

Mobilization of an ICEclc-like element as a potential mechanism for the spread of IMP-13 carbapenemase in *Pseudomonas aeruginosa*

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PII: S2213-7165(24)00465-X
DOI: <https://doi.org/10.1016/j.jgar.2024.12.006>
Reference: JGAR 2448



To appear in: *Journal of Global Antimicrobial Resistance*

Received date: 30 June 2024
Revised date: 1 December 2024
Accepted date: 7 December 2024

Please cite this article as: Léa Bientz , Ulysse Guyet , Jennifer Guiraud , Mathieu Metifiot , Mikeldi Moulieras , Sabine Aillerie , Laure Coulange-Mayonnove , Bachir Boureima Abdou , Alexis Groppi , Macha Nikolski , Cécile Bébéar , Sabine Pereyre , Véronique Dubois , Mobilization of an ICEclc-like element as a potential mechanism for the spread of IMP-13 carbapenemase in *Pseudomonas aeruginosa*, *Journal of Global Antimicrobial Resistance* (2024), doi: <https://doi.org/10.1016/j.jgar.2024.12.006>

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Highlights

- The *bla*_{IMP-13} gene is closely related to the *Pseudomonas aeruginosa* ST621 clone
- The *bla*_{IMP-13} gene is mainly located on an Integrative and Conjugative Element (ICE)
- ICE*clc*-like element carrying the *bla*_{IMP-13} gene is functional and mobile
- ICE transfer mobilizes some adjacent parental genes

To be submitted to *Journal of Global Antimicrobial Resistance*

As an original article

2024

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SHORT TITLE: Spread of *bla*_{IMP-13} carried on ICE in *P. aeruginosa*

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Abstract

Carbapenem-resistant *Pseudomonas aeruginosa* is a global public health concern. IMP-13 is a carbapenemase that was described for the first time in 2001 but is often underestimated due to poor hydrolysis of carbapenems and a lack of molecular detection. The aim of this study was to characterize the genetic support of *bla*_{IMP-13} in *P. aeruginosa* and to assess the ability of mobile genetic elements to disseminate this resistance. A retrospective analysis conducted between 2010 and 2020 revealed eight multiresistant *P. aeruginosa* isolates by their production of the carbapenemase IMP-13 in Bordeaux. Additionally, three of the studied isolates exhibited high-level resistance to imipenem and imipenem-relebactam that was linked to an insertion sequence in the *oprD* gene. Successful mating was achieved, and transconjugants and parental clinical isolate genomes were sequenced. All clinical isolates were found to be ST621 strains. The data revealed that *bla*_{IMP-13} was carried on an Integrative and Conjugative Element (ICE_{clc}-like) of 88589 bp with a 62% GC content harboring 85 CDSs, and was inserted at the tRNA^{Gly} locus PA0729.1. The ICE was identical in the eight studied clinical isolates and in all the ST621 strains found in the databases. The conjugation rate was low, at approximately 10⁻⁸ transconjugants per donor and ICE transfer appeared to mobilize some adjacent parental genes located immediately downstream of the ICE. In conclusion, these results suggest that even if the spread of *bla*_{IMP-13} is mainly due to an epidemic ST621 clone, the mobilization of a *bla*_{IMP-13}-carrying ICE_{clc}-like element is possible and should not be underestimated.

Keywords

Pseudomonas aeruginosa, IMP-13 carbapenemase, ICE_{clc}-like element, horizontal gene transfer, antibiotic resistance

1. Introduction

Pseudomonas aeruginosa is a Gram-negative bacterium widely found in the environment, animals and humans. It is an opportunistic pathogen that causes mainly hospital-acquired infections, such as pneumonia, bacteremia, urinary tract infections, and burn wound infections, in immunocompromised patients as well as community infections [1,2]. *P. aeruginosa* has an intrinsically high level of antibiotic resistance due to different mechanisms, such as chromosomal cephalosporinase, efflux pumps and low outer membrane permeability, and the occurrence of chromosomal mutations allows additional resistance to be acquired, for example in the *ampC*, *oprD* (β -lactams resistance), and *gyrA* and *parC* genes (fluoroquinolones resistance) [2]. Antibiotic resistance can also be acquired through horizontal gene transfer (HGT), mediated by plasmids, integrative and conjugative elements (ICEs) or other mobile genetic elements (MGEs). HGT mainly involves the acquisition of genes encoding β -lactamases, especially carbapenemases which hydrolyze most β -lactams, including carbapenems [3]. The most common carbapenemases in *P. aeruginosa* are metallo- β -lactamases (MBLs), such as the VIM (Verona Integron-encoded Metallo- β -lactamase) and IMP (IMiPenemase) types, followed by the class A GES (Guiana Extended-Spectrum) type [4]. The first IMP-13-producing *P. aeruginosa* strain was described in 2001 in Rome, Italy [5]. Since then, the *bla*_{IMP-13} gene has been mainly recovered in Europe [6–10] but also in Tunisia [11] and South America [12,13]. This gene is most commonly found in *P. aeruginosa* but has also been described in *Pseudomonas monteilii* [14] and various *Enterobacterales* [11,13]. For multidrug resistant (MDR)-*P. aeruginosa*, treatment options may be limited, and the last resort antibiotics used are new combinations (ceftolozane-tazobactam, ceftazidime-avibactam (CZA), imipenem-relebactam, meropenem-vaborbactam, and aztreonam-

avibactam), cefiderocol, and colistin [15]. Thus, carbapenem-resistant *P. aeruginosa* have been considered “high-priority” bacteria by the World Health Organization (WHO) since 2024.

Integrative and conjugative elements (ICEs) are MGEs integrated into the chromosome, capable of excising, self-transferring by conjugation and reintegrating into another chromosome [16]. The contribution of ICEs to the propagation of carbapenemase-encoding genes has been understudied, although the frequent presence of these genes in ICEs has recently been reported in *P. aeruginosa* [17]. The most important ICE family found in *P. aeruginosa* is ICE_{clc}, which carries a bacteriophage P4 tyrosine recombinase and is a derivative of the ICE found in *Pseudomonas knackmussii* strain B13 [17,18]

In this study, we reported the spread of an IMP-13 carbapenemase-producing *P. aeruginosa* ST621 clone in several wards in Bordeaux, France, and analyzed the mobilization of the ICE_{clc}-like element carrying the *bla*_{IMP-13} gene by successful mating assays.

