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Pathogenicity Genomic Island-Associated CrpP-Like Fluoroquinolone-Modifying Enzymes among *Pseudomonas aeruginosa* Clinical Isolates in Europe

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ABSTRACT Many transferable quinolone resistance mechanisms have been identified in Gram-negative bacteria. The plasmid-encoded 65-amino-acid-long ciprofloxacin-modifying enzyme CrpP was recently identified in *Pseudomonas aeruginosa* isolates. We analyzed a collection of 100 clonally unrelated and multidrug-resistant *P. aeruginosa* clinical isolates, among which 46 were positive for *crpP*-like genes, encoding five CrpP variants conferring variable levels of reduced susceptibility to fluoroquinolones. These *crpP*-like genes were chromosomally located as part of pathogenicity genomic islands.

KEYWORDS *Pseudomonas aeruginosa*, CrpP, fluoroquinolones, ciprofloxacin

Bacterial resistance to quinolones and fluoroquinolones mainly results from decreased outer membrane permeability, overexpression of efflux pumps, and/or amino acid substitutions in the sequences of the chromosomally encoded topoisomerases that are the targets of these antibiotics (1). However, a series of acquired and transferable mechanisms have been identified in Gram-negative bacteria, including quinolone target protection conferred by Qnr-type pentapeptide proteins, enzymatic inactivation of the antibiotic through the action of the AAC(6′)-Ib-cr acetyltransferase (coresistance to aminoglycosides), and plasmid-encoded efflux pumps, such as OqxAB or QepA (2, 3). Nevertheless, the latter horizontally transferred resistance determinants have rarely been identified in *Pseudomonas aeruginosa*, in which resistance to fluoroquinolones is mainly driven by mutations in topoisomerases, efflux overproduction, and outer membrane permeability defects (4–6).

Recently, a plasmid-encoded 65-amino-acid-long ciprofloxacin-modifying enzyme, CrpP (renamed CrpP1 here for clarity), was identified in a single clinical *P. aeruginosa* isolate from Mexico (7). The *crpP1* gene was located in a 123-kb conjugative plasmid (pUM505) coharboring virulence and heavy-metal resistance genes (8). CrpP confers reduced susceptibility (7.5-fold) to ciprofloxacin (CIP) once produced in *Escherichia coli* J53-3. In *P. aeruginosa*, this enzyme confers decreased susceptibility (4-fold decrease of MIC values) to CIP, norfloxacin, and moxifloxacin but only when transferring the *crpP1*-positive pUM505 natural plasmid, since expression of *crpP1* from a recombinant plasmid once transformed in *P. aeruginosa* did not have any significant effect. Very recently, homologues of the *crpP1* gene that shared low-level nucleotide identity (corresponding proteins being 10% to 43% identical) were identified among *Enterobacterales* from Mexico, with corresponding proteins exhibiting conserved residues Thr8, Asp9, Lys33, Gly34, and Cys40; these amino acids are possibly key residues in respect to the CrpP activity (9). It was demonstrated that

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TABLE 1 MICs of fluoroquinolones for the *P. aeruginosa* and *E. coli* recombinant strains examined in this study

	MIC (μg/ml) in:											
	Escherichia coli TOP10						*Pseudomonas aeruginosa* PAO1					
		CrpP variant						CrpP variant				
Antibiotic	TOP10	CrpP1	CrpP2	CrpP3	CrpP4	CrpP5	PAO1	CrpP1	CrpP2	CrpP3	CrpP4	CrpP5
Ciprofloxacin	0.002	0.002	0.015	0.015	0.008	0.015	0.25	0.25	0.5	0.25	0.25	0.25
Levofloxacin	0.004	0.004	0.015	0.015	0.015	0.015	1	1	2	2	2	1

CrpP1 exhibits ATP-dependent phosphorylation activity toward CIP, explaining the decreased antibacterial activity of this antibiotic (7). Considering the single report of the *crpP1* gene in *P. aeruginosa*, our aim was to evaluate the dissemination of this novel resistance determinant.

