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Letter to the Editor

High-copy IncP-2 megaplasmid carrying *bla*$_{AFM}$ in clinical difficult-to-treat resistance *Pseudomonas aeruginosa*: Associated with high-level cefiderocol resistance

Dear Editor,

We read with interest the article by Soueges S and colleagues, which described that a novel siderophore cephalosporin, cefiderocol (FDC), has demonstrated strong antibacterial efficacy against gram-negative bacteria.[1] Since the high susceptibility rate among Difficult-to-Treat Resistance *Pseudomonas aeruginosa* (DTR-PA),[2] FDC showed a promising clinical application prospects.

Currently, there are limited reports on low-level FDC resistance (MIC=4–16 mg/L) in clinical *P. aeruginosa*.[3] The knowledge regarding high-level FDC resistance (MIC > 128 mg/L) in *P. aeruginosa* is derived from in vitro induction experiments, which may not fully reflect clinical conditions.[4] In this study, we identified 5 FDC-resistant clinical strains producing *Alcaligenes faecalis* metallo-beta-lactamase (AFM) from 225 carbapenem-resistant *P. aeruginosa* (CRPA) isolates collected between 2016 and 2023, prior to the implementation of FDC in China (Fig. S1). All these 5 FDC-resistant strains were resistant or intermediate to other commonly used antibiotics except for colistin, which could be classified as DTR-PA (Table S1). ZYPA162 and ZYPA192, belonging to ST275, exhibited high-level resistance to FDC (MIC > 256 mg/L). SRMPA3860 (ST262), SRMPA1914 (ST262), and WTJH17 (ST260) showed low-level resistance to FDC (MIC = 4 mg/L). The two high-level FDC-resistant strains were both isolated from the same clinical patient (post-duodenectomy peritoneal infection) at different time from peritoneal fluid (Fig. 1A). Genome sequence analysis revealed only 6 SNPs between ZYPA162 and ZYPA192 (ST275) (Fig. S1).

All these 5 FDC-resistant clinical CRPA strains were found to carry IncP-2 megaplasmid harboring *bla*$_{AFM}$. We further performed plasmid curing, conjugation, and *bla*$_{AFM}$ cloning experiments, as well as molecular simulation docking between AFM-2 protein (predicted by Alphafold3) and FDC (Fig. 1B, C). Conjugation experiment results revealed that the plasmids from all three low-level resistant strains including SRMPA3860, SRMPA1914, and WTJH17, were conjugable with very low conjugation efficiency ($P < 2 \times 10^{8}$) (Fig. 1D). In contrast, plasmids from high-level resistant strains (ZYPA162 and ZYPA192) were non-conjugable. Comparative sequence analysis showed that pZYPA162 and pZYPA192 lacked conjugative transfer genes (Fig. S2). Following plasmid curing, the MIC of FDC for plasmid-cured strain 162D decreased to 0.0625 mg/L, and for plasmid-cured strain 3860D decreased to 0.125 mg/L, representing an over 32-fold reduction. Based on our preliminary research methods,[5,6] we cloned four reported *bla*$_{AFM}$ subtypes into the pGK1900 vector as described in the literature. Complementation with the pGK_AFM-2 plasmid resulted in a FDC MIC increase to 32 mg/L for both 162D/pGK_AFM-2 and 3860D/pGK_AFM-2, and to 16 mg/L for PAO1. Due to the concentration of subtype mutations in *bla*$_{AFM}$ in the N-terminal signal peptide region, its resistance level to FDC was similar (Fig. S3). Molecular docking results indicated that the binding free energy between AFM-2 and FDC was −9.3 kcal/mol (Fig. 1C).

