506.5	*Pseudomonas aeruginosa*	P6_East_of_England_6_IMP_1_03_09	25204	NA	NA	NA
IMP-1	NZ_LYTV01000132.1	8.0e-145	506.1	*Pseudomonas aeruginosa*	TRN6622	2924	2012	Thailand	NA
IMP-10	NZ_BCBB01000121.1	3.2e-144	506.9	*Pseudomonas sp.*	NBRC 111125	20110	NA	NA	NA
IMP-10	NZ_BCBS01000290.1	2.5e-145	507.3	*Pseudomonas sp.*	NBRC 111142	2076	NA	NA	NA
IMP-10	NZ_BCBT01000045.1	6.9e-144	507.3	*Pseudomonas sp.*	NBRC 111143	56542	NA	NA	NA
IMP-13	NZ_JTUA01000030.1	6.2e-143	505.4	*Pseudomonas aeruginosa*	AZPAE14862	133844	2007	India: Chennai	Urinary tract infection
IMP-13	NZ_CVUU01000081.1	2.7e-145	506.1	*Pseudomonas aeruginosa*	P23_East_of_England_6_IMP_13_07_10	987	NA	NA	NA
IMP-13	NZ_CWEU01000115.1	2.7e-145	506.1	*Pseudomonas aeruginosa*	P9_East_of_England_6_IMP_13_08_09	987	NA	NA	NA
IMP-13	NZ_NFGR01000020.1	6.2e-143	505.4	*Pseudomonas aeruginosa*	S137_C02_RS	134100	2013/2014	Italy: Lecco	Respiratory Sample
IMP-13	NZ_NFGQ01000074.1	2.4e-144	505.4	*Pseudomonas aeruginosa*	S143_C02_RS	5135	2013/2014	Italy: Lecco	Respiratory Sample
IMP-13	NZ_NFGP01000019.1	6.2e-143	505.4	*Pseudomonas aeruginosa*	S220_C06_RS	134100	2013/2014	Italy: Modena	Respiratory Sample
IMP-13	NZ_NFGO01000041.1	1.8e-143	505.4	*Pseudomonas aeruginosa*	S247_C06_RS	38967	2013/2014	Italy: Modena	Respiratory Sample
IMP-13	NZ_NFFS01000020.1	6.2e-143	505.4	*Pseudomonas aeruginosa*	S668_C14_BS	134100	2013/2014	Italy: Roma	Blood
IMP-13	NZ_NFFQ01000021.1	6.2e-143	505.4	*Pseudomonas aeruginosa*	S700_C14_RS	134100	2013/2014	Italy: Roma	Respiratory Sample
IMP-13	NZ_NFFM01000067.1	1.8e-144	505.4	*Pseudomonas aeruginosa*	S769_C16_RS	3873	2013/2014	Italy: Casarano	Respiratory Sample
IMP-13	NZ_NFFL01000020.1	6.2e-143	505.4	*Pseudomonas aeruginosa*	S782_C16_RS	134000	2013/2014	Italy: Casarano	Respiratory Sample
IMP-13	NZ_NFFK01000022.1	6.2e-143	505.4	*Pseudomonas aeruginosa*	S787_C16_RS	134000	2013/2014	Italy: Casarano	Respiratory Sample

IMP-13	NZ_NFFI01000020.1	6.2e-143	505.4	*Pseudomonas aeruginosa*	S811_C17_BS	134000	2013/2014	Italy: Napoli	Blood
IMP-13	NZ_NFFE01000043.1	1.8e-143	505.4	*Pseudomonas aeruginosa*	S829_C17_RS	38725	2013/2014	Italy: Napoli	Respiratory Sample
IMP-14	NZ_MPBS01000001.1	7.2e-141	504.2	*Pseudomonas aeruginosa*	AR_0092	6963676	NA	NA	NA
IMP-14	NZ_JXBE01000114.1	1.6e-144	504.2	*Pseudomonas aeruginosa*	ST260	1535	2012	Australia: Victoria	Blood culture
IMP-15	NZ_JTZG01000017.1	1.9e-145	511.9	*Pseudomonas aeruginosa*	AZPAE13872	38616	2010	Mexico	NA
IMP-18	NZ_JTZQ01000083.1	8.0e-144	503.1	*Pseudomonas aeruginosa*	AZPAE13756	3455	2009	Canada	Respiratory tract infection
IMP-18	NZ_CVVU01000084.1	6.5e-144	503.1	*Pseudomonas aeruginosa*	P19_London_7_VIM_2_05_10	2825	NA	NA	NA
IMP-19	NZ_NFFT01000053.1	5.5e-145	507.3	*Pseudomonas aeruginosa*	S658_C13_RS	4525	2013/2014	Italy: Ancona	Respiratory Sample
IMP-26	NZ_JTXD01000152.1	1.5e-145	508.4	*Pseudomonas aeruginosa*	AZPAE14702	2812	2012	Philippines	Respiratory tract infection
IMP-26	NZ_NIJE01000027.1	4.6e-144	508.4	*Pseudomonas aeruginosa*	PAS1	84097	2009	Malaysia: Kuala Lumpur	Wound
IMP-26	NZ_NIJB01000026.1	6.3e-144	508.1	*Pseudomonas aeruginosa*	PAS4	87244	2009	Malaysia: Kuala Lumpur	Urine
IMP-26	NZ_NIIY01000031.1	4.6e-144	508.4	*Pseudomonas aeruginosa*	PAS7	83381	2010	Malaysia: Kuala Lumpur	Urine
IMP-34	NZ_AP014622.1	8.3e-142	507.3	*Pseudomonas aeruginosa*	NCGM 1900	6814936	NA	NA	NA
IMP-34	NZ_AP014622.1	8.3e-142	507.3	*Pseudomonas aeruginosa*	NCGM 1900	6814936	NA	NA	NA
IMP-34	NZ_AP014646.1	8.4e-142	507.3	*Pseudomonas aeruginosa*	NCGM 1984	6850954	NA	NA	NA
IMP-34	NZ_AP014646.1	8.4e-142	507.3	*Pseudomonas aeruginosa*	NCGM 1984	6850954	NA	NA	NA
IMP-45	NZ_MKEO01001262.1	8.3e-147	512.3	*Pseudomonas aeruginosa*	M140A	2177	2012	China: Guangzhou, Guangdong	Urine
IMP-45	NZ_CP016215.1	1.6e-144	512.3	*Pseudomonas aeruginosa*	PA121617	423017	2012	China: Guangzhou	Sputum
IMP-45	NZ_MKEM01000369.1	1.5e-146	512.3	*Pseudomonas aeruginosa*	PA13SY16	3876	2013	China: Guangzhou, Guangdong	Urine
IMP-45	NZ_MBPN01000205.1	1.5e-146	512.3	*Pseudomonas sp.*	WCHP16	3842	2015	China: Sichuan	Sewage
IMP-56	NZ_JTXQ01000099.1	1.2e-144	504.6	*Pseudomonas aeruginosa*	AZPAE14688	1534	2012	Mexico	NA

IMP-62	NZ_JTXR01000035.1	7.3e-147	513.1	*Pseudomonas aeruginosa*	AZPAE14687	3272	2012	Mexico	Respiratory tract infection
IMP-7	NZ_MPCR01000099.1	3.8e-145	506.1	*Pseudomonas aeruginosa*	PAC17	1380	2014	Malaysia	Blood
IMP-7	NZ_MWWM01000109.1	4.2e-145	506.9	*Pseudomonas aeruginosa*	UQCCR 393788042 K AB94	2644	2009	Australia: Brisbane	Blood
IMP-8	NZ_MJMC01000004.1	1.3e-144	509.2	*Pseudomonas aeruginosa*	PA77	39882	2010	Germany	NA
IMP-9	NC_022344.1	5.5e-144	510.8	*Pseudomonas aeruginosa*	PA96	500839	NA	NA	NA
KPC-2	NZ_LODN01000080.1	1.0e-166	582.4	*Pseudomonas aeruginosa*	AATYA	33551	2014	USA	NA
KPC-2	NZ_LQXW01000026.1	1.8e-166	582.4	*Pseudomonas sp.*	ABFPK	59517	2014	USA	NA
KPC-2	NZ_CP027168.1	1.7e-166	582.4	*Pseudomonas aeruginosa*	AR_0356	57053	NA	NA	NA
KPC-2	NZ_JTWN01000034.1	3.5e-167	582.4	*Pseudomonas aeruginosa*	AZPAE14719	11794	2012	Colombia	Respiratory tract infection
KPC-2	NZ_JTWM01000123.1	3.5e-167	582.4	*Pseudomonas aeruginosa*	AZPAE14720	11794	2012	Colombia	Urinary tract infection
KPC-2	NZ_JTWL01000087.1	5.1e-167	582.4	*Pseudomonas aeruginosa*	AZPAE14721	17094	2012	Colombia	Intra-abdominal tract infection
KPC-2	NZ_NOKO01000029.1	3.4e-167	582.4	*Pseudomonas aeruginosa*	CCBH17348	11227	2014	Brazil	Blood
KPC-2	NZ_CP026386.1	1.9e-164	582.4	*Pseudomonas sp.*	PONIH3	6313552	2014	USA	NA
KPC-2	NZ_CP026386.1	1.9e-164	582.4	*Pseudomonas sp.*	PONIH3	6313552	2014	USA	NA
NDM-1	NZ_PESJ01000063.1	3.1e-156	544.7	*Pseudomonas sp.*	MR 02	4495	2016	India	River
NDM-1	NZ_CP020703.1	4.8e-153	544.7	*Pseudomonas aeruginosa*	PASGNDM345	6893164	2015	Singapore	Sputum
NDM-1	NZ_NDFQ01000052.1	1.6e-156	544.7	*Pseudomonas aeruginosa*	PASGNDM544	2365	2015	Singapore	Endotracheal tube (ETT) aspirate
NDM-1	NZ_NDFR01000042.1	1.6e-156	544.7	*Pseudomonas aeruginosa*	PASGNDM571	2365	2015	Singapore	Urine
NDM-1	NZ_NDFS01000048.1	1.8e-156	544.7	*Pseudomonas aeruginosa*	PASGNDM583	2597	2015	Singapore	Urine
NDM-1	NZ_NDFT01000055.1	1.8e-156	544.7	*Pseudomonas aeruginosa*	PASGNDM586	2597	2015	Singapore	Urine
NDM-1	NZ_NDFV01000059.1	1.6e-156	544.7	*Pseudomonas aeruginosa*	PASGNDM587	2365	2015	Singapore	Foot wound swab

NDM-1	NZ_NDFU01000049.1	1.6e-156	544.7	*Pseudomonas aeruginosa*	PASGNDM590	2310	2015	Singapore	Urine
NDM-1	NZ_NDFW01000065.1	1.6e-156	544.7	*Pseudomonas aeruginosa*	PASGNDM591	2365	2015	Singapore	Urine
NDM-1	NZ_NDFX01000043.1	1.6e-156	544.7	*Pseudomonas aeruginosa*	PASGNDM592	2365	2015	Singapore	Urine
NDM-1	NZ_NDFY01000046.1	1.6e-156	544.7	*Pseudomonas aeruginosa*	PASGNDM593	2365	2015	Singapore	Urine
NDM-1	NZ_CP020704.1	4.8e-153	544.7	*Pseudomonas aeruginosa*	PASGNDM699	6985102	2015	Singapore	Sputum
SPM-1	NZ_AFXJ01000001.1	2.2e-158	562.4	*Pseudomonas aeruginosa*	19BR	6742964	NA	NA	NA
SPM-1	NZ_AFXK01000001.1	2.2e-158	562.4	*Pseudomonas aeruginosa*	213BR	6719211	NA	NA	NA
SPM-1	NZ_AFXI01000001.1	2.2e-158	562.4	*Pseudomonas aeruginosa*	9BR	6801503	NA	NA	NA
SPM-1	NZ_MPBU01000007.1	7.0e-159	562.4	*Pseudomonas aeruginosa*	AR_0064	2188636	NA	NA	NA
SPM-1	NZ_JTVP01000053.1	4.5e-162	562.4	*Pseudomonas aeruginosa*	AZPAE14819	1419	2004	Brazil: Sao Paulo	Urinary tract infection
SPM-1	NZ_JTVN01000070.1	4.5e-162	562.4	*Pseudomonas aeruginosa*	AZPAE14821	1419	2004	Brazil: Sao Paulo	Urinary tract infection
SPM-1	NZ_JTUI01000094.1	4.5e-162	562.4	*Pseudomonas aeruginosa*	AZPAE14853	1417	2007	Brazil: Curitiba	Respiratory tract infection
SPM-1	NZ_JTRS01000063.1	4.5e-162	562.4	*Pseudomonas aeruginosa*	AZPAE14923	1417	2008	Brazil: Sao Paulo	Respiratory tract infection
SPM-1	NZ_CP021380.1	2.3e-158	562.4	*Pseudomonas aeruginosa*	CCBH4851	7060875	2008	Brazil	Catheter tip
SPM-1	NZ_CP021380.1	2.3e-158	562.4	*Pseudomonas aeruginosa*	CCBH4851	7060875	2008	Brazil	Catheter tip
SPM-1	NZ_CP015001.1	2.2e-158	562.4	*Pseudomonas aeruginosa*	PA1088	6721480	1997	Brazil: Sao Paulo, SP	Urine
SPM-1	NZ_CP015003.1	2.2e-158	562.4	*Pseudomonas aeruginosa*	PA11803	7006578	2011	Brazil: Sao Paulo, SP	Bloodstream
SPM-1	NZ_LVXB01000001.1	1.1e-158	562.4	*Pseudomonas aeruginosa*	PA12117	3316996	2012	Brazil: Sao Paulo, SP	Bloodstream
SPM-1	NZ_PHSS01000091.1	4.2e-162	562.4	*Pseudomonas aeruginosa*	PA151	1306	2011	Brazil: Sao Paulo	River
SPM-1	NZ_PHST01000144.1	1.1e-161	562.4	*Pseudomonas aeruginosa*	PA19	3393	2010	Brazil: Sao Paulo	River
SPM-1	NZ_LVWC01000001.1	2.2e-158	562.4	*Pseudomonas aeruginosa*	PA3448	6788539	2003	Brazil: Sao Paulo -SP	bloodstream

SPM-1	NZ_LVWC01000001.1	2.2e-158	562.4	*Pseudomonas aeruginosa*	PA3448	6788539	2003	Brazil: Sao Paulo -SP	Bloodstream
SPM-1	NZ_CP014999.1	2.2e-158	562.4	*Pseudomonas aeruginosa*	PA7790	7018690	2006	Brazil: Sao Paulo, SP	Tracheal aspirate
SPM-1	NZ_CP015002.1	2.2e-158	562.4	*Pseudomonas aeruginosa*	PA8281	6928736	2007	Brazil: Sao Paulo, SP	Tracheal aspirate
SPM-1	NZ_CP015002.1	2.2e-158	562.4	*Pseudomonas aeruginosa*	PA8281	6928736	2007	Brazil: Sao Paulo, SP	Tracheal aspirate
VIM-1	NC_019906.1	1.1e-150	530.4	*Pseudomonas putida*	HB3267	80360	NA	NA	NA
VIM-1	NZ_NFGN01000084.1	3.6e-152	530.4	*Pseudomonas aeruginosa*	S252_C06_RS	2641	2013/2014	Italy: Modena	Respiratory Sample
VIM-1	NZ_NFGM01000055.1	3.4e-152	530.4	*Pseudomonas aeruginosa*	S292_C06_RS	2541	2013/2014	Italy: Modena	Respiratory Sample
VIM-1	NZ_NFGC01000059.1	1.3e-151	530.4	*Pseudomonas aeruginosa*	S461_C10_RS	9365	2013/2014	Italy: Firenze	Respiratory Sample
VIM-1	NZ_NFGB01000058.1	4.8e-152	530.4	*Pseudomonas aeruginosa*	S518_C10_BS	3551	2013/2014	Italy: Firenze	Blood
VIM-1	NZ_NFGV01000057.1	2.2e-151	530.4	*Pseudomonas aeruginosa*	S53_C01_BS	16165	2013/2014	Italy: Milano	Blood
VIM-1	NZ_NFFZ01000073.1	4.4e-152	530.4	*Pseudomonas aeruginosa*	S567_C10_BS	3236	2013/2014	Italy: Firenze	Blood
VIM-1	NZ_NFFZ01000073.1	4.4e-152	530.4	*Pseudomonas aeruginosa*	S567_C10_BS	3236	2013/2014	Italy: Firenze	Blood
VIM-1	NZ_NFGU01000072.1	2.2e-151	530.4	*Pseudomonas aeruginosa*	S57_C01_BS	16191	2013/2014	Italy: Milano	Blood
VIM-1	NZ_NFFY01000029.1	1.1e-150	530.4	*Pseudomonas aeruginosa*	S611_C13_RS	78563	2013/2014	Italy: Ancona	Respiratory Sample
VIM-1	NZ_NFFX01000040.1	4.7e-151	530.4	*Pseudomonas aeruginosa*	S619_C13_RS	35200	2013/2014	Italy: Ancona	Respiratory Sample
VIM-1	NZ_NFFV01000038.1	6.5e-151	530.4	*Pseudomonas aeruginosa*	S626_C13_RS	48362	2013/2014	Italy: Ancona	Respiratory Sample
VIM-1	NZ_NFFU01000013.1	2.8e-150	530.4	*Pseudomonas aeruginosa*	S650_C13_BS	207944	2013/2014	Italy: Ancona	Blood
VIM-1	NZ_NFFO01000062.1	4.0e-151	530.4	*Pseudomonas aeruginosa*	S742_C15_BS	29466	2013/2014	Italy: San Giovanni Rotondo	Blood
VIM-1	NZ_NFFG01000090.1	6.5e-152	530.4	*Pseudomonas aeruginosa*	S823_C17_RS	4788	2013/2014	Italy: Napoli	Respiratory Sample
VIM-1	NZ_NFFC01000079.1	5.1e-152	530.4	*Pseudomonas aeruginosa*	S854_C18_BS	3763	2013/2014	Italy: Cosenza	Blood
VIM-11	NZ_JTZD01000113.1	8.4e-152	528.5	*Pseudomonas aeruginosa*	AZPAE13879	1646	2010	Argentina	NA

VIM-11	NZ_JYGC02000004.1	2.6e-149	528.9	*Pseudomonas aeruginosa*	MRSN 20176	657351	2013	Afghanistan	Surveillance swab
VIM-2	NZ_MWUI01000032.1	8.9e-151	530.0	*Pseudomonas stutzeri*	40D2	50445	2012	Bangladesh: Dhaka	Inflamed tissue lesion of infected patient during surgery
VIM-2	NZ_MPBQ01000001.1	7.7e-149	530.0	*Pseudomonas aeruginosa*	AR_0100	4354749	NA	NA	NA
VIM-2	NZ_MPBN01000001.1	1.2e-148	530.0	*Pseudomonas aeruginosa*	AR_0108	6879367	NA	NA	NA
VIM-2	NZ_CP027174.1	1.2e-148	530.0	*Pseudomonas aeruginosa*	AR_0230	7012922	NA	NA	NA
VIM-2	NZ_JTZQ01000101.1	1.7e-152	530.0	*Pseudomonas aeruginosa*	AZPAE13756	973	2009	Canada	Respiratory tract infection
VIM-2	NZ_JTZM01000008.1	9.4e-150	530.0	*Pseudomonas aeruginosa*	AZPAE13853	531755	2010	India	NA
VIM-2	NZ_JTZK01000020.1	3.3e-150	530.0	*Pseudomonas aeruginosa*	AZPAE13858	185470	2010	India	NA
VIM-2	NZ_JTZE01000060.1	3.4e-152	530.0	*Pseudomonas aeruginosa*	AZPAE13877	1932	2010	Romania	NA
VIM-2	NZ_JTYE01000112.1	1.7e-152	530.0	*Pseudomonas aeruginosa*	AZPAE14463	974	2011	Colombia: Bogota	Urinary tract infection
VIM-2	NZ_JTXF01000066.1	1.4e-150	530.0	*Pseudomonas aeruginosa*	AZPAE14700	81597	2012	Philippines	Respiratory tract infection
VIM-2	NZ_JTXC01000105.1	4.0e-152	530.0	*Pseudomonas aeruginosa*	AZPAE14703	2264	2012	Philippines	Intra-abdominal tract infection
VIM-2	NZ_JTXA01000187.1	4.0e-152	530.0	*Pseudomonas aeruginosa*	AZPAE14705	2264	2012	Greece	Urinary tract infection
VIM-2	NZ_JTWY01000131.1	1.7e-152	530.0	*Pseudomonas aeruginosa*	AZPAE14707	971	2012	Greece	Respiratory tract infection
VIM-2	NZ_JTWU01000220.1	1.7e-152	530.0	*Pseudomonas aeruginosa*	AZPAE14712	943	2012	Venezuela	Intra-abdominal tract infection
VIM-2	NZ_JTWT01000168.1	1.7e-152	530.0	*Pseudomonas aeruginosa*	AZPAE14713	972	2012	Venezuela	Intra-abdominal tract infection
VIM-2	NZ_JTWS01000232.1	1.7e-152	530.0	*Pseudomonas aeruginosa*	AZPAE14714	972	2012	Venezuela	Intra-abdominal tract infection
VIM-2	NZ_JTWQ01000139.1	3.2e-152	530.0	*Pseudomonas aeruginosa*	AZPAE14716	1814	2012	Venezuela	Intra-abdominal tract infection
VIM-2	NZ_JTWO01000202.1	4.4e-152	530.0	*Pseudomonas aeruginosa*	AZPAE14718	2520	2012	USA	Respiratory tract infection
VIM-2	NZ_JTWI01000104.1	1.7e-152	530.0	*Pseudomonas aeruginosa*	AZPAE14724	972	2012	Italy	Intra-abdominal tract infection
VIM-2	NZ_JTWD01000173.1	1.7e-152	530.0	*Pseudomonas aeruginosa*	AZPAE14729	972	2012	Italy	Urinary tract infection

VIM-2	NZ_JTVX01000068.1	1.1e-151	530.0	*Pseudomonas aeruginosa*	AZPAE14811	6396	2004	India: Mumbai	Respiratory tract infection
VIM-2	NZ_JTRT01000042.1	7.2e-152	530.0	*Pseudomonas aeruginosa*	AZPAE14922	4067	2009	France: Paris	Respiratory tract infection
VIM-2	NZ_JTRM01000079.1	3.4e-152	530.0	*Pseudomonas aeruginosa*	AZPAE14929	1932	2009	Germany: Aachen	Urinary tract infection
VIM-2	NZ_JTQI01000054.1	3.5e-152	530.4	*Pseudomonas aeruginosa*	AZPAE14959	2614	2009	India: Mumbai	Intra-abdominal tract infection
VIM-2	NZ_JTPK01000055.1	7.2e-152	530.0	*Pseudomonas aeruginosa*	AZPAE14984	4068	2010	France: Paris	Urinary tract infection
VIM-2	NZ_JTNR01000060.1	2.3e-151	530.0	*Pseudomonas aeruginosa*	AZPAE15029	12782	2011	France: Paris	Respiratory tract infection
VIM-2	NZ_LFDH01000007.1	4.0e-150	530.0	*Pseudomonas aeruginosa*	BK6	227447	2013	India: Madurai	Cornea from keratitis patient
VIM-2	NZ_LFMP01000001.1	4.9e-149	530.0	*Pseudomonas aeruginosa*	BTP032	2771368	2014	USA: Rochester, Minnesota	Associated Infection
VIM-2	NZ_LFMV01000038.1	1.5e-149	530.0	*Pseudomonas aeruginosa*	BTP038	838992	2014	USA: Rochester, Minnesota	Associated Infection
VIM-2	NZ_CP011317.1	1.3e-148	530.0	*Pseudomonas aeruginosa*	Carb01 63	7497593	2012	Netherlands:Rotterdam	Microbial feature
VIM-2	NZ_NBVZ01000065.1	1.7e-151	530.4	*Pseudomonas aeruginosa*	DZ-B1	12785	2014	China: Shandong	Feces swab
VIM-2	NZ_CWGH010000042.1	1.6e-151	530.0	*Pseudomonas aeruginosa*	E1_London_17_VIM_2_02_09	9036	NA	NA	NA
VIM-2	NZ_CVVZ01000076.1	1.7e-151	530.0	*Pseudomonas aeruginosa*	E10_London_26_VIM_2_06_13	9854	NA	NA	NA
VIM-2	NZ_CVVM01000189.1	2.6e-151	530.0	*Pseudomonas aeruginosa*	E11_London_26_VIM_2_06_13	15035	NA	NA	NA
VIM-2	NZ_CVUX01000007.1	2.2e-151	530.0	*Pseudomonas aeruginosa*	E12_London_26_VIM_2_06_13	12608	NA	NA	NA
VIM-2	NZ_CVVQ01000050.1	2.8e-152	530.0	*Pseudomonas aeruginosa*	E13_London_26_VIM_2_06_13	1571	NA	NA	NA
VIM-2	NZ_CWGG010000041.1	1.8e-152	530.0	*Pseudomonas aeruginosa*	E14_London_26_VIM_2_06_13	1017	NA	NA	NA
VIM-2	NZ_CVWG01001514.1	6.0e-151	530.0	*Pseudomonas aeruginosa*	E16_London_17_VIM_2_04_14	33943	NA	NA	NA
VIM-2	NZ_CVUZ01000153.1	2.8e-152	530.0	*Pseudomonas aeruginosa*	E2_London_17_VIM_2_02_09	1571	NA	NA	NA
VIM-2	NZ_CVVB01000030.1	1.6e-151	530.0	*Pseudomonas aeruginosa*	E3_London_17_VIM_2_03_09	9035	NA	NA	NA
VIM-2	NZ_CVUK01000042.1	1.8e-152	530.0	*Pseudomonas aeruginosa*	E4_London_17_VIM_2_03_09	1020	NA	NA	NA

