**Identification of potential antimicrobial targets of *Pseudomonas aeruginosa* biofilms through a novel screening approach**

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**Abstract**

*Pseudomonas aeruginosa* is an opportunistic pathogen of considerable medical importance owing to its pronounced antibiotic tolerance and association with cystic fibrosis and other life-threatening diseases. The aim of this study was to highlight the genes responsible for *P. aeruginosa* biofilm tolerance to antibiotics and thereby identify potential new targets for the development of drugs against biofilm-related infections. By developing a novel screening approach and utilizing a public *P. aeruginosa* transposon insertion library, several biofilm relevant genes were identified. The Pf phage gene(*PA0720*) and flagellin gene (*fliC*) conferred biofilm-specific tolerance to gentamicin. Compared with the reference biofilms, the biofilms formed by *PA0720* and *fliC* mutants were completely eliminated with a fourfold lower gentamicin concentration. Furthermore, the *mreC*, *pprB*, *coxC*, and *PA3785* genes were demonstrated to play major roles in enhancing biofilm tolerance to gentamicin. The analysis of biofilm-relevant genes performed in this study provides important novel insights into the understanding of *P. aeruginosa* antibiotic tolerance, which will facilitate the detection of antibiotic resistance and the development of anti-biofilm strategies against *P. aeruginosa*.

**Importance**

*Pseudomonas aeruginosa* is an opportunistic pathogen of high medical importance and is one of the main pathogens responsible for the mortality of patients with cystic fibrosis. In addition to inherited antibiotic resistance, *P. aeruginosa* can form biofilms, defined as surface-attached aggregates of bacterial cells embedded in a self-produced matrix of polymeric substances. Biofilms protect bacteria from antibiotic treatments and represent a major reason for antibiotic failure in the treatment of chronic infections caused by cystic fibrosis. Therefore, it is crucial to develop new therapeutic strategies aimed at specifically eradicating biofilms. The aim of this study was to generalize a novel screening method for biofilm research and to identify the possible genes involved in *P. aeruginosa* biofilm tolerance to antibiotics, both of which can improve the understanding of biofilm-related infections and allow for the identification of relevant therapeutic targets for drug development.

**Keywords:** Biofilm formation, biofilm tolerance, PA0720, FliC, PA3785

**Introduction**

Since the 1950s, antimicrobial drugs have been mainly developed in screens using planktonic bacteria (1). However, more than 80% of human chronic infections are associated with biofilms (2). Biofilms are communities of microorganisms embedded in a self-produced matrix of extracellular polymeric substances adhering to each other and/or to a surface (2). Sessile bacteria in biofilms are protected from immune system defenses and can tolerate up to 1000 times higher antibiotic concentrations than planktonic cells, requiring doses that cannot be administered in humans (3). New therapeutic options are urgently needed to combat biofilm-related infections, especially those caused by the multi-drug resistant pathogen *Pseudomonas aeruginosa* (4, 5). We hypothesized that studying biofilm-relevant genes can advance the mechanistic understanding of *P. aeruginosa* biofilm tolerance to antibiotics, and highlight new targets for anti-biofilm drug development.

Biofilm-relevant genes were selected from a transcriptomic study by Whiteley *et al*., in which 73 genes were upregulated or downregulated by more than twofold in *P. aeruginosa* biofilms compared with planktonic cells (6). Of the 73 genes, 42 were functionally characterized using a *P. aeruginosa* MPAO1 transposon mutant library (7) and our *in vitro* assay system (8, 9) (see **Table S1** for additional information on the selected transposon mutants). The antibiotic tolerance of *P. aeruginosa* biofilms was tested using gentamicin, an aminoglycoside commonly used to treat *P. aeruginosa* infections (10) (**Figure 1 and Table 1**), and the last-resort antibiotic colistin (**Figure S1**; see detailed results in **Figures S2** **to** **S4**). To account for the potential influence of the transposon Tn5 background (9), the MPAO1 mutant missing the *fiuA* and *arnB* genes, which encode for heterologous siderophores (11) and colistin resistance (12), respectively, were selected as control reference strains. As previously shown (9, 13), the inactivation of *fiuA* or *arnB* in *P. aeruginosa* MPAO1 did not impact biofilm formation and tolerance toward gentamicin and led to phenotypes representative of most analyzed mutants (**Figure 1**).