A collection of 100 *P. aeruginosa* isolates recovered in France and Switzerland during 2000 to 2015 were selected for this study, including extended-spectrum β-lactamase ($n = 17$) and carbapenemase ($n = 73$) producers. A PCR approach was used for screening the *crpP* gene among these isolates, using designed primers crpP-F (5′-AGAGCGG GATCGATCAGAAAT-3′) and crpP-R (5′-ACGAGGTGCAGTGTGTCAAA-3′), followed by sequencing of the corresponding amplicons (Microsynth, Balgach, Switzerland). A total of 46 isolates were positive for *crpP*-like genes, and 5 CrpP variants were identified, namely, CrpP1 (referring to the sequence previously reported from Mexico [7]) and 4 additional variants exhibiting few amino acid substitutions: CrpP2 (Lys4Arg and Gly7Asp), CrpP3 (Gly7Asp), CrpP4 (Gly7His, Phe16Tyr and Ile26Leu), and CrpP5 (Lys4Arg, Gly7Asp and Phe55Tyr).

Multilocus sequencing typing (MLST) was performed, and sequence type assignation was done by using the MLST database for *P. aeruginosa* (https://pubmlst.org/paeruginosa/). Sequence typing of all the *crpP*-positive isolates showed the most prevalent types to be ST235, ST111, ST233, and ST273 (24%, 17.4%, 6.5%, and 4.3%, respectively).

To evaluate the impact of the different CrpP enzymes on quinolone susceptibility, a series of cloning and expression experiments were performed. The *crpP*-like genes were cloned into shuttle vector pUCp24 using primers *crpP*-XbaI-F (5′-TCTAGAAGAGCGGG ATCGATCAGAAAT-3′) and *crpP*-SacI-R (5′-GAGCTCACGAGGTGCAGTGTGTCAAA-3′) and then transformed in both *E. coli* TOP10 and *P. aeruginosa* PAO1 that did not harbor any *crpP* gene, as described (10). MICs were determined for the different recombinant strains by the broth microdilution method using cation-adjusted Mueller-Hinton (MH) broth. This showed similar decreased susceptibility to CIP for the *E. coli* strains producing CrpP2, CrpP3, CrpP4, and CrpP5 but not CrpP1. Indeed, CrpP1 did not decrease the susceptibility of *E. coli* TOP10 to CIP, even though it was reported to do so in a previous study that used *E. coli* J53-3 as the recipient strain (7). Of note, CrpP1 did not have as much effect on quinolones as on the other variants tested. In particular, production of CrpP1 in *E. coli* TOP10 did not decrease the susceptibility to levofloxacin (LEV) and CIP. MIC values were 7.5-fold higher upon production of CrpP2, CrpP3, and CrpP5 and 4-fold higher with CrpP4. However, only a 2-fold increase in MIC of CIP was observed for the *P. aeruginosa* recombinant strain producing CrpP2, but MICs remained unchanged for the other *P. aeruginosa* recombinant strains. Instead, a 3.75-fold increase in the MIC of LEV was observed once CrpP2, CrpP3, CrpP4, and CrpP5 were produced in *E. coli* isolates, although no change was observed with CrpP1 (Table 1). The MIC of LEV was increased by 2-fold once CrpP2, CrpP3, and CrpP5 were produced in *P. aeruginosa*. *P. aeruginosa* PAO1isolates expressing CrpP2, CrpP3, and CrpP4 were resistant to LEV according to the 2020 EUCAST breakpoint value for resistance (>1 μg/ml).

To identify the genetic location of the *crpP*-like genes in the positive isolates, we performed whole-genome sequencing (WGS) on 7 of the 46 *crpP*-positive isolates as representatives of the different ST types. DNAs were extracted by use of a DNeasy