VIM-2	NZ_CVWE01000645.1	1.8e-152	530.0	*Pseudomonas aeruginosa*	E5_London_17_VIM_2_12_12	1012	NA	NA	NA
VIM-2	NZ_CVWD01000239.1	1.8e-152	530.0	*Pseudomonas aeruginosa*	E6_London_17_VIM_2_12_12	1002	NA	NA	NA
VIM-2	NZ_CVWB01000082.1	1.8e-151	530.0	*Pseudomonas aeruginosa*	E7_London_9_VIM_2_02_13	10104	NA	NA	NA
VIM-2	NZ_CVUW01000087.1	1.8e-152	530.0	*Pseudomonas aeruginosa*	E8_London_17_VIM_2_04_13	1009	NA	NA	NA
VIM-2	NZ_CVUN01000192.1	2.6e-151	530.0	*Pseudomonas aeruginosa*	E9_London_17_VIM_2_04_13	15035	NA	NA	NA
VIM-2	NZ_NINS01000056.1	6.0e-151	530.0	*Pseudomonas aeruginosa*	FFUP_PS_105	33810	2012	Portugal	Urine
VIM-2	NZ_NINQ01000001.1	5.4e-151	530.0	*Pseudomonas aeruginosa*	FFUP_PS_12	30772	2002	Portugal	NA
VIM-2	NZ_NINR01000084.1	4.8e-151	530.0	*Pseudomonas aeruginosa*	FFUP_PS_144	27056	2013	Portugal	Urine
VIM-2	NZ_NINU01000134.1	1.2e-151	529.3	*Pseudomonas aeruginosa*	FFUP_PS_35	4035	2002	Portugal	Urine
VIM-2	NZ_NINT01000013.1	1.5e-150	530.0	*Pseudomonas aeruginosa*	FFUP_PS_37	87430	2008	Portugal	Bronchial secretions
VIM-2	NZ_PJQP01000061.1	4.9e-152	530.0	*Pseudomonas sp.*	FFUP_PS_41	2763	2008	Portugal: Porto	Endotracheal tube secretions
VIM-2	NZ_NINN01000021.1	1.7e-150	530.0	*Pseudomonas aeruginosa*	FFUP_PS_65	99153	2010	Portugal	Urine
VIM-2	NZ_NINP01000047.1	4.7e-151	530.0	*Pseudomonas aeruginosa*	FFUP_PS_CB5	26506	2002	Portugal	Bronchial aspirate
VIM-2	NZ_NINO01000017.1	5.7e-151	530.0	*Pseudomonas aeruginosa*	FFUP_PS_CB58	32345	2004	Portugal	Urine
VIM-2	NZ_AEVV03000013.1	5.4e-151	530.0	*Pseudomonas aeruginosa*	HB13	30558	NA	NA	NA
VIM-2	NZ_NINV01000065.1	2.7e-151	530.0	*Pseudomonas aeruginosa*	HSV3483	15428	1995	Portugal	Urine
VIM-2	NZ_NWBV01000195.1	3.5e-152	530.0	*Pseudomonas aeruginosa*	ICBBVIM-2	1962	2017	Brazil: Sao Paulo	Oral swab
VIM-2	NZ_MZND01000183.1	1.7e-152	530.0	*Pseudomonas aeruginosa*	ICBDVIM-2	941	2017	Brazil: Sao Paulo	Ear
VIM-2	NZ_NWBW010000093.1	1.7e-152	530.0	*Pseudomonas aeruginosa*	ICBSVIM-2	947	2017	Brazil: Sao Paulo	Environmental swab
VIM-2	NZ_CP010893.1	6.4e-151	530.0	*Pseudomonas sp.*	MRSN12121	36379	NA	USA	Urine
VIM-2	NZ_BCBD01000273.1	6.9e-152	529.6	*Pseudomonas sp.*	NBRC 111127	3009	NA	NA	NA

VIM-2	NZ_BCBE01000104.1	3.1e-151	530.0	*Pseudomonas sp.*	NBRC 111128	17527	NA	NA	NA
VIM-2	NZ_CVVA01000017.1	1.8e-152	530.0	*Pseudomonas aeruginosa*	P10_London_6_VIM_2_09_09	1022	NA	NA	NA
VIM-2	NZ_CVVH01000182.1	1.6e-151	530.0	*Pseudomonas aeruginosa*	P11_London_17_VIM_2_10_09	9036	NA	NA	NA
VIM-2	NZ_CVUP01000182.1	2.6e-151	530.0	*Pseudomonas aeruginosa*	P12_London_17_VIM_2_11_09	15035	NA	NA	NA
VIM-2	NZ_CVUL01000160.1	2.6e-151	530.0	*Pseudomonas aeruginosa*	P13_London_14_VIM_2_12_09	15035	NA	NA	NA
VIM-2	NZ_CVVT01000092.1	2.7e-152	530.0	*Pseudomonas aeruginosa*	P14_London_17_VIM_2_01_10	1514	NA	NA	NA
VIM-2	NZ_CVVD01000036.1	1.8e-152	530.0	*Pseudomonas aeruginosa*	P16_Lonon_17_VIM_2_02_10	1012	NA	NA	NA
VIM-2	NZ_CVUV01000039.1	1.1e-151	530.0	*Pseudomonas aeruginosa*	P17_North_West_14_VIM_2_03_10	6417	NA	NA	NA
VIM-2	NZ_CVVY01000315.1	2.7e-152	530.0	*Pseudomonas aeruginosa*	P18_London_17_VIM_2_04_10	1524	NA	NA	NA
VIM-2	NZ_CVVU01000098.1	2.9e-152	530.0	*Pseudomonas aeruginosa*	P19_London_7_VIM_2_05_10	1625	NA	NA	NA
VIM-2	NZ_CVUO01000054.1	1.8e-152	530.0	*Pseudomonas aeruginosa*	P20_London_17_VIM_2_05_10	1018	NA	NA	NA
VIM-2	NZ_CVVE01000152.1	2.6e-151	530.0	*Pseudomonas aeruginosa*	P21_London_17_VIM_2_06_10	15035	NA	NA	NA
VIM-2	NZ_CVVI01000002.1	1.0e-151	530.0	*Pseudomonas aeruginosa*	P22_London_17_VIM_2_06_10	5825	NA	NA	NA
VIM-2	NZ_CVUR01000052.1	1.8e-152	530.0	*Pseudomonas aeruginosa*	P24_London_17_VIM_2_08_10	1032	NA	NA	NA
VIM-2	NZ_CVVL01000058.1	1.8e-152	530.0	*Pseudomonas aeruginosa*	P25_London_12_VIM_2_08_10	1020	NA	NA	NA
VIM-2	NZ_CVVN01000218.1	2.6e-151	530.0	*Pseudomonas aeruginosa*	P26_Wales_1_VIM_2_11_10	15035	NA	NA	NA
VIM-2	NZ_CVVX01000292.1	2.6e-151	530.0	*Pseudomonas aeruginosa*	P27_Wales_1_VIM_2_02_11	15035	NA	NA	NA
VIM-2	NZ_CVVO01000009.1	1.5e-151	530.0	*Pseudomonas aeruginosa*	P29_London_12_VIM_2_05_11	8275	NA	NA	NA
VIM-2	NZ_CVVV01000124.1	2.8e-152	530.0	*Pseudomonas aeruginosa*	P3_North_West_16_VIM_2_07_06	1571	NA	NA	NA
VIM-2	NZ_CVVK01000198.1	2.6e-151	530.0	*Pseudomonas aeruginosa*	P30_South_East_2_VIM_2_10_11	15035	NA	NA	NA
VIM-2	NZ_CVVS01000315.1	2.6e-151	530.0	*Pseudomonas aeruginosa*	P31_Wales_1_VIM_2_11_11	15035	NA	NA	NA

VIM-2	NZ_CVVJ01000185.1	2.6e-151	530.0	*Pseudomonas aeruginosa*	P32_London_17_VIM_2_10_11	15035	NA	NA	NA
VIM-2	NZ_CVVG01000173.1	2.6e-151	530.0	*Pseudomonas aeruginosa*	P33_London_28_VIM_2_02_12	15035	NA	NA	NA
VIM-2	NZ_CVVF01000171.1	2.6e-151	530.0	*Pseudomonas aeruginosa*	P34_London_28_VIM_2_02_12	15035	NA	NA	NA
VIM-2	NZ_CVUS01000017.1	2.5e-151	530.0	*Pseudomonas aeruginosa*	P35_London_26_VIM_2_05_12	14212	NA	NA	NA
VIM-2	NZ_CVVP01000224.1	2.6e-151	530.0	*Pseudomonas aeruginosa*	P36_West_Midlands_5_VIM_2_06_12	15035	NA	NA	NA
VIM-2	NZ_CVUJ01000038.1	1.8e-152	530.0	*Pseudomonas aeruginosa*	P37_London_28_VIM_2_07_12	1043	NA	NA	NA
VIM-2	NZ_CVUT01000041.1	1.8e-152	530.0	*Pseudomonas aeruginosa*	P38_London_12_VIM_2_08_12	1018	NA	NA	NA
VIM-2	NZ_CVWA01000091.1	2.7e-152	530.0	*Pseudomonas aeruginosa*	P4_London_1_VIM_2_10_07	1524	NA	NA	NA
VIM-2	NZ_CVUM01000027.1	1.8e-152	530.0	*Pseudomonas aeruginosa*	P40_Scotland_4_VIM_2_09_12	1026	NA	NA	NA
VIM-2	NZ_CVWH01000032.1	1.8e-152	530.0	*Pseudomonas aeruginosa*	P42_1_London_26_VIM_2_10_12	1020	NA	NA	NA
VIM-2	NZ_CVWI01000150.1	2.6e-151	530.0	*Pseudomonas aeruginosa*	P42_2_London_28_VIM_2_11_12	15035	NA	NA	NA
VIM-2	NZ_CVWJ01000041.1	1.8e-152	530.0	*Pseudomonas aeruginosa*	P43_1_London_9_VIM_2_11_12	1022	NA	NA	NA
VIM-2	NZ_CVWK01000175.1	2.6e-151	530.0	*Pseudomonas aeruginosa*	P43_2_London_9_VIM_2_11_12	15035	NA	NA	NA
VIM-2	NZ_CVWL01000379.1	2.6e-151	530.0	*Pseudomonas aeruginosa*	P44_Wales_1_VIM_2_11_12	15035	NA	NA	NA
VIM-2	NZ_CVWO01000426.1	2.7e-152	530.0	*Pseudomonas aeruginosa*	P45_London_17_VIM_2_12_12	1516	NA	NA	NA
VIM-2	NZ_CVWN01000191.1	6.9e-152	530.0	*Pseudomonas aeruginosa*	P47_London_12_VIM_2_12_12	3897	NA	NA	NA
VIM-2	NZ_CVWT01000424.1	7.0e-152	530.0	*Pseudomonas aeruginosa*	P48_London_17_VIM_2_01_13	3984	NA	NA	NA
VIM-2	NZ_CVWQ01000099.1	1.4e-150	530.0	*Pseudomonas aeruginosa*	P49_London_7_VIM_2_01_13	79069	NA	NA	NA
VIM-2	NZ_CVWS01000440.1	2.1e-152	530.4	*Pseudomonas aeruginosa*	P5_London_26_VIM_2_01_09	1572	NA	NA	NA
VIM-2	NZ_CVWP01000175.1	2.6e-151	530.0	*Pseudomonas aeruginosa*	P50_London_9_VIM_2_01_13	15035	NA	NA	NA
VIM-2	NZ_CVWR01000046.1	2.6e-151	530.0	*Pseudomonas aeruginosa*	P51_1_London_9_VIM_2_02_13	15035	NA	NA	NA

VIM-2	NZ_CVWU01000051.1	1.8e-152	530.0	*Pseudomonas aeruginosa*	P51_2_London_11_VIM_2_02_13	1027	NA	NA	NA
VIM-2	NZ_CVWV01000016.1	2.6e-151	530.0	*Pseudomonas aeruginosa*	P52_1_London_26_VIM_2_02_13	15035	NA	NA	NA
VIM-2	NZ_CVWW010000030.1	2.6e-151	530.0	*Pseudomonas aeruginosa*	P52_2_London_26_VIM_2_02_13	15035	NA	NA	NA
VIM-2	NZ_CWER01000165.1	8.2e-152	530.0	*Pseudomonas aeruginosa*	P53_London_9_VIM_2_02_13	4645	NA	NA	NA
VIM-2	NZ_CWFR01000312.1	1.0e-151	530.0	*Pseudomonas aeruginosa*	P54_1_London_24_VIM_2_04_13	5826	NA	NA	NA
VIM-2	NZ_CVXL01000023.1	2.4e-151	530.0	*Pseudomonas aeruginosa*	P54_2_London_24_VIM_2_04_13	13396	NA	NA	NA
VIM-2	NZ_CVWX01000168.1	2.6e-151	530.0	*Pseudomonas aeruginosa*	P55_London_26_VIM_2_05_13	15035	NA	NA	NA
VIM-2	NZ_CVXC01000069.1	1.8e-152	530.0	*Pseudomonas aeruginosa*	P56_London_12_VIM_2_07_13	1020	NA	NA	NA
VIM-2	NZ_CVWZ01000040.1	1.8e-152	530.0	*Pseudomonas aeruginosa*	P57_South_East_3_VIM_2_09_13	1040	NA	NA	NA
VIM-2	NZ_CVXN01000005.1	2.6e-151	530.0	*Pseudomonas aeruginosa*	P59_Wales_1_VIM_2_09_13	15035	NA	NA	NA
VIM-2	NZ_CVXD01000052.1	1.8e-152	530.0	*Pseudomonas aeruginosa*	P60_London_6_VIM_2_11_13	1042	NA	NA	NA
VIM-2	NZ_CVXB01000200.1	2.6e-151	530.0	*Pseudomonas aeruginosa*	P61_London_9_VIM_2_11_13	15035	NA	NA	NA
VIM-2	NZ_CWEO01000045.1	2.5e-151	530.0	*Pseudomonas aeruginosa*	P62_London_9_VIM_2_01_14	14204	NA	NA	NA
VIM-2	NZ_CWFH01000038.1	2.3e-151	530.0	*Pseudomonas aeruginosa*	P63_London_25_VIM_2_03_14	12782	NA	NA	NA
VIM-2	NZ_CVXP01000128.1	1.8e-152	530.0	*Pseudomonas aeruginosa*	P7_London_17_VIM_2_06_09	1002	NA	NA	NA
VIM-2	NZ_CWET01000138.1	1.8e-152	530.0	*Pseudomonas aeruginosa*	P8_1_South_East_10_VIM_2_07_09	999	NA	NA	NA
VIM-2	NZ_CVXH01000194.1	2.6e-151	530.0	*Pseudomonas aeruginosa*	P8_2_London_17_VIM_2_07_13	15035	NA	NA	NA
VIM-2	NZ_AOIH01000060.1	3.7e-152	530.0	*Pseudomonas aeruginosa*	PA21_ST175	2117	2010	Spain	Blood
VIM-2	NZ_CP017293.1	1.2e-148	530.0	*Pseudomonas aeruginosa*	PA83	6816227	2013	Germany	NA
VIM-2	NZ_CP017073.1	8.1e-149	530.4	*Pseudomonas putida*	PP112420	6031212	2011	China: Guangzhou	Urine
VIM-2	NZ_CP016955.1	1.3e-148	530.0	*Pseudomonas aeruginosa*	RIVM-EMC2982	7380063	NA	Netherlands: Bilthoven	NA

VIM-2	NZ_CP011370.1	2.8e-150	530.0	*Pseudomonas aeruginosa*	S04 90	159187	2013	Netherlands: Rotterdam	Microbial material
VIM-2	NZ_NFGK01000057.1	2.4e-151	530.0	*Pseudomonas aeruginosa*	S422_C09_BS	13810	2013/2014	Italy: Udine	Blood
VIM-2	NZ_NFGJ01000105.1	1.8e-152	530.0	*Pseudomonas aeruginosa*	S426_C09_BS	1036	2013/2014	Italy: Udine	Blood
VIM-2	NZ_NFGI01000040.1	2.4e-151	530.0	*Pseudomonas aeruginosa*	S432_C09_RS	13810	2013/2014	Italy: Udine	Respiratory Sample
VIM-2	NZ_NFGH01000056.1	2.4e-151	530.0	*Pseudomonas aeruginosa*	S434_C09_BS	13810	2013/2014	Italy: Udine	Blood
VIM-2	NZ_NFGG01000040.1	2.4e-151	530.0	*Pseudomonas aeruginosa*	S435_C09_BS	13710	2013/2014	Italy: Udine	Blood
VIM-2	NZ_NFGF01000042.1	2.4e-151	530.0	*Pseudomonas aeruginosa*	S440_C09_BS	13710	2013/2014	Italy: Udine	Blood
VIM-2	NZ_NFGE01000042.1	2.4e-151	530.0	*Pseudomonas aeruginosa*	S442_C09_BS	13710	2013/2014	Italy: Udine	Blood
VIM-2	NZ_NFGD01000045.1	2.4e-151	530.0	*Pseudomonas aeruginosa*	S443_C09_RS	13710	2013/2014	Italy: Udine	Respiratory Sample
VIM-2	NZ_NFGA01000029.1	1.7e-150	530.0	*Pseudomonas aeruginosa*	S558_C10_BS	97921	2013/2014	Italy: Firenze	Blood
VIM-2	NZ_NFGT01000092.1	2.0e-152	530.0	*Pseudomonas aeruginosa*	S61_C01_BS	1111	2013/2014	Italy: Milano	Blood
VIM-2	NZ_NFFR01000057.1	3.9e-152	530.0	*Pseudomonas aeruginosa*	S669_C14_BS	2201	2013/2014	Italy: Roma	Blood
VIM-2	NZ_NFFP01000094.1	4.6e-152	530.0	*Pseudomonas aeruginosa*	S708_C14_RS	2606	2013/2014	Italy: Roma	Respiratory Sample
VIM-2	NZ_NFFN01000017.1	2.6e-150	530.0	*Pseudomonas aeruginosa*	S749_C15_RS	146236	2013/2014	Italy: San Giovanni Rotondo	Respiratory Sample
VIM-2	NZ_NFFJ01000075.1	1.1e-151	530.4	*Pseudomonas aeruginosa*	S794_C17_BS	8276	2013/2014	Italy: Napoli	Blood
VIM-2	NZ_NFFH01000037.1	6.2e-151	530.4	*Pseudomonas aeruginosa*	S819_C17_BS	45581	2013/2014	Italy: Napoli	Blood
VIM-2	NZ_NFFF01000041.1	6.2e-151	530.4	*Pseudomonas aeruginosa*	S827_C17_BS	45581	2013/2014	Italy: Napoli	Blood
VIM-2	NZ_NFFD01000041.1	6.2e-151	530.4	*Pseudomonas aeruginosa*	S830_C17_BS	45581	2013/2014	Italy: Napoli	Blood
VIM-2	NZ_LYTW01000096.1	8.0e-152	530.0	*Pseudomonas aeruginosa*	TRN6633	4566	2013	Russia	NA
VIM-2	NZ_LYTX01000091.1	8.0e-152	530.0	*Pseudomonas aeruginosa*	TRN6635	4563	2013	Russia	NA
VIM-2	NZ_CP008739.1	9.2e-149	530.4	*Pseudomonas aeruginosa*	VRFPA04	6818030	2013	India:Chennai	Corneal button from patient with corneal keratitis

VIM-2	NZ_MBPN01000207.1	6.5e-152	530.0	*Pseudomonas sp.*	WCHP16	3690	2015	China: Sichuan	Sewage
VIM-2	NZ_LLVI01000133.1	2.7e-152	530.0	*Pseudomonas aeruginosa*	WH-SGI-V-07322	1508	2003	Colombia	Blood
VIM-2	NZ_LLVL01000137.1	2.7e-152	530.0	*Pseudomonas aeruginosa*	WH-SGI-V-07325	1508	1999	Colombia	Urine
VIM-2	NZ_LLQC01000091.1	3.8e-152	530.0	*Pseudomonas aeruginosa*	WH-SGI-V-07496	2165	2005	USA	Hospital
VIM-2	NZ_LLQZ01000051.1	1.3e-150	530.0	*Pseudomonas aeruginosa*	WH-SGI-V-07628	76200	2005	USA	Hospital
VIM-36	NZ_LLSF01000116.1	3.4e-152	530.4	*Pseudomonas aeruginosa*	WH-SGI-V-07685	2498	2005	USA	Hospital
VIM-4	NZ_MPBV01000001.1	1.7e-149	531.6	*Pseudomonas aeruginosa*	AR_0054	2863301	NA	NA	NA
VIM-4	NZ_JTWZ01000116.1	1.9e-152	531.6	*Pseudomonas aeruginosa*	AZPAE14706	3202	2012	Greece	Intra-abdominal tract infection
VIM-5	NZ_JTZJ01000113.1	2.9e-152	529.3	*Pseudomonas aeruginosa*	AZPAE13860	952	2010	India	NA
VIM-5	NZ_JTTX01000070.1	8.6e-152	529.3	*Pseudomonas aeruginosa*	AZPAE14865	2861	2007	India: Chennai	Respiratory tract infection
VIM-5	NZ_JTSP01000126.1	5.3e-152	529.3	*Pseudomonas aeruginosa*	AZPAE14900	1760	2008	India: Chennai	Intra-abdominal tract infection
VIM-5	NZ_PJCP01000041.1	4.1e-151	529.3	*Pseudomonas guariconensis*	MR119	13479	2014	Nigeria: Ibadan	Soil
VIM-5	NZ_PJCL01000002.1	1.5e-149	529.3	*Pseudomonas plecoglossicida*	MR134	491336	2014	Nigeria: Ibadan	Soil
VIM-5	NZ_PJCL01000056.1	9.5e-152	529.3	*Pseudomonas plecoglossicida*	MR134	3169	2014	Nigeria: Ibadan	Soil
VIM-5	NZ_PJCM01000001.1	1.5e-149	529.3	*Pseudomonas plecoglossicida*	MR135	491336	2014	Nigeria: Ibadan	Soil
VIM-5	NZ_PJCQ01000042.1	3.2e-151	529.3	*Pseudomonas guariconensis*	MR144	10655	2014	Nigeria: Ibadan	Soil
VIM-5	NZ_PJCR01000038.1	4.1e-151	529.3	*Pseudomonas guariconensis*	MR149	13479	2014	Nigeria: Ibadan	Soil
VIM-5	NZ_PJCO01000068.1	2.4e-151	529.3	*Pseudomonas plecoglossicida*	MR170	7887	2014	Nigeria: Ibadan	Soil
VIM-5	NZ_PJCJ01000004.1	1.5e-149	529.3	*Pseudomonas plecoglossicida*	MR69	491416	2014	Nigeria: Ibadan	Soil
VIM-5	NZ_PJCK01000003.1	1.5e-149	529.3	*Pseudomonas plecoglossicida*	MR70	491062	2014	Nigeria: Ibadan	Soil
VIM-5	NZ_PJCN01000069.1	2.4e-151	529.3	*Pseudomonas plecoglossicida*	MR83	7887	2014	Nigeria: Ibadan	Soil

VIM-6	NZ_LOHJ01000053.1	1.1e-150	528.9	*Pseudomonas aeruginosa*	105857	28969	2008	USA: Fort Sam Houston	Right knee
VIM-6	NZ_JYGB02000018.1	1.2e-150	528.9	*Pseudomonas aeruginosa*	MRSN 17623	30188	NA	USA	NA

NA, not available.