The expression level of *bla*$_{AFM}$ was significantly higher in the high-level resistant strain ZYPA162 compared to SRMPA3860 ($p < 0.001$) determined by RT-qPCR and RNA-seq (Fig. 1E). Since all *bla*$_{AFM}$ in this study were all located on the IncP-2 plasmid, we further analyzed 100 IncP-2 plasmid sequences from the NCBI database. A prominent characteristic of global IncP-2 plasmids was the high prevalence of carbapenem resistance genes (74%), with metallo-β-lactamase genes being the most frequent (66%) (Fig. S2). The analysis of gene copy numbers revealed that the plasmid pZYPA162 exhibited a significantly higher copy number compared to pSRMPA3860 ($p < 0.001$) (Fig. 1E). Notably, no significant differences were observed between the copy number of the *bla*$_{AFM}$ gene cluster and the plasmid replicon gene within each strain. Since the genetic environment surrounding *bla*$_{AFM}$ was consistent across all plasmids with the same promoter sequence,[5,6] we speculated that the differential expression level of *bla*$_{AFM}$ was attributable to variations in plasmid copy number.

Due to research suggesting that the production of siderophore could affect the MIC value of FDC,[4] we used a special Chrome Azurole S(CAS) medium to determine the production of siderophore. The siderophore production of ZYPA162 was lower than that observed in SRMPA3860 ($p < 0.001$). However, the FDC-induced resistant strain SRMPA3860_FDC512 exhibited a significantly increased siderophore production compared to its wild-type (WT) strain, SRMPA3860 ($p < 0.001$) (Fig. S2). No significant differences were detected in the expression levels of TonB-dependent transporters (TBDTs) and siderophore between ZYPA162 and SRMPA3860. In contrast, all TBDT-related genes were significantly upregulated in SRMPA3860_FDC512 compared to SRMPA3860 (Fig. S4). It seems that the pressure of FDC may lead to different resistance strategies.

In addition to the rare high-level resistance to FDC, ZYPA162 also displayed a distinct small colony variant (SCV) phenotype (Fig. S2). In contrast, its plasmid-cured strain, 162D, exhibited restored normal colony morphology. Furthermore, ZYPA162 demonstrated significantly reduced swarming, twitching, and swimming motilities compared to 162D ($p < 0.001$) (Fig. 1F). The biofilm formation ability of the plasmid-cured strain 162D increased significantly ($p < 0.001$) (Fig. 1D). In *Galleria mellonella* model, ZYPA162 displayed higher virulence than 162D ($p < 0.001$) (Fig. 1G). In 162D, *lasI* expression was significantly upregulated, leading to enhanced activation of the Rhl quorum sensing system, which affected biofilm formation and motility according to a previous study.[7] Compared to 162D, the