2. Materials and methods

2.1. Bacterial isolates

Between 2010 and 2020, 88 MDR isolates of *P. aeruginosa* were collected from non-cystic fibrosis patients at the University Hospital of Bordeaux (France) and 3 from the strain collection of the Bacteriology Laboratory of the University of Bordeaux. The isolated species were identified using MALDI-TOF mass spectrometry (Bruker, Champs-sur-Marne, France). A spontaneous rifampin-resistant mutant (Rif^R) of *P. aeruginosa* PAO1 was used as the recipient strain in conjugation and transformation experiments and was used with the PAO1 reference strain as a control for antimicrobial susceptibility assays. All strains were grown at 37 °C in aerobiosis on Mueller Hinton (MH) agar (Bio-Rad, Marnes-la-Coquette, France) or broth (Bio-Rad, Marnes-la-Coquette, France).

2.2. Antimicrobial susceptibility testing

Antibiotic susceptibility was determined using 13 antimicrobial agents (ticarcillin, ticarcillin/clavulanic acid, piperacillin, piperacillin/tazobactam, cefepime, ceftazidime, aztreonam, imipenem, meropenem, tobramycin, amikacin, ciprofloxacin, and levofloxacin; Bio-Rad) by the disk diffusion method according to the guidelines of the European Committee on Antimicrobial Susceptibility Testing and Antibigram Committee of the French Society of Microbiology (EUCAST-CASFM 2023) (<https://www.sfm-microbiologie.org/>). Antibiotic minimum inhibitory concentrations (MICs) were determined using the broth microdilution (BMD) method for imipenem (Merck, Lyon, France), cefiderocol and colistin (UMIC® Biocentric, Bandol, France) and using MIC test strips for ceftolozane-tazobactam, ceftazidime-avibactam, imipenem-relebactam (Liofilchem®, I2A, Montpellier, France) and imipenem (ETEST®, bioMérieux, Marcy l'Etoile, France). The strains were considered “susceptible”, “susceptible, increased exposure” or “resistant” to the tested drugs according to the EUCAST-CASFM 2023 breakpoints.

The *in vitro* synergy of ceftazidime-avibactam and aztreonam (ATM) was studied using the MIC test-strip-based synergy method as previously described [19] with some modifications: the first antibiotic strip was removed after 1 hour of incubation, and the second strip was applied at the same location so that the MICs of the two antibiotics overlapped. The fractional inhibitory concentration (FIC) index was calculated as follows: $FIC_{CZA} + FIC_{ATM}$, where FIC_{CZA} is $MIC_{CZA+ATM}/MIC_{CZA}$ and FIC_{ATM} is $MIC_{ATM+CZA}/MIC_{ATM}$.

2.3. Detection of carbapenemases

Carbapenemase production was detected by various commercial assays according to the manufacturer's instructions, immunochromatography kits RESIST-5 O.O.K.N.V., RESIST-5

O.K.N.V.I., IMP K-SeT (Coris BioConcept, Gembloux, Belgium), PCR Xpert[®] Carba-R, GXCARBAR-10, GXCARBAR-CE-10, GXCARBARP-CE-10 (Cepheid, Maurens-Scopont, France), the colorimetric test MAST[®] CARBA PAcE (Mast Group, Amiens, France) and Imipenem/EDTA combined with ETEST[®] (bioMérieux, Marcy l'Etoile, France).

A multiplex PCR protocol was used to detect the presence of carbapenemase-encoding genes with the primers MultiIMP-for, MultiIMP-rev, MultiVIM-for, MultiVIM-rev, MultiKPC-for, and MultiKPC-rev [20,21]. A specific PCR for *bla*_{IMP-13} was designed with primers IMP13-F/IMP13-R (**Table 1**), and the PCR cycling parameters were as follows: initial denaturation at 95 °C for 2 min; 35 cycles of 95 °C for 40 s, 55 °C for 40 s and 72 °C for 1 min; and a final elongation step at 72 °C for 10 min.

2.4. Gene transfer experiments and plasmid content analysis

Genetic support was studied by mating assays in which the clinical strain Pa6523 was used as a donor and Pa6761, an Rif^R mutant of the *P. aeruginosa* PAO1 strain, was used as a recipient. Briefly, suspensions of 0.5 McF of donor and recipient strains were prepared, and a dilution (1/200) was made for each strain in 50 mL of MH broth before incubation at 35 °C ± 2 °C with agitation.

Mating experiments were performed at different times during the exponential growth phase of the donor strain, as determined by measuring the optical density at 600 nm. At each time point, 500 µL of donor and recipient cultures were mixed at a 1:1 ratio and then centrifuged, and 100 µL was added on a 0.45 µm nitrocellulose filter placed on an MH agar plate. After 24 h of incubation at 35 °C ± 2 °C, the colonies on the filter were placed in 1 mL of MH broth and thoroughly mixed, and 100 µL was plated onto selective media. Transconjugants were selected on agar plates containing 128 mg/L rifampicin and 8 mg/L ceftazidime. Each potential

transconjugant was screened by antibiotic susceptibility testing via the disk diffusion method, and the presence of *bla*_{IMP-13} was checked via PCR. The transfer frequency was expressed as the number of transconjugants per donor cell.

To study the excision, circularization and integration of the ICE, PCR was performed at different growth times. The ICE circular form and the donor locus free were investigated with the primers ICE_IMP-13_CDS_deb_R/ICE_IMP-13_CDS_fin_F (amplicon of 688 bp) and PA0729_2R/PA0729_1F (1475 bp) (**Table 1, Figure S1**). To assess ICE integration and determine the start and end sites, the PA0729_2R/ICE_IMP-13_CDS_deb_R (749 bp) and ICE_IMP-13_CDS_fin_F/PA0729_1F (1433 bp) primer pairs were used, respectively (**Table 1, Figure S1**).

