

The Role of PaFicT in *Pseudomonas aeruginosa* Persister Cell Formation

Dawson Fogen^{1, 2*}

1. Department of Microbiology, University of Hawaii at Manoa, Honolulu, Hawaii 96822, USA.

2. Department of Molecular Biosciences and Bioengineering, University of Hawaii at Manoa, Honolulu, Hawaii 96822, USA.

Submitted 19 October 2021; Accepted 9 April 2022; Published 6 June 2022

The opportunistic pathogen *Pseudomonas aeruginosa* (*Pa*) is a major concern for immunocompromised and cystic fibrosis patients. Chronic lung infections caused by *Pa* are generally considered incurable, in part, due to the bacteria's ability to form persister cells. These variants are categorized as being phenotypically dormant and highly tolerant to antibiotic treatment. Currently, the mechanisms involved in *Pa* persister cell formation is poorly understood. One promising candidate is the *Pa* filamentation induced by cAMP (FIC) domain containing toxin (PaFicT), which like other FIC toxins transiently inhibits cell growth. Genetic knockout and complementation by single copy chromosomal insertion was used to characterize *paficT* involvement in *Pa* persister cell formation. Toxicity and the PaFicT active site were examined by overexpression of wild-type and mutant protein variants. Antibiotic tolerance of PaFicT-induced *Pa* persister cells, was measured by minimum inhibitory concentration (MIC) analysis and compared to parental mostly non-persister populations. Deletion of *paficT* resulted in a 7.2-fold reduction in persister cell formation, which was fully complemented by re-insertion of the gene. Expression of PaFicT significantly increased persister cell formation by 5.9-fold, and this phenotype required a functional FIC active site motif. Unlike growing cell populations, PaFicT-induced persister cells were unaffected by 4 h treatment with $10 \times$ MIC meropenem and showed an increased survival of 6.2×10^5 -fold to tobramycin under the same conditions. Alternatively, survival of both persisters and parental, mostly non-persister, populations were below detectable levels following amikacin treatment. Results indicate a potential major involvement of PaFicT in *Pa* persister cell formation and multidrug tolerance.

Key words: Drug resistance, *Pseudomonas*, cystic fibrosis

The opportunistic pathogen *Pseudomonas aeruginosa* (*Pa*) is the primary causative agent of lung infection and mortality in cystic fibrosis (CF) patients (1). During infection of the CF lung, *Pa* produces increased numbers of persister cells (2). These specialized cells are

* Corresponding author: Department of Microbiology, University of Hawaii at Manoa, Honolulu, Hawaii 96822, USA.
Email: dfogen@hawaii.edu

This work is published as an open access article distributed under the terms of the Creative Commons Attribution 4.0 License (<http://creativecommons.org/licenses/by-nc/4/>). Non-commercial uses of the work are permitted, provided the original work is properly cited.

phenotypically dormant variants able to survive antibiotic treatment (3). Although aspects of persister cells are beginning to be revealed, the mechanisms involved in the formation of these variants remains unclear. In *Pa*, it has been shown that disruption of various global regulators (RNA polymerase-binding transcription factor DksA, alternative sigma factors RpoS and RpoN, (p)ppGpp synthesis SpoT and RelA), the carbamoyl phosphate synthetase CarB, and the putative D-N-acetylase DnpA, all affect persister cell formation (4–8). However, an emerging mechanism implicated in persister cell formation involves toxin-antitoxin (TA) systems (3). In this model, bacteria are thought to enter a dormant, persister state, through the inactivation of essential cellular process by the toxin, which can be reversed or inhibited by the antitoxin (9). While the role of TA systems in *Pa* persister cell formation is unknown, one promising candidate is PaFicT. Harms and colleagues showed that PaFicT, and other FIC toxins, AMPylate the ATP-binding site of DNA gyrase and topoisomerase IV, thereby inhibiting their biological function (10). The same study showed that ectopic expression of FIC toxins causes reversible inhibition of cell growth by disrupting DNA topology (10).

In this study, the role of PaFicT in *Pa* persister cell formation was characterized. Results detailed here provide the first direct evidence for the involvement of PaFicT in *Pa* persister formation. Deletion of *paficT* from the genome significantly decreased persister cell formation during colony biofilm growth, a phenotype fully complemented by re-introduction of *paficT* back into the chromosome. Furthermore, overexpression of PaFicT significantly increased persister cell formation through the catalytic activity of the FIC domain, and these variants were highly tolerant to clinically relevant antibiotics.