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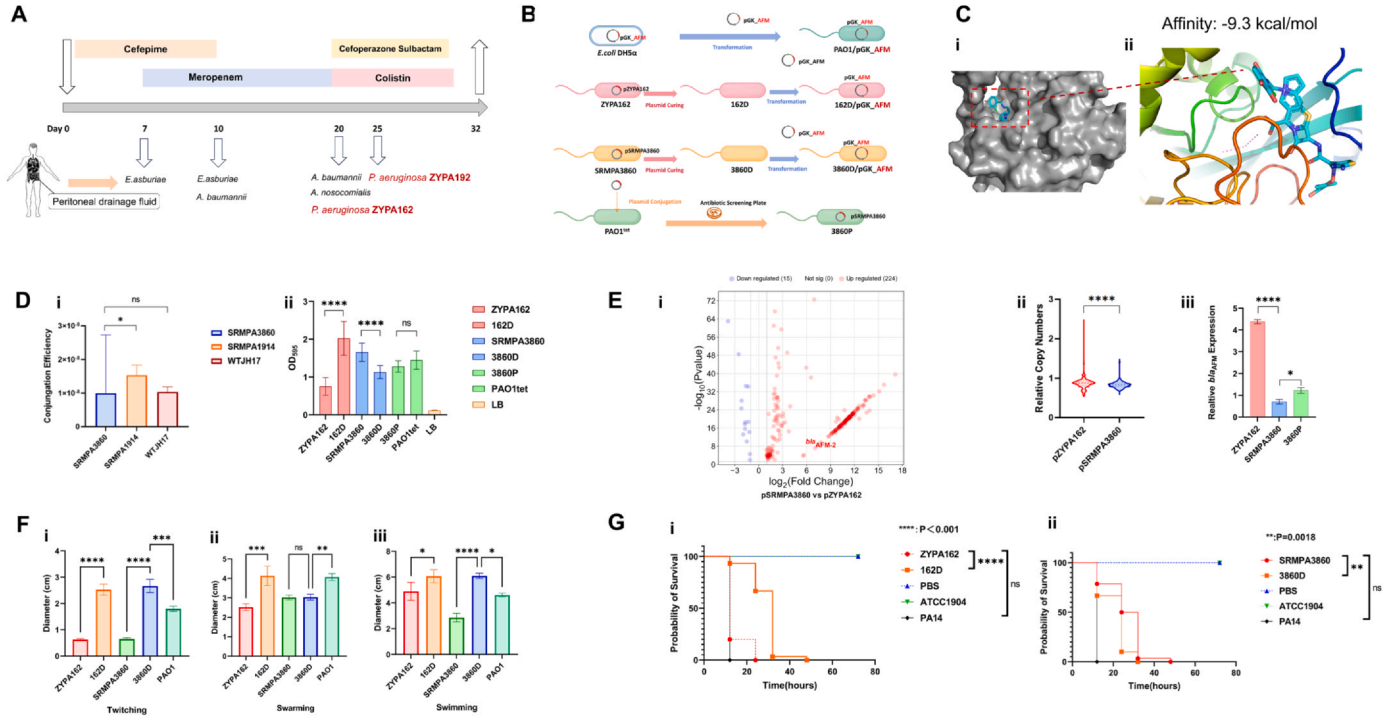

Fig. 1. A. High-level FDC resistant strains antibiotic use history. The patient was a 64-year-old male who developed an infection after a duodenal repair surgery, with multiple pathogens isolated from the peritoneal fluid; B. A brief introduction of plasmid experiments. We cloned four bla_{AFM} subtypes into the pGK1900 vector and complemented them into PAO1 and clinical plasmid-cured strains. Tetracycline-resistant $PAO1_{tet}$ was used as the recipient strain. Conjugation experiments were performed on selection plates containing 800 mg/L tetracycline and 128 mg/L ceftazidime; C. The binding pocket of FDC with AFM predicted by Autodock (i). The binding free energy between FDC and AFM was approximately −9.3 kcal/mol (ii); D. The conjugation efficiency of clinical plasmids was relatively low (i). After plasmid curing, the biofilm formation of ZYPA162 was significantly increased (ii); E. The expression level of bla_{AFM} in ZYPA162 was very high (i), which might be due to the high copy number of the plasmid it carried (ii). This resulted in higher expression compared to SRMPA3860 and 3860P (iii), leading to a high level of resistance to FDC; F. The motility ability of the strain. The motility of the plasmid-cured strain 162D was significantly higher than that of the wild-type strain ZYPA162; G. The virulence of the plasmid-cured strain 162D was significantly lower than that of the wild-type strain ZYPA162, but this phenomenon was not observed in SRMPA3860.