Plasmid DNA was extracted using the ZyppyTM Plasmid Miniprep Kit (Zymo Research) or an alkaline-lysis method [21]. Putative plasmid DNA extracts (5µl) were analyzed by agarose electrophoresis and electroporated into Rif^R *P. aeruginosa* PAO1 in a 2mm gap electroporation cuvette, using a MicroPulser Electroporator at 200 ohms, 25 µFD and 2,5 V (Bio-Rad, Marnes-la-Coquette, France), with selection on agar plates supplemented with 8 mg/L ceftazidime.

2.5. Whole-genome sequencing

Genomic DNA was extracted using the DNeasy[®] PowerSoil[®] Pro Kit (Qiagen, Courtaboeuf, France), according to manufacturer's instructions, except for the sample quantity corresponding to two 10µl loops of bacterial culture on agar plate. Sequencing was performed by Illumina technology (using a Nextera XT DNA Library Preparation Kit and a NextSeq 2000 or NovaSeq 6000 instrument) and by Oxford Nanopore technology (using an Oxford Nanopore Rapid Barcoding Kit (SQK-RBK114.96) with a R10.4.1 flowcell and a MinION system MK1C) for the 8 clinical isolates and one transconjugant (Pa6716) to obtain more accurate results, and only by

Oxford Nanopore technology for two transconjugants (Pa7077 and Pa7078) and the recipient strain (PAO1 Rif^R).

Genomes were assembled from Nanopore long-read data using Epi2me laboratories with the wf-bacterial-genome Workflow v1.2.0 (<https://github.com/epi2me-labs/wf-bacterial-genomes>) and then Unicycler v0.5.1 (<https://github.com/rrwick/Unicycler>) when Illumina short-read data were also available. For each genome, we performed a multilocus sequence typing (MLST) analysis (<https://github.com/tseemann/mlst>) on all the genomes available in the NCBI database. Automatic gene prediction and structural and functional annotation of the genomes were performed using Prokka v1.14.5 [22] and for complete sequence of ICEPa6523, we also used BAKTA v1.4.2 [23]. Antimicrobial resistance genes were identified using AMRFinderPlus v3.10.30 [24] with a minimum identity of 90% and a minimum coverage of 50% of the reference protein. chewBBACA software v2.8.5 [25] was used to type the strains according to the *P. aeruginosa* core genome MLST (gMLST) scheme from Tönnies et al., 2021 [26].

Genomes were analyzed using the following bioinformatics tools: NCBI BLAST tool (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>), Jalview (v.2.11.3.2) [27], SnapGene[®] software (www.snapgene.com), and DNADynamo alignment software (Blue Tractor Software, UK, v.1.633) (<https://www.bluetractorsoftware.com/DynamoDemo.htm>). Genome comparisons were performed using BLAST and visualized by Easyfig 3.0.0 [28].

Default parameters were used for all software cited.

3. Results - Discussion

3.1. Phenotypic characterization of IMP-13-producing *P. aeruginosa*

Between March and July 2020, four MDR *P. aeruginosa* isolates (Pa6567, Pa6536, Pa6566, and Pa6559) with carbapenem and ceftolozane-tazobactam resistance were collected at the

University Hospital of Bordeaux. Carbapenemase detection was negative according to the RESIST-5 O.O.K.N.V., Xpert® Carba-R kits and in-house multiplex PCR. The results of the imipenem/EDTA combined ETEST® were inconclusive. Nevertheless, the MAST® CARBA PAcE assay was positive, which led us to send the isolates to the French National Reference Center for Antibiotic Resistance (Besançon), where an IMP-13 enzyme was detected in all isolates. Since 2021, new tests, RESIST-5 O.O.K.N.V.I. and IMP K-SeT, have become available and gave positive results for our four isolates, highlighting the difficulty in detecting IMP-13 before this date.

In light of this, a retrospective analysis was conducted to identify MDR *P. aeruginosa* isolates collected between 2010 and 2020 in the Bordeaux hospital laboratory or present in the strain collection of the Bacteriology Laboratory of the University of Bordeaux (ARMYNE team). Data extraction revealed 29 isolates with resistance profiles similar to those of the IMP-13-producing isolates, among which 17 were preserved. Among the 17 preserved strains, four additional IMP-13-producing isolates (Pa5247, Pa5957, Pa6466, and Pa6523) were discovered via *bla*_{IMP-13}-specific PCR, giving a total of eight IMP-13-producing isolates analyzed together. These isolates were collected from stool (3), blood (1), urine (3), and sputum (1). Interestingly, five of them were recovered from patients hospitalized in the same hematology department, two were from other hospital units (orthopedic traumatology and cardiology), and one was from a private laboratory. The Pa6523 and Pa6567 strains were isolated from the same patient within a three-month time interval.

The eight isolates were all resistant to ticarcillin, ticarcillin-clavulanic acid, cefepime, ceftazidime, tobramycin, ciprofloxacin and levofloxacin and to the last-resort antibiotics ceftolozane-tazobactam, ceftazidime-avibactam, and imipenem-relebactam (**Table 2**). The susceptibilities to piperacillin, piperacillin-tazobactam, aztreonam, imipenem, meropenem and

amikacin varied. However, all the isolates were susceptible to colistin and cefiderocol. The combination of CZA/ATM had synergistic effects on the four isolates tested ($\text{FIC}_{\text{index}} \leq 0,5$) despite reduced susceptibility to aztreonam. As expected, this combination was particularly useful against the aztreonam-resistant isolate Pa6466.

The IMP-13 carbapenemase has been described as difficult to detect [9], and the decreased susceptibility to carbapenems may be moderate; IMP-13-producing isolates were sometimes classified as “susceptible” according to the CLSI and EUCAST breakpoints [7,9]. This was the case in this study for five isolates (**Table 2**) for which susceptibility was determined by the disk diffusion method. However, the MICs determined by the E-test and confirmed by the BMD were consistent with a resistant phenotype.