Materials and methods

Bacterial strains and culturing conditions

A list of strains utilized throughout this manuscript are shown in Table 1. *Pa* strains and DNA fragments were derived from the PAO1 sequenced strain (11). Unless otherwise indicated bacteria were cultured under aerobic conditions (200 rpm) at 37 °C in Luria-Bertani (LB) broth or LB agar. Electrocompetent *E. coli* and *Pa* cells were prepared in 1mM HEPES and 1 mM MgSO₄, respectively. When required, antibiotics were added to the media for selection and plasmid maintenance as follows: for *E. coli*, 100 µg/ml ampicillin or 15 µg/ml gentamycin was used. For *Pa*, 150 µg/ml gentamycin, 50 µg/ml triclosan or 250 µg/ml carbenicillin was used. Unless otherwise indicated *E. coli* strains containing inducible *paficT* or its derivatives were grown in media supplemented with 1% (wt/v) glucose to limit leaky expression. Prior to experimentation, single colonies were cultured without antibiotics at 37 °C, 200 rpm in 3 ml LB broth or biofilm minimal medium (BMM) (12).

For colony biofilm growth, *Pa* strains were grown to mid-log phase in LB broth, harvested by centrifugation (2 min at 10,000 × *g*), resuspended and washed three times with BMM. Cell suspensions were diluted in fresh BMM to OD₆₀₀ of 0.4. Colony biofilm was generated by spotting 10 µl of diluted culture on a 12-well clear flat bottom multiwell culture plate containing 2 ml BMM and 1% (w/v) agar per well and incubating at 30 °C for 48 h.

Strain construction

Table 1 represents detailed list and notes on strains constructed and utilized in this study. Briefly, an unmarked deletion of *paficT* in the PAO1 genome (PA-Δ*paficT*) was generated using the previously described Flp-*FRT* recombination system (13). The complemented strain (PA-Δ*paficT*_{comp}) was achieved by inserting the native promoter-driven *paficT* gene back into the genome of PA-Δ*paficT* using the same method. Generation

Table 1. Strains used in this study

Strain	Genotype [description]	Reference
<i>P. aeruginosa</i>		
PAO1 (PA-wt)	PAO1 [prototroph]	(11)
PA- Δ <i>paficT</i>	PAO1- Δ <i>paficT</i> :FRT [unmarked deletion of <i>paficT</i>]	This study
PA- Δ <i>paficT</i> -comp	PAO1- Δ <i>paficT</i> :FRT/attTn7: <i>P_{nat}</i> - <i>paficTA</i> -Gm ^R [mutant containing native promoter driven <i>paficTA</i>]	This study
PA- <i>paficT</i> - <i>P_{lac}</i>	PAO1- Δ <i>paficT</i> :FRT/attTn7: <i>lacI</i> - <i>P_{lac}</i> - <i>paficT</i> -Gm ^R [mutant containing inducible <i>paficT</i>]	This study
PA- <i>paficT</i> (H136A)- <i>P_{lac}</i>	PAO1- Δ <i>paficT</i> :FRT/attTn7: <i>lacI</i> - <i>P_{lac}</i> - <i>paficT</i> (H136A)-Gm ^R [mutant containing inducible <i>paficT</i> (H136A)]	This study
<i>E. coli</i>		
EPMaX10B	F- λ - <i>mcrA</i> Δ (<i>mrr</i> - <i>hsdRMS</i> - <i>mcrBC</i>) ϕ 80 <i>dlacZ</i> Δ M15 Δ <i>lacX</i> 74 <i>deoR</i> <i>recA1</i> <i>endA1</i> <i>araD</i> 139 Δ (<i>ara</i> , <i>leu</i>)7697 <i>galU</i> <i>galK</i> <i>rpsL</i> <i>nupG</i> [restriction system and endonuclease deficient competent cells]	Bio-Rad
EC- <i>paficT</i> - <i>P_{lac}</i>	EPMaX10B/attTn7: <i>lacI</i> - <i>P_{lac}</i> - <i>paficT</i> -Gm ^R [EPMaX10B containing inducible <i>paficT</i>]	This study
EC- <i>paficT</i> (H136A)- <i>P_{lac}</i>	EPMaX10B/attTn7: <i>lacI</i> - <i>P_{lac}</i> - <i>paficT</i> (H136A)-Gm ^R [EPMaX10B containing inducible <i>paficT</i> (H136A)]	This study
EC- <i>paficT</i> (H180A)- <i>P_{lac}</i>	EPMaX10B/attTn7: <i>lacI</i> - <i>P_{lac}</i> - <i>paficT</i> (H180A)-Gm ^R [EPMaX10B containing inducible <i>paficT</i> (H180A)]	This study

wt: wild-type; Gm^R: gentamicin resistant; *P_{nat}*: native promoter; *lacI*: lac repressor; *P_{lac}*: lac promoter.