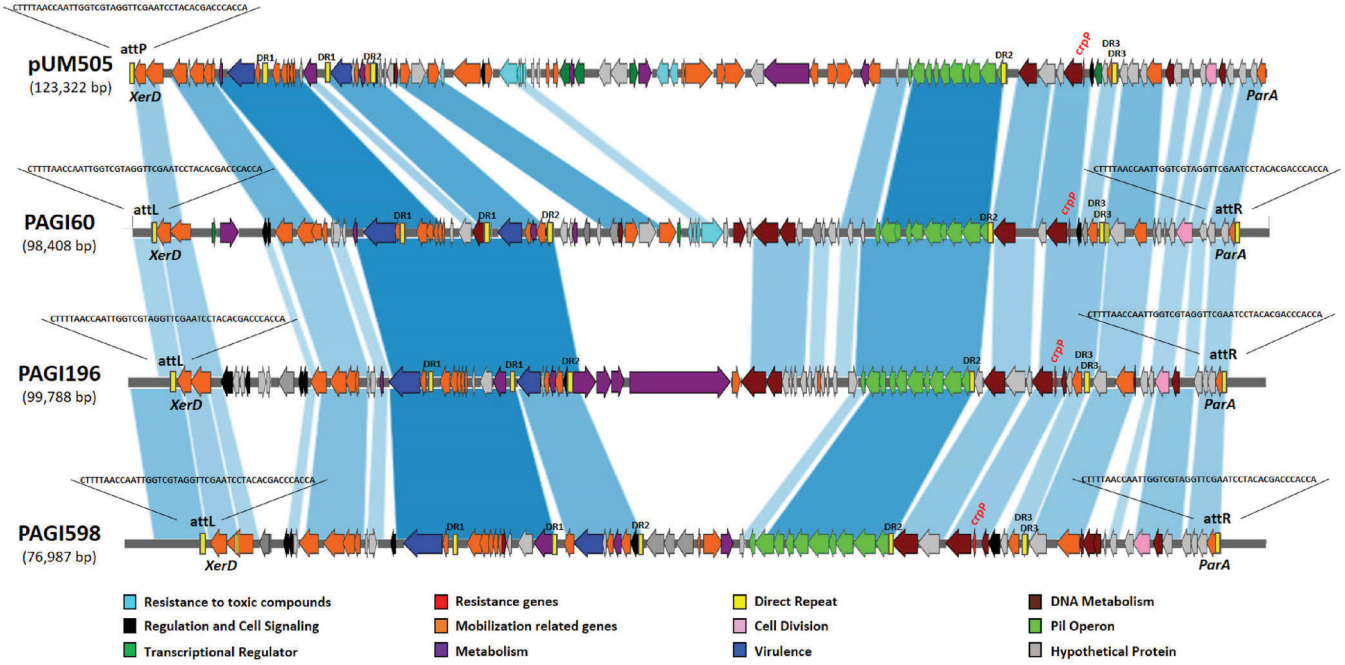

FIG 1 Sequence comparison between pUM505 and PAGIs (60, 196, and 598). Arrows and arrowheads indicate open reading frames and their direction of transcription. Shading between bars indicates portions of sequences that align with each other. The map was drawn using Kablammo software (http://kablammo.wasmuthlab.org/).

blood and tissue kit using the QIAcube apparatus (Qiagen). Genomic libraries were assessed using a Nextera XT library preparation kit (Illumina, Inc., San Diego, CA), and sequencing was performed using an Illumina MiniSeq system with 300-bp paired-end reads and a coverage of 50 times. The generated FastQ data were compiled and analyzed using the CLC Genomic Workbench (version 7.5.1; CLC Bio, Aarhus, Denmark). Reads were assembled *de novo* with automatic bubble and word size, and contigs with a minimum contig length of 800 nucleotides were generated using the mapping-mode map reads back to contigs. Analysis of WGS data showed that the *crpP*-like genes were always located on the chromosome of the corresponding isolates within large fragments (~70 to 100 kb) bracketed by two direct repeats named *attL* and *attR* of 45 bp each. At both extremities of this whole fragment, tyrosine recombinase (XerD) and ParA protein-encoding genes were identified, respectively. These two proteins are known to be linked to the mobilization and integration, respectively, of pathogenicity genomic islands (PAGI) (11). The presence of these genes together with the presence of the *attL* and *attR* direct repeats bracketing the entire DNA fragment strongly suggested that the *crpP* genes were located into chromosomal PAGI (Fig. 1). The PAGIs presented one conserved region, encompassing the *crpP* gene, the *pil* operon, a series of virulence genes, and mobilization-related genes. In addition, they actually contained a variable region, in which the same genes encoding resistance to toxic compounds as originally reported in plasmid pUM505 were detected in two isolates (R60 and R90).