3.2. Genotypic relationships and AMR profiles of clinical isolates

Genome analysis revealed that all the isolates were identified as ST621, corresponding to the major *P. aeruginosa* ST that produces IMP-13 carbapenemase [4,6,7,9,17](**Table 3**). cgMLST analysis revealed a close epidemiological relationship among the five isolates collected in 2020 from the hematologic and orthopedic units (≤ 7 allele differences) (**Figure 1**). The isolates collected in 2019, 2015 and 2011 had allelic distances of 18, 12 and 59 alleles from the closest isolate, respectively. This suggests that the spread of IMP-13 may be linked, at least in part, to clonal strain diffusion. The acquired antibiotic resistance genes and the main chromosomal resistance mutations detected by WGS are shown in **Table 3**. In addition to the presence of the *bla*_{IMP-13} gene, all isolates possessed the *aac(6')-Ib4* gene, which encodes for aminoglycoside resistance (tobramycin, netilmicin, amikacin), and the *crpP*-like gene, which has been described as being involved in a novel ciprofloxacin resistance mechanism [29]. Five isolates also acquired the genes encoding the aminoglycoside-modifying enzymes APH(3')-VIa, which confers

resistance to kanamycin, neomycin, amikacin, and isepamicin and was initially identified in *Acinetobacter baumannii*, and AAC(3)-IIIb, which is specific to *P. aeruginosa* and confers resistance to kanamycin, neomycin, gentamicin, and tobramycin [30]. The presence of the latter enzymes was related to a higher level of resistance to amikacin. The genes encoding the chromosomal β -lactamases PDC-5 and OXA-914 were found in each isolate. Fluoroquinolone resistance-associated mutations were observed in *gyrA* (T83I, D87H) and *parC* (S87L). Sequence analysis of the *oprD* gene encoding the outer membrane porin responsible for the passive uptake of carbapenems, revealed that the eight isolates possessed an *oprD*_5 variant [31]. Moreover, insertion sequences (ISs) were found in the *oprD* gene or in its promoter for the three isolates with imipenem MICs greater than 32 mg/L (Pa6466, Pa6566, Pa6559). Actually, an identical IS30-like sequence of 1113 bp was inserted at + 8 bases after the initiator codon ATG of *oprD* in Pa6559 and at -13 in the promoter in Pa6566 (**Figure S2**). In Pa6466, an IS5-like sequence of 1120 bp was observed at +220 bp. Indeed, OprD defects can be mediated by indels, recombination events such as the insertion of IS elements, nonsense mutations, start- or stop-loss mutations, promoter disruptions, or amino acid substitutions affecting the porin's pore width, charge, or structural integrity leading carbapenem resistance [31].

The *bla*_{IMP-13} gene, frequently associated with the *aac(6')-Ib4* aminoglycoside resistance gene cassette, was found to be carried on a different class I integron harbored by a different Tn3-like transposon, mostly InPSG and Tn5051-like [7,8,12,32]. In all clinical isolates studied here, the *bla*_{IMP-13} gene was carried on a class I integron next to the *aac4A* gene on a previously described Tn5051-like transposon [32], accession no. AJ628135. Nevertheless, the horizontal transfer of the *bla*_{IMP-13} gene has been understudied in the literature. Mating and transformation assays have been mostly unsuccessful, suggesting a chromosomal location of the gene [6,7]. However, the

location of the *bla*_{IMP-13} has sometimes also been described on a plasmid [8,12,33]. Recently, using bioinformatics predictions, it was shown that the *bla*_{IMP-13} gene is located on an ICE [4,17].

3.3. *bla*_{IMP-13} is carried on an ICE*clc*-like element

Mating experiments with rifampicin-resistant *P. aeruginosa* PAO1 (Pa6761) demonstrated horizontal transfer of the *bla*_{IMP-13} gene from the Pa6523 clinical isolate. The transfer rate was very low, with 10⁻⁸ transconjugants per donor. Plasmid DNA preparations from the donor isolate Pa6523 did not reveal any plasmid DNA in gel electrophoresis, and the transformation experiments failed. Therefore, horizontal gene transfer experiments suggested that the MGE carrying the *bla*_{IMP-13} gene may be an ICE.

A comparative whole-genome analysis of the recipient Pa6761 cells with three transconjugants (Pa6716, Pa7077, and Pa7078) and the donor Pa6523 cells revealed the presence of a large fragment of 88589 bp inserted at 3' end of the tRNA^{Gly} gene (locus PA0729.1), with an insertion site of 20 bp (**Figure 2**), and was identified as an ICE designated ICEPa6523 in this study. As expected, the transconjugants were found to be ST549 strains (as PAO1) and possessed only the resistance genes (*bla*_{IMP-13}, *aac*(6')-Ib4, *qacEΔ1*, and *sul1*) carried by a class1 integron, located on Tn5051-like, itself inserted on ICEPa6523 (**Table 3**), and conferring resistance to beta-lactams and aminoglycosides (**Table 2**).

NCBI BLASTn analysis alignment of ICEPa6523 showed 100% query coverage and 100% nucleotide identity with two complete genomes: *P. aeruginosa* GenBank accession number CP034354.1 (ranging from nt 1343223 to nt 1431811), which was isolated in Belgium in 2011, and *P. aeruginosa* strain 3541, GenBank accession number OX638610.1 (ranging from nt 2363617 to nt 2275029), which was collected in Bulgaria in 2018 [6]. ICEPa6523 was found to

belong to the ICE*clc* family; its GC content was 62%, and it harbored 85 CDSs and a tyrosine-type recombinase (**Table S1**). ICEPa6523 exhibiting 87,40% identity and 49% query cover when compared with ICE*clc* (accession number AJ617740) and it was also similar to other ICE*clc*-like: CE6441 (MF168946) (100% identity and 94% cover query), ICE6440 (MF168944) (99,74% identity and 86% cover query), ICEPa690 (KY852375) (86,77 % identity and 51% cover query), harboring carbapenemase genes *bla*_{VIM-2}, *bla*_{VIM-2} and *bla*_{GES-6} respectively. ICEPa6523 was found in a single copy in the eight clinical isolates, as in the transconjugant, and was always inserted next to the same tRNA^{Gly} gene. A comparison of our eight isolates with the complete genome of OX638610 revealed differences in at least 19 alleles, and CP034354 was slightly further distant (**Figure 1**).