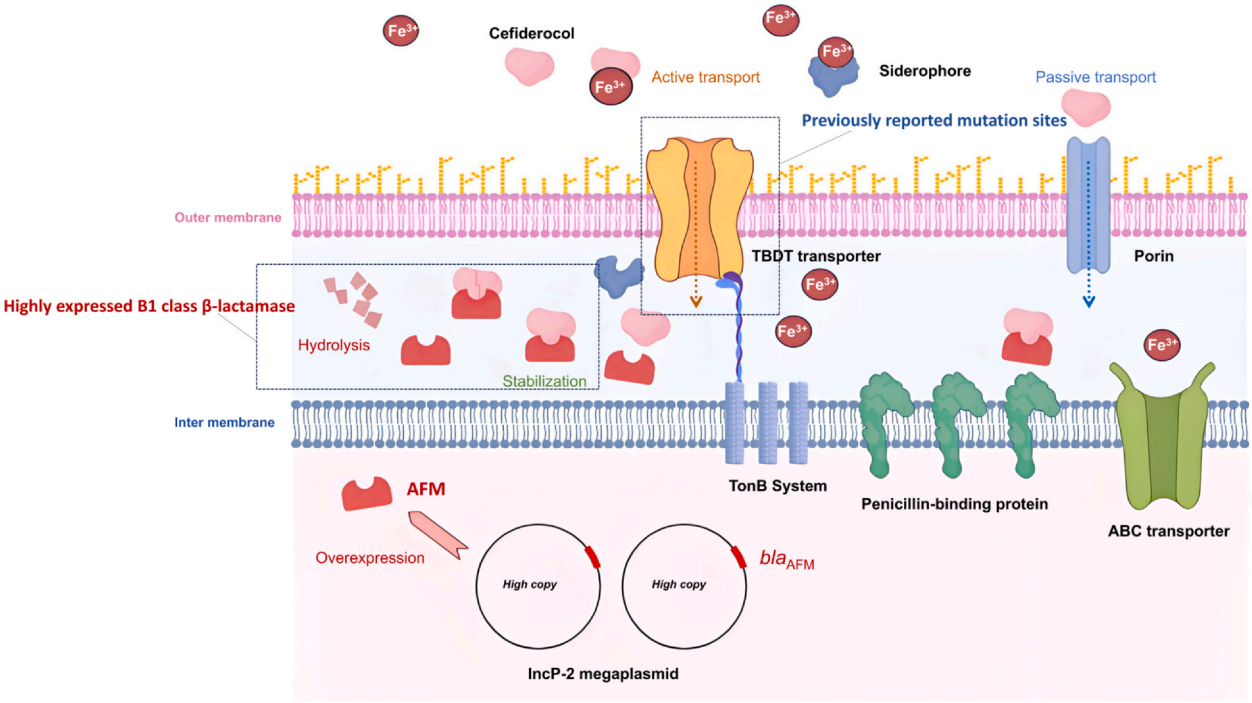

Fig. 2. Pattern diagram of high-level FDC resistant *P. aeruginosa*: overexpression of bla_{AFM} caused by high copy IncP-2 megaplasmid. High levels of AFM hydrolyze FDC that entered the bacterial periplasmic space.

expression levels of the type III secretion system (T3SS) effector components *exoS/T/Y* and *exsB/C/D/E* were significantly increased in the WT strain ZYPA162 ($p < 0.0001$) (Fig. S7). Due to the main virulence factors of *P. aeruginosa* was the T3SS effector,[8] we speculated that the presence of plasmid pZYPA162 increased the production of these effectors, thereby enhancing the virulence of the strain. The association mechanism between megaplasmid, SCV, and *lasI* requires further investigation.

For the first time, we have identified high-level FDC resistance in AFM-producing DTR-PA strains in clinical patients. The resistance was attributed to the increased copy number of IncP-2 megaplasmids, which significantly elevated the expression of bla_{AFM} (Fig. 2B). The plasmid affected the bacteria's virulence and led to SCV phenotype, through the regulation of the *lasI* gene, which affected the Rhl system and T3SS effectors. Clinical monitoring of AFM-producing DTR-PA is essential to mitigate the dissemination of these strains.

Ethical approval

This study has been reviewed by Clinical Research Ethics Committe of the First Affliated Hospital, Zhejiang University School of Medicine, and the reference number is IIT20210120B.

Sequence information

I have deposited the sequences to NCBI database (BioProject Number: PRJNA1001145).

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Declaration of Competing Interest

No potential conflict of interest was reported by the author(s).

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jinf.2025.106422.

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