To evaluate whether the PAG1-like structures are functional in terms of mobilization, PCR assays were performed to determine their putative circularization. Outward primers designed from the sequences located at the extremities of the structures, i.e., PAGIact-F (5′-ATTTCCCTACACCACCCTTG-3′) and PAGIact-R (5′-TTCGAGCAAAGAGTGCT GTT-3′), were used for that purpose. Positive PCR results were obtained, showing that a circular form of these PAGI-like elements was present and confirming their functionality as mobile structures.

Several features identified here were also found in the sequence of plasmid

pUM505. In pUM505, a single copy of the *attL* sequence was present (corresponding sequence, 15,649 to 15,693 bp); and the *parA* gene (14,687 to 15,553 bp) and *xerD* integrase gene (16,030 to 17,331 bp), both encoding proteins necessary for the mobilization of pathogenic islands, were also identified. Furthermore, because the so-called pUM505 plasmid did not harbor any replicase gene, we hypothesize that plasmid pUM505 was actually a product of the circularized form of this newly described pathogenic island in the original description (8).

In summary, we report the spread of *crpP*-like genes in a large series of *P. aeruginosa* isolates from Europe. CrpP-like enzymes, despite not conferring clinical resistance *per se* but only reduced susceptibility to fluoroquinolones once produced in *E. coli* isolates, may be considered transferable mechanisms of fluoroquinolone resistance that may further be transferred to other strains. Acquisition of these genes found in clonally unrelated *P. aeruginosa* strains was likely mediated by the acquisition of a PAGI-like element. While this work was in progress, a study performed by Ruiz (12) revealed that CrpP-like-encoding genes were frequently identified within *P. aeruginosa* genomes according to an *in silico* analysis of sequences available from GenBank, which is in line with our observations.

Interestingly, we show here that susceptibility to fluoroquinolone molecules might vary slightly depending on the nature of the CrpP protein, but without conferring a high level of resistance. Supporting our data, modifications in conserved amino acids within the CrpP protein sequence were recently reported to affect their respective enzymatic activities (13). Furthermore, apart from their impact on fluoroquinolone susceptibility, acquisition of the PAGI-like elements in *P. aeruginosa* is of significant concern, considering that many of them coharbor genes encoding resistance to heavy metals and encode genes for virulence factors (14, 15).

Accession number(s). The sequences of PAGIs have been deposited in the GenBank database under accession numbers MT074669 (PAGI-R60), MT074670 (PAGI-R90), MT074671 (PAGI-R104), MT074672 (PAGI-R135), MT074673 (PAGI-R184), MT074674 (PAGI-R196), and MT074675 (PAGI-R598). GenBank accession numbers for CrpP variants are WP_033179079 (CrpP1), WP_031644039 (CrpP2), WP_003109353 (CrpP3), WP_071533909 (CrpP4) and WP_071580329 (CrpP5).