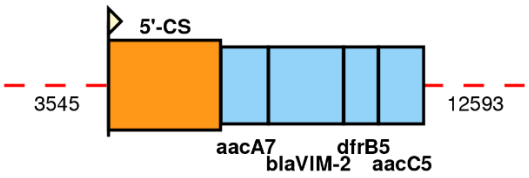


Figure S1. Genetic environment of a novel genomic island (GI) harboring *bla*$_{VIM-2}$ in *P. aeruginosa* strain AZPAE13853. Gene cassettes are shown by pale blue boxes, the conserved sequence (5'-CS) of the integron as orange boxes. Gaps >50 bp are indicated by dashed red lines and the length in bp given. Transposons IRs are shown as flags, with the flat side at the outer boundary of the transposon.

(((GCF_001180625.1_P11_London_17_VIM_2_10_09_genomic/1-146106:0.0,GCF_001180725.1_P16_Lonon_17_VIM_2_02_10_genomic/1-146106:0.0):0.00055,((GCF_001180465.1_E2_London_17_VIM_2_02_09_genomic/1-146106:0.00055,GCF_001180605.1_P10_London_6_VIM_2_09_09_genomic/1-146106:0.00055)0.732:0.00055,((GCF_001180505.1_E4_London_17_VIM_2_03_09_genomic/1-146106:0.00055,(((GCF_001180825.1_P21_London_17_VIM_2_06_10_genomic/1-146106:0.00055,(GCF_001181165.1_P38_London_12_VIM_2_08_12_genomic/1-146106:0.00055,(GCF_001180985.1_P29_London_12_VIM_2_05_11_genomic/1-146106:0.00055,GCF_001181385.1_P47_London_12_VIM_2_12_12_genomic/1-146106:0.00181)0.828:0.00055)0.876:0.00055)0.680:0.00055,(GCF_001180905.1_P25_London_12_VIM_2_08_10_genomic/1-146106:0.00055,(GCF_001180765.1_P18_London_17_VIM_2_04_10_genomic/1-146106:0.00055,GCF_001181805.1_P56_London_12_VIM_2_07_13_genomic/1-146106:0.00055)0.692:0.00055)0.913:0.00055)0.767:0.00055,((GCF_001180565.1_P8_1_South_East_10_VIM_2_07_09_genomic/1-146106:0.00055,GCF_001181825.1_P8_2_London_17_VIM_2_07_13_genomic/1-146106:0.00055)0.736:0.00055,(GCF_001180665.1_P13_London_14_VIM_2_12_09_genomic/1-146106:0.00055,(GCF_001181845.1_P57_South_East_3_VIM_2_09_13_genomic/1-146106:0.00055,(GCF_001181345.1_E6_London_17_VIM_2_12_12_genomic/1-146106:0.00072,GCF_001182025.1_E16_London_17_VIM_2_04_14_genomic/1-146106:0.00177)0.996:0.00055)0.740:0.00055)0.908:0.00055)0.924:0.00055)0.749:0.00055)0.704:0.00055,(GCF_001180645.1_P12_London_17_VIM_2_11_09_genomic/1-146106:0.00055,GCF_001180805.1_P20_London_17_VIM_2_05_10_genomic/1-146106:0.00055)0.732:0.00055)0.738:0.00055)0.732:0.00055)0.000:0.00055,GCF_001180685.1_P14_London_17_VIM_2_01_10_genomic/1-146106:0.00055,((((((GCF_001181125.1_P36_West_Midlands_5_VIM_2_06_12_genomic/1-146106:0.00055,((GCF_002136275.1_ASM213627v1_genomic/1-146106:0.00055,(GCF_002136005.1_ASM213600v1_genomic/1-146106:0.00190,(GCF_002136415.1_ASM213641v1_genomic/1-146106:0.00055,(GCF_000794925.1_AZPAE14729_genomic/1-146106:0.00055,GCF_002135755.1_ASM213575v1_genomic/1-146106:0.00055)0.973:0.00055)0.697:0.00055)0.958:0.00055)0.795:0.00055,GCF_001180385.1_P3_North_West_16_VIM_2_07_06_genomic/1-146106:0.00128)0.809:0.00055)0.689:0.00055,((((GCF_000981825.1_ASM98182v1_genomic/1-146106:0.00055,GCF_002085605.1_ASM208560v1_genomic/1-146106:0.00055)0.939:0.00055,(((GCF_000796845.1_AZPAE14714_genomic/1-146106:0.00055,GCF_000797085.1_AZPAE14713_genomic/1-146106:0.00055)0.944:0.00055,((GCF_000797395.1_AZPAE13756_genomic/1-146106:0.00055,GCF_001180785.1_P19_London_7_VIM_2_05_10_genomic/1-146106:0.00055)1.000:0.00066,GCF_000796365.1_AZPAE14463_genomic/1-146106:0.00055)0.000:0.00055)0.889:0.00055,(GCF_001469695.1_ASM146969v1_genomic/1-146106:0.00055,(GCF_001454445.1_WH-SGI-V-07322_genomic/1-146106:0.00055,GCF_001454495.1_WH-SGI-V-07325_genomic/1-146106:0.00055)0.941:0.00055)0.788:0.00055)0.809:0.00055)0.884:0.00055,(GCF_001181085.1_P34_London_28_VIM_2_02_12_genomic/1-146106:0.00055,(GCF_000795865.1_AZPAE14724_genomic/1-146106:0.00055,GCF_000796025.1_AZPAE14707_genomic/1-146106:0.00055)0.977:0.00055)0.952:0.00055)0.504:0.00055,(GCF_001181205.1_P40_Scotland_4_VIM_2_09_12_genomic/1-146106:0.00055,((GCF_001180945.1_P27_Wales_1_VIM_2_02_11_genomic/1-146106:0.00055,GCF_001181885.1_P59_Wales_1_VIM_2_09_13_genomic/1-146106:0.00055)0.776:0.00055,(GCF_001180925.1_P26_Wales_1_VIM_2_11_10_genomic/1-146106:0.00055,GCF_001182945.1_P44_Wales_1_VIM_2_11_12_genomic/1-146106:0.00055)0.462:0.00055)0.987:0.00055)0.846:0.00055)0.723:0.00055)0.889:0.00055,(((GCF_001181425.1_P49_London_7_VIM_2_01_13_genomic/1-146106:0.00055,(GCF_002135955.1_ASM213595v1_genomic/1-146106:0.00055,(GCF_002135805.1_ASM213580v1_genomic/1-146106:0.00372,(GCF_002188565.1_ASM218856v1_genomic/1-146106:0.00055,(GCF_002188625.1_ASM218862v1_genomic/1-146106:0.00402,(GCF_000795525.1_AZPAE13860_genomic/1-146106:0.00203,(((GCF_001875165.1_ASM187516v1_genomic/1-146106:0.00055,(GCF_001086755.1_ASM108675v1_genomic/1-146106:0.00055,GCF_001874795.1_ASM187479v1_genomic/1-146106:0.00055)0.996:0.00055)1.000:0.04530,(GCF_002411815.1_ASM241181v1_genomic/1-146106:0.00055,(GCF_002411865.1_ASM241186v1_genomic/1-146106:0.00055,(GCF_002411845.1_ASM24118

4v1_genomic/1-146106:0.00055,GCF_002411785.1_ASM241178v1_genomic/1-146106:0.0005
5)0.831:0.00055)0.926:0.00055)1.000:0.05076)1.000:0.01922,((GCF_000626655.2_ASM6
2665v2_genomic/1-146106:0.05830,GCF_002135965.1_ASM213596v1_genomic/1-146106:0.0
6218)1.000:0.00878,(((((GCF_002188695.1_ASM218869v1_genomic/1-146106:0.00141,(GC
F_000937495.2_ASM93749v2_genomic/1-146106:0.00055,GCF_001601665.1_ASM160166v1_ge
nomic/1-146106:0.00055)1.000:0.02197)1.000:0.05158,(GCF_002968755.1_ASM296875v1_
genomic/1-146106:0.81947,reference/1-146106:0.00274)1.000:0.04778)1.000:0.01569,
(GCF_000291745.1_ASM29174v1_genomic/1-146106:0.05992,(GCF_000763245.2_ASM76324v2
_genomic/1-146106:0.00111,((((GCF_000789905.1_AZPAE14853_genomic/1-146106:0.0005
5,GCF_000795235.1_AZPAE14821_genomic/1-146106:0.00055)0.985:0.00055,((((GCF_0028
35725.1_ASM283572v1_genomic/1-146106:0.00055,GCF_001870265.1_ASM187026v1_genomic
/1-146106:0.00055)0.944:0.00055,GCF_001792855.1_ASM179285v1_genomic/1-146106:0.0
0055)0.882:0.00055,((GCF_000791205.1_AZPAE14923_genomic/1-146106:0.00055,GCF_002
835765.1_ASM283576v1_genomic/1-146106:0.00055)0.891:0.00055,GCF_001792875.1_ASM1
79287v1_genomic/1-146106:0.00055)0.962:0.00055)0.995:0.00055,GCF_001875185.1_ASM
187518v1_genomic/1-146106:0.00055)0.819:0.00055)0.938:0.00055,((GCF_000223945.1_
ASM22394v2_genomic/1-146106:0.00055,((GCF_000223925.1_ASM22392v2_genomic/1-14610
6:0.00055,GCF_001802735.1_ASM180273v1_genomic/1-146106:0.00055)0.996:0.00055,(GC
F_001806505.1_ASM180650v1_genomic/1-146106:0.00055,GCF_000795205.1_AZPAE14819_ge
nomic/1-146106:0.00055)0.737:0.00055)0.970:0.00055)0.841:0.00055,GCF_001792835.1
_ASM179283v1_genomic/1-146106:0.00055)0.000:0.00055)0.963:0.00055,GCF_000223965.
1_ASM22396v2_genomic/1-146106:0.00055)1.000:0.00195)1.000:0.06361)1.000:0.00941)
0.991:0.00394,(((GCF_002029925.1_ASM202992v1_genomic/1-146106:0.00051,((GCF_0022
15345.1_ASM221534v1_genomic/1-146106:0.00055,((GCF_000795905.1_AZPAE14718_genomi
c/1-146106:0.00055,(GCF_001875215.1_ASM187521v1_genomic/1-146106:0.00055,GCF_002
968695.1_ASM296869v1_genomic/1-146106:0.00055)0.000:0.00055)1.000:0.00055,((GCF_
002351425.1_ASM235142v1_genomic/1-146106:0.00055,GCF_002351435.1_ASM235143v1_gen
omic/1-146106:0.00055)0.999:0.00055,(GCF_000789535.1_AZPAE14929_genomic/1-146106
:0.00055,(GCF_002135795.1_ASM213579v1_genomic/1-146106:0.00060,GCF_000795665.1_A
ZPAE13877_genomic/1-146106:0.00055)0.892:0.00055)0.964:0.00055)0.885:0.00055)0.7
49:0.00055)0.458:0.00055,((GCF_000792145.1_AZPAE15029_genomic/1-146106:0.00055,(
GCF_000791185.1_AZPAE14922_genomic/1-146106:0.00055,GCF_000792325.1_AZPAE14984_g
enomic/1-146106:0.00055)0.941:0.00055)1.000:0.00055,GCF_000795045.1_AZPAE14811_g
enomic/1-146106:0.00055)0.991:0.00055)1.000:0.00055)1.000:0.06926,(GCF_000817865
.1_ASM81786v1_genomic/1-146106:0.00106,(GCF_002135875.1_ASM213587v1_genomic/1-14
6106:0.00055,GCF_002135925.1_ASM213592v1_genomic/1-146106:0.00055)1.000:0.00055)
1.000:0.05803)1.000:0.01307,(GCF_002251125.1_ASM225112v1_genomic/1-146106:0.0789
0,((GCF_002753655.1_ASM275365v1_genomic/1-146106:0.15069,(GCF_002915615.1_ASM291
561v1_genomic/1-146106:0.09351,((GCF_000473745.2_ASM47374v3_genomic/1-146106:0.0
1225,(GCF_001086725.1_ASM108672v1_genomic/1-146106:0.00068,(GCF_000795465.1_AZPA
E13853_genomic/1-146106:0.00055,GCF_000795505.1_AZPAE13858_genomic/1-146106:0.00
055)1.000:0.00055)1.000:0.00906)1.000:0.08669,((((GCF_001547955.1_ASM154795v1_ge
nomic/1-146106:0.00066,(GCF_002242855.1_ASM224285v1_genomic/1-146106:0.00055,(GC
F_000790185.1_AZPAE14865_genomic/1-146106:0.00063,GCF_000791085.1_AZPAE14900_gen
omic/1-146106:0.00055)1.000:0.00055)1.000:0.00055)1.000:0.09056,(GCF_001548335.1
_ASM154833v1_genomic/1-146106:0.08377,(GCF_000791485.1_AZPAE14948_genomic/1-1461
06:0.00055,GCF_002188615.1_ASM218861v1_genomic/1-146106:0.00055)1.000:0.08602)1.
000:0.01385)0.588:0.00731,((((GCF_002135685.1_ASM213568v1_genomic/1-146106:0.000
55,(GCF_002135675.1_ASM213567v1_genomic/1-146106:0.00061,(GCF_002136355.1_ASM213
635v1_genomic/1-146106:0.00067,GCF_002136365.1_ASM213636v1_genomic/1-146106:0.00
073)0.888:0.00055)0.393:0.00055)1.000:0.00055,((GCF_001750425.1_ASM175042v1_geno
mic/1-146106:0.00145,(GCF_000795535.1_AZPAE14716_genomic/1-146106:0.00055,GCF_00

0797205.1_AZPAE14712_genomic/1-146106:0.00055)0.999:0.00055)1.000:0.00055,(GCF_0
02104595.1_ASM210459v1_genomic/1-146106:0.00055,((((((GCF_002134915.1_ASM213491v
1_genomic/1-146106:0.00055,GCF_002134985.1_ASM213498v1_genomic/1-146106:0.00055)
0.730:0.00055,((GCF_002134975.1_ASM213497v1_genomic/1-146106:0.00055,GCF_0021348
35.1_ASM213483v1_genomic/1-146106:0.00053)0.948:0.00055,GCF_002134965.1_ASM21349
6v1_genomic/1-146106:0.00055)0.806:0.00055)0.731:0.00055,GCF_002134905.1_ASM2134
90v1_genomic/1-146106:0.00055)0.720:0.00055,GCF_002134885.1_ASM213488v1_genomic/
1-146106:0.00055)0.716:0.00055,GCF_002134895.1_ASM213489v1_genomic/1-146106:0.00
055)0.000:0.00055,(GCF_002104615.1_ASM210461v1_genomic/1-146106:0.00055,GCF_0021
34845.1_ASM213484v1_genomic/1-146106:0.00055)0.742:0.00055)0.000:0.00055)1.000:0
.00055)1.000:0.00055)0.561:0.00055,GCF_002211545.1_ASM221154v1_genomic/1-146106:
0.00068)1.000:0.08130,(GCF_000791735.1_AZPAE14959_genomic/1-146106:0.02074,(GCF_
002088215.1_ASM208821v1_genomic/1-146106:0.00055,(GCF_002136535.1_ASM213653v1_ge
nomic/1-146106:0.00321,GCF_002136295.1_ASM213629v1_genomic/1-146106:0.00055)1.00
0:0.00055)1.000:0.02057)1.000:0.06119)1.000:0.01416)0.990:0.00691,(((GCF_0009885
05.1_ASM98850v1_genomic/1-146106:0.00074,GCF_001452445.1_WH-SGI-V-07685_genomic/
1-146106:0.00098)1.000:0.08422,(GCF_000937465.2_ASM93746v2_genomic/1-146106:0.00
762,GCF_001750225.1_ASM175022v1_genomic/1-146106:0.00266)1.000:0.08197)1.000:0.0
1452,((GCF_000794735.1_AZPAE14700_genomic/1-146106:0.00054,(GCF_002205355.1_ASM2
20535v1_genomic/1-146106:0.00132,GCF_002192475.1_ASM219247v1_genomic/1-146106:0.
00055)1.000:0.00063)1.000:0.08666,(GCF_001086635.1_ASM108663v1_genomic/1-146106:
0.00685,((GCF_002136335.1_ASM213633v1_genomic/1-146106:0.00055,GCF_002188505.1_A
SM218850v1_genomic/1-146106:0.00055)1.000:0.00055,(((GCF_002189115.1_ASM218911v1
_genomic/1-146106:0.00055,(GCF_001451785.1_WH-SGI-V-07496_genomic/1-146106:0.000
55,GCF_001452055.1_WH-SGI-V-076137_genomic/1-146106:0.00055)1.000:0.00055)0.948:
0.00055,(((GCF_000794945.1_AZPAE14730_genomic/1-146106:0.00147,(GCF_000829255.1_
ASM82925v1_genomic/1-146106:0.0,GCF_000829275.1_ASM82927v1_genomic/1-146106:0.0)
:0.00055)1.000:0.00055,(GCF_000795685.1_AZPAE13879_genomic/1-146106:0.00055,(GCF
_000797025.1_AZPAE14719_genomic/1-146106:0.00055,(GCF_000796245.1_AZPAE14721_gen
omic/1-146106:0.00055,GCF_000795955.1_AZPAE14720_genomic/1-146106:0.00055)0.973:
0.00055)1.000:0.00055)0.905:0.00055)0.902:0.00055,(((GCF_000737795.1_PA_ST235_v.
05_genomic/1-146106:0.00055,GCF_000284555.1_ASM28455v1_genomic/1-146106:0.00055)
0.718:0.00055,((GCF_001921095.1_TRN6622_genomic/1-146106:0.00055,(GCF_002201395.
1_ASM220139v1_genomic/1-146106:0.00055,(GCF_002201405.1_ASM220140v1_genomic/1-14
6106:0.00055,GCF_002201455.1_ASM220145v1_genomic/1-146106:0.00055)0.152:0.00055)
1.000:0.00055)1.000:0.00055,(((GCF_001623945.1_ASM162394v1_genomic/1-146106:0.00
055,(GCF_002406345.1_ASM240634v1_genomic/1-146106:0.00055,GCF_001623985.1_ASM162
398v1_genomic/1-146106:0.00055)0.407:0.00055)0.984:0.00055,((GCF_002136435.1_ASM
213643v1_genomic/1-146106:0.01061,GCF_002406265.1_ASM240626v1_genomic/1-146106:0
.00055)0.314:0.00055,(GCF_002136245.1_ASM213624v1_genomic/1-146106:0.00055,(GCF_
002136285.1_ASM213628v1_genomic/1-146106:0.00055,GCF_002136525.1_ASM213652v1_gen
omic/1-146106:0.00055)0.992:0.00055)0.963:0.00055)0.964:0.00055)0.905:0.00055,GC
F_001921165.1_TRN6637_genomic/1-146106:0.00055)1.000:0.00055)0.987:0.00055)0.999
:0.00055,(GCF_000796165.1_AZPAE14706_genomic/1-146106:0.00051,(GCF_000794785.1_A
ZPAE14702_genomic/1-146106:0.00055,(GCF_000794805.1_AZPAE14703_genomic/1-146106:
0.00055,GCF_000795805.1_AZPAE14705_genomic/1-146106:0.00055)1.000:0.00055)0.988:
0.00055)1.000:0.00055)1.000:0.00055)0.996:0.00055)0.913:0.00055,(((GCF_001874805
.1_ASM187480v1_genomic/1-146106:0.00055,(GCF_001874975.1_ASM187497v1_genomic/1-1
46106:0.00055,(GCF_001921105.1_TRN6633_genomic/1-146106:0.00055,GCF_001921125.1_
TRN6635_genomic/1-146106:0.00055)0.974:0.00055)1.000:0.00055)0.917:0.00055,GCF_0
01921065.1_TRN6601_genomic/1-146106:0.00055)0.204:0.00055,(GCF_002002765.1_ASM20
0276v1_genomic/1-146106:0.00055,(GCF_002135635.1_ASM213563v1_genomic/1-146106:0.

00101,GCF_002136455.1_ASM213645v1_genomic/1-146106:0.00055)0.901:0.00055)0.751:0
.00055)0.776:0.00055)0.903:0.00055)1.000:0.00337)1.000:0.08405)1.000:0.01308)0.9
53:0.00701)1.000:0.01001)1.000:0.01293)1.000:0.01756)1.000:0.10089,(GCF_00213570
5.1_ASM213570v1_genomic/1-146106:0.00083,(GCF_000790105.1_AZPAE14862_genomic/1-1
46106:0.00055,((((GCF_002136195.1_ASM213619v1_genomic/1-146106:0.00055,GCF_00213
6515.1_ASM213651v1_genomic/1-146106:0.00055)0.974:0.00055,(GCF_002135715.1_ASM21
3571v1_genomic/1-146106:0.00055,GCF_002135625.1_ASM213562v1_genomic/1-146106:0.0
0073)0.944:0.00055)0.945:0.00055,GCF_002135615.1_ASM213561v1_genomic/1-146106:0.
00055)0.715:0.00055,(GCF_002136375.1_ASM213637v1_genomic/1-146106:0.00055,((GCF_
002136205.1_ASM213620v1_genomic/1-146106:0.00055,GCF_002136495.1_ASM213649v1_gen
omic/1-146106:0.00055)0.996:0.00055,(GCF_002135765.1_ASM213576v1_genomic/1-14610
6:0.00055,GCF_002135835.1_ASM213583v1_genomic/1-146106:0.00055)0.999:0.00055)0.9
04:0.00055)0.734:0.00055)0.429:0.00055)1.000:0.00104)1.000:0.06117)1.000:0.02853
)1.000:0.01118)0.787:0.00407)1.000:0.00400,(((GCF_002135885.1_ASM213588v1_genomi
c/1-146106:0.00150,(GCF_002188535.1_ASM218853v1_genomic/1-146106:0.00059,GCF_002
188585.1_ASM218858v1_genomic/1-146106:0.00055)1.000:0.00055)1.000:0.06240,(GCF_0
01679685.1_ASM167968v1_genomic/1-146106:0.01515,(GCF_002188545.1_ASM218854v1_gen
omic/1-146106:0.00094,(GCF_000215775.4_PA13_miseq_genomic/1-146106:0.00055,(GCF_
000342145.1_PA_21_ST175v1_genomic/1-146106:0.00055,((((GCF_002136065.1_ASM213606
v1_genomic/1-146106:0.00054,(GCF_002136015.1_ASM213601v1_genomic/1-146106:0.0,GC
F_002136055.1_ASM213605v1_genomic/1-146106:0.0):0.00055)0.000:0.00055,(GCF_00213
6445.1_ASM213644v1_genomic/1-146106:0.00055,GCF_002136115.1_ASM213611v1_genomic/
1-146106:0.00055)0.000:0.00055)0.000:0.00055,((GCF_002136125.1_ASM213612v1_genom
ic/1-146106:0.00055,GCF_002136135.1_ASM213613v1_genomic/1-146106:0.00055)0.976:0
.00055,GCF_002136075.1_ASM213607v1_genomic/1-146106:0.00055)0.885:0.00055)1.000:
0.00055,(((GCF_002136165.1_ASM213616v1_genomic/1-146106:0.00055,GCF_002136215.1_
ASM213621v1_genomic/1-146106:0.00221)1.000:0.00055,GCF_002135995.1_ASM213599v1_g
enomic/1-146106:0.00055)0.949:0.00055,GCF_002135845.1_ASM213584v1_genomic/1-1461
06:0.00055)0.104:0.00055)0.912:0.00055)1.000:0.00055)0.995:0.00055)1.000:0.01667
)1.000:0.04354)1.000:0.01101,((GCF_002188645.1_ASM218864v1_genomic/1-146106:0.05
585,(GCF_000794515.1_AZPAE14687_genomic/1-146106:0.00055,GCF_000795625.1_AZPAE13
872_genomic/1-146106:0.00055)1.000:0.05912)1.000:0.01008,(GCF_000794505.1_AZPAE1
4688_genomic/1-146106:0.00123,GCF_002094805.1_ASM209480v1_genomic/1-146106:0.001
61)1.000:0.06840)0.993:0.00409)1.000:0.00404)0.984:0.00369)1.000:0.01170)1.000:0
.06227)1.000:0.00408)0.644:0.00055)0.923:0.00055)0.826:0.00055)0.923:0.00055)0.9
41:0.00055,(((GCF_001180345.1_P1_London_28_IMP_1_04_05_genomic/1-146106:0.00055,
((GCF_001180745.1_P17_North_West_14_VIM_2_03_10_genomic/1-146106:0.00055,GCF_001
181065.1_P33_London_28_VIM_2_02_12_genomic/1-146106:0.00055)0.885:0.00055,(GCF_0
01181025.1_P31_Wales_1_VIM_2_11_11_genomic/1-146106:0.00055,((((GCF_001180445.1_
E1_London_17_VIM_2_02_09_genomic/1-146106:0.00055,GCF_001181765.1_E13_London_26_
VIM_2_06_13_genomic/1-146106:0.00055)1.000:0.00055,(GCF_001181565.1_P52_2_London
_26_VIM_2_02_13_genomic/1-146106:0.00055,GCF_001181725.1_E11_London_26_VIM_2_06_
13_genomic/1-146106:0.00055)0.724:0.00055)0.972:0.00055,(GCF_001180405.1_P4_Lond
on_1_VIM_2_10_07_genomic/1-146106:0.00055,GCF_001181045.1_P32_London_17_VIM_2_10
_11_genomic/1-146106:0.00094)0.425:0.00055)0.463:0.00055,((GCF_001181465.1_P43_2
_London_9_VIM_2_11_12_genomic/1-146106:0.00055,(GCF_001181265.1_P43_1_London_9_V
IM_2_11_12_genomic/1-146106:0.00055,GCF_001181585.1_P53_London_9_VIM_2_02_13_gen
omic/1-146106:0.00063)0.000:0.00055)0.802:0.00055,GCF_001181485.1_P51_1_London_9
_VIM_2_02_13_genomic/1-146106:0.00055)0.996:0.00055)0.924:0.00055)0.845:0.00055)
1.000:0.00055)0.908:0.00055,(GCF_001180585.1_P9_East_of_England_6_IMP_13_08_09_g
enomic/1-146106:0.00055,GCF_001180865.1_P23_East_of_England_6_IMP_13_07_10_genom
ic/1-146106:0.00055)0.714:0.00055)0.970:0.00055,(GCF_001180525.1_P6_East_of_Engl