ST621 is the major ST of *P. aeruginosa* that produces IMP-13 carbapenemase. Indeed, in the PATRIC-BV-BRC database [34], among 24 *P. aeruginosa* strains carrying the *bla*_{IMP-13} gene, 19 were ST621, and *bla*_{IMP-13} was always located on ICEPa6523. Interestingly, the same ICE was found in two ST357 strains collected from Bucharest (JAMKXY000000000.1 and JAMKYE000000000.1) [35], suggesting that this ICE could be mobilized from one strain to another. In two ST111 strains [10], an ICE was found, but it was slightly different from ICEPa6523. Finally, in a ST235 isolate, *bla*_{IMP-13} was described as being on a plasmid without an ICE sequence [12] (**Table S3**). In the literature, other less frequent STs with no complete genome available have been reported, for which *bla*_{IMP-13} was associated with a plasmid or without identifying genetic support [7,8,12,33].

3.4. Mobilization of ICEPa6523

Surprisingly, among the transconjugants obtained from three different experiments, some adjacent genes located immediately downstream of the ICE were also mobilized. The

transconjugant Pa6716 possessed the minimal form of ICEPa6523, with the core segment of attachment sites (att) clearly identified with identical sequences on both sides of the ICE (GATTCCCTTCGCCCCGCTCCA) (**Figure 2**). The transconjugant Pa7078 was very similar to Pa6716, with only attR differing by three nucleotides (GATTCCCTCTACCCGCTCCA). Interestingly, in the transconjugant Pa7077, in addition to ICEPa6523 acquisition, 38 adjacent genes (30493 bp) were also transferred for a total transferred sequence length of more than 119 kb. The mobilized adjacent genes encoded a tyrosine recombinase and 28 hypothetical proteins (**Table S2**), and shared 98.9% identity 83% query coverage with the corresponding sequences of the PAO1 recipient strain. Surprisingly, it seems that concomitantly with the insertion of the ICE and adjacent genes, there was a sequence exchange or homologous recombination with the PAO1 sequence. In other words, the 30 kb region of the clinical strain has replaced the corresponding PAO1 region (**Figure S3**).

Moreover, the study of ICE excision by PCR detection of the locus free and of circularized forms of ICEPa6523 was unsuccessful, suggesting that horizontal transfer was an extremely rare event or that the circularized forms were labile or of different sizes. The mobilization of ICEPa6523 appears to be complex and difficult to highlight due to its low transfer rate. The determination of inductive experimental conditions for improving the transfer rate should help to understand the mobilization mechanisms involved.

HGT is a major process in the dissemination of antimicrobial resistance genes [3], and bioinformatics analyses have recently shown the involvement of ICEs in the spread of antibiotic resistance, especially in *P. aeruginosa* [16,17]. Sometimes, ICEs were reported using experimental methods, *e.g.*, for 388 of the 2065 ICEs described in the ICEberg 3.0 database (update Jun 1, 2023) [36]. Among the nine ICEs carrying carbapenemase genes in *P. aeruginosa*, three (carrying VIM-4, GES-6 or NDM-1) were experimentally studied, but only *bla*_{GES-6}

containing ICE (ICE*Pae*690) was successfully transferred to a spontaneous rifampicin-resistant mutant of *P. aeruginosa* PAO1 [37]. In the literature, two divergent ICE predictions were deduced using bioinformatics tools in the CP034354 genome: (i) a 102416 bp ICE localized between nt 1343196 and nt 1445611 [38] and (ii) an 88590 bp ICE inserted in the PA0714 hypothetical protein with no details on its precise localization in the genome [4]. These variations underscore the limits of bioinformatic tools for ICE prediction and show the importance of experimental results. In addition, we highlighted here the possible translocation of genes adjacent to ICE*Pa*6523, as is the case with ISs such as ISE*cp1* or IS91 with a one-ended transposition mechanism [39], or cis-mobilization, as described in *Streptococcus* sp. [16,40].

Furthermore, the ICE*clc*-type transfer mechanism is better known in *Pseudomonas knackmussii* and *Pseudomonas putida* than in *P. aeruginosa* according to several studies [18,41–43]. It appears that the genes involved in the control of ICE*clc* activation (*mfsR-marR-tciR* operon and the *alpA* and *bisR* genes) in *P. knackmussii* are different from those in *P. aeruginosa* [44]. Homologs of the *tciR*, *alpA* and *bisR* genes but not the *mfsR* and *marR* genes have been found in ICE*Pa*6523, which could explain the low conjugation rates.

4. Conclusion

We confirmed in this study that the *bla*_{IMP-13} gene is closely related to the *P. aeruginosa* ST621 clone. Nevertheless, to the best of our knowledge, we have demonstrated for the first time the functionality and mobility of an ICE*clc*-like element carrying a *bla*_{IMP-13} gene by successful mating assays. Although the mechanism of ICE mobilization may be secondary to the spread of resistance, it should not be underestimated.

Author contributions

L. B. designed the study, analyzed the data, and wrote the main manuscript text. V. D. obtained the funding for the research and contributed to the design of the study and the interpretation of the results. U. G. performed the bioinformatic analysis, and A. G. and M. N. provided their bioinformatic expertise. M. Me. performed the nanopore data analysis. S. P., J. G., B. B., and C. B. provided their ICE expertise. M. Mo., S. A. and L. C. provided technical support. V. D. and S. P. critically revised the manuscript. All authors participated in the data analysis, reviewed the manuscript and approved the final version.

Acknowledgments

Libraries were prepared and sequenced on a NextSeq 2000 system at the PGTB (doi:10.15454/1.5572396583599417E12) with the help of Erwan Guichoux. Sequencing on a NovaSeq 6000 system was performed at the GeT-PlaGe core facility, INRAE Toulouse. We thank Patricia Recordon-Pinson and Claudio Plaisant for help during library preparation.

Declarations

Funding: This work was supported by MAGITICS, JPI-AMR.