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REFERENCES

1. Hooper DC, Jacoby GA. 2016. Topoisomerase inhibitors: fluoroquinolone mechanisms of action and resistance. Cold Spring Harb Perspect Med 6:a025320. https://doi.org/10.1101/cshperspect.a025320.
2. Martinez-Martinez L, Pascual A, Jacoby GA. 1998. Quinolone resistance from a transferable plasmid. Lancet 351:797–799. https://doi.org/10.1016/S0140-6736(97)07322-4.
3. Ruiz J, Pons MJ, Gomes C. 2012. Transferable mechanisms of quinolone resistance. Int J Antimicrob Agents 40:196–203. https://doi.org/10.1016/j.ijantimicag.2012.02.011.
4. Rehman A, Patrick WM, Lamont IL. 2019. Mechanisms of ciprofloxacin resistance in *Pseudomonas aeruginosa*: new approaches to an old problem. J Med Microbiol 68:1–10. https://doi.org/10.1099/jmm.0.000873.
5. El-Badawy MF, Alrobaian MM, Shohayeb MM, Abdelwahab SF. 2019. Investigation of six plasmid-mediated quinolone resistance genes among clinical isolates of pseudomonas: a genotypic study in Saudi Arabia. Infect Drug Resist 12:915–923. https://doi.org/10.2147/IDR.S203288.
6. Hamed SM, Elkhatib WF, El-Mahallawy HA, Helmy MM, Ashour MS, Aboshanab K. 2018. Multiple mechanisms contributing to ciprofloxacin resistance among Gram negative bacteria causing infections to cancer patients. Sci Rep 8:12268. https://doi.org/10.1038/s41598-018-30756-4.
7. Chávez-Jacobo VM, Hernández- Ramírez KC, Romo-Rodríguez P, Pérez-Gallardo RV, Campos-García J, Gutiérrez-Corona JF, García-Merinos JP, Meza-Carmen V, Silva-Sánchez J, Ramírez-Díaz MI. 2018. CrpP is a novel ciprofloxacin-modifying enzyme encoded by the *Pseudomonas aeruginosa* pUM505 plasmid. Antimicrob Agents Chemother 62:e02629-17. https://doi.org/10.1128/AAC.02629-17.
8. Ramírez-Díaz MI, Díaz-Magaña A, Meza-Carmen V, Johnstone L, Cervantes C, Rensing C. 2011. Nucleotide sequence of *Pseudomonas aeruginosa* conjugative plasmid pUM505 containing virulence and heavy-metal resistance genes. Plasmid 66:7–18. https://doi.org/10.1016/j.plasmid.2011.03.002.
9. Chavez-Jacobo VM, Hernández- Ramírez KC, Silva-Sanchez J, Garza-Ramos U, Barrios-Camacho H, Ortiz-Alvarado R, Cervantes C, Meza-Carmen V, Ramírez-Díaz MI. 2019. Prevalence of the *crpP* gene conferring decreased ciprofloxacin susceptibility in enterobacterial clinical isolates from Mexican hospitals. J Antimicrob Chemother 74:1253–1259. https://doi.org/10.1093/jac/dky562.
10. Ortiz de la Rosa JM, Nordmann P, Poirel L. 2019. ESBLs and resistance to ceftazidime/avibactam and ceftolozane/tazobactam combinations in *Escherichia coli* and *Pseudomonas aeruginosa*. J Antimicrob Chemother 74:1934–1939. https://doi.org/10.1093/jac/dkz149.
11. Qiu X, Gurkar AU, Lory S. 2006. Interstrain transfer of the large pathogenicity island (PAPI-1) of *Pseudomonas aeruginosa*. Proc Natl Acad Sci U S A 103:19830–19835. https://doi.org/10.1073/pnas.0606810104.
12. Ruiz J. 2019. CrpP, a passenger or a hidden stowaway in the *Pseudomonas aeruginosa* genome? J Antimicrob Chemother 74:3397–3399. https://doi.org/10.1093/jac/dkz316.
13. Chávez-Jacobo VM, Garcia Merinos JP, López Y, Meza-Carmen V,

Ramírez-Diaz MI. 2020. Identification of essential residues for ciprofloxacin resistance of ciprofloxacin-modifying enzyme (CrpP) of pUM505. Microbiology https://doi.org/10.1099/mic.0.000889.
14. Hernández-Ramírez KC, Chávez-Jacobo VM, Valle-Maldonado MI, Patiño-Medina JA, Díaz-Pérez SP, Jácome-Galarza IE, Ortiz-Alvarado R, Meza-Carmen V, Ramírez-Díaz MI. 2017. Plasmid pUM505 encodes a toxin-antitoxin system conferring plasmid stability and increased *Pseudomonas aeruginosa* virulence. Microb Pathog 112:259–268. https://doi.org/10.1016/j.micpath.2017.09.060.
15. Hernández-Ramírez KC, Reyes-Gallegos RI, Chávez-Jacobo VM, Díaz-Magaña A, Meza-Carmen V, Ramírez-Díaz MI. 2018. A plasmid-encoded mobile genetic element from *Pseudomonas aeruginosa* that confers heavy metal resistance and virulence. Plasmid 98:15–22. https://doi.org/10.1016/j.plasmid.2018.07.003.