and_6_IMP_1_03_09_genomic/1-146106:0.00055,GCF_001180365.1_P2_London_28_IMP_1_06_05_genomic/1-146106:0.00070)0.958:0.00055)0.981:0.00055)0.985:0.00055,((GCF_001181905.1_P60_London_6_VIM_2_11_13_genomic/1-146106:0.00055,((GCF_001182005.1_P63_London_25_VIM_2_03_14_genomic/1-146106:0.00055,(GCF_001181545.1_P51_2_London_11_VIM_2_02_13_genomic/1-146106:0.00055,(GCF_001181445.1_P50_London_9_VIM_2_01_13_genomic/1-146106:0.00055,GCF_001181965.1_P62_London_9_VIM_2_01_14_genomic/1-146106:0.00055)0.732:0.00055)0.867:0.00055)0.750:0.00055,(GCF_001181505.1_E7_London_9_VIM_2_02_13_genomic/1-146106:0.00055,GCF_001181925.1_P61_London_9_VIM_2_11_13_genomic/1-146106:0.00110)0.927:0.00055)0.769:0.00055)0.998:0.00055,GCF_001181005.1_P30_South_East_2_VIM_2_10_11_genomic/1-146106:0.00055)0.222:0.00055)0.796:0.00055)0.414:0.00055,GCF_001180845.1_P22_London_17_VIM_2_06_10_genomic/1-146106:0.00055)0.742:0.00055,GCF_001180885.1_P24_London_17_VIM_2_08_10_genomic/1-146106:0.00055)0.000:0.00055,((((GCF_001181605.1_P54_1_London_24_VIM_2_04_13_genomic/1-146106:0.00058,GCF_001181665.1_P54_2_London_24_VIM_2_04_13_genomic/1-146106:0.00055)0.999:0.00055,((GCF_001181325.1_E5_London_17_VIM_2_12_12_genomic/1-146106:0.00055,(GCF_001181305.1_P45_London_17_VIM_2_12_12_genomic/1-146106:0.00055,(GCF_001181245.1_P42_1_London_26_VIM_2_10_12_genomic/1-146106:0.00055,GCF_001181745.1_E12_London_26_VIM_2_06_13_genomic/1-146106:0.00055)1.000:0.00096)1.000:0.00055)1.000:0.00055,(GCF_001181405.1_P48_London_17_VIM_2_01_13_genomic/1-146106:0.00055,GCF_001180425.1_P5_London_26_VIM_2_01_09_genomic/1-146106:0.00141)0.305:0.00055)0.931:0.00055)0.857:0.00055,GCF_001181645.1_E9_London_17_VIM_2_04_13_genomic/1-146106:0.00055)0.898:0.00055,(((GCF_001181525.1_P52_1_London_26_VIM_2_02_13_genomic/1-146106:0.00055,GCF_001181625.1_E8_London_17_VIM_2_04_13_genomic/1-146106:0.00055)0.999:0.00055,((GCF_001181105.1_P35_London_26_VIM_2_05_12_genomic/1-146106:0.00055,GCF_001181145.1_P37_London_28_VIM_2_07_12_genomic/1-146106:0.00055)0.935:0.00055,(GCF_001180545.1_P7_London_17_VIM_2_06_09_genomic/1-146106:0.00055,(GCF_001181785.1_E14_London_26_VIM_2_06_13_genomic/1-146106:0.00055,(GCF_001181285.1_P42_2_London_28_VIM_2_11_12_genomic/1-146106:0.00055,(GCF_001181685.1_P55_London_26_VIM_2_05_13_genomic/1-146106:0.00055,GCF_001181705.1_E10_London_26_VIM_2_06_13_genomic/1-146106:0.00055)0.543:0.00055)0.852:0.00055)0.793:0.00055)0.782:0.00055)0.923:0.00055)0.947:0.00055,GCF_001180485.1_E3_London_17_VIM_2_03_09_genomic/1-146106:0.00055)0.788:0.00055)0.916:0.00055)0.000:0.00055);

Final discussion and future directions

The work presented in this thesis analyzed the genetic environment of CEG and the mechanisms involved in the spread of these AR genes in *P. aeruginosa* isolates from Portuguese hospitals and from a worldwide collection of publicly available *Pseudomonas* genomes. 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 promoter of carbapenem resistance in *P. aeruginosa*, transferable resistance by MGE is becoming increasingly relevant [85, 86, 114, 255]. The acquisition of class I integrons harboring gene cassettes which confer resistance to several antibiotics have prompted the dissemination of MDR *P. aeruginosa* isolates.

We identified 28 CEG in our collection from Portuguese hospitals, all encoding for VIM-2 with the exception of a bla_{GES-6} gene. These genes were linked to 8 class I integrons. In58 already described among different species was the most prevalent integron to be here identified [265–268]. Besides bla_{VIM-2}, In58 comprises the aminoglycoside resistance genes *aacA7*, *aacA4* and *aacC1*. The remarkable structural heterogeneity of class I integrons here identified could be explained by i) the acquisition of different integrons by representatives of several STs already circulating in hospital settings and/or ii) integrase-mediated gene cassette shuffling or other recombination events that took place during the evolution of a given ST. Indeed, the majority of these integrons are derived from the In4 lineage, which presents an IS*6100* downstream the 3'CS region [112, 133, 269]. Due to the known genetic rearrangement ability of this insertion sequence, an additional recombination potential can be expected for these integrons [269–271].

Although integrons are not mobilizable *per se*, the association with MGE promotes its spread. Several studies have reported the presence of CEG-harboring integrons among the chromosome of *P. aeruginosa* [86]. Out of the 28 CEG here identified, 9 had a plasmid and 19 a chromosomal location. So one can speculate that these later integrons may be associated with MGE that tend to integrate into the chromosome, such as GIs, ICE and/or bacteriophages. Since the genetic context of these elements was rarely inspected [176], we investigated not only the plasmid backbones but also the MGE involved with the integration of CEG in the chromosome. Data obtained in this project point to the presence of CEG-bearing ICE among *P. aeruginosa* clinical isolates from high-risk clones belonging to STs 111 and 235. Three complete ICE were here characterized, ranging from 80 to 90-kb long. Two bla_{VIM-2} (ST111 and ST235) and one bla_{GES-6}-harboring (ST235) ICE were identified in our collection. Interestingly, we found a Matryoshka-like arrangement on the ICE from the ST235 isolate, comprising a novel bla_{VIM-2}-harboring transposon within the boundaries of the ICE. Acquisition of In56 (only harboring bla_{VIM-2}) by this ICE was most likely mediated by insertion of this transposon. ICE carrying bla_{VIM-2} were highly related between them, but their backbone present low similarity with the one housing bla_{GES-6}. Also, highly similar

regions comprising the backbone of these elements were identified on the genome of international *P. aeruginosa* strains deposited on NCBI. These results emphasize both the ability of these MGE to disseminate at a local and global level. Taking these observations to a wider level, we analyzed the link between CEG and ICE among all *Pseudomonas* spp. genomes available in NCBI (accessed on 24/04/18). Out of the more than 4000 genomes, we identified CEG in less than 10% of the genomes, which is consistent with previous findings stating that chromosomal mutations are the most important drivers of AR in *P. aeruginosa*. Surprisingly, we were able to identity a wide array of ICE associated with several CEG and with specific ICE families.

Plasmids also played a part in the HGT of CEG-harboring integrons. We fully sequenced the first In58-harboring plasmid, named pJB12 and isolated from a strain belonging to the high-risk clone ST175. As for the *bla*$_{VIM-2}$-harboring ICE from ST235, we also found a different *bla*$_{VIM-2}$-harboring transposon within the boundaries of the c.a. 30kb-long plasmid. The backbone of this plasmid was also identified in other isolates of our collection that also belong to ST175. Plasmids of the same size were identified in isolates belonging to ST179 and ST282. The former was highly related to pJB12 and was associated with In103, an In58-like integron lacking the *aacC1* gene, while the later presented an In58 integron but with a different plasmid backbone structure. These c.a. 30kb-long plasmids were not self-conjugative, but may be mobilized by the presence of an helper plasmid [145]. Interestingly, the In58 integron was also identified in a 450kb-long plasmid identified in an isolate from our collection belonging to the high-risk clone ST253, and named pJB37. As for pJB12, the acquisition of In58 by pJB37 was most likely achieved by the transposition of a new complex transposon structure. The data here presented represents the first description of a *bla*$_{VIM-2}$-harboring megaplasmid in *P. aeruginosa*. This IncP-2 plasmid was transferrable by conjugation to a spontaneous rifampicin-resistant mutant of *P. aeruginosa* PAO1 strain. The backbone of pJB37 was highly related to a previously reported IncP-2 *bla*$_{IMP-9}$-harboring megaplasmid identified in a *P. aeruginosa* clinical isolate [162]. This structure was also identified in other isolates belonging to ST253 from our collection.

Besides characterizing the AR genes among CEG-bearing MGE, we also explored the presence of virulence factors. We described the backbone of an *exoU*-carrying GI in FFUP_PS_690 strain, belonging to ST235. The exoS^{-}/exoU^{+} genotype is related to specific clonal lineages, such as ST235 [20, 256]. ExoU is a potent cytotoxin with phospholipase activity, which is frequently related to nosocomial-acquired pneumonia, ear infections and keratitis [185, 187]. The *exoU* gene is less prevalent than other type-III secretion system effector genes in the *P. aeruginosa* genome, which may be explained by its recent acquisition by HGT into particular lineages [188, 256]. We also explored the virulence determinants carried by the megaplasmid pJB37, such as those encoding for twitching

motility proteins PilT and PilG, type IV fimbrial assembly ATPase PilB and a chemotaxis (*che*) operon. *pilB*, *pilG* and *pilT* genes are involved in several traits, such as the biogenesis and mechanical function of type IV pili, twitching motility and biofilm formation [272]. The *che* operon is required for flagella-mediated chemotaxis in *P. aeruginosa* and is similar to the Pil-Chp system [162, 272]. This system is also involved in the regulation of intracellular levels of cyclic AMP, a messenger that activates Vfr. Vfr is a cyclic AMP-binding protein that plays a vital role in several virulence traits, such as the expression of type IV pili [162].

An intriguing question that arises from this and previous studies is why the class I integrons tend to be integrated into the chromosome rather than inserted in plasmids. In the near future, we intend to target this enigma by performing an *in silico* analysis of CEG deposited in NCBI and by analyzing specific traits that may help to explain this preference. We also plan to explore the mechanisms that mediated the acquisition of the bla_{VIM-2}-harboring transposons by ICE*Pae*CB5, pJB12 and pJB37. Triparental mating assays to assess if the c.a. 30kb-long plasmids identified in our collection are indeed mobilized by helper plasmids will also be performed. It would also be interesting to explore the reasons why chromosomal mutations are more important for resistance to carbapenems than acquisition of MGE 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* [209, 215–217].

Besides exploring the different MGE involved in the spread of CEG among *P. aeruginosa* isolates in Portuguese hospitals, our work also analyzed the different STs associated with these isolates. We identified the internationally-recognized high-risk clones, namely STs 111, 175, 235, 244 and 253. Besides these internationally successful clones and sporadic clonal types, the most frequently encountered clone in our collection belonged to ST179. VIM-2-producing ST179 was also detected in several patients with chronic respiratory infections from Spanish hospitals [261, 273]. These findings probably account for a clonal spread of this ST in the Iberian Peninsula. Also, most of the isolates from ST179 were identified in the same hospital throughout different years, exposing the long-term persistence of this clone.

High-risk clones appear to be more capable of acquiring and/or maintaining AR genes when comparing with other clones. However, the reason 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 MGE [119, 250, 258, 262]. 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. We plan to undergo comparative genomic studies to identity the presence/absence of specific genes in high-risk clones and in other *P. aeruginosa* strains, in order 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 inspect if high-risk clones exhibit a different gene expression profile [274, 275]. Another question that needs to be addressed is regarding the nature of high-risk clones [251]: are these clones a previously minor strain that recently succeeded, with resistance as a key driver, and/or a previously successful antibiotic-susceptible strain that received recent attention 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 ICE. With the advent of WGS, the real contribution of these MGE for the dissemination of CEG 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 mandatory to identify the main hospital reservoirs of MDR *P. aeruginosa*, which may include plumbing systems, ventilators and/or long-term colonization of patients. It is vital to have a deeper understanding of these MDR organisms so that better infection prevention and control strategies to limit their future spread can be implemented. The genomic era and integrated molecular epidemiology approaches may help on outbreak investigations and long-term persistence surveillance of MDR *P. aeruginosa* in real-time [119, 276, 277].

References

1. Moradali MF, Ghods S, Rehm BHA. *Pseudomonas aeruginosa* Lifestyle: A Paradigm for Adaptation, Survival, and Persistence. *Front Cell Infect Microbiol* 2017; **7**: 39.
2. Silby MW, Winstanley C, Godfrey SAC, Levy SB, Jackson RW. *Pseudomonas* genomes: diverse and adaptable. *FEMS Microbiol Rev* 2011; **35**: 652–680.
3. Nguyen L, Garcia J, Gruenberg K, MacDougall C. Multidrug-Resistant *Pseudomonas* Infections: Hard to Treat, But Hope on the Horizon? *Curr Infect Dis Rep* 2018; **20**: 23.
4. Bassetti M, Vena A, Croxatto A, Righi E, Guery B. How to manage *Pseudomonas aeruginosa* infections. *Drugs Context* 2018; **7**: 1–18.
5. Burrows LL. The Therapeutic Pipeline for *Pseudomonas aeruginosa* Infections. *ACS Infect Dis* . 2018. , **4**: 1041–1047
6. Boucher HW, Talbot GH, Bradley JS, Edwards JE, Gilbert D, Rice LB, et al. Bad Bugs, No Drugs: No ESKAPE! An Update from the Infectious Diseases Society of America. *Clin Infect Dis* 2009; **48**: 1–12.
7. World Health Organization. Global priority list of antibiotic-resistant bacteria to guide research, discovery, and development of new antibiotics. http://www.who.int/news-room/detail/27-02-2017-who-publishes-list-of-bacteria-for-which-new-antibiotics-are-urgently-needed. Accessed 11 Aug 2018.
8. Rosenthal VD, Al-Abdely HM, El-Kholy AA, AlKhawaja SAA, Leblebicioglu H, Mehta Y, et al. International Nosocomial Infection Control Consortium report, data summary of 50 countries for 2010-2015: Device-associated module. *Am J Infect Control* 2016; **44**: 1495–1504.
9. European Centre for Disease Prevention and Control. Healthcare-associated infections acquired in intensive care units - Annual Epidemiological Report for 2016. 2018. Accessed 20 Aug 2018.
10. European Centre for Disease Prevention and Control (ECDC). Point prevalence survey reports: European long-term care facilities. https://ecdc.europa.eu/en/infectious-diseases-public-health/healthcare-associated-infections-long-term-care-facilities-0. Accessed 20 Aug 2018.
11. European Centre for Disease Prevention and Control (ECDC). Point prevalence survey report: Acute care hospitals. https://ecdc.europa.eu/en/healthcare-associated-infections-acute-care-hospitals/surveillance-disease-data/report. Accessed 20 Aug 2018.
12. Balasubramanian D, Schneper L, Kumari H, Mathee K. A dynamic and intricate regulatory network determines *Pseudomonas aeruginosa* virulence. *Nucleic Acids Res* 2013; **41**: 1–20.
13. Balasubramanian D, Kumari H, Mathee K. *Pseudomonas aeruginosa* AmpR: an acute-chronic switch regulator. *Pathog Dis* 2014; **73**: n/a-n/a.
14. Lee J, Zhang L. The hierarchy quorum sensing network in *Pseudomonas aeruginosa*. *Protein Cell* 2015; **6**: 26–41.
15. Mikkelsen H, Sivaneson M, Filloux A. Key two-component regulatory systems that control biofilm formation in *Pseudomonas aeruginosa*. *Environ Microbiol* 2011; **13**: 1666–1681.
16. Chambonnier G, Roux L, Redelberger D, Fadel F, Filloux A, Sivaneson M, et al. The Hybrid

Histidine Kinase LadS Forms a Multicomponent Signal Transduction System with the GacS/GacA Two-Component System in *Pseudomonas aeruginosa*. *PLOS Genet* 2016; **12**: e1006032.

17. Koeppen K, Hampton TH, Jarek M, Scharfe M, Gerber SA, Mielcarz DW, et al. A Novel Mechanism of Host-Pathogen Interaction through sRNA in Bacterial Outer Membrane Vesicles. *PLOS Pathog* 2016; **12**: e1005672.
18. Darch SE, Simoska O, Fitzpatrick M, Barraza JP, Stevenson KJ, Bonnecaze RT, et al. Spatial determinants of quorum signaling in a *Pseudomonas aeruginosa* infection model. *Proc Natl Acad Sci U S A* 2018; **115**: 4779–4784.
19. Breidenstein EBM, de la Fuente-Núñez C, Hancock REW. *Pseudomonas aeruginosa*: all roads lead to resistance. *Trends Microbiol* 2011; **19**: 419–26.
20. Juan C, Peña C, Oliver A. Host and Pathogen Biomarkers for Severe *Pseudomonas aeruginosa* Infections. *J Infect Dis* 2017; **215**: S44–S51.
21. Stover CK, Pham XQ, Erwin AL, Mizoguchi SD, Warrener P, Hickey MJ, et al. Complete genome sequence of *Pseudomonas aeruginosa* PAO1, an opportunistic pathogen. *Nature* 2000; **406**: 959–964.
22. Mathee K, Narasimhan G, Valdes C, Qiu X, Matewish JM, Koehrsen M, et al. Dynamics of *Pseudomonas aeruginosa* genome evolution. *Proc Natl Acad Sci* 2008; **105**: 3100–3105.
23. Moran NA, McLaughlin HJ, Sorek R. The Dynamics and Time Scale of Ongoing Genomic Erosion in Symbiotic Bacteria. *Science* 2009; **323**: 379–382.
24. Moran NA. Microbial minimalism: genome reduction in bacterial pathogens. *Cell* 2002; **108**: 583–6.
25. Kung VL, Ozer EA, Hauser AR. The accessory genome of *Pseudomonas aeruginosa*. *Microbiol Mol Biol Rev* 2010; **74**: 621–41.
26. Valot B, Guyeux C, Rolland JY, Mazouzi K, Bertrand X, Hocquet D. What It Takes to Be a *Pseudomonas aeruginosa*? The Core Genome of the Opportunistic Pathogen Updated. *PLoS One* 2015; **10**: e0126468.
27. Klockgether J, Cramer N, Wiehlmann L, Davenport CF, Tümmler B. *Pseudomonas aeruginosa* Genomic Structure and Diversity. *Front Microbiol* 2011; **2**: 150.
28. Fischer S, Klockgether J, Morán Losada P, Chouvarine P, Cramer N, Davenport CF, et al. Intraclonal genome diversity of the major *Pseudomonas aeruginosa* clones C and PA14. *Environ Microbiol Rep* 2016; **8**: 227–34.
29. Hilker R, Munder A, Klockgether J, Losada PM, Chouvarine P, Cramer N, et al. Interclonal gradient of virulence in the *P seudomonas aeruginosa* pangenome from disease and environment. *Environ Microbiol* 2015; **17**: 29–46.
30. McInerney JO, McNally A, O'Connell MJ. Why prokaryotes have pangenomes. *Nat Microbiol* 2017; **2**: 17040.
31. Freschi L, Bertelli C, Jeukens J, Moore MP, Kukavica-Ibrulj I, Emond-Rheault J-G, et al. Genomic characterisation of an international *Pseudomonas aeruginosa* reference panel indicates that the two major groups draw upon distinct mobile gene pools. *FEMS Microbiol*

Lett 2018; **365**.

32. Touchon M, Hoede C, Tenaillon O, Barbe V, Baeriswyl S, Bidet P, et al. Organised Genome Dynamics in the *Escherichia coli* Species Results in Highly Diverse Adaptive Paths. *PLoS Genet* 2009; **5**: e1000344.
33. Boissy R, Ahmed A, Janto B, Earl J, Hall BG, Hogg JS, et al. Comparative supragenomic analyses among the pathogens *Staphylococcus aureus*, *Streptococcus pneumoniae*, and *Haemophilus influenzae* using a modification of the finite supragenome model. *BMC Genomics* 2011; **12**: 187.
34. Touchon M, Hoede C, Tenaillon O, Barbe V, Baeriswyl S, Bidet P, et al. Organised Genome Dynamics in the *Escherichia coli* Species Results in Highly Diverse Adaptive Paths. *PLoS Genet* 2009; **5**: e1000344.
35. Yoon S-H, Ha S-M, Kwon S, Lim J, Kim Y, Seo H, et al. Introducing EzBioCloud: a taxonomically united database of 16S rRNA gene sequences and whole-genome assemblies. *Int J Syst Evol Microbiol* 2017; **67**: 1613–1617.
36. Wattam AR, Davis JJ, Assaf R, Boisvert S, Brettin T, Bun C, et al. Improvements to PATRIC, the all-bacterial Bioinformatics Database and Analysis Resource Center. *Nucleic Acids Res* 2017; **45**: D535–D542.
37. Winsor GL, Griffiths EJ, Lo R, Dhillon BK, Shay JA, Brinkman FSL. Enhanced annotations and features for comparing thousands of *Pseudomonas* genomes in the *Pseudomonas* genome database. *Nucleic Acids Res* 2016; **44**: D646–D653.
38. Freschi L, Jeukens J, Kukavica-Ibrulj I, Boyle B, Dupont M-J, Laroche J, et al. Clinical utilization of genomics data produced by the international *Pseudomonas aeruginosa* consortium. *Front Microbiol* 2015; **6**: 1036.
39. Turner KH, Everett J, Trivedi U, Rumbaugh KP, Whiteley M. Requirements for *Pseudomonas aeruginosa* Acute Burn and Chronic Surgical Wound Infection. *PLoS Genet* 2014; **10**: e1004518.
40. Cornforth DM, Dees JL, Ibberson CB, Huse HK, Mathiesen IH, Kirketerp-Møller K, et al. *Pseudomonas aeruginosa* transcriptome during human infection. *Proc Natl Acad Sci U S A* 2018; **115**: E5125–E5134.
41. Lassek C, Berger A, Zühlke D, Wittmann C, Riedel K. Proteome and carbon flux analysis of *Pseudomonas aeruginosa* clinical isolates from different infection sites. *Proteomics* 2016; **16**: 1381–1385.
42. Tognon M, Köhler T, Gdaniec BG, Hao Y, Lam JS, Beaume M, et al. Co-evolution with *Staphylococcus aureus* leads to lipopolysaccharide alterations in *Pseudomonas aeruginosa*. *ISME J* 2017; **11**: 2233–2243.
43. Limoli DH, Whitfield GB, Kitao T, Ivey ML, Davis MR, Grahl N, et al. *Pseudomonas aeruginosa* Alginate Overproduction Promotes Coexistence with *Staphylococcus aureus* in a Model of Cystic Fibrosis Respiratory Infection. *MBio* 2017; **8**: e00186-17.
44. Orazi G, O'Toole GA. *Pseudomonas aeruginosa* Alters Staphylococcus aureus Sensitivity to Vancomycin in a Biofilm Model of Cystic Fibrosis Infection. *MBio* 2017; **8**.