Competing Interests: None

Ethical Approval: Not applicable

Sequence Information: Raw DNA sequencing and assembled genomes data from Nanopore technology are available under BioProject accession number PRJNA1106512 and Raw DNA sequencing from Illumina technology are available under BioProject accession number PRJNA1106824. The complete nucleotide sequence of ICEPa6523 has been also deposited at NCBI GenBank under accession number PQ227710.

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Figure 1. Minimum spanning tree of the eight *P. aeruginosa* clinical isolates, OX638610 and CP034354 genomes based on cgMLST.

Each circle represents a unique cgMLST allele profile. The numbers in circles correspond to the isolates, and each motif represents a different collection site. The numbers on the connecting lines correspond to the number of different alleles. Complete genome OX638610 and CP034354 which contained the same ICE_{Pa6523} were added for comparison with our clinical isolate genomes.

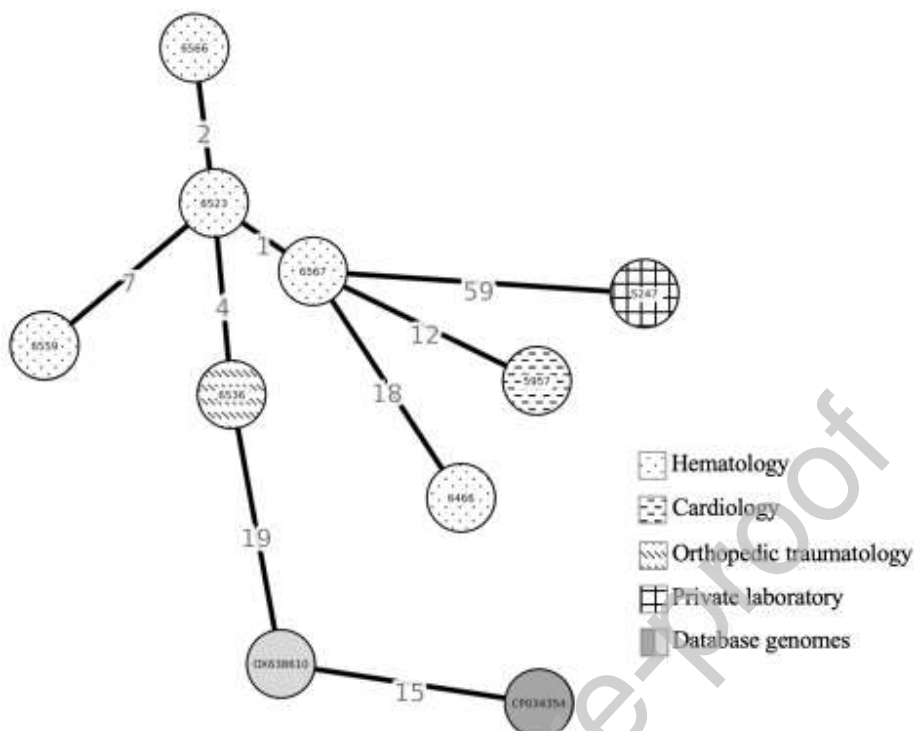


Figure 2. Schematic representation of the ICEPa6523 insertion in transconjugant and parental clinical isolates.

Linear comparison of ICEPa6523 genetic surroundings among the genomes of the three transconjugants, donor and recipient cells. The CDSs are shown as arrows, with the arrowhead indicating the direction of transcription. ICEPa6523 is represented in green; the sequence of the donor cell is in blue, and the recipient cell sequence is in orange; the *bla*_{IMP-13} gene is shown in red. The profile of gene similarity between genomes is shown as a gray color spectrum as a percentage. The black boxes represent *attB*, *attL* and *attR* site

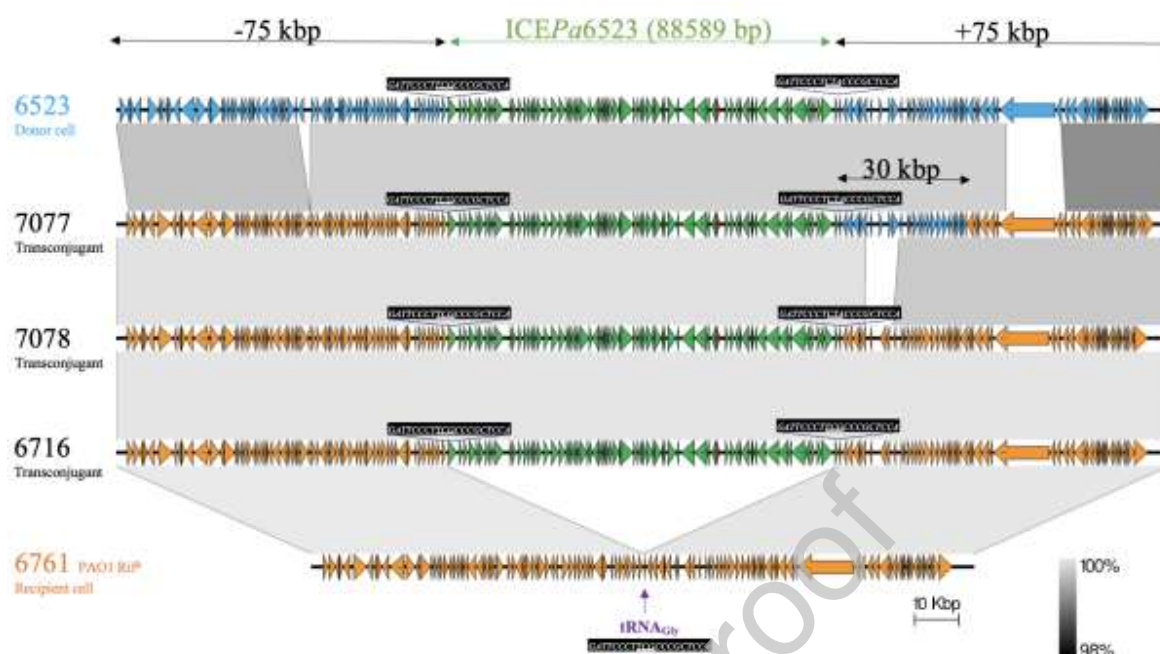


Table 1. Sequences of primers used for single and multiplex PCR for carbapenemase gene detection and ICEPa6523 mobilization.