of a controllable *paficT* expression strain (PA-*paficT*-*P_{lac}*) was done by first constructing and then inserting an inducible *paficT* fragment (*lacI*-*P_{lac}*-*paficT*) into the PA- Δ *paficT* genome via the mini-Tn7 based integration system (14). Site-directed mutagenesis of PaFicT residue His-136 to Ala-136 (PA-*paficT*(H136A)-*P_{lac}*) was performed using the Single-Primer Reactions IN Parallel method (15). Construction of *E. coli* strains with inducible chromosomal expression of *paficT* or its derivatives (EC-*paficT*-*P_{lac}*, EC-*paficT*(H136A)-*P_{lac}*, and EC-*paficT*(H180A)-*P_{lac}*) was generated as described above.

Growth and survival of *E. coli* ectopically expressing PaFicT or its derivatives

E. coli strains were grown as outlined above. Resulting colonies were resuspended in LB broth, washed twice, and diluted in LB to an OD₆₀₀ of 0.4. Cultures were further diluted 50-fold in LB containing 15 μ g/ml gentamycin and 1mM IPTG (inducing). Following dilution 150 μ L was transferred to individual wells of a sterile, 96-well flat-bottom polystyrene microplate (Corning, USA).

Growth curves were performed using an ELx808 Absorbance Microplate Reader (BioTek Instruments, USA) at 37° C under continuous shaking conditions. Data was obtained by measuring optical density at 630 nm. Cell viability was determined by plating cultures on LB agar containing 1% glucose and 15 μ g/ml gentamycin.

Antibiotic susceptibility test

Susceptibility of bacteria to antibiotics was determined by minimal inhibitory concentrations (MIC) using the agar microdilution method (16).

Identification and isolation of persister cells

Cells from colony biofilm were resuspended in BMM and diluted to OD₆₀₀ of approximately 0.1. Diluted cell suspension was incubated with 65 μ g/ml norfloxacin (130 x MIC), at 37 °C, with agitation. Aliquots were removed at various time points, and norfloxacin activity was inhibited with 1% (w/v) MgCl₂ (17). Survival was expressed as a percentage of time 0 samples and measured by CFU enumeration using the drop plate method (18) on LB agar, 1% MgCl₂. For overexpression experiments, strains were grown to mid-log phase

in LB, diluted 1:5 in new media, and incubated in inducing (0.1 mM IPTG) or non-inducing (no IPTG) conditions for 2 h. Persister fraction was determined as described above, and data expressed as a percentage compared to uninduced samples.

Confirmation of persister cell dormant state and non-heritability

Cells surviving 24 h norfloxacin treatment were harvested by centrifugation ($10,000 \times g$ for 2 min), washed twice in BMM ($10,000 \times g$ for 2 min), resuspended in $1/10^{\text{th}}$ the original volume of BMM containing 65 $\mu\text{g/ml}$ norfloxacin, and incubated at 37 °C with agitation. Survival of the isolated bacteria was determined as above for 0, 16, and 24 h time points. Testing for heritability was done by measuring the antibiotic susceptibility of mid-log phase cultures derived from either the parental or persister populations.

Drug tolerance of PaFicT-induced persister cells

PA-paficT-P_{lac} was grown to mid-log phase in LB. Persister cells were induced by IPTG and isolated using methods described above. A mostly non-persister cell population was prepared by growing PA-wt in BMM to mid-log phase then harvesting the cells by centrifugation at $10,000 \times g$ for 2 min and resuspended in BMM to OD₆₀₀ of 0.1. Samples of mostly non-persister cells and PaFicT-induced persister cells were incubated at 37 °C, 200 rpm for 4 h in the presence of $10 \times \text{MIC}$ antibiotics and survival was enumerated as before.

Protein modeling

The amino acid sequence of PaFicT was obtained from the Pseudomonas Genome Database (19) and used for protein modeling via the phyre2 web portal (20). PyMol Molecular Graphics System software (Schrödinger, LLC.) was utilized for visualization and modeling.

Statistical analysis

Differences between two means were identified by unpaired Student's *t*-test. One-way ANOVA, followed by Tukey's test, was utilized when comparing more than two means. All

statistical analysis was performed using GraphPad Prism version 6.0h for Mac, GraphPad Software, La Jolla California USA, (www.graphpad.com).