45. O'Neill J. Tackling drug-resistant infections globally: final report and recommendations. *Rev Antimicrob Resist* 2016. Accessed 15 Aug 2018.
46. European Centre for Disease Prevention and Control (ECDC). Antimicrobial resistance surveillance in Europe 2016. https://ecdc.europa.eu/en/publications-data/antimicrobial-resistance-surveillance-europe-2016. Accessed 15 Aug 2018.
47. World Health Organization. Global action plan on antimicrobial resistance. *WHO* 2015. Accessed 15 Aug 2018.
48. Temkin E, Fallach N, Almagor J, Gladstone BP, Tacconelli E, Carmeli Y, et al. Estimating the number of infections caused by antibiotic-resistant *Escherichia coli* and *Klebsiella pneumoniae* in 2014: a modelling study. *Lancet Glob Heal* 2018; **6**: e969–e979.
49. Versporten A, Zarb P, Caniaux I, Gros M-F, Drapier N, Miller M, et al. Antimicrobial consumption and resistance in adult hospital inpatients in 53 countries: results of an internet-based global point prevalence survey. *Lancet Glob Heal* 2018; **6**: e619–e629.
50. Centre for Disease Prevention and Control (CDC). Antibiotic Prescribing and Use in the U.S. | Antibiotic Use | CDC.
51. Political Declaration of the High-Level Meeting of the General Assembly on Antimicrobial Resistance : draft resolution / submitted by the President of the General Assembly. 2016. UN,. , 6 p.
52. Brochado AR, Telzerow A, Bobonis J, Banzhaf M, Mateus A, Selkrig J, et al. Species-specific activity of antibacterial drug combinations. *Nature* 2018; **559**: 259–263.
53. Livermore DM. The 2018 Garrod Lecture: Preparing for the Black Swans of resistance. *J Antimicrob Chemother* 2018.
54. Maier L, Pruteanu M, Kuhn M, Zeller G, Telzerow A, Anderson EE, et al. Extensive impact of non-antibiotic drugs on human gut bacteria. *Nature* 2018; **555**: 623–628.
55. Ventola CL. The antibiotic resistance crisis: part 1: causes and threats. *P T* 2015; **40**: 277–83.
56. Waglechner N, Wright GD. Antibiotic resistance: it's bad, but why isn't it worse? *BMC Biol* 2017; **15**: 84.
57. Davies J, Davies D. Origins and evolution of antibiotic resistance. *Microbiol Mol Biol Rev* 2010; **74**: 417–33.
58. Núñez-Núñez M, Navarro MD, Palomo V, Rajendran NB, del Toro MD, Voss A, et al. The methodology of surveillance for antimicrobial resistance and healthcare-associated infections in Europe (SUSPIRE): a systematic review of publicly available information. *Clin Microbiol Infect* 2018; **24**: 105–109.
59. Durão P, Balbontín R, Gordo I. Evolutionary Mechanisms Shaping the Maintenance of Antibiotic Resistance. *Trends Microbiol* 2018; **26**: 677–691.
60. The Global Risks Report 2017 | World Economic Forum. https://www.weforum.org/reports/the-global-risks-report-2017. Accessed 15 Aug 2018.
61. Magiorakos A-P, Srinivasan A, Carey RB, Carmeli Y, Falagas ME, Giske CG, et al. Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an

international expert proposal for interim standard definitions for acquired resistance. *Clin Microbiol Infect* 2012; **18**: 268–81.

62. Baltrus DA. Exploring the costs of horizontal gene transfer. *Trends Ecol Evol* 2013; **28**: 489–495.
63. Sommer MOA, Munck C, Toft-Kehler RV, Andersson DI. Prediction of antibiotic resistance: time for a new preclinical paradigm? *Nat Rev Microbiol* 2017; **15**: 689–696.
64. Cantón R, Ruiz-Garbajosa P. Co-resistance: an opportunity for the bacteria and resistance genes. *Curr Opin Pharmacol* 2011; **11**: 477–485.
65. Schenk MF, Szendro IG, Salverda MLM, Krug J, de Visser JAGM. Patterns of Epistasis between Beneficial Mutations in an Antibiotic Resistance Gene. *Mol Biol Evol* 2013; **30**: 1779–1787.
66. Estrela S, Brown SP. Community interactions and spatial structure shape selection on antibiotic resistant lineages. *PLOS Comput Biol* 2018; **14**: e1006179.
67. Lehtinen S, Blanquart F, Croucher NJ, Turner P, Lipsitch M, Fraser C. Evolution of antibiotic resistance is linked to any genetic mechanism affecting bacterial duration of carriage. *Proc Natl Acad Sci U S A* 2017; **114**: 1075–1080.
68. Yokoyama M, Stevens E, Laabei M, Bacon L, Heesom K, Bayliss S, et al. Epistasis analysis uncovers hidden antibiotic resistance-associated fitness costs hampering the evolution of MRSA. *Genome Biol* 2018; **19**: 94.
69. Bell G, MacLean C. The Search for 'Evolution-Proof' Antibiotics. *Trends Microbiol* 2018; **26**: 471–483.
70. Knopp M, Andersson DI. Predictable Phenotypes of Antibiotic Resistance Mutations. *MBio* 2018; **9**: e00770-18.
71. Frost I, Smith WPJ, Mitri S, Millan AS, Davit Y, Osborne JM, et al. Cooperation, competition and antibiotic resistance in bacterial colonies. *ISME J* 2018; **12**: 1582–1593.
72. Knöppel A, Näsvall J, Andersson DI. Evolution of Antibiotic Resistance without Antibiotic Exposure. *Antimicrob Agents Chemother* 2017; **61**.
73. D'Costa VM, King CE, Kalan L, Morar M, Sung WWL, Schwarz C, et al. Antibiotic resistance is ancient. *Nature* 2011; **477**: 457–461.
74. Clemente JC, Pehrsson EC, Blaser MJ, Sandhu K, Gao Z, Wang B, et al. The microbiome of uncontacted Amerindians. *Sci Adv* 2015; **1**: e1500183–e1500183.
75. Karkman A, Do TT, Walsh F, Virta MPJ. Antibiotic-Resistance Genes in Waste Water. *Trends Microbiol* 2018; **26**: 220–228.
76. Dantas G, Sommer MOA, Oluwasegun RD, Church GM. Bacteria Subsisting on Antibiotics. *Science* 2008; **320**: 100–103.
77. Munck C, Albertsen M, Telke A, Ellabaan M, Nielsen PH, Sommer MOA. Limited dissemination of the wastewater treatment plant core resistome. *Nat Commun* 2015; **6**: 8452.
78. Sommer MOA, Dantas G, Church GM. Functional Characterization of the Antibiotic Resistance Reservoir in the Human Microflora. *Science* 2009; **325**: 1128–1131.

79. Forsberg KJ, Patel S, Gibson MK, Lauber CL, Knight R, Fierer N, et al. Bacterial phylogeny structures soil resistomes across habitats. *Nature* 2014; **509**: 612–6.
80. D'Costa VM, McGrann KM, Hughes DW, Wright GD. Sampling the antibiotic resistome. *Science* 2006; **311**: 374–7.
81. Crofts TS, Gasparrini AJ, Dantas G. Next-generation approaches to understand and combat the antibiotic resistome. *Nat Rev Microbiol* 2017; **15**: 422–434.
82. Benveniste R, Davies J. Aminoglycoside antibiotic-inactivating enzymes in actinomycetes similar to those present in clinical isolates of antibiotic-resistant bacteria. *Proc Natl Acad Sci U S A* 1973; **70**: 2276–80.
83. Forsberg KJ, Reyes A, Wang B, Selleck EM, Sommer MOA, Dantas G. The Shared Antibiotic Resistome of Soil Bacteria and Human Pathogens. *Science* 2012; **337**: 1107–1111.
84. Knapp CW, Dolfing J, Ehlert PAI, Graham DW. Evidence of Increasing Antibiotic Resistance Gene Abundances in Archived Soils since 1940. *Environ Sci Technol* 2010; **44**: 580–587.
85. Lister PD, Wolter DJ, Hanson ND. Antibacterial-resistant *Pseudomonas aeruginosa*: clinical impact and complex regulation of chromosomally encoded resistance mechanisms. *Clin Microbiol Rev* 2009; **22**: 582–610.
86. Oliver A, Mulet X, López-Causapé C, Juan C. The increasing threat of *Pseudomonas aeruginosa* high-risk clones. *Drug Resist Updat* 2015; **21**–**22**: 41–59.
87. Juan C, Torrens G, González-Nicolau M, Oliver A. Diversity and regulation of intrinsic β-lactamases from non-fermenting and other Gram-negative opportunistic pathogens. *FEMS Microbiol Rev* 2017; **41**: 781–815.
88. Chevalier S, Bouffartigues E, Bodilis J, Maillot O, Lesouhaitier O, Feuilloley MGJ, et al. Structure, function and regulation of *Pseudomonas aeruginosa* porins. *FEMS Microbiol Rev* 2017; **41**: 698–722.
89. Hancock RE. Resistance mechanisms in *Pseudomonas aeruginosa* and other nonfermentative gram-negative bacteria. *Clin Infect Dis* 1998; **27 Suppl 1**: S93-9.
90. Martinez JL, Sánchez MB, Martínez-Solano L, Hernandez A, Garmendia L, Fajardo A, et al. Functional role of bacterial multidrug efflux pumps in microbial natural ecosystems. *FEMS Microbiol Rev* 2009; **33**: 430–449.
91. Du D, Wang-Kan X, Neuberger A, van Veen HW, Pos KM, Piddock LJ V., et al. Multidrug efflux pumps: structure, function and regulation. *Nat Rev Microbiol* 2018; **16**: 523–539.
92. Li X-Z, Plésiat P, Nikaido H. The Challenge of Efflux-Mediated Antibiotic Resistance in Gram-Negative Bacteria. *Clin Microbiol Rev* 2015; **28**: 337–418.
93. Girlich D, Naas T, Nordmann P. Biochemical characterization of the naturally occurring oxacillinase OXA-50 of *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother* 2004; **48**: 2043–8.
94. Fajardo A, Hernando-Amado S, Oliver A, Ball G, Filloux A, Martinez JL. Characterization of a novel Zn^{2+}-dependent intrinsic imipenemase from *Pseudomonas aeruginosa*. *J Antimicrob Chemother* 2014; **69**: 2972–2978.

95. Livermore DM. Interplay of impermeability and chromosomal beta-lactamase activity in imipenem-resistant *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother* 1992; **36**: 2046–8.
96. Skurnik D, Roux D, Cattoir V, Danilchanka O, Lu X, Yoder-Himes DR, et al. Enhanced in vivo fitness of carbapenem-resistant *oprD* mutants of *Pseudomonas aeruginosa* revealed through high-throughput sequencing. *Proc Natl Acad Sci* 2013; **110**: 20747–20752.
97. Juan C, Moya B, Perez JL, Oliver A. Stepwise Upregulation of the *Pseudomonas aeruginosa* Chromosomal Cephalosporinase Conferring High-Level β-Lactam Resistance Involves Three AmpD Homologues. *Antimicrob Agents Chemother* 2006; **50**: 1780–1787.
98. Cabot G, Ocampo-Sosa AA, Tubau F, Macia MD, Rodríguez C, Moya B, et al. Overexpression of AmpC and Efflux Pumps in *Pseudomonas aeruginosa* Isolates from Bloodstream Infections: Prevalence and Impact on Resistance in a Spanish Multicenter Study. *Antimicrob Agents Chemother* 2011; **55**: 1906–1911.
99. Moya B, Dötsch A, Juan C, Blázquez J, Zamorano L, Haussler S, et al. β-Lactam Resistance Response Triggered by Inactivation of a Nonessential Penicillin-Binding Protein. *PLoS Pathog* 2009; **5**: e1000353.
100. Rodríguez-Martínez J-M, Poirel L, Nordmann P. Extended-spectrum cephalosporinases in *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother* 2009; **53**: 1766–71.
101. Berrazeg M, Jeannot K, Ntsogo Enguéné VY, Broutin I, Loeffert S, Fournier D, et al. Mutations in β-Lactamase AmpC Increase Resistance of *Pseudomonas aeruginosa* Isolates to Antipseudomonal Cephalosporins. *Antimicrob Agents Chemother* 2015; **59**: 6248–6255.
102. Gifford DR, Furió V, Papkou A, Vogwill T, Oliver A, MacLean RC. Identifying and exploiting genes that potentiate the evolution of antibiotic resistance. *Nat Ecol Evol* 2018; **2**: 1033–1039.
103. Bruchmann S, Dötsch A, Nouri B, Chaberny IF, Häussler S. Quantitative contributions of target alteration and decreased drug accumulation to *Pseudomonas aeruginosa* fluoroquinolone resistance. *Antimicrob Agents Chemother* 2013; **57**: 1361–8.
104. Fernández L, Hancock REW. Adaptive and mutational resistance: role of porins and efflux pumps in drug resistance. *Clin Microbiol Rev* 2012; **25**: 661–81.
105. Livermore DM. Of *Pseudomonas*, porins, pumps and carbapenems. *J Antimicrob Chemother* 2001; **47**: 247–50.
106. Dößelmann B, Willmann M, Steglich M, Bunk B, Nübel U, Peter S, et al. Rapid and Consistent Evolution of Colistin Resistance in Extensively Drug-Resistant *Pseudomonas aeruginosa* during Morbidostat Culture. *Antimicrob Agents Chemother* 2017; **61**: e00043-17.
107. Lee J-Y, Park YK, Chung ES, Na IY, Ko KS. Evolved resistance to colistin and its loss due to genetic reversion in *Pseudomonas aeruginosa*. *Sci Rep* 2016; **6**: 25543.
108. Jochumsen N, Marvig RL, Damkiær S, Jensen RL, Paulander W, Molin S, et al. The evolution of antimicrobial peptide resistance in *Pseudomonas aeruginosa* is shaped by strong epistatic interactions. *Nat Commun* 2016; **7**: 13002.
109. Scientific Opinion on Carbapenem resistance in food animal ecosystems. *EFSA J* 2013; **11**.

110. European Centre for Disease Prevention and Control (ECDC). Annual Epidemiological Report for 2016 - Antimicrobial consumption. 2018. Accessed 20 Aug 2018.
111. Queenan AM, Bush K. Carbapenemases: the versatile beta-lactamases. *Clin Microbiol Rev* 2007; **20**: 440–58, table of contents.
112. Partridge SR, Kwong SM, Firth N, Jensen SO. Mobile Genetic Elements Associated with Antimicrobial Resistance. *Clin Microbiol Rev* 2018; **31**: e00088-17.
113. Diene SM, Rolain J-M. Carbapenemase genes and genetic platforms in Gram-negative bacilli: *Enterobacteriaceae*, *Pseudomonas* and *Acinetobacter* species. *Clin Microbiol Infect* 2014; **20**: 831–838.
114. Castanheira M, Deshpande LM, Costello A, Davies TA, Jones RN. Epidemiology and carbapenem resistance mechanisms of carbapenem-non-susceptible *Pseudomonas aeruginosa* collected during 2009-11 in 14 European and Mediterranean countries. *J Antimicrob Chemother* 2014; **69**: 1804–1814.
115. Thaller MC, Borgianni L, Di Lallo G, Chong Y, Lee K, Dajcs J, et al. Metallo-beta-lactamase production by *Pseudomonas otitidis*: a species-related trait. *Antimicrob Agents Chemother* 2011; **55**: 118–23.
116. Suzuki M, Suzuki S, Matsui M, Hiraki Y, Kawano F, Shibayama K. A subclass B3 metallo-β-lactamase found in *Pseudomonas alcaligenes*. *J Antimicrob Chemother* 2014; **69**: 1430–1432.
117. Cornaglia G, Giamarellou H, Rossolini GM. Metallo-β-lactamases: a last frontier for β-lactams? *Lancet Infect Dis* 2011; **11**: 381–93.
118. McGeary RP, Tan DT, Schenk G. Progress toward inhibitors of metallo-β-lactamases. *Future Med Chem* 2017; **9**: 673–691.
119. Turton JF, Wright L, Underwood A, Witney AA, Chan Y-T, Al-Shahib A, et al. High-Resolution Analysis by Whole-Genome Sequencing of an International Lineage (Sequence Type 111) of *Pseudomonas aeruginosa* Associated with Metallo-Carbapenemases in the United Kingdom. *J Clin Microbiol* 2015; **53**: 2622–31.
120. Nascimento APB, Ortiz MF, Martins WMBS, Morais GL, Fehlberg LCC, Almeida LGP, et al. Intraclonal Genome Stability of the Metallo-β-lactamase SPM-1-producing *Pseudomonas aeruginosa* ST277, an Endemic Clone Disseminated in Brazilian Hospitals. *Front Microbiol* 2016; **7**: 1946.
121. Poirel L, Rodríguez-Martínez J-M, Al Naiemi N, Debets-Ossenkopp YJ, Nordmann P. Characterization of DIM-1, an integron-encoded metallo-beta-lactamase from a *Pseudomonas stutzeri* clinical isolate in the Netherlands. *Antimicrob Agents Chemother* 2010; **54**: 2420–4.
122. Berglund F, Marathe NP, Österlund T, Bengtsson-Palme J, Kotsakis S, Flach C-F, et al. Identification of 76 novel B1 metallo-β-lactamases through large-scale screening of genomic and metagenomic data. *Microbiome* 2017; **5**: 134.
123. Salabi A El, Toleman MA, Weeks J, Bruderer T, Frei R, Walsh TR. First report of the metallo-beta-lactamase SPM-1 in Europe. *Antimicrob Agents Chemother* 2010; **54**: 582.

124. Hopkins KL, Findlay J, Mustafa N, Pike R, Parsons H, Wright L, et al. SPM-1 metallo-β-lactamase-producing *Pseudomonas aeruginosa* ST277 in the UK. *J Med Microbiol* 2016; **65**: 696–697.
125. Naas T, Bonnin RA, Cuzon G, Villegas M-V, Nordmann P. Complete sequence of two KPC-harbouring plasmids from *Pseudomonas aeruginosa*. *J Antimicrob Chemother* 2013; **68**: 1757–1762.
126. Dai X, Zhou D, Xiong W, Feng J, Luo W, Luo G, et al. The IncP-6 Plasmid p10265-KPC from *Pseudomonas aeruginosa* Carries a Novel ΔIS*Ec33*-Associated bla_{KPC-2} Gene Cluster. *Front Microbiol* 2016; **7**: 310.
127. Meunier D, Doumith M, Findlay J, Mustafa N, Mallard K, Anson J, et al. Carbapenem resistance mediated by $bla_{OXA-181}$ in *Pseudomonas aeruginosa*. *J Antimicrob Chemother* 2016; **71**: 2056–2057.
128. Sevillano E, Gallego L, García-Lobo JM. First detection of the OXA-40 carbapenemase in P. *aeruginosa* isolates, located on a plasmid also found in *A. baumannii*. *Pathol Biol* 2009; **57**: 493–495.
129. El Garch F, Bogaerts P, Bebrone C, Galleni M, Glupczynski Y. OXA-198, an acquired carbapenem-hydrolyzing class D beta-lactamase from *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother* 2011; **55**: 4828–33.
130. Langille MGI, Hsiao WWL, Brinkman FSL. Detecting genomic islands using bioinformatics approaches. *Nat Rev Microbiol* 2010; **8**: 373–382.
131. Oliveira PH, Touchon M, Cury J, Rocha EPC. The chromosomal organization of horizontal gene transfer in bacteria. *Nat Commun* 2017; **8**: 841.
132. Rankin DJ, Rocha EPC, Brown SP. What traits are carried on mobile genetic elements and why? *Heredity (Edinb)* 2011; **106**: 1–10.
133. Partridge SR. Analysis of antibiotic resistance regions in Gram-negative bacteria. *FEMS Microbiol Rev* 2011; **35**: 820–855.
134. Domingues S, da Silva GJ, Nielsen KM. Integrons. *Mob Genet Elements* 2012; **2**: 211–223.
135. Baquero F. From pieces to patterns: evolutionary engineering in bacterial pathogens. *Nat Rev Microbiol* 2004; **2**: 510–518.
136. Roberts AP, Chandler M, Courvalin P, Guédon G, Mullany P, Pembroke T, et al. Revised nomenclature for transposable genetic elements. *Plasmid* 2008; **60**: 167–73.
137. Roberts AP, Mullany P. Tn*916* -like genetic elements: a diverse group of modular mobile elements conferring antibiotic resistance. *FEMS Microbiol Rev* 2011; **35**: 856–871.
138. Petrovski S, Blackmore DW, Jackson KL, Stanisich VA. Mercury(II)-resistance transposons Tn*502* and Tn*512*, from *Pseudomonas* clinical strains, are structurally different members of the Tn*5053* family. *Plasmid* 2011; **65**: 58–64.
139. Ding Y, Teo JWP, Drautz-Moses DI, Schuster SC, Givskov M, Yang L. Acquisition of resistance to carbapenem and macrolide-mediated quorum sensing inhibition by *Pseudomonas aeruginosa* via ICE_{Tn4371}6385. *Commun Biol* 2018; **1**: 57.
140. Cuzon G, Naas T, Nordmann P. Functional characterization of Tn*4401*, a Tn*3*-based

transposon involved in *bla*$_{KPC}$ gene mobilization. *Antimicrob Agents Chemother* 2011; **55**: 5370–3.

141. Tseng S-P, Tsai J-C, Teng L-J, Hsueh P-R. Dissemination of transposon Tn*6001* in carbapenem-non-susceptible and extensively drug-resistant *Pseudomonas aeruginosa* in Taiwan. *J Antimicrob Chemother* 2009; **64**: 1170–1174.
142. Di Pilato V, Pollini S, Rossolini GM. Tn*6249* , a New Tn*6162* Transposon Derivative Carrying a Double-Integron Platform and Involved with Acquisition of the *bla*$_{VIM-1}$ Metallo-β-Lactamase Gene in *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother* 2015; **59**: 1583–1587.
143. Di Pilato V, Pollini S, Rossolini GM. Characterization of plasmid pAX22, encoding VIM-1 metallo-β-lactamase, reveals a new putative mechanism of In70 integron mobilization. *J Antimicrob Chemother* 2014; **69**: 67–71.
144. Toleman MA, Biedenbach D, Bennett D, Jones RN, Walsh TR. Genetic characterization of a novel metallo- -lactamase gene, *bla*$_{IMP-13}$, harboured by a novel Tn*5051*-type transposon disseminating carbapenemase genes in Europe: report from the SENTRY worldwide antimicrobial surveillance programme. *J Antimicrob Chemother* 2003; **52**: 583–590.
145. Smillie C, Garcillán-Barcia MP, Francia MV, Rocha EPC, de la Cruz F. Mobility of plasmids. *Microbiol Mol Biol Rev* 2010; **74**: 434–52.
146. Garcillán-Barcia MP, Alvarado A, de la Cruz F. Identification of bacterial plasmids based on mobility and plasmid population biology. *FEMS Microbiol Rev* 2011; **35**: 936–956.
147. Rozwandowicz M, Brouwer MSM, Fischer J, Wagenaar JA, Gonzalez-Zorn B, Guerra B, et al. Plasmids carrying antimicrobial resistance genes in *Enterobacteriaceae*. *J Antimicrob Chemother* 2018; **73**: 1121–1137.
148. San Millan A, MacLean RC. Fitness Costs of Plasmids: a Limit to Plasmid Transmission. *Microbiol Spectr* 2017; **5**.
149. San Millan A. Evolution of Plasmid-Mediated Antibiotic Resistance in the Clinical Context. *Trends Microbiol* 2018; **0**.
150. Halary S, Leigh JW, Cheaib B, Lopez P, Bapteste E. Network analyses structure genetic diversity in independent genetic worlds. *Proc Natl Acad Sci U S A* 2010; **107**: 127–32.
151. Ramsay JP, Firth N. Diverse mobilization strategies facilitate transfer of non-conjugative mobile genetic elements. *Curr Opin Microbiol* 2017; **38**: 1–9.
152. Lopatkin AJ, Meredith HR, Srimani JK, Pfeiffer C, Durrett R, You L. Persistence and reversal of plasmid-mediated antibiotic resistance. *Nat Commun* 2017; **8**: 1689.
153. Díaz-Orejas R, Espinosa M, Yeo CC. The Importance of the Expendable: Toxin–Antitoxin Genes in Plasmids and Chromosomes. *Front Microbiol* 2017; **8**: 1479.
154. Baxter JC, Funnell BE. Plasmid Partition Mechanisms. *Microbiol Spectr* 2014; **2**.
155. Loftie-Eaton W, Bashford K, Quinn H, Dong K, Millstein J, Hunter S, et al. Compensatory mutations improve general permissiveness to antibiotic resistance plasmids. *Nat Ecol Evol* 2017; **1**: 1354–1363.
156. Svara F, Rankin DJ. The evolution of plasmid-carried antibiotic resistance. *BMC Evol Biol*

2011; **11**: 130.