Primer	Sequence (5'-3')	Reference
MultiIMP-for	TTGACACTCCATTTACDG	Dallenne et al.
MultiIMP-rev	GATYGAGAATTAAGCCACYCT	Dallenne et al.
MultiVIM-for	GATGGTGTCTGGTCGCATA	Dallenne et al.
MultiVIM-rev	CGAATGCGCAGCACCAG	Dallenne et al.
MultiKPC-for	CATTCAAGGGCTTCTTGCTGC	Dallenne et al.
MultiKPC-rev	ACGACGGCATAGTCATTTGC	Dallenne et al.
IMP13-F	ACACGGTTTACTGGTGXTTG	This study
IMP13-R	GGTTTAATAAAGCAACCACC	This study
PA0729_1F	GTTTCGCCAGTCACTTCATC	This study
PA0729_2R	TTCCACACCATCCGCAATG	This study
ICE_IMP-13_CDS_fin_F	CCGCACAATCGCGTATCAAG	This study
ICE_IMP-13_CDS_deb_R	AAGTGAAGCGGAAGTGCCAG	This study

Table 2. Antibiotic susceptibility of eight clinical IMP-13-producing *P. aeruginosa* isolates, a rifampicin-resistant PAO1 strain and three transconjugants from mating assays.

Isolate	Collecti on date	Samp le	Unit	Diameter (mm)												MIC (mg/L)					FIC inde x		
				TI	TC	PI	PT	FE	CA	AT	IP	ME	TO	AM	CI	LV	C/T	CZ	IP	I/		FD	CS
				C	C	P	Z	P	Z	M	M	R	B	K	P	X	A	M	R	C		T	
Pa524 7	Nov. 2011	Urine	Private laboratory	6	6	21	22	11	6	29	21	20	6	22	6	6	>25 6	>25 6	8	4	1	1	ND

Pa595	Sep. 7	2015	Urine	Cardiolog y	6	6	16	16	11	6	25	24	23	6	20	6	6	>25	>25	8	4	2	2	ND
Pa646	Aug. 6	2019	Sputum	Hematolog y	6	6	6	6	6	6	6	6	6	6	10	6	6	>25	>25	>3	>3	0,5	1	0,008
Pa652	Jan. 3*	2020	Stool	Hematolog y	6	6	11	12	8	6	19	20	22	6	14	6	6	>25	>25	16	8	1	1	0,25
Pa656	Mar. 7*	2020	Stool	Hematolog y	6	6	10	11	6	6	19	21	21	6	14	6	6	>25	>25	8	4	1	2	ND
				Orthopedic traumatology	6	6	10	14	8	6	24	22	22	6	21	6	6	>25	>25	16	4	1	1	ND
Pa653	May 6	2020	Urine	Hematolog y	6	6	10	12	6	6	19	6	6	6	14	6	6	>25	>25	>3	>3	1	1	0,5
Pa656	Jun. 6	2020	Stool	Hematolog y	6	6	11	14	9	6	15	6	6	6	12	6	6	>25	>25	>3	>3	1	2	0,5
Pa655	Jul. 9	2020	Blood	Hematolog y	6	6	11	14	9	6	15	6	6	6	12	6	6	>25	>25	>3	>3	1	2	0,5
					6	6	11	14	9	6	15	6	6	6	12	6	6	>25	>25	>3	>3	1	2	0,5
PAO1	-	-	-	-	28	27	27	31	31	25	30	30	30	26	24	36	27	ND	ND	N	N	ND	ND	ND
PAO1	-	-	-	-	20	18	28	31	34	24	28	35	35	26	28	38	30	ND	ND	N	N	ND	ND	ND
rif^R	-	-	-	-	20	18	28	31	34	24	28	35	35	26	28	38	30	ND	ND	N	N	ND	ND	ND
(R)	-	-	-	-	20	18	28	31	34	24	28	35	35	26	28	38	30	ND	ND	N	N	ND	ND	ND
Pa671	-	-	-	-	6	6	22	24	8	6	33	24	14	6	25	39	30	ND	ND	N	N	ND	ND	ND
6 (Tc)	-	-	-	-	6	6	24	24	8	6	28	25	15	6	25	39	30	ND	ND	N	N	ND	ND	ND
Pa707	-	-	-	-	6	6	24	24	8	6	28	25	15	6	25	39	30	ND	ND	N	N	ND	ND	ND
7 (Tc)	-	-	-	-	6	6	21	25	10	6	27	23	15	6	25	38	28	ND	ND	N	N	ND	ND	ND
Pa707	-	-	-	-	6	6	21	25	10	6	27	23	15	6	25	38	28	ND	ND	N	N	ND	ND	ND
8 (Tc)	-	-	-	-	6	6	21	25	10	6	27	23	15	6	25	38	28	ND	ND	N	N	ND	ND	ND

TIC, ticarcillin; TCC, ticarcillin/clavulanic acid; PIP, piperacillin; PTZ, piperacillin/tazobactam; FEP, cefepime; CAZ, ceftazidime; ATM, aztreonam; IPM, imipenem; MER, meropenem; TOB, tobramycin; AMK, amikacin; CIP, ciprofloxacin; LVX, levofloxacin; C/T, ceftolozane/tazobactam; CZA, ceftazidime/avibactam; I/R, imipenem/relebactam; FDC, cefiderocol; CST, colistin. MIC, minimum inhibitory concentration. In light gray: susceptible; in gray: susceptible increased exposure; in dark gray: resistant (according to CASFM 2023/EUCAST); FIC, fractional inhibitory concentration; D, donor cell in mating assay; R, recipient cell (rifampicin-resistant PAO1) in mating assay; Tc, transconjugant; ND: not determined.

*Isolates collected from the same patient.

Table 3. Genotypic characteristics of the isolates in this study.