Results

Expression of PaFicT is toxic to *E. coli* through catalytic activity of the PaFicT FIC domain

Enzymatic activity of FIC proteins requires a invariant histidine located at the beginning of the FIC active site motif [HxFx(D/E)GNGR] (21). In this study, the invariant His residue of PaFicT was studied using single copy chromosomally insertion of an inducible PaFicT encoding gene in *E. coli*. As expected, inducing expression of PaFicT by addition of 1 mM IPTG was highly toxic in *E. coli* (Figure 1). Furthermore, in agreement with the literature, PaFicT toxicity was abolished when the invariant His residue (H136) was replaced with alanine (Figure 1). Importantly, this elimination of PaFicT activity in *E. coli* was not caused by simple His-Ala substitution as replacing the neighboring His residue (H180), located outside the active site motif did not abolish toxicity (Figure 1) Together these results confirm the toxic nature and active site of PaFicT.

Deletion of *paficT* decreases persister cell formation

To characterize *paficT* persister cell formation, a method for isolating these variants was optimized. Exposing *Pa* cells obtained from colony biofilm to $130 \times$ the MIC of norfloxacin (Table 2) resulted in biphasic kill curve (22), with an initial 1-2 h fast killing phase followed by a relatively stable persister phase (Figure 2A). To test if cells in the persister phase were dormant, viable cells post 24 h treatment were isolated and again exposed to the antibiotic for an additional 24 h. As anticipated, no substantial decrease in survival occurred when persister cells were exposed to the antibiotic (Figure 2B). Furthermore, exponentially growing cultures derived from persister, and parental populations were equally susceptible to various antibiotics.