157. San Millan A, Toll-Riera M, Escudero JA, Cantón R, Coque TM, MacLean RC. Sequencing of plasmids pAMBL1 and pAMBL2 from *Pseudomonas aeruginosa* reveals a *bla*$_{VIM-1}$ amplification causing high-level carbapenem resistance. *J Antimicrob Chemother* 2015; **70**: 3000–3003.
158. Bonnin RA, Poirel L, Nordmann P, Eikmeyer FG, Wibberg D, Pühler A, et al. Complete sequence of broad-host-range plasmid pNOR-2000 harbouring the metallo-β-lactamase gene *bla*$_{VIM-2}$ from *Pseudomonas aeruginosa*. *J Antimicrob Chemother* 2013; **68**: 1060–1065.
159. Vilacoba E, Quiroga C, Pistorio M, Famiglietti A, Rodríguez H, Kovensky J, et al. A *bla*$_{VIM-2}$ plasmid disseminating in extensively drug-resistant clinical *Pseudomonas aeruginosa* and Serratia marcescens isolates. *Antimicrob Agents Chemother* 2014; **58**: 7017–8.
160. Li H, Toleman MA, Bennett PM, Jones RN, Walsh TR. Complete Sequence of p07-406, a 24,179-base-pair plasmid harboring the *bla*$_{VIM-7}$ metallo-beta-lactamase gene in a *Pseudomonas aeruginosa* isolate from the United States. *Antimicrob Agents Chemother* 2008; **52**: 3099–105.
161. Feng W, Zhou D, Wang Q, Luo W, Zhang D, Sun Q, et al. Dissemination of IMP-4-encoding pIMP-HZ1-related plasmids among Klebsiella pneumoniae and *Pseudomonas aeruginosa* in a Chinese teaching hospital. *Sci Rep* 2016; **6**: 33419.
162. Xiong J, Alexander DC, Ma JH, Déraspe M, Low DE, Jamieson FB, et al. Complete sequence of pOZ176, a 500-kilobase IncP-2 plasmid encoding IMP-9-mediated carbapenem resistance, from outbreak isolate *Pseudomonas aeruginosa* 96. *Antimicrob Agents Chemother* 2013; **57**: 3775–82.
163. Thomas CM, Haines AS. Plasmids of the Genus *Pseudomonas*. *Pseudomonas*. 2004. Springer US, Boston, MA, pp 197–231.
164. Tazzyman SJ, Bonhoeffer S. Why There Are No Essential Genes on Plasmids. *Mol Biol Evol* 2014; **32**: msu293.
165. Orlek A, Stoesser N, Anjum MF, Doumith M, Ellington MJ, Peto T, et al. Plasmid Classification in an Era of Whole-Genome Sequencing: Application in Studies of Antibiotic Resistance Epidemiology. *Front Microbiol* 2017; **8**: 182.
166. Arredondo-Alonso S, Willems RJ, van Schaik W, Schürch AC. On the (im)possibility of reconstructing plasmids from whole-genome short-read sequencing data. *Microb Genomics* 2017; **3**: e000128.
167. Lanza VF, de Toro M, Garcillán-Barcia MP, Mora A, Blanco J, Coque TM, et al. Plasmid Flux in Escherichia coli ST131 Sublineages, Analyzed by Plasmid Constellation Network (PLACNET), a New Method for Plasmid Reconstruction from Whole Genome Sequences. *PLoS Genet* 2014; **10**: e1004766.
168. Vielva L, de Toro M, Lanza VF, de la Cruz F, Berger B. PLACNETw: a web-based tool for plasmid reconstruction from bacterial genomes. *Bioinformatics* 2017; **33**: 3796–3798.
169. Antipov D, Hartwick N, Shen M, Raiko M, Lapidus A, Pevzner PA. plasmidSPAdes:

assembling plasmids from whole genome sequencing data. *Bioinformatics* 2016; **32**: btw493.

170. Rozov R, Brown Kav A, Bogumil D, Shterzer N, Halperin E, Mizrahi I, et al. Recycler: an algorithm for detecting plasmids from *de novo* assembly graphs. *Bioinformatics* 2016; **33**: btw651.
171. Bellanger X, Payot S, Leblond-Bourget N, Guédon G. Conjugative and mobilizable genomic islands in bacteria: evolution and diversity. *FEMS Microbiol Rev* 2014; **38**: 720–760.
172. Chen J, Novick RP. Phage-mediated intergeneric transfer of toxin genes. *Science* 2009; **323**: 139–41.
173. Karlin S. Detecting anomalous gene clusters and pathogenicity islands in diverse bacterial genomes. *Trends Microbiol* 2001; **9**: 335–43.
174. Vernikos GS, Parkhill J. Resolving the structural features of genomic islands: A machine learning approach. *Genome Res* 2008; **18**: 331–342.
175. Bertelli C, Laird MR, Williams KP, Lau BY, Hoad G, Winsor GL, et al. IslandViewer 4: expanded prediction of genomic islands for larger-scale datasets. *Nucleic Acids Res* 2017; **45**: W30–W35.
176. Stokes HW, Martinez E, Chowdhury PR, Djordjevic S. Class 1 integron-associated spread of resistance regions in *Pseudomonas aeruginosa*: plasmid or chromosomal platforms? *J Antimicrob Chemother* 2012; **67**: 1799–1800.
177. Roy Chowdhury P, Scott M, Worden P, Huntington P, Hudson B, Karagiannis T, et al. Genomic islands 1 and 2 play key roles in the evolution of extensively drug-resistant ST235 isolates of *Pseudomonas aeruginosa*. *Open Biol* 2016; **6**.
178. Chowdhury PR, Scott MJ, Djordjevic SP. Genomic islands 1 and 2 carry multiple antibiotic resistance genes in *Pseudomonas aeruginosa* ST235, ST253, ST111 and ST175 and are globally dispersed. *J Antimicrob Chemother* . 2017. , **72**: 620–622
179. Martinez E, Marquez C, Ingold A, Merlino J, Djordjevic SP, Stokes HW, et al. Diverse mobilized class 1 integrons are common in the chromosomes of pathogenic *Pseudomonas aeruginosa* clinical isolates. *Antimicrob Agents Chemother* 2012; **56**: 2169–72.
180. Hong JS, Yoon EJ, Lee H, Jeong SH, Lee K. Clonal dissemination of *Pseudomonas aeruginosa* sequence type 235 isolates carrying $bla_{\text{IMP-6}}$ and emergence of $bla_{\text{GES-24}}$ and $bla_{\text{IMP-10}}$ on novel genomic islands PAGI-15 and -16 in South Korea. *Antimicrob Agents Chemother* 2016; **60**: 7216–7223.
181. Winstanley C, Langille MGI, Fothergill JL, Kukavica-Ibrulj I, Paradis-Bleau C, Sanschagrin F, et al. Newly introduced genomic prophage islands are critical determinants of in vivo competitiveness in the Liverpool Epidemic Strain of *Pseudomonas aeruginosa*. *Genome Res* 2009; **19**: 12–23.
182. Jani M, Mathee K, Azad RK. Identification of Novel Genomic Islands in Liverpool Epidemic Strain of *Pseudomonas aeruginosa* Using Segmentation and Clustering. *Front Microbiol* 2016; **7**: 1210.
183. Harrison EM, Carter MEK, Luck S, Ou H-Y, He X, Deng Z, et al. Pathogenicity islands

PAPI-1 and PAPI-2 contribute individually and synergistically to the virulence of *Pseudomonas aeruginosa* strain PA14. *Infect Immun* 2010; **78**: 1437–46.

184. He J, Baldini RL, Déziel E, Saucier M, Zhang Q, Liberati NT, et al. The broad host range pathogen *Pseudomonas aeruginosa* strain PA14 carries two pathogenicity islands harboring plant and animal virulence genes. *Proc Natl Acad Sci U S A* 2004; **101**: 2530–5.
185. Sawa T, Hamaoka S, Kinoshita M, Kainuma A, Naito Y, Akiyama K, et al. *Pseudomonas aeruginosa* Type III Secretory Toxin ExoU and Its Predicted Homologs. *Toxins (Basel)* 2016; **8**: 307.
186. Dortet L, Lombardi C, Cretin F, Dessen A, Filloux A. Pore-forming activity of the *Pseudomonas aeruginosa* type III secretion system translocon alters the host epigenome. *Nat Microbiol* 2018; **3**: 378–386.
187. Diaz MH, Hauser AR. *Pseudomonas aeruginosa* cytotoxin ExoU is injected into phagocytic cells during acute pneumonia. *Infect Immun* 2010; **78**: 1447–56.
188. Kulasekara BR, Kulasekara HD, Wolfgang MC, Stevens L, Frank DW, Lory S. Acquisition and evolution of the *exoU* locus in *Pseudomonas aeruginosa*. *J Bacteriol* 2006; **188**: 4037–50.
189. Toleman MA, Walsh TR. Combinatorial events of insertion sequences and ICE in Gram-negative bacteria. *FEMS Microbiol Rev* 2011; **35**: 912–935.
190. Johnson CM, Grossman AD. Integrative and Conjugative Elements (ICE): What They Do and How They Work. *Annu Rev Genet* 2015; **49**: 577–601.
191. Carraro N, Poulin D, Burrus V. Replication and Active Partition of Integrative and Conjugative Elements (ICE) of the SXT/R391 Family: The Line between ICE and Conjugative Plasmids Is Getting Thinner. *PLOS Genet* 2015; **11**: e1005298.
192. Guglielmini J, Quintais L, Garcillán-Barcia MP, de la Cruz F, Rocha EPC. The Repertoire of ICE in Prokaryotes Underscores the Unity, Diversity, and Ubiquity of Conjugation. *PLoS Genet* 2011; **7**: e1002222.
193. Cury J, Touchon M, Rocha EPC. Integrative and conjugative elements and their hosts: composition, distribution and organization. *Nucleic Acids Res* 2017; **45**: 8943–8956.
194. Burrus V. Mechanisms of stabilization of integrative and conjugative elements. *Curr Opin Microbiol* 2017; **38**: 44–50.
195. Delavat F, Miyazaki R, Carraro N, Pradervand N, van der Meer JR. The hidden life of integrative and conjugative elements. *FEMS Microbiol Rev* 2017; **41**: 512–537.
196. Wozniak RAF, Waldor MK. Integrative and conjugative elements: mosaic mobile genetic elements enabling dynamic lateral gene flow. *Nat Rev Microbiol* 2010; **8**: 552–563.
197. Flannagan SE, Clewell DB. Conjugative transfer of Tn*916* in *Enterococcus faecalis*: *trans* activation of homologous transposons. *J Bacteriol* 1991; **173**: 7136–41.
198. Haskett TL, Terpolilli JJ, Bekuma A, O'Hara GW, Sullivan JT, Wang P, et al. Assembly and transfer of tripartite integrative and conjugative genetic elements. *Proc Natl Acad Sci U S A* 2016; **113**: 12268–12273.
199. Guédon G, Libante V, Coluzzi C, Payot S, Leblond-Bourget N. The Obscure World of

Integrative and Mobilizable Elements, Highly Widespread Elements that Pirate Bacterial Conjugative Systems. *Genes (Basel)* 2017; **8**: 337.

200. Ravatn R, Studer S, Springael D, Zehnder AJ, van der Meer JR. Chromosomal integration, tandem amplification, and deamplification in *Pseudomonas putida* F1 of a 105-kilobase genetic element containing the chlorocatechol degradative genes from *Pseudomonas* sp. Strain B13. *J Bacteriol* 1998; **180**: 4360–9.
201. Gaillard M, Vallaeys T, Vorhölter FJ, Minoia M, Werlen C, Sentchilo V, et al. The *clc* element of *Pseudomonas* sp. strain B13, a genomic island with various catabolic properties. *J Bacteriol* 2006; **188**: 1999–2013.
202. Larbig KD, Christmann A, Johann A, Klockgether J, Hartsch T, Merkl R, et al. Gene islands integrated into $tRNA^{Gly}$ genes confer genome diversity on a *Pseudomonas aeruginosa* clone. *J Bacteriol* 2002; **184**: 6665–80.
203. Ryan MP, Pembroke JT, Adley CC. Novel Tn*4371*-ICE like element in *Ralstonia pickettii* and Genome mining for comparative elements. *BMC Microbiol* 2009; **9**: 242.
204. Fonseca EL, Marin MA, Encinas F, Vicente ACP. Full characterization of the integrative and conjugative element carrying the metallo-β-lactamase bla_{SPM-1} and bicyclomycin *bcr1* resistance genes found in the pandemic *Pseudomonas aeruginosa* clone SP/ST277. *J Antimicrob Chemother* 2015; **70**: 2547–2550.
205. Bi D, Xu Z, Harrison EM, Tai C, Wei Y, He X, et al. ICEberg: a web-based resource for integrative and conjugative elements found in Bacteria. *Nucleic Acids Res* 2012; **40**: D621-6.
206. Guglielmini J, de la Cruz F, Rocha EPC. Evolution of Conjugation and Type IV Secretion Systems. *Mol Biol Evol* 2013; **30**: 315–331.
207. Guglielmini J, Néron B, Abby SS, Garcillán-Barcia MP, de la Cruz F, Rocha EPC. Key components of the eight classes of type IV secretion systems involved in bacterial conjugation or protein secretion. *Nucleic Acids Res* 2014; **42**: 5715–27.
208. Andersson DI, Hughes D. Antibiotic resistance and its cost: is it possible to reverse resistance? *Nat Rev Microbiol* 2010; **8**: 260–271.
209. Vogwill T, MacLean RC. The genetic basis of the fitness costs of antimicrobial resistance: a meta-analysis approach. *Evol Appl* 2015; **8**: 284–295.
210. San Millan A, Toll-Riera M, Qi Q, Betts A, Hopkinson RJ, McCullagh J, et al. Integrative analysis of fitness and metabolic effects of plasmids in *Pseudomonas aeruginosa* PAO1. *ISME J* 2018; 1.
211. Rodriguez-Beltran J, Hernandez-Beltran JCR, DelaFuente J, Escudero JA, Fuentes-Hernandez A, MacLean RC, et al. Multicopy plasmids allow bacteria to escape from fitness trade-offs during evolutionary innovation. *Nat Ecol Evol* 2018; **2**: 873–881.
212. Sander P, Springer B, Prammananan T, Sturmfels A, Kappler M, Pletschette M, et al. Fitness cost of chromosomal drug resistance-conferring mutations. *Antimicrob Agents Chemother* 2002; **46**: 1204–11.
213. Böttger EC, Springer B, Pletschette M, Sander P. Fitness of antibiotic-resistant

microorganisms and compensatory mutations. *Nat Med* 1998; **4**: 1343–1344.

214. Andersson DI, Hughes D. Microbiological effects of sublethal levels of antibiotics. *Nat Rev Microbiol* 2014; **12**: 465–478.
215. Ward H, Perron GG, MacLean RC. The cost of multiple drug resistance in *Pseudomonas aeruginosa*. *J Evol Biol* 2009; **22**: 997–1003.
216. Trindade S, Sousa A, Xavier KB, Dionisio F, Ferreira MG, Gordo I. Positive Epistasis Drives the Acquisition of Multidrug Resistance. *PLoS Genet* 2009; **5**: e1000578.
217. Kassen R. The experimental evolution of specialists, generalists, and the maintenance of diversity. *J Evol Biol* 2002; **15**: 173–190.
218. Melnyk AH, McCloskey N, Hinz AJ, Dettman J, Kassen R. Evolution of Cost-Free Resistance under Fluctuating Drug Selection in *Pseudomonas aeruginosa*. *mSphere* 2017; **2**.
219. Olivares Pacheco J, Alvarez-Ortega C, Alcalde Rico M, Martínez JL. Metabolic Compensation of Fitness Costs Is a General Outcome for Antibiotic-Resistant *Pseudomonas aeruginosa* Mutants Overexpressing Efflux Pumps. *MBio* 2017; **8**.
220. Kloesges T, Popa O, Martin W, Dagan T. Networks of Gene Sharing among 329 Proteobacterial Genomes Reveal Differences in Lateral Gene Transfer Frequency at Different Phylogenetic Depths. *Mol Biol Evol* 2011; **28**: 1057–1074.
221. Andam CP, Gogarten JP. Biased gene transfer in microbial evolution. *Nat Rev Microbiol* 2011; **9**: 543–555.
222. Hooper SD, Mavromatis K, Kyrpides NC. Microbial co-habitation and lateral gene transfer: what transposases can tell us. *Genome Biol* 2009; **10**: R45.
223. Soucy SM, Huang J, Gogarten JP. Horizontal gene transfer: building the web of life. *Nat Rev Genet* 2015; **16**: 472–482.
224. Tuller T, Girshovich Y, Sella Y, Kreimer A, Freilich S, Kupiec M, et al. Association between translation efficiency and horizontal gene transfer within microbial communities. *Nucleic Acids Res* 2011; **39**: 4743–4755.
225. Polz MF, Alm EJ, Hanage WP. Horizontal gene transfer and the evolution of bacterial and archaeal population structure. *Trends Genet* 2013; **29**: 170–175.
226. Olsen RH, Wright CD. Interaction of *Pseudomonas* and *Enterobacteriaceae* plasmids in *Aeromonas salmonicida*. *J Bacteriol* 1976; **128**: 228–34.
227. Cohen O, Gophna U, Pupko T. The Complexity Hypothesis Revisited: Connectivity Rather Than Function Constitutes a Barrier to Horizontal Gene Transfer. *Mol Biol Evol* 2011; **28**: 1481–1489.
228. Treangen TJ, Rocha EPC. Horizontal Transfer, Not Duplication, Drives the Expansion of Protein Families in Prokaryotes. *PLoS Genet* 2011; **7**: e1001284.
229. Popa O, Hazkani-Covo E, Landan G, Martin W, Dagan T. Directed networks reveal genomic barriers and DNA repair bypasses to lateral gene transfer among prokaryotes. *Genome Res* 2011; **21**: 599–609.
230. Popa O, Dagan T. Trends and barriers to lateral gene transfer in prokaryotes. *Curr Opin*

Microbiol 2011; **14**: 615–623.

231. Harrison E, Brockhurst MA. Plasmid-mediated horizontal gene transfer is a coevolutionary process. *Trends Microbiol* 2012; **20**: 262–7.
232. Gogarten JP, Townsend JP. Horizontal gene transfer, genome innovation and evolution. *Nat Rev Microbiol* 2005; **3**: 679–687.
233. Broaders E, Gahan CGM, Marchesi JR. Mobile genetic elements of the human gastrointestinal tract. *Gut Microbes* 2013; **4**: 271–280.
234. Loftie-Eaton W, Yano H, Burleigh S, Simmons RS, Hughes JM, Rogers LM, et al. Evolutionary Paths That Expand Plasmid Host-Range: Implications for Spread of Antibiotic Resistance. *Mol Biol Evol* 2016; **33**: 885–897.
235. Millan AS, Peña-Miller R, Toll-Riera M, Halbert Z V., McLean AR, Cooper BS, et al. Positive selection and compensatory adaptation interact to stabilize non-transmissible plasmids. *Nat Commun* 2014; **5**: 5208.
236. San Millan A, Escudero JA, Gifford DR, Mazel D, MacLean RC. Multicopy plasmids potentiate the evolution of antibiotic resistance in bacteria. *Nat Ecol Evol* 2016; **1**: 0010.
237. Harrison E, Guymer D, Spiers AJ, Paterson S, Brockhurst MA. Parallel Compensatory Evolution Stabilizes Plasmids across the Parasitism-Mutualism Continuum. *Curr Biol* 2015; **25**: 2034–2039.
238. Stevenson C, Hall JPJ, Brockhurst MA, Harrison E. Plasmid stability is enhanced by higher-frequency pulses of positive selection. *Proceedings Biol Sci* 2018; **285**.
239. Kottara A, Hall JPJ, Harrison E, Brockhurst MA. Variable plasmid fitness effects and mobile genetic element dynamics across *Pseudomonas* species. *FEMS Microbiol Ecol* 2018; **94**.
240. San Millan A, Heilbron K, MacLean RC. Positive epistasis between co-infecting plasmids promotes plasmid survival in bacterial populations. *ISME J* 2014; **8**: 601–612.
241. Smith J. Superinfection drives virulence evolution in experimental populations of bacteria and plasmids. *Evolution (N Y)* 2011; **65**: 831–841.
242. Sundqvist M, Geli P, Andersson DI, Sjolund-Karlsson M, Runehagen A, Cars H, et al. Little evidence for reversibility of trimethoprim resistance after a drastic reduction in trimethoprim use. *J Antimicrob Chemother* 2010; **65**: 350–360.
243. Yen P, Papin JA. History of antibiotic adaptation influences microbial evolutionary dynamics during subsequent treatment. *PLOS Biol* 2017; **15**: e2001586.
244. Alekshun MN, Levy SB. Molecular Mechanisms of Antibacterial Multidrug Resistance. *Cell* 2007; **128**: 1037–1050.
245. Starikova I, Al-Haroni M, Werner G, Roberts AP, Sorum V, Nielsen KM, et al. Fitness costs of various mobile genetic elements in *Enterococcus faecium* and *Enterococcus faecalis*. *J Antimicrob Chemother* 2013; **68**: 2755–2765.
246. Delavat F, Mitri S, Pelet S, van der Meer JR. Highly variable individual donor cell fates characterize robust horizontal gene transfer of an integrative and conjugative element. *Proc Natl Acad Sci U S A* 2016; **113**: E3375-83.
247. Miyazaki R, Yano H, Sentchilo V, van der Meer JR. Physiological and transcriptome

changes induced by *Pseudomonas putida* acquisition of an integrative and conjugative element. *Sci Rep* 2018; **8**: 5550.

248. Gaillard M, Pernet N, Vogne C, Hagenbüchle O, van der Meer JR. Host and invader impact of transfer of the *clc* genomic island into *Pseudomonas aeruginosa* PAO1. *Proc Natl Acad Sci U S A* 2008; **105**: 7058–63.
249. Pirnay J-P, Bilocq F, Pot B, Cornelis P, Zizi M, Van Eldere J, et al. *Pseudomonas aeruginosa* Population Structure Revisited. *PLoS One* 2009; **4**: e7740.
250. Kos VN, Déraspe M, McLaughlin RE, Whiteaker JD, Roy PH, Alm RA, et al. The resistome of *Pseudomonas aeruginosa* in relationship to phenotypic susceptibility. *Antimicrob Agents Chemother* 2015; **59**: 427–36.
251. Woodford N, Turton JF, Livermore DM. Multiresistant Gram-negative bacteria: the role of high-risk clones in the dissemination of antibiotic resistance. *FEMS Microbiol Rev* 2011; **35**: 736–755.
252. Sánchez-Diener I, Zamorano L, López-Causapé C, Cabot G, Mulet X, Peña C, et al. Interplay among Resistance Profiles, High-Risk Clones, and Virulence in the *Caenorhabditis elegans Pseudomonas aeruginosa* Infection Model. *Antimicrob Agents Chemother* 2017; **61**.
253. Cabot G, Ocampo-Sosa AA, Domínguez MA, Gago JF, Juan C, Tubau F, et al. Genetic Markers of Widespread Extensively Drug-Resistant *Pseudomonas aeruginosa* High-Risk Clones. *Antimicrob Agents Chemother* 2012; **56**: 6349–6357.
254. Cabot G, López-Causapé C, Ocampo-Sosa AA, Sommer LM, Domínguez MÁ, Zamorano L, et al. Deciphering the resistome of the widespread *Pseudomonas aeruginosa* sequence type 175 international high-risk clone through whole-genome sequencing. *Antimicrob Agents Chemother* 2016; **60**: 7415–7423.
255. López-Causapé C, Cabot G, del Barrio-Tofiño E, Oliver A. The Versatile Mutational Resistome of *Pseudomonas aeruginosa*. *Front Microbiol* 2018; **9**: 685.
256. Treepong P, Kos VN, Guyeux C, Blanc DS, Bertrand X, Valot B, et al. Global emergence of the widespread *Pseudomonas aeruginosa* ST235 clone. *Clin Microbiol Infect* 2018; **24**: 258–266.
257. Maatallah M, Cheriaa J, Backhrouf A, Iversen A, Grundmann H, Do T, et al. Population Structure of *Pseudomonas aeruginosa* from Five Mediterranean Countries: Evidence for Frequent Recombination and Epidemic Occurrence of CC235. *PLoS One* 2011; **6**: e25617.
258. van Belkum A, Soriaga LB, LaFave MC, Akella S, Veyrieras J-B, Barbu EM, et al. Phylogenetic Distribution of CRISPR-Cas Systems in Antibiotic-Resistant *Pseudomonas aeruginosa*. *MBio* 2015; **6**: e01796-15.
259. Miyoshi-Akiyama T, Tada T, Ohmagari N, Viet Hung N, Tharavichitkul P, Pokhrel BM, et al. Emergence and Spread of Epidemic Multidrug-Resistant *Pseudomonas aeruginosa*. *Genome Biol Evol* 2017; **9**: 3238–3245.
260. Scotta C, Juan C, Cabot G, Oliver A, Lalucat J, Bennasar A, et al. Environmental microbiota represents a natural reservoir for dissemination of clinically relevant metallo-beta-

lactamases. *Antimicrob Agents Chemother* 2011; **55**: 5376–9.