**Mutants with increased biofilm formation and tolerance to gentamicin**

The screening identified several genes that promote biofilm formation and tolerance to gentamicin once inactivated. Mutations in the genes encoding the rod shape-determining protein MreC (14), the response regulator PprB (15), and the cytochrome c oxidase subunit CoxC (16) increased the *P. aeruginosa* biofilm biomass by approximately fourfold compared with the reference mutant (**Figure 1A**). These three mutants also showed high biofilm recovery after treatment with 100 μg/mL gentamicin (**Figure 1B**). Further characterizations revealed similar minimal bactericidal concentrations of biofilms (MBC-B) of gentamicin for *fiuA* and *pprB* mutants (**Table 1**) but a significantly higher recovery of *pprB* mutants after exposure to a sub-MBC-B of gentamicin (**Figures S2 and S3**). The latter observation concurs with previous studies which showed that *pprB* overexpression increases membrane permeability and aminoglycoside susceptibility (15). The *coxC* mutant exhibited a lower MBC-B of gentamicin than the *fiuA* mutant (**Table 1**) and a similar MBC of planktonic cells (MBC-P) and higher biofilm recovery after exposure to a sub-MBC-B of gentamicin. Inactivating the genes encoding the aa3-type cytochrome c oxidase (i.e., *coxB* and *coxA*) did not influence biofilm formation or tolerance to gentamicin (**Figure 1**). Extensive research is needed to decipher the precise roles of *mreC*, *pprB*, and *coxC* in antibiotic resistance and biofilm formation. However, our results suggest that the decreased *pprB* and *coxC* expression levels in *P. aeruginosa* biofilms (6) represent an active mechanism of tolerance against gentamicin.

**Mutants with altered tolerance to gentamicin but unchanged biofilm formation**

Our screening results revealed that the inactivation of the hypothetical protein PA3785, the bacteriophage protein PA0720, and the flagellin FliC significantly altered biofilm tolerance to gentamicin independently to biofilm biomass, planktonic resistance and growth rate (**Figure 1 and S4 and Table 1**).

The conserved hypothetical protein encoded by the *PA3785* gene appeared to be important for biofilm tolerance to gentamicin (**Figure 1B**). Despite the unchanged MBC-B value, *PA3785* mutant biofilms exhibited the highest recovery among all tested mutants after exposure to a sub-MBC-B of gentamicin (**Figure 1B and S2**). The *PA3785* gene was downregulated in *P. aeruginosa* biofilms compared with planktonic cells and upregulated fivefold higher in tobramycin-treated biofilms than in untreated biofilms (6). Its exact function remains unknown, and its role in *P. aeruginosa* remains to be elucidated through further research.

Filamentous Pf1-like bacteriophages (Pf phages) play major roles in biofilm physiology and antibiotic tolerance (17, 18) and correlate with increased antibiotic resistance in *P. aeruginosa* isolates from patients with cystic fibrosis (CF) (19). Encoding a single-stranded DNA binding protein, *PA0720* is part of the Pf phage operon integrated in the *P. aeruginosa* genome (20). Our study suggests that *PA0720* can confer biofilm-mediated tolerance of *P. aeruginosa* MPAO1 to gentamicin. Inactivating *PA0720* did not impact the planktonic resistance toward gentamicin, but led to a fourfold decrease in MBC-B (**Table 1**). Gentamicin tolerance was only reduced by inactivating *PA0720* but not *PA0728*, which is essential to produce Pf phages (21), or any other Pf phage genes (**Figure 1B**). Therefore, these results highlight the potential role of *PA0720* in *P. aeruginosa* physiology besides its role in Pf phage production. It is especially interesting that several transcriptomic and proteomic studies have found *PA0720* to be one of the few genes systematically upregulated in *P. aeruginosa* biofilms (6, 22–24). In summary, *PA0720* represents a promising target for drug development and has potential value as a clinically relevant marker for prediction of *P. aeruginosa* biofilm tolerance to gentamicin.

Our screening further revealed that the inactivation of *fliC*, which encodes flagellin type B, decreased the *P. aeruginosa* biofilm tolerance to gentamicin (**Figures 1B and S3**). The MBC-B of gentamicin was fourfold lower for the *fliC* mutant (400 µg/mL) than for the *fiuA* mutant (1600 µg/mL), while the MBC-P was similar (**Table 1**). The biofilm tolerance to colistin was not affected by the inactivation of *fliC* (**Figure S1**), which suggests a tolerance mechanism specific to gentamicin. In contrast to other motility gene mutants, *fliC* inactivation did not reduce the biofilm biomass of *P. aeruginosa* (**Figure 1A**), agreeing with a previous study which showed that the non-motile *fliC* mutant produced higher biofilm biomass owing to an increased ability to adhere on abiotic surfaces than the wild-type (25). *FliC* was downregulated in biofilms (6) and chronic CF infections (26), which was attributed to an adaptive response to avoid phagocytic recognition and clearance (26). We hypothesize that *fliC* repression contributes to the biofilm-specific tolerance to antibiotics. However, further work is required to understand the precise role of FliC in biofilm physiology and assess its potential value for developing anti-biofilm strategies.