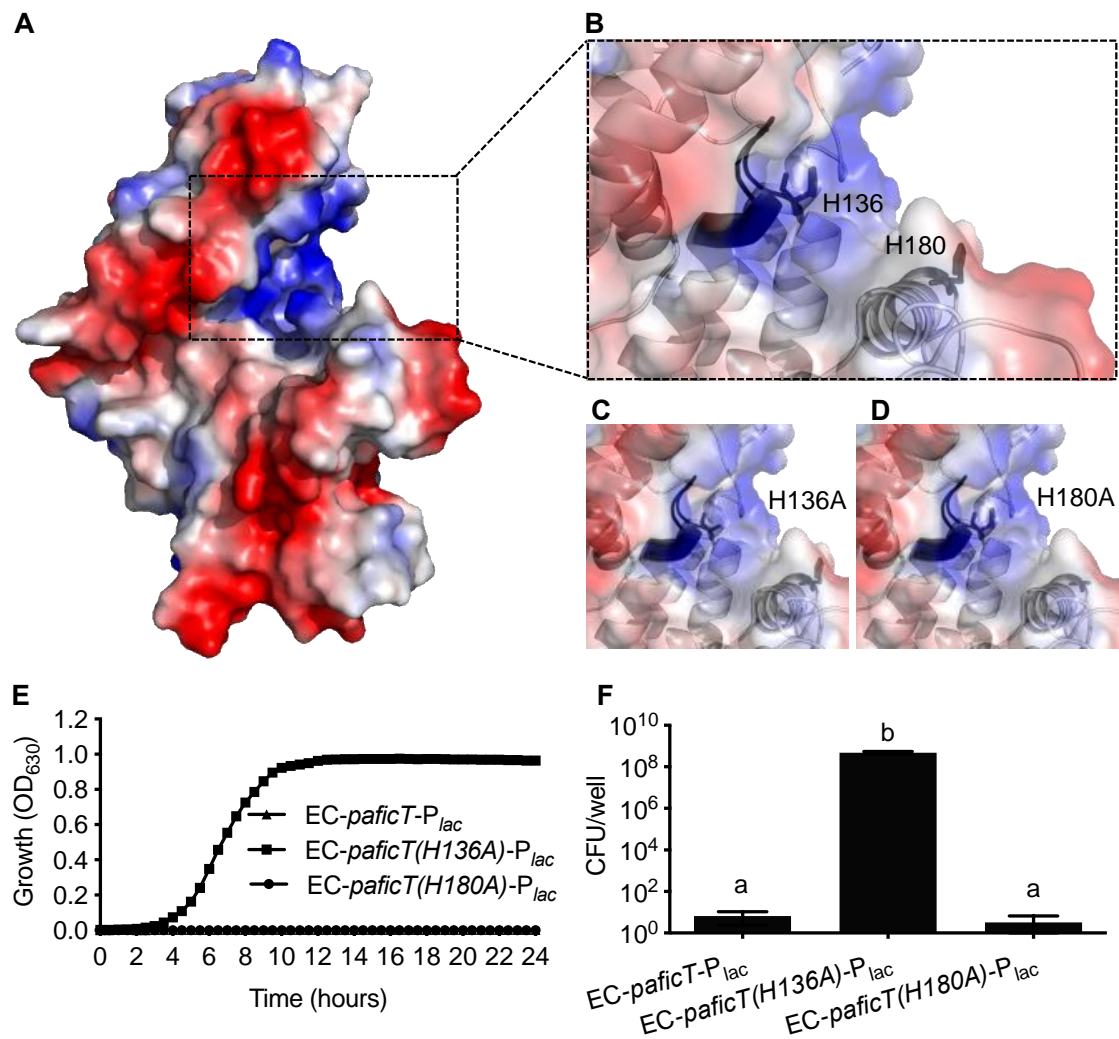


Fig. 1. Toxicity of PaFicT and derivatives in *E. coli*. A: charge/smooth surface representation of the predicted PaFicT structure generated using the PyMol Adaptive Poisson-Boltzmann Solver (red-blue indicates negative-positive electrostatic potential, respectively); B: zoomed in version of modeled active site. Conserved FIC motif catalytic loop [HxFx(D/E)GNGR] are shown in black and grey stick model, respectively; C: active site of the predicted PaFicT structure with H136A substitution within the conserved FIC motif; D: active site of PaFicT with point mutation causing H180A replacement outside of the conserved FIC motif; E and F: single copy chromosomal insertion of *paficT* or its derivatives into *E. coli* under the control of an inducible *P_{lac}* promoter; E: *E. coli* growth during ectopic expression of PaFicT or its derivatives under inducing conditions (1 mM IPTG). Data represents the mean of three independent replicates (\pm SEM); F: *E. coli* CFU following 24 h ectopic expression under inducing conditions. Data represents the mean of six independent replicates (\pm SEM). Differences between means were calculated by one-way ANOVA followed by Tukey's test and indicated by means without a common letter ($P < 0.0001$).

Table 2. MIC (μ g/ml) of various antibiotics on parental and [persister] populations:

Strain	NOR	AMK	MEM	TOB
PA-wt	0.5 [0.5]	8 [8]	4 [4]	1 [1]
PA- Δ <i>paficT</i>	0.5 [0.5]	16 [16]	4 [4]	1 [1]
PA- Δ <i>paficT</i> -comp	0.5 [0.5]	16 [16]	8 [8]	1 [1]
PA- <i>paficT</i> -P _{lac}	0.5 [0.5]	16 [16]	8 [8]	1 [1]
PA- <i>paficT</i> (H136A)-P _{lac}	0.5 [0.5]	16 [16]	8 [8]	1 [1]

Data obtained are the results of triplicate experiments on Mueller Hinton Agar (MHA). NOR: norfloxacin; AMK: amikacin; MEM: meropenem; TOB: tobramycin.

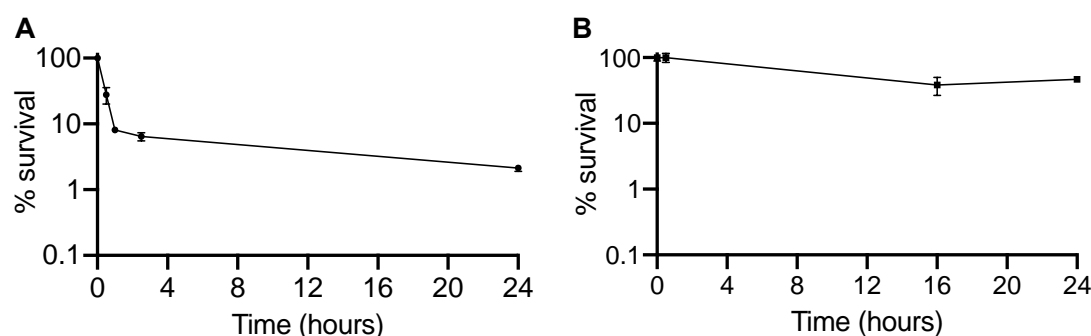


Fig. 2. Persister cell isolation and confirmation in *Pa* colony biofilm population. A: biphasic kill curve of PA-wt upon exposure to norfloxacin at 130 x MIC. PA-wt colony biofilm cells were resuspended in BMM and incubated in the presence of 130 x MIC norfloxacin. Cell viability was determined throughout by plating on LB agar containing 1% MgCl₂; B: confirmation of persister cells dormant state. Cells surviving 24 h norfloxacin treatment were isolated and further exposed to the antibiotic at 130 x MIC. Survival was determined throughout by plating on LB agar containing 1% MgCl₂ and stable cell viability indicated a dormant state of the persister cells. Data are expressed as a percentage compared to time 0 and represent the mean of three independent replicates (\pm SEM)