261. Juan C, Zamorano L, Mena A, Alberti S, Perez JL, Oliver A. Metallo-β-lactamase-producing *Pseudomonas putida* as a reservoir of multidrug resistance elements that can be transferred to successful *Pseudomonas aeruginosa* clones. *J Antimicrob Chemother* 2010; **65**: 474–478.
262. Bondy-Denomy J, Davidson AR. To acquire or resist: the complex biological effects of CRISPR-Cas systems. *Trends Microbiol* 2014; **22**: 218–25.
263. Mulet X, Cabot G, Ocampo-Sosa AA, Domínguez MA, Zamorano L, Juan C, et al. Biological Markers of *Pseudomonas aeruginosa* Epidemic High-Risk Clones. *Antimicrob Agents Chemother* 2013; **57**: 5527–5535.
264. Wong A, Kassen R. Parallel evolution and local differentiation in quinolone resistance in *Pseudomonas aeruginosa*. *Microbiology* 2011; **157**: 937–944.
265. Poirel L, Lambert T, Türkoglü S, Ronco E, Gaillard J, Nordmann P. Characterization of Class 1 integrons from *Pseudomonas aeruginosa* that contain the *bla*$_{VIM-2}$ carbapenem-hydrolyzing beta-lactamase gene and of two novel aminoglycoside resistance gene cassettes. *Antimicrob Agents Chemother* 2001; **45**: 546–52.
266. Santos C, Caetano T, Ferreira S, Mendo S. Tn*5090*-like class 1 integron carrying *bla*$_{VIM-2}$ in a *Pseudomonas putida* strain from Portugal. *Clin Microbiol Infect* 2010; **16**: 1558–1561.
267. Cardoso O, Alves AF, Leitão R. Metallo-β-lactamase VIM-2 in *Pseudomonas aeruginosa* isolates from a cystic fibrosis patient. *Int J Antimicrob Agents* 2008; **31**: 375–379.
268. Quinteira S, Peixe L. Multiniche screening reveals the clinically relevant metallo-beta-lactamase VIM-2 in *Pseudomonas aeruginosa* far from the hospital setting: an ongoing dispersion process? *Appl Environ Microbiol* 2006; **72**: 3743–5.
269. Partridge SR, Brown HJ, Stokes HW, Hall RM. Transposons Tn*1696* and Tn*21* and Their Integrons In4 and In2 Have Independent Origins. *Antimicrob Agents Chemother* 2001; **45**: 1263–1270.
270. Coyne S, Courvalin P, Galimand M. Acquisition of multidrug resistance transposon Tn*6061* and IS*6100*-mediated large chromosomal inversions in *Pseudomonas aeruginosa* clinical isolates. *Microbiology* 2010; **156**: 1448–1458.
271. Targant H, Doublet B, Aarestrup FM, Cloeckaert A, Madec J-Y. IS*6100*-mediated genetic rearrangement within the complex class 1 integron In104 of the Salmonella genomic island 1. *J Antimicrob Chemother* 2010; **65**: 1543–1545.
272. Burrows LL. *Pseudomonas aeruginosa* Twitching Motility: Type IV Pili in Action. *Annu Rev Microbiol* 2012; **66**: 493–520.
273. Gomila M, Del Carmen Gallegos M, Fernández-Baca V, Pareja A, Pascual M, Díaz-Antolín P, et al. Genetic diversity of clinical *Pseudomonas aeruginosa* isolates in a public hospital in Spain. *BMC Microbiol* 2013; **13**: 138.
274. Khaledi A, Schniederjans M, Pohl S, Rainer R, Bodenhofer U, Xia B, et al. Transcriptome Profiling of Antimicrobial Resistance in *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother* 2016; **60**: 4722–33.

275. Bruchmann S, Muthukumarasamy U, Pohl S, Preusse M, Bielecka A, Nicolai T, et al. Deep transcriptome profiling of clinical *K lebsiella pneumoniae* isolates reveals strain and sequence type-specific adaptation. *Environ Microbiol* 2015; **17**: 4690–4710.
276. Quick J, Cumley N, Wearn CM, Niebel M, Constantinidou C, Thomas CM, et al. Seeking the source of *Pseudomonas aeruginosa* infections in a recently opened hospital: an observational study using whole-genome sequencing. *BMJ Open* 2014; **4**: e006278.
277. Davis RJ, Jensen SO, Van Hal S, Espedido B, Gordon A, Farhat R, et al. Whole Genome Sequencing in Real-Time Investigation and Management of a *Pseudomonas aeruginosa* Outbreak on a Neonatal Intensive Care Unit. *Infect Control Hosp Epidemiol* 2015; **36**: 1058–1064.

Appendixes

OXFORD UNIVERSITY PRESS LICENSE TERMS AND CONDITIONS

Aug 26, 2018

This Agreement between João Botelho ("You") and Oxford University Press ("Oxford University Press") consists of your license details and the terms and conditions provided by Oxford University Press and Copyright Clearance Center.

License Number	4414270172700
License date	Aug 22, 2018
Licensed content publisher	Oxford University Press
Licensed content publication	Nucleic Acids Research
Licensed content title	A dynamic and intricate regulatory network determines *Pseudomonas aeruginosa* virulence
Licensed content author	Balasubramanian, Deepak; Schneper, Lisa
Licensed content date	Nov 10, 2012
Type of Use	Thesis/Dissertation
Institution name	
Title of your work	CARBAPENEMASE PRODUCERS OF PSEUDOMONAS AERUGINOSA: LIGHTS ON NEW MOBILE GENETIC ELEMENTS
Publisher of your work	Faculdade de Farmácia, Universidade do Porto
Expected publication date	Dec 2018
Permissions cost	0.00 EUR
Value added tax	0.00 EUR
Total	0.00 EUR
Title	CARBAPENEMASE PRODUCERS OF PSEUDOMONAS AERUGINOSA: LIGHTS ON NEW MOBILE GENETIC ELEMENTS
Instructor name	Luísa Peixe
Institution name	Faculdade de Farmácia, Universidade do Porto
Expected presentation date	Dec 2018
Portions	Figure 1
Requestor Location	João Botelho Rua de Jorge Viterbo Ferreira, 228 Porto, other Portugal Attn:
Publisher Tax ID	GB125506730
Billing Type	Invoice
Billing Address	João Botelho Rua de Jorge Viterbo Ferreira, 228

Porto, Portugal
Attn:

Total 0.00 EUR

Terms and Conditions

STANDARD TERMS AND CONDITIONS FOR REPRODUCTION OF MATERIAL FROM AN OXFORD UNIVERSITY PRESS JOURNAL

1. Use of the material is restricted to the type of use specified in your order details.
2. This permission covers the use of the material in the English language in the following territory: world. If you have requested additional permission to translate this material, the terms and conditions of this reuse will be set out in clause 12.
3. This permission is limited to the particular use authorized in (1) above and does not allow you to sanction its use elsewhere in any other format other than specified above, nor does it apply to quotations, images, artistic works etc that have been reproduced from other sources which may be part of the material to be used.
4. No alteration, omission or addition is made to the material without our written consent. Permission must be re-cleared with Oxford University Press if/when you decide to reprint.
5. The following credit line appears wherever the material is used: author, title, journal, year, volume, issue number, pagination, by permission of Oxford University Press or the sponsoring society if the journal is a society journal. Where a journal is being published on behalf of a learned society, the details of that society must be included in the credit line.
6. For the reproduction of a full article from an Oxford University Press journal for whatever purpose, the corresponding author of the material concerned should be informed of the proposed use. Contact details for the corresponding authors of all Oxford University Press journal contact can be found alongside either the abstract or full text of the article concerned, accessible from www.oxfordjournals.org Should there be a problem clearing these rights, please contact journals.permissions@oup.com
7. If the credit line or acknowledgement in our publication indicates that any of the figures, images or photos was reproduced, drawn or modified from an earlier source it will be necessary for you to clear this permission with the original publisher as well. If this permission has not been obtained, please note that this material cannot be included in your publication/photocopies.
8. While you may exercise the rights licensed immediately upon issuance of the license at the end of the licensing process for the transaction, provided that you have disclosed complete and accurate details of your proposed use, no license is finally effective unless and until full payment is received from you (either by Oxford University Press or by Copyright Clearance Center (CCC)) as provided in CCC's Billing and Payment terms and conditions. If full payment is not received on a timely basis, then any license preliminarily granted shall be deemed automatically revoked and shall be void as if never granted. Further, in the event that you breach any of these terms and conditions or any of CCC's Billing and Payment terms and conditions, the license is automatically revoked and shall be void as if never granted. Use of materials as described in a revoked license, as well as any use of the materials beyond the scope of an unrevoked license, may constitute copyright infringement and Oxford University Press reserves the right to take any and all action to protect its copyright in the materials.
9. This license is personal to you and may not be sublicensed, assigned or transferred by you to any other person without Oxford University Press's written permission.

10. Oxford University Press reserves all rights not specifically granted in the combination of (i) the license details provided by you and accepted in the course of this licensing transaction, (ii) these terms and conditions and (iii) CCC's Billing and Payment terms and conditions.
11. You hereby indemnify and agree to hold harmless Oxford University Press and CCC, and their respective officers, directors, employs and agents, from and against any and all claims arising out of your use of the licensed material other than as specifically authorized pursuant to this license.
12. Other Terms and Conditions:
v1.4

Questions? customercare@copyright.com or +1-855-239-3415 (toll free in the US) or +1-978-646-2777.

SPRINGER NATURE LICENSE TERMS AND CONDITIONS

Aug 26, 2018

This Agreement between João Botelho ("You") and Springer Nature ("Springer Nature") consists of your license details and the terms and conditions provided by Springer Nature and Copyright Clearance Center.

License Number	4414280526505
License date	Aug 22, 2018
Licensed Content Publisher	Springer Nature
Licensed Content Publication	Nature Reviews Microbiology
Licensed Content Title	Detecting genomic islands using bioinformatics approaches
Licensed Content Author	Morgan G. I. Langille, William W. L. Hsiao, Fiona S. L. Brinkman
Licensed Content Date	May 1, 2010
Licensed Content Volume	8
Licensed Content Issue	5
Type of Use	Thesis/Dissertation
Requestor type	academic/university or research institute
Format	print and electronic
Portion	figures/tables/illustrations
Number of figures/tables /illustrations	1
High-res required	no
Will you be translating?	no
Circulation/distribution	<501
Author of this Springer Nature content	no
Title	CARBAPENEMASE PRODUCERS OF PSEUDOMONAS AERUGINOSA: LIGHTS ON NEW MOBILE GENETIC ELEMENTS
Instructor name	Luísa Peixe
Institution name	Faculdade de Farmácia, Universidade do Porto
Expected presentation date	Dec 2018
Portions	Figure 1
Requestor Location	João Botelho Rua de Jorge Viterbo Ferreira, 228 Porto, other Portugal Attn:
Billing Type	Invoice

Billing Address	João Botelho Rua de Jorge Viterbo Ferreira, 228 Porto, Portugal Attn: João Botelho
Total	0.00 EUR

Terms and Conditions

Springer Nature Terms and Conditions for RightsLink Permissions

Springer Nature Customer Service Centre GmbH (the Licensor) hereby grants you a non-exclusive, world-wide licence to reproduce the material and for the purpose and requirements specified in the attached copy of your order form, and for no other use, subject to the conditions below:

1. The Licensor warrants that it has, to the best of its knowledge, the rights to license reuse of this material. However, you should ensure that the material you are requesting is original to the Licensor and does not carry the copyright of another entity (as credited in the published version).

   If the credit line on any part of the material you have requested indicates that it was reprinted or adapted with permission from another source, then you should also seek permission from that source to reuse the material.

2. Where **print only** permission has been granted for a fee, separate permission must be obtained for any additional electronic re-use.

3. Permission granted **free of charge** for material in print is also usually granted for any electronic version of that work, provided that the material is incidental to your work as a whole and that the electronic version is essentially equivalent to, or substitutes for, the print version.

4. A licence for 'post on a website' is valid for 12 months from the licence date. This licence does not cover use of full text articles on websites.

5. Where **'reuse in a dissertation/thesis'** has been selected the following terms apply: Print rights of the final author's accepted manuscript (for clarity, NOT the published version) for up to 100 copies, electronic rights for use only on a personal website or institutional repository as defined by the Sherpa guideline (www.sherpa.ac.uk/romeo/).

6. Permission granted for books and journals is granted for the lifetime of the first edition and does not apply to second and subsequent editions (except where the first edition permission was granted free of charge or for signatories to the STM Permissions Guidelines http://www.stm-assoc.org/copyright-legal-affairs/permissions/permissions-guidelines/), and does not apply for editions in other languages unless additional translation rights have been granted separately in the licence.

7. Rights for additional components such as custom editions and derivatives require additional permission and may be subject to an additional fee. Please apply to Journalpermissions@springernature.com/bookpermissions@springernature.com for these rights.

8. The Licensor's permission must be acknowledged next to the licensed material in print. In electronic form, this acknowledgement must be visible at the same time as the figures/tables/illustrations or abstract, and must be hyperlinked to the journal/book's homepage. Our required acknowledgement format is in the Appendix below.

9. Use of the material for incidental promotional use, minor editing privileges (this does not include cropping, adapting, omitting material or any other changes that affect the meaning,

intention or moral rights of the author) and copies for the disabled are permitted under this licence.

10. Minor adaptations of single figures (changes of format, colour and style) do not require the Licensor's approval. However, the adaptation should be credited as shown in Appendix below.

Appendix — Acknowledgements:

For Journal Content:
Reprinted by permission from [**the Licensor**]: [**Journal Publisher** (e.g. Nature/Springer/Palgrave)] [**JOURNAL NAME**] [**REFERENCE CITATION** (Article name, Author(s) Name), [**COPYRIGHT**] (year of publication)

For **Advance Online Publication papers:**
Reprinted by permission from [**the Licensor**]: [**Journal Publisher** (e.g. Nature/Springer/Palgrave)] [**JOURNAL NAME**] [**REFERENCE CITATION** (Article name, Author(s) Name), [**COPYRIGHT**] (year of publication), advance online publication, day month year (doi: 10.1038/sj.[JOURNAL ACRONYM].)

For Adaptations/Translations:
Adapted/Translated by permission from [**the Licensor**]: [**Journal Publisher** (e.g. Nature/Springer/Palgrave)] [**JOURNAL NAME**] [**REFERENCE CITATION** (Article name, Author(s) Name), [**COPYRIGHT**] (year of publication)

Note: For any republication from the British Journal of Cancer, the following credit line style applies:

Reprinted/adapted/translated by permission from [**the Licensor**]: on behalf of Cancer Research UK: : [**Journal Publisher** (e.g. Nature/Springer/Palgrave)] [**JOURNAL NAME**] [**REFERENCE CITATION** (Article name, Author(s) Name), [**COPYRIGHT]** (year of publication)

For **Advance Online Publication** papers:
Reprinted by permission from The [**the Licensor**]: on behalf of Cancer Research UK: [**Journal Publisher** (e.g. Nature/Springer/Palgrave)] [**JOURNAL NAME**] [**REFERENCE CITATION** (Article name, Author(s) Name), [**COPYRIGHT**] (year of publication), advance online publication, day month year (doi: 10.1038/sj.[JOURNAL ACRONYM])

For Book content:
Reprinted/adapted by permission from [**the Licensor**]: [**Book Publisher** (e.g. Palgrave Macmillan, Springer etc) [**Book Title**] by [**Book author**(s)] [**COPYRIGHT**] (year of publication)

Other Conditions:

Version 1.1

Questions? customercare@copyright.com or +1-855-239-3415 (toll free in the US) or +1-978-646-2777.

Title: Mobility of Plasmids

Author: Chris Smillie,M. Pilar Garcillán-Barcia,M. Victoria Francia,Eduardo P. C. Rocha,Fernando de la Cruz

Publication: Microbiology and Molecular Biology Reviews

Publisher: American Society for Microbiology

Date: Aug 30, 2010

Permissions Request

ASM authorizes an advanced degree candidate to republish the requested material in his/her doctoral thesis or dissertation. If your thesis, or dissertation, is to be published commercially, then you must reapply for permission.

BACK CLOSE WINDOW

ELSEVIER LICENSE
TERMS AND CONDITIONS

Aug 26, 2018

This Agreement between João Botelho ("You") and Elsevier ("Elsevier") consists of your license details and the terms and conditions provided by Elsevier and Copyright Clearance Center.

License Number	4414281414639
License date	Aug 22, 2018
Licensed Content Publisher	Elsevier
Licensed Content Publication	Drug Resistance Updates
Licensed Content Title	The increasing threat of Pseudomonas aeruginosa high-risk clones
Licensed Content Author	Antonio Oliver,Xavier Mulet,Carla López-Causapé,Carlos Juan
Licensed Content Date	July–August 2015
Licensed Content Volume	21
Licensed Content Issue	n/a
Licensed Content Pages	19
Start Page	41
End Page	59
Type of Use	reuse in a thesis/dissertation
Intended publisher of new work	other
Portion	figures/tables/illustrations
Number of figures/tables /illustrations	1
Format	both print and electronic
Are you the author of this Elsevier article?	No
Will you be translating?	No
Original figure numbers	Figure 3
Title of your thesis/dissertation	CARBAPENEMASE PRODUCERS OF PSEUDOMONAS AERUGINOSA: LIGHTS ON NEW MOBILE GENETIC ELEMENTS
Publisher of new work	Faculdade de Farmácia, Universidade do Porto
Author of new work	Luísa Peixe
Expected completion date	Dec 2018
Estimated size (number of pages)	1
Requestor Location	João Botelho Rua de Jorge Viterbo Ferreira, 228 Porto, other

	Portugal Attn:
Publisher Tax ID	GB 494 6272 12
Total	0.00 EUR

Terms and Conditions

INTRODUCTION

1. The publisher for this copyrighted material is Elsevier. By clicking "accept" in connection with completing this licensing transaction, you agree that the following terms and conditions apply to this transaction (along with the Billing and Payment terms and conditions established by Copyright Clearance Center, Inc. ("CCC"), at the time that you opened your Rightslink account and that are available at any time at http://myaccount.copyright.com).

GENERAL TERMS

2. Elsevier hereby grants you permission to reproduce the aforementioned material subject to the terms and conditions indicated.
3. Acknowledgement: If any part of the material to be used (for example, figures) has appeared in our publication with credit or acknowledgement to another source, permission must also be sought from that source. If such permission is not obtained then that material may not be included in your publication/copies. Suitable acknowledgement to the source must be made, either as a footnote or in a reference list at the end of your publication, as follows:
"Reprinted from Publication title, Vol /edition number, Author(s), Title of article / title of chapter, Pages No., Copyright (Year), with permission from Elsevier [OR APPLICABLE SOCIETY COPYRIGHT OWNER]." Also Lancet special credit - "Reprinted from The Lancet, Vol. number, Author(s), Title of article, Pages No., Copyright (Year), with permission from Elsevier."
4. Reproduction of this material is confined to the purpose and/or media for which permission is hereby given.
5. Altering/Modifying Material: Not Permitted. However figures and illustrations may be altered/adapted minimally to serve your work. Any other abbreviations, additions, deletions and/or any other alterations shall be made only with prior written authorization of Elsevier Ltd. (Please contact Elsevier at permissions@elsevier.com). No modifications can be made to any Lancet figures/tables and they must be reproduced in full.
6. If the permission fee for the requested use of our material is waived in this instance, please be advised that your future requests for Elsevier materials may attract a fee.
7. Reservation of Rights: Publisher reserves all rights not specifically granted in the combination of (i) the license details provided by you and accepted in the course of this licensing transaction, (ii) these terms and conditions and (iii) CCC's Billing and Payment terms and conditions.
8. License Contingent Upon Payment: While you may exercise the rights licensed immediately upon issuance of the license at the end of the licensing process for the transaction, provided that you have disclosed complete and accurate details of your proposed use, no license is finally effective unless and until full payment is received from you (either by publisher or by CCC) as provided in CCC's Billing and Payment terms and conditions. If full payment is not received on a timely basis, then any license preliminarily granted shall be deemed automatically revoked and shall be void as if never granted. Further, in the event

that you breach any of these terms and conditions or any of CCC's Billing and Payment terms and conditions, the license is automatically revoked and shall be void as if never granted. Use of materials as described in a revoked license, as well as any use of the materials beyond the scope of an unrevoked license, may constitute copyright infringement and publisher reserves the right to take any and all action to protect its copyright in the materials.

9. Warranties: Publisher makes no representations or warranties with respect to the licensed material.

10. Indemnity: You hereby indemnify and agree to hold harmless publisher and CCC, and their respective officers, directors, employees and agents, from and against any and all claims arising out of your use of the licensed material other than as specifically authorized pursuant to this license.

11. No Transfer of License: This license is personal to you and may not be sublicensed, assigned, or transferred by you to any other person without publisher's written permission.

12. No Amendment Except in Writing: This license may not be amended except in a writing signed by both parties (or, in the case of publisher, by CCC on publisher's behalf).

13. Objection to Contrary Terms: Publisher hereby objects to any terms contained in any purchase order, acknowledgment, check endorsement or other writing prepared by you, which terms are inconsistent with these terms and conditions or CCC's Billing and Payment terms and conditions. These terms and conditions, together with CCC's Billing and Payment terms and conditions (which are incorporated herein), comprise the entire agreement between you and publisher (and CCC) concerning this licensing transaction. In the event of any conflict between your obligations established by these terms and conditions and those established by CCC's Billing and Payment terms and conditions, these terms and conditions shall control.

14. Revocation: Elsevier or Copyright Clearance Center may deny the permissions described in this License at their sole discretion, for any reason or no reason, with a full refund payable to you. Notice of such denial will be made using the contact information provided by you. Failure to receive such notice will not alter or invalidate the denial. In no event will Elsevier or Copyright Clearance Center be responsible or liable for any costs, expenses or damage incurred by you as a result of a denial of your permission request, other than a refund of the amount(s) paid by you to Elsevier and/or Copyright Clearance Center for denied permissions.

LIMITED LICENSE

The following terms and conditions apply only to specific license types:

15. **Translation**: This permission is granted for non-exclusive world **English** rights only unless your license was granted for translation rights. If you licensed translation rights you may only translate this content into the languages you requested. A professional translator must perform all translations and reproduce the content word for word preserving the integrity of the article.

16. **Posting licensed content on any Website**: The following terms and conditions apply as follows: Licensing material from an Elsevier journal: All content posted to the web site must maintain the copyright information line on the bottom of each image; A hyper-text must be included to the Homepage of the journal from which you are licensing at http://www.sciencedirect.com/science/journal/xxxxx or the Elsevier homepage for books at http://www.elsevier.com; Central Storage: This license does not include permission for a scanned version of the material to be stored in a central repository such as that provided by

Heron/XanEdu.
Licensing material from an Elsevier book: A hyper-text link must be included to the Elsevier homepage at http://www.elsevier.com . All content posted to the web site must maintain the copyright information line on the bottom of each image.

Posting licensed content on Electronic reserve: In addition to the above the following clauses are applicable: The web site must be password-protected and made available only to bona fide students registered on a relevant course. This permission is granted for 1 year only. You may obtain a new license for future website posting.
17. **For journal authors:** the following clauses are applicable in addition to the above:
Preprints:
A preprint is an author's own write-up of research results and analysis, it has not been peer-reviewed, nor has it had any other value added to it by a publisher (such as formatting, copyright, technical enhancement etc.).
Authors can share their preprints anywhere at any time. Preprints should not be added to or enhanced in any way in order to appear more like, or to substitute for, the final versions of articles however authors can update their preprints on arXiv or RePEc with their Accepted Author Manuscript (see below).
If accepted for publication, we encourage authors to link from the preprint to their formal publication via its DOI. Millions of researchers have access to the formal publications on ScienceDirect, and so links will help users to find, access, cite and use the best available version. Please note that Cell Press, The Lancet and some society-owned have different preprint policies. Information on these policies is available on the journal homepage.
Accepted Author Manuscripts: An accepted author manuscript is the manuscript of an article that has been accepted for publication and which typically includes author-incorporated changes suggested during submission, peer review and editor-author communications.
Authors can share their accepted author manuscript:

- immediately
  - via their non-commercial person homepage or blog
  - by updating a preprint in arXiv or RePEc with the accepted manuscript
  - via their research institute or institutional repository for internal institutional uses or as part of an invitation-only research collaboration work-group
  - directly by providing copies to their students or to research collaborators for their personal use
  - for private scholarly sharing as part of an invitation-only work group on commercial sites with which Elsevier has an agreement
- After the embargo period
  - via non-commercial hosting platforms such as their institutional repository
  - via commercial sites with which Elsevier has an agreement

In all cases accepted manuscripts should:

- link to the formal publication via its DOI
- bear a CC-BY-NC-ND license - this is easy to do
- if aggregated with other manuscripts, for example in a repository or other site, be shared in alignment with our hosting policy not be added to or enhanced in any way to

appear more like, or to substitute for, the published journal article.