**Mutants with altered biofilm formation but unchanged antibiotic tolerance**

The potential link between the AMR phenotype and biofilm production is controversial (27, 28). Our results reveal that no clear correlation exists. Some mutants showed antibiotic tolerance relating to higher biofilm production, whereas others did not follow this trend (**Figure 1C**). Concurring with a previous study (25), our screening shows that compared with the reference mutant, the mutations in the motility genes (*fliD*, *cupA1*, *cupA2*, and *pilA*) led to significantly less biofilm biomass (**Figure 1A**) but did not alter biofilm tolerance to gentamicin (**Figure 1B**). Moreover, mutation in the sigma factor RpoS (29) increased the biofilm biomass by 250% (**Figure 1A**), but did not increase tolerance to gentamicin (**Figure 1B**) and displayed high sensitivity to colistin compared with the reference mutant (**Figure S1**). These results suggest that biofilm biomass alone is not a good indicator for AMR phenotype.

**Conclusion**

This study developed a novel screening method for biofilm research and identified candidate genes involved in biofilm antibiotic tolerance, thereby improving the understanding of biofilm-related infections and identifying relevant therapeutic targets. The screening results suggest that the level of biofilm biomass or planktonic cell resistance of a given strain is not a strong indicator of antibiotic failure. The inactivation of the Pf phage *PA0720* and flagellin *fliC* significantly reduced the gentamicin tolerance of *P. aeruginosa* biofilms, without impacting the biofilm biomass or MBC-P. This study discovered that novel factors such as *pprB*, *coxC*, and *PA3785* are involved in the gentamicin tolerance of *P. aeruginosa* biofilms. Thus, it highlights promising targets to develop anti-biofilm treatments and relevant markers to predict gentamicin failure in the treatment of biofilm infections. The transposon mutant phenotypes remain to be confirmed with knockout strains. The present study highlights potential leads for future research in biofilms physiology and anti-biofilm treatment.

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**Competing interests**

The authors declare no competing interest.

**Author contributions**

J.D.P.V., H.C.M. and Q.R. designed the project experiments. J.D.P.V. and S.A. performed experiments. J.D.P.V., Q.R. and H.C.M. interpreted the data with the contribution of A.R.V., C.H.A., F.S. and J.S.W. The manuscript was written by J.D.P.V. and Q.R. with scientific revision from A.R.V., C.H.A., F.S., J.S.W. and H.C.M.

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**Figure Legends**



**Figure 1.** Influence of biofilm-associated genes on (A) biofilm formation and (B) biofilm tolerance to gentamicin in *P. aeruginosa* MPAO1. (A) Biofilm biomass was quantified using crystal violet (CV) staining after 24 h growth in M9 medium under static conditions at 37°C. (B) Biofilm tolerance to gentamicin was quantified by measuring the turbidity of the biofilm suspension after 24 h gentamicin treatment at 100 μg/mL and 24 h recovery in fresh M9 medium. Biofilm recovery was expressed relative to untreated biofilms (defined as 100%). The results represent the mean ± standard deviation (SD) of two independent biological repeats (three for the *fiuA*, *arnB*, and *PA0720* mutants), with (A) eight and (B) four technical repeats each. Student *t*-tests were performed with \*\* *P* < 0.01 and \*\*\* *P* < 0.001, using the biomass and recovery of the *fiuA* mutant as references. The arrows in front of each gene indicate whether the gene is upregulated (green) or downregulated (red) in *P. aeruginosa* biofilm cells compared with planktonic cells (6)*.* (C) The phenotypic distribution of all tested *P. aeruginosa* mutants (obtained by combining the results from A and B). The green symbols represent the mutants used as reference. The red symbols indicate mutants with significantly different biofilm tolerance to gentamicin, compared with the reference *fiuA* mutant, with *P* < 0.001.

**Table 1.** Gentamicin susceptibility of the biofilm and planktonic cells of *P. aeruginosa* MPAO1 transposon mutants missing a functional *fiuA*, *PA0720*, *fliC*, *coxC*, *pprB* or *PA3785* gene. The MBC of gentamicin for planktonic cells (MBC-P) was determined by spotting the cell suspension on brain heart infusion (BHI) agar after gentamicin treatment. The MBC of gentamicin for biofilm cells (MBC-B) was determined by spotting the cell suspension on BHI agar after gentamicin treatment and recovery. Results are presented as the mean from two independent experiments with two technical repeats each.

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| Gene inactivated in *P. aeruginosa* MPAO1 | MBC-P of gentamicin (μg/mL) | MBC-B of gentamicin (μg/mL) |
| *fiuA* | 4 | 1600 |
| *PA0720* | 4-8 | 400 |
| *fliC* | 4-8 | 400 |
| *coxC* | 4 | 800 |
| *pprB* | 8 | 1600 |
| *PA3785* | - | 1600 |