Isolate	ST	Acquired antibiotic resistance genes		β-lactamases		Modifications** associated with resistance				
		Carrying by ICE Pa6523	Other	PDC	OXA	<i>gyrA</i>	<i>parC</i>	<i>parE</i>	<i>oprD</i>	
Pa5247	62	<i>bla_{IMP-13}</i> , <i>aac(6')-Ib4</i> , <i>qacEΔ1</i> , <i>sul1</i>	<i>crpP</i>	PDC-5	OXA-914	T83I	WT	S87L	WT	oprD_5
	1	<i>bla_{IMP-13}</i> , <i>aac(6')-Ib4</i> , <i>qacEΔ1</i> , <i>sul1</i>	<i>crpP</i>	PDC-5	OXA-914	T83I, D87H	WT	S87L	WT	oprD_5
Pa5957	62	<i>bla_{IMP-13}</i> , <i>aac(6')-Ib4</i> , <i>qacEΔ1</i> , <i>sul1</i>	<i>crpP</i> , <i>aph(3')-VIa</i> , <i>aac(3)-IIIb</i>	PDC-5	OXA-914	T83I, D87H	WT	S87L	WT	oprD_5 (IS insertion)
	1	<i>bla_{IMP-13}</i> , <i>aac(6')-Ib4</i> , <i>qacEΔ1</i> , <i>sul1</i>	<i>crpP</i> , <i>aph(3')-VIa</i> , <i>aac(3)-IIIb</i>	PDC-5	OXA-914	T83I, D87H	WT	S87L	WT	oprD_5
Pa6466	62	<i>bla_{IMP-13}</i> , <i>aac(6')-Ib4</i> , <i>qacEΔ1</i> , <i>sul1</i>	<i>crpP</i> , <i>aph(3')-VIa</i> , <i>aac(3)-IIIb</i>	PDC-5	OXA-914	T83I, D87H	WT	S87L	WT	oprD_5 (IS insertion)
	1	<i>bla_{IMP-13}</i> , <i>aac(6')-Ib4</i> , <i>qacEΔ1</i> , <i>sul1</i>	<i>crpP</i> , <i>aph(3')-VIa</i> , <i>aac(3)-IIIb</i>	PDC-5	OXA-914	T83I, D87H	WT	S87L	WT	oprD_5 (IS insertion)
Pa6523*	62	<i>bla_{IMP-13}</i> , <i>aac(6')-Ib4</i> , <i>qacEΔ1</i> , <i>sul1</i>	<i>crpP</i> , <i>aph(3')-VIa</i> , <i>aac(3)-IIIb</i>	PDC-5	OXA-914	T83I, D87H	WT	S87L	WT	oprD_5 (IS insertion)
	1	<i>bla_{IMP-13}</i> , <i>aac(6')-Ib4</i> , <i>qacEΔ1</i> , <i>sul1</i>	<i>crpP</i> , <i>aph(3')-VIa</i> , <i>aac(3)-IIIb</i>	PDC-5	OXA-914	T83I, D87H	WT	S87L	WT	oprD_5 (IS insertion)
Pa6567*	62	<i>bla_{IMP-13}</i> , <i>aac(6')-Ib4</i> , <i>qacEΔ1</i> , <i>sul1</i>	<i>crpP</i>	PDC-5	OXA-914	T83I, D87H	WT	S87L	WT	oprD_5
	1	<i>bla_{IMP-13}</i> , <i>aac(6')-Ib4</i> , <i>qacEΔ1</i> , <i>sul1</i>	<i>crpP</i>	PDC-5	OXA-914	T83I, D87H	WT	S87L	WT	oprD_5
Pa6536	62	<i>bla_{IMP-13}</i> , <i>aac(6')-Ib4</i> , <i>qacEΔ1</i> , <i>sul1</i>	<i>crpP</i> , <i>aph(3')-VIa</i> , <i>aac(3)-IIIb</i>	PDC-5	OXA-914	T83I, D87H	WT	S87L	WT	oprD_5 (IS insertion)
	1	<i>bla_{IMP-13}</i> , <i>aac(6')-Ib4</i> , <i>qacEΔ1</i> , <i>sul1</i>	<i>crpP</i> , <i>aph(3')-VIa</i> , <i>aac(3)-IIIb</i>	PDC-5	OXA-914	T83I, D87H	WT	S87L	WT	oprD_5 (IS insertion)
Pa6566	62	<i>bla_{IMP-13}</i> , <i>aac(6')-Ib4</i> , <i>qacEΔ1</i> , <i>sul1</i>	<i>crpP</i> , <i>aph(3')-VIa</i> , <i>aac(3)-IIIb</i>	PDC-5	OXA-914	T83I, D87H	WT	S87L	WT	oprD_5 (IS insertion)
	1	<i>bla_{IMP-13}</i> , <i>aac(6')-Ib4</i> , <i>qacEΔ1</i> , <i>sul1</i>	<i>crpP</i> , <i>aph(3')-VIa</i> , <i>aac(3)-IIIb</i>	PDC-5	OXA-914	T83I, D87H	WT	S87L	WT	oprD_5 (IS insertion)

	54	None	None	PDC-1	OXA-50	WT	WT	WT	WT	WT
PAO1	9									
PAO1	54	None	None	PDC-1	OXA-50	WT	WT	WT	WT	WT
rif^R (R)	9									
Pa6716 (Tc)	54	<i>bla_{IMP-13}, aac(6')-Ib4, qacEΔ1, sulI</i>	None	PDC-1	OXA-50	WT	WT	WT	WT	WT
Pa7077 (Tc)	54	<i>bla_{IMP-13}, aac(6')-Ib4, qacEΔ1, sulI</i>	None	PDC-1	OXA-50	WT	WT	WT	WT	WT
Pa7078 (Tc)	54	<i>bla_{IMP-13}, aac(6')-Ib4, qacEΔ1, sulI</i>	None	PDC-1	OXA-50	WT	WT	WT	WT	WT
	9									

D, Donor cell used in the mating assay; R, recipient cell (Rifampicin-resistant PAO1) used in the mating assay; Tc, transconjugant; WT, wild type (protein reference PAO1 NC_002516); IS, insertion sequence.

* Isolates collected from the same patient.

** Modifications were mutations or insertion sequences

Graphical abstract