Published journal article (JPA): A published journal article (PJA) is the definitive final record of published research that appears or will appear in the journal and embodies all value-adding publishing activities including peer review co-ordination, copy-editing, formatting, (if relevant) pagination and online enrichment.
Policies for sharing publishing journal articles differ for subscription and gold open access articles:
Subscription Articles: If you are an author, please share a link to your article rather than the full-text. Millions of researchers have access to the formal publications on ScienceDirect, and so links will help your users to find, access, cite, and use the best available version.
Theses and dissertations which contain embedded PJAs as part of the formal submission can be posted publicly by the awarding institution with DOI links back to the formal publications on ScienceDirect.
If you are affiliated with a library that subscribes to ScienceDirect you have additional private sharing rights for others' research accessed under that agreement. This includes use for classroom teaching and internal training at the institution (including use in course packs and courseware programs), and inclusion of the article for grant funding purposes.
Gold Open Access Articles: May be shared according to the author-selected end-user license and should contain a CrossMark logo, the end user license, and a DOI link to the formal publication on ScienceDirect.
Please refer to Elsevier's posting policy for further information.
18. **For book authors** the following clauses are applicable in addition to the above:
Authors are permitted to place a brief summary of their work online only. You are not allowed to download and post the published electronic version of your chapter, nor may you scan the printed edition to create an electronic version. **Posting to a repository:** Authors are permitted to post a summary of their chapter only in their institution's repository.
19. **Thesis/Dissertation**: If your license is for use in a thesis/dissertation your thesis may be submitted to your institution in either print or electronic form. Should your thesis be published commercially, please reapply for permission. These requirements include permission for the Library and Archives of Canada to supply single copies, on demand, of the complete thesis and include permission for Proquest/UMI to supply single copies, on demand, of the complete thesis. Should your thesis be published commercially, please reapply for permission. Theses and dissertations which contain embedded PJAs as part of the formal submission can be posted publicly by the awarding institution with DOI links back to the formal publications on ScienceDirect.

Elsevier Open Access Terms and Conditions
You can publish open access with Elsevier in hundreds of open access journals or in nearly 2000 established subscription journals that support open access publishing. Permitted third party re-use of these open access articles is defined by the author's choice of Creative Commons user license. See our open access license policy for more information.
Terms & Conditions applicable to all Open Access articles published with Elsevier:
Any reuse of the article must not represent the author as endorsing the adaptation of the article nor should the article be modified in such a way as to damage the author's honour or reputation. If any changes have been made, such changes must be clearly indicated.
The author(s) must be appropriately credited and we ask that you include the end user

license and a DOI link to the formal publication on ScienceDirect.
If any part of the material to be used (for example, figures) has appeared in our publication with credit or acknowledgement to another source it is the responsibility of the user to ensure their reuse complies with the terms and conditions determined by the rights holder.
Additional Terms & Conditions applicable to each Creative Commons user license:
CC BY: The CC-BY license allows users to copy, to create extracts, abstracts and new works from the Article, to alter and revise the Article and to make commercial use of the Article (including reuse and/or resale of the Article by commercial entities), provided the user gives appropriate credit (with a link to the formal publication through the relevant DOI), provides a link to the license, indicates if changes were made and the licensor is not represented as endorsing the use made of the work. The full details of the license are available at http://creativecommons.org/licenses/by/4.0.
CC BY NC SA: The CC BY-NC-SA license allows users to copy, to create extracts, abstracts and new works from the Article, to alter and revise the Article, provided this is not done for commercial purposes, and that the user gives appropriate credit (with a link to the formal publication through the relevant DOI), provides a link to the license, indicates if changes were made and the licensor is not represented as endorsing the use made of the work. Further, any new works must be made available on the same conditions. The full details of the license are available at http://creativecommons.org/licenses/by-nc-sa/4.0.
CC BY NC ND: The CC BY-NC-ND license allows users to copy and distribute the Article, provided this is not done for commercial purposes and further does not permit distribution of the Article if it is changed or edited in any way, and provided the user gives appropriate credit (with a link to the formal publication through the relevant DOI), provides a link to the license, and that the licensor is not represented as endorsing the use made of the work. The full details of the license are available at http://creativecommons.org/licenses/by-nc-nd/4.0.
Any commercial reuse of Open Access articles published with a CC BY NC SA or CC BY NC ND license requires permission from Elsevier and will be subject to a fee.
Commercial reuse includes:

- Associating advertising with the full text of the Article
- Charging fees for document delivery or access
- Article aggregation
- Systematic distribution via e-mail lists or share buttons

Posting or linking by commercial companies for use by customers of those companies.

20. **Other Conditions**:

v1.9

Questions? customercare@copyright.com or +1-855-239-3415 (toll free in the US) or +1-978-646-2777.

OXFORD UNIVERSITY PRESS LICENSE TERMS AND CONDITIONS

Aug 26, 2018

This Agreement between João Botelho ("You") and Oxford University Press ("Oxford University Press") consists of your license details and the terms and conditions provided by Oxford University Press and Copyright Clearance Center.

License Number	4414261488703
License date	Aug 22, 2018
Licensed content publisher	Oxford University Press
Licensed content publication	Journal of Antimicrobial Chemotherapy
Licensed content title	Two decades of *bla*$_{VIM-2}$-producing *Pseudomonas aeruginosa* dissemination: an interplay between mobile genetic elements and successful clones
Licensed content author	Botelho, João; Grosso, Filipa
Licensed content date	Jan 23, 2018
Type of Use	Thesis/Dissertation
Institution name	
Title of your work	CARBAPENEMASE PRODUCERS OF PSEUDOMONAS AERUGINOSA: LIGHTS ON NEW MOBILE GENETIC ELEMENTS
Publisher of your work	Faculdade de Farmácia, Universidade do Porto
Expected publication date	Dec 2018
Permissions cost	0.00 EUR
Value added tax	0.00 EUR
Total	0.00 EUR
Title	CARBAPENEMASE PRODUCERS OF PSEUDOMONAS AERUGINOSA: LIGHTS ON NEW MOBILE GENETIC ELEMENTS
Instructor name	Luísa Peixe
Institution name	Faculdade de Farmácia, Universidade do Porto
Expected presentation date	Dec 2018
Portions	Full text
Requestor Location	João Botelho Rua de Jorge Viterbo Ferreira, 228 Porto, other Portugal Attn:
Publisher Tax ID	GB125506730
Billing Type	Invoice
Billing Address	João Botelho Rua de Jorge Viterbo Ferreira, 228

Porto, Portugal
Attn:

Total 0.00 EUR

Terms and Conditions

STANDARD TERMS AND CONDITIONS FOR REPRODUCTION OF MATERIAL FROM AN OXFORD UNIVERSITY PRESS JOURNAL

1. Use of the material is restricted to the type of use specified in your order details.
2. This permission covers the use of the material in the English language in the following territory: world. If you have requested additional permission to translate this material, the terms and conditions of this reuse will be set out in clause 12.
3. This permission is limited to the particular use authorized in (1) above and does not allow you to sanction its use elsewhere in any other format other than specified above, nor does it apply to quotations, images, artistic works etc that have been reproduced from other sources which may be part of the material to be used.
4. No alteration, omission or addition is made to the material without our written consent. Permission must be re-cleared with Oxford University Press if/when you decide to reprint.
5. The following credit line appears wherever the material is used: author, title, journal, year, volume, issue number, pagination, by permission of Oxford University Press or the sponsoring society if the journal is a society journal. Where a journal is being published on behalf of a learned society, the details of that society must be included in the credit line.
6. For the reproduction of a full article from an Oxford University Press journal for whatever purpose, the corresponding author of the material concerned should be informed of the proposed use. Contact details for the corresponding authors of all Oxford University Press journal contact can be found alongside either the abstract or full text of the article concerned, accessible from www.oxfordjournals.org Should there be a problem clearing these rights, please contact journals.permissions@oup.com
7. If the credit line or acknowledgement in our publication indicates that any of the figures, images or photos was reproduced, drawn or modified from an earlier source it will be necessary for you to clear this permission with the original publisher as well. If this permission has not been obtained, please note that this material cannot be included in your publication/photocopies.
8. While you may exercise the rights licensed immediately upon issuance of the license at the end of the licensing process for the transaction, provided that you have disclosed complete and accurate details of your proposed use, no license is finally effective unless and until full payment is received from you (either by Oxford University Press or by Copyright Clearance Center (CCC)) as provided in CCC's Billing and Payment terms and conditions. If full payment is not received on a timely basis, then any license preliminarily granted shall be deemed automatically revoked and shall be void as if never granted. Further, in the event that you breach any of these terms and conditions or any of CCC's Billing and Payment terms and conditions, the license is automatically revoked and shall be void as if never granted. Use of materials as described in a revoked license, as well as any use of the materials beyond the scope of an unrevoked license, may constitute copyright infringement and Oxford University Press reserves the right to take any and all action to protect its copyright in the materials.
9. This license is personal to you and may not be sublicensed, assigned or transferred by you

to any other person without Oxford University Press's written permission.
10. Oxford University Press reserves all rights not specifically granted in the combination of (i) the license details provided by you and accepted in the course of this licensing transaction, (ii) these terms and conditions and (iii) CCC's Billing and Payment terms and conditions.
11. You hereby indemnify and agree to hold harmless Oxford University Press and CCC, and their respective officers, directors, employs and agents, from and against any and all claims arising out of your use of the licensed material other than as specifically authorized pursuant to this license.
12. Other Terms and Conditions:
v1.4

Questions? customercare@copyright.com or +1-855-239-3415 (toll free in the US) or +1-978-646-2777.

AMERICAN SOCIETY FOR MICROBIOLOGY

Title: Characterization of the pJB12 Plasmid from Pseudomonas aeruginosa Reveals Tn*6352*, a Novel Putative Transposon Associated with Mobilization of the bla_{VIM-2}-Harboring In58 Integron

Author: João Botelho,Filipa Grosso,Luísa Peixe

Publication: Antimicrobial Agents and Chemotherapy

Publisher: American Society for Microbiology

Date: Apr 24, 2017

Permissions Request

Authors in ASM journals retain the right to republish discrete portions of his/her article in any other publication (including print, CD-ROM, and other electronic formats) of which he or she is author or editor, provided that proper credit is given to the original ASM publication. ASM authors also retain the right to reuse the full article in his/her dissertation or thesis. For a full list of author rights, please see: http://journals.asm.org/site/misc/ASM_Author_Statement.xhtml

OXFORD UNIVERSITY PRESS LICENSE TERMS AND CONDITIONS

Aug 26, 2018

This Agreement between João Botelho ("You") and Oxford University Press ("Oxford University Press") consists of your license details and the terms and conditions provided by Oxford University Press and Copyright Clearance Center.

License Number	4414261139813
License date	Aug 22, 2018
Licensed content publisher	Oxford University Press
Licensed content publication	Journal of Antimicrobial Chemotherapy
Licensed content title	The complete nucleotide sequence of an IncP-2 megaplasmid unveils a mosaic architecture comprising a putative novel *bla*$_{VIM-2}$-harbouring transposon in *Pseudomonas aeruginosa*
Licensed content author	Botelho, João; Grosso, Filipa
Licensed content date	May 12, 2017
Type of Use	Thesis/Dissertation
Institution name	
Title of your work	CARBAPENEMASE PRODUCERS OF PSEUDOMONAS AERUGINOSA: LIGHTS ON NEW MOBILE GENETIC ELEMENTS
Publisher of your work	Faculdade de Farmácia, Universidade do Porto
Expected publication date	Dec 2018
Permissions cost	0.00 EUR
Value added tax	0.00 EUR
Total	0.00 EUR
Title	CARBAPENEMASE PRODUCERS OF PSEUDOMONAS AERUGINOSA: LIGHTS ON NEW MOBILE GENETIC ELEMENTS
Instructor name	Luísa Peixe
Institution name	Faculdade de Farmácia, Universidade do Porto
Expected presentation date	Dec 2018
Portions	Full text
Requestor Location	João Botelho Rua de Jorge Viterbo Ferreira, 228 Porto, other Portugal Attn:
Publisher Tax ID	GB125506730
Billing Type	Invoice
Billing Address	João Botelho Rua de Jorge Viterbo Ferreira, 228

Porto, Portugal
Attn:

Total 0.00 EUR

Terms and Conditions

STANDARD TERMS AND CONDITIONS FOR REPRODUCTION OF MATERIAL FROM AN OXFORD UNIVERSITY PRESS JOURNAL

1. Use of the material is restricted to the type of use specified in your order details.
2. This permission covers the use of the material in the English language in the following territory: world. If you have requested additional permission to translate this material, the terms and conditions of this reuse will be set out in clause 12.
3. This permission is limited to the particular use authorized in (1) above and does not allow you to sanction its use elsewhere in any other format other than specified above, nor does it apply to quotations, images, artistic works etc that have been reproduced from other sources which may be part of the material to be used.
4. No alteration, omission or addition is made to the material without our written consent. Permission must be re-cleared with Oxford University Press if/when you decide to reprint.
5. The following credit line appears wherever the material is used: author, title, journal, year, volume, issue number, pagination, by permission of Oxford University Press or the sponsoring society if the journal is a society journal. Where a journal is being published on behalf of a learned society, the details of that society must be included in the credit line.
6. For the reproduction of a full article from an Oxford University Press journal for whatever purpose, the corresponding author of the material concerned should be informed of the proposed use. Contact details for the corresponding authors of all Oxford University Press journal contact can be found alongside either the abstract or full text of the article concerned, accessible from www.oxfordjournals.org Should there be a problem clearing these rights, please contact journals.permissions@oup.com
7. If the credit line or acknowledgement in our publication indicates that any of the figures, images or photos was reproduced, drawn or modified from an earlier source it will be necessary for you to clear this permission with the original publisher as well. If this permission has not been obtained, please note that this material cannot be included in your publication/photocopies.
8. While you may exercise the rights licensed immediately upon issuance of the license at the end of the licensing process for the transaction, provided that you have disclosed complete and accurate details of your proposed use, no license is finally effective unless and until full payment is received from you (either by Oxford University Press or by Copyright Clearance Center (CCC)) as provided in CCC's Billing and Payment terms and conditions. If full payment is not received on a timely basis, then any license preliminarily granted shall be deemed automatically revoked and shall be void as if never granted. Further, in the event that you breach any of these terms and conditions or any of CCC's Billing and Payment terms and conditions, the license is automatically revoked and shall be void as if never granted. Use of materials as described in a revoked license, as well as any use of the materials beyond the scope of an unrevoked license, may constitute copyright infringement and Oxford University Press reserves the right to take any and all action to protect its copyright in the materials.

9. This license is personal to you and may not be sublicensed, assigned or transferred by you to any other person without Oxford University Press's written permission.
10. Oxford University Press reserves all rights not specifically granted in the combination of (i) the license details provided by you and accepted in the course of this licensing transaction, (ii) these terms and conditions and (iii) CCC's Billing and Payment terms and conditions.
11. You hereby indemnify and agree to hold harmless Oxford University Press and CCC, and their respective officers, directors, employs and agents, from and against any and all claims arising out of your use of the licensed material other than as specifically authorized pursuant to this license.
12. Other Terms and Conditions:
v1.4

Questions? customercare@copyright.com or +1-855-239-3415 (toll free in the US) or +1-978-646-2777.

OXFORD UNIVERSITY PRESS LICENSE TERMS AND CONDITIONS

Aug 26, 2018

This Agreement between João Botelho ("You") and Oxford University Press ("Oxford University Press") consists of your license details and the terms and conditions provided by Oxford University Press and Copyright Clearance Center.

License Number	4414261386970
License date	Aug 22, 2018
Licensed content publisher	Oxford University Press
Licensed content publication	Journal of Antimicrobial Chemotherapy
Licensed content title	Characterization of a new genetic environment associated with GES-6 carbapenemase from a *Pseudomonas aeruginosa* isolate belonging to the high-risk clone ST235
Licensed content author	Botelho, João; Grosso, Filipa
Licensed content date	Sep 25, 2014
Type of Use	Thesis/Dissertation
Institution name	
Title of your work	CARBAPENEMASE PRODUCERS OF PSEUDOMONAS AERUGINOSA: LIGHTS ON NEW MOBILE GENETIC ELEMENTS
Publisher of your work	Faculdade de Farmácia, Universidade do Porto
Expected publication date	Dec 2018
Permissions cost	0.00 EUR
Value added tax	0.00 EUR
Total	0.00 EUR
Title	CARBAPENEMASE PRODUCERS OF PSEUDOMONAS AERUGINOSA: LIGHTS ON NEW MOBILE GENETIC ELEMENTS
Instructor name	Luísa Peixe
Institution name	Faculdade de Farmácia, Universidade do Porto
Expected presentation date	Dec 2018
Portions	Full text
Requestor Location	João Botelho Rua de Jorge Viterbo Ferreira, 228 Porto, other Portugal Attn:
Publisher Tax ID	GB125506730
Billing Type	Invoice
Billing Address	João Botelho Rua de Jorge Viterbo Ferreira, 228

Porto, Portugal
Attn:

Total 0.00 EUR

Terms and Conditions

STANDARD TERMS AND CONDITIONS FOR REPRODUCTION OF MATERIAL FROM AN OXFORD UNIVERSITY PRESS JOURNAL

1. Use of the material is restricted to the type of use specified in your order details.
2. This permission covers the use of the material in the English language in the following territory: world. If you have requested additional permission to translate this material, the terms and conditions of this reuse will be set out in clause 12.
3. This permission is limited to the particular use authorized in (1) above and does not allow you to sanction its use elsewhere in any other format other than specified above, nor does it apply to quotations, images, artistic works etc that have been reproduced from other sources which may be part of the material to be used.
4. No alteration, omission or addition is made to the material without our written consent. Permission must be re-cleared with Oxford University Press if/when you decide to reprint.
5. The following credit line appears wherever the material is used: author, title, journal, year, volume, issue number, pagination, by permission of Oxford University Press or the sponsoring society if the journal is a society journal. Where a journal is being published on behalf of a learned society, the details of that society must be included in the credit line.
6. For the reproduction of a full article from an Oxford University Press journal for whatever purpose, the corresponding author of the material concerned should be informed of the proposed use. Contact details for the corresponding authors of all Oxford University Press journal contact can be found alongside either the abstract or full text of the article concerned, accessible from www.oxfordjournals.org Should there be a problem clearing these rights, please contact journals.permissions@oup.com
7. If the credit line or acknowledgement in our publication indicates that any of the figures, images or photos was reproduced, drawn or modified from an earlier source it will be necessary for you to clear this permission with the original publisher as well. If this permission has not been obtained, please note that this material cannot be included in your publication/photocopies.
8. While you may exercise the rights licensed immediately upon issuance of the license at the end of the licensing process for the transaction, provided that you have disclosed complete and accurate details of your proposed use, no license is finally effective unless and until full payment is received from you (either by Oxford University Press or by Copyright Clearance Center (CCC)) as provided in CCC's Billing and Payment terms and conditions. If full payment is not received on a timely basis, then any license preliminarily granted shall be deemed automatically revoked and shall be void as if never granted. Further, in the event that you breach any of these terms and conditions or any of CCC's Billing and Payment terms and conditions, the license is automatically revoked and shall be void as if never granted. Use of materials as described in a revoked license, as well as any use of the materials beyond the scope of an unrevoked license, may constitute copyright infringement and Oxford University Press reserves the right to take any and all action to protect its copyright in the materials.
9. This license is personal to you and may not be sublicensed, assigned or transferred by you

to any other person without Oxford University Press's written permission.
10. Oxford University Press reserves all rights not specifically granted in the combination of (i) the license details provided by you and accepted in the course of this licensing transaction, (ii) these terms and conditions and (iii) CCC's Billing and Payment terms and conditions.
11. You hereby indemnify and agree to hold harmless Oxford University Press and CCC, and their respective officers, directors, employs and agents, from and against any and all claims arising out of your use of the licensed material other than as specifically authorized pursuant to this license.
12. Other Terms and Conditions:
v1.4

Questions? customercare@copyright.com or +1-855-239-3415 (toll free in the US) or +1-978-646-2777.

OXFORD UNIVERSITY PRESS LICENSE TERMS AND CONDITIONS

Aug 26, 2018

This Agreement between João Botelho ("You") and Oxford University Press ("Oxford University Press") consists of your license details and the terms and conditions provided by Oxford University Press and Copyright Clearance Center.

License Number	4414261267871
License date	Aug 22, 2018
Licensed content publisher	Oxford University Press
Licensed content publication	Journal of Antimicrobial Chemotherapy
Licensed content title	Unravelling the genome of a *Pseudomonas aeruginosa* isolate belonging to the high-risk clone ST235 reveals an integrative conjugative element housing a *bla*$_{GES-6}$ carbapenemase
Licensed content author	Botelho, João; Grosso, Filipa
Licensed content date	Oct 4, 2017
Type of Use	Thesis/Dissertation
Institution name	
Title of your work	CARBAPENEMASE PRODUCERS OF PSEUDOMONAS AERUGINOSA: LIGHTS ON NEW MOBILE GENETIC ELEMENTS
Publisher of your work	Faculdade de Farmácia, Universidade do Porto
Expected publication date	Dec 2018
Permissions cost	0.00 EUR
Value added tax	0.00 EUR
Total	0.00 EUR
Title	CARBAPENEMASE PRODUCERS OF PSEUDOMONAS AERUGINOSA: LIGHTS ON NEW MOBILE GENETIC ELEMENTS
Instructor name	Luísa Peixe
Institution name	Faculdade de Farmácia, Universidade do Porto
Expected presentation date	Dec 2018
Portions	Full text
Requestor Location	João Botelho Rua de Jorge Viterbo Ferreira, 228 Porto, other Portugal Attn:
Publisher Tax ID	GB125506730
Billing Type	Invoice
Billing Address	João Botelho Rua de Jorge Viterbo Ferreira, 228

Porto, Portugal
Attn:

Total 0.00 EUR

Terms and Conditions

STANDARD TERMS AND CONDITIONS FOR REPRODUCTION OF MATERIAL FROM AN OXFORD UNIVERSITY PRESS JOURNAL

1. Use of the material is restricted to the type of use specified in your order details.
2. This permission covers the use of the material in the English language in the following territory: world. If you have requested additional permission to translate this material, the terms and conditions of this reuse will be set out in clause 12.
3. This permission is limited to the particular use authorized in (1) above and does not allow you to sanction its use elsewhere in any other format other than specified above, nor does it apply to quotations, images, artistic works etc that have been reproduced from other sources which may be part of the material to be used.
4. No alteration, omission or addition is made to the material without our written consent. Permission must be re-cleared with Oxford University Press if/when you decide to reprint.
5. The following credit line appears wherever the material is used: author, title, journal, year, volume, issue number, pagination, by permission of Oxford University Press or the sponsoring society if the journal is a society journal. Where a journal is being published on behalf of a learned society, the details of that society must be included in the credit line.
6. For the reproduction of a full article from an Oxford University Press journal for whatever purpose, the corresponding author of the material concerned should be informed of the proposed use. Contact details for the corresponding authors of all Oxford University Press journal contact can be found alongside either the abstract or full text of the article concerned, accessible from www.oxfordjournals.org Should there be a problem clearing these rights, please contact journals.permissions@oup.com
7. If the credit line or acknowledgement in our publication indicates that any of the figures, images or photos was reproduced, drawn or modified from an earlier source it will be necessary for you to clear this permission with the original publisher as well. If this permission has not been obtained, please note that this material cannot be included in your publication/photocopies.
8. While you may exercise the rights licensed immediately upon issuance of the license at the end of the licensing process for the transaction, provided that you have disclosed complete and accurate details of your proposed use, no license is finally effective unless and until full payment is received from you (either by Oxford University Press or by Copyright Clearance Center (CCC)) as provided in CCC's Billing and Payment terms and conditions. If full payment is not received on a timely basis, then any license preliminarily granted shall be deemed automatically revoked and shall be void as if never granted. Further, in the event that you breach any of these terms and conditions or any of CCC's Billing and Payment terms and conditions, the license is automatically revoked and shall be void as if never granted. Use of materials as described in a revoked license, as well as any use of the materials beyond the scope of an unrevoked license, may constitute copyright infringement and Oxford University Press reserves the right to take any and all action to protect its copyright in the materials.
9. This license is personal to you and may not be sublicensed, assigned or transferred by you

to any other person without Oxford University Press's written permission.
10. Oxford University Press reserves all rights not specifically granted in the combination of (i) the license details provided by you and accepted in the course of this licensing transaction, (ii) these terms and conditions and (iii) CCC's Billing and Payment terms and conditions.
11. You hereby indemnify and agree to hold harmless Oxford University Press and CCC, and their respective officers, directors, employs and agents, from and against any and all claims arising out of your use of the licensed material other than as specifically authorized pursuant to this license.
12. Other Terms and Conditions:
v1.4

Questions? customercare@copyright.com or +1-855-239-3415 (toll free in the US) or +1-978-646-2777.