(Table 2), confirming survival of persisters was not from inherited resistance. Throughout the remainder of this study, cell viability following exposure to 130 \times MIC norfloxacin (65 μ g/ml) for 15 h was used to infer persister fraction of a population

Following 48 h growth under colony biofilm conditions, *Pa* persister cells accounted for 2.1% of the total wild-type population (Figure 3). In comparison, a 7.2-fold reduction in persister cells was observed when colony biofilm was comprised of the *paFicT* deletion strain PA- $\Delta*paFicT* and this decrease in persister cell formation was fully complemented by single copy, chromosomal insertion of *paFicT* back into the genome (Figure 3). Together the data indicated that a majority (86%) of *Pa* persister cells formed during colony biofilm growth required *paFicT*.$

Expression of PaFicT induces persister cell formation, and this phenotype requires a functional FIC active site motif

To further study the role of PaFicT in persister cell formation, a toxin overexpression mutant was constructed with a single copy of *paFicT* controlled by an inducible promoter inserted into the chromosome of PA- Δ *paFicT*, resulting in PA-*paFicT*-P_{lac}. Mid-log phase cultures of PA-*paFicT*-P_{lac} were exposed for 2 h to inducing (0.1 mM

IPTG), or non-inducing (no IPTG) conditions and the subsequent number of persister cells formed was measured. Results showed induction of PaFicT significantly increased persister cell formation by 5.9-fold compared to the non-inducing condition (Figure 4). Alternatively, when wild-type *Pa* was incubated in the presence or absence of 0.1 mM IPTG no significant difference in persister cell formation was observed (Figure 4).

It is known that strong ectopic expression of numerous unrelated proteins can result in increased persister cell formation through the accumulation of misfolded proteins (23). It was therefore of interest to examine if the increased persister cells observed during PaFicT overexpression resulted from FIC toxin specific activity, or some unrelated consequence of PaFicT expression. To investigate this, the PA-*paFicT*(H136A)-P_{lac} strain containing an inducible, catalytically inactive, PaFicT(H136A) was constructed, and persister cells formed following 2 h exposure to inducing and non-inducing conditions was measured. Results showed that the overexpression of PaFicT(H136A) did not significantly alter the formation of persister cells (Figure 4). Together, these data show that the expression of PaFicT induces persister cells formation in *Pa*, and this phenomenon requires FIC toxin enzymatic activity.

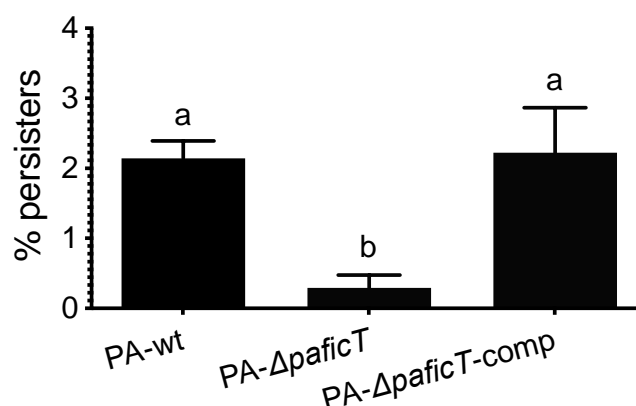


Fig. 3. PaFicT is involved in *Pa* persister cell formation. Persister cell formation during colony biofilm growth of strains: PA-wt, PA-ΔpaficT, and PA-ΔpaficT-comp. Following growth, total viable cells were determined by resuspending the cells and plating on LB agar. Total persister cells were determined by incubating resuspended colony biofilms in 130 x MIC norfloxacin for 15 h and subsequently plating samples on LB agar containing 1% MgCl₂. Persister cell data are expressed as a percentage of total viable cells and represents the mean of three independent replicates (± SEM). Significant differences between the means were calculated using one-way ANOVA followed by Tukey's test and indicated by means without a common letter ($P < 0.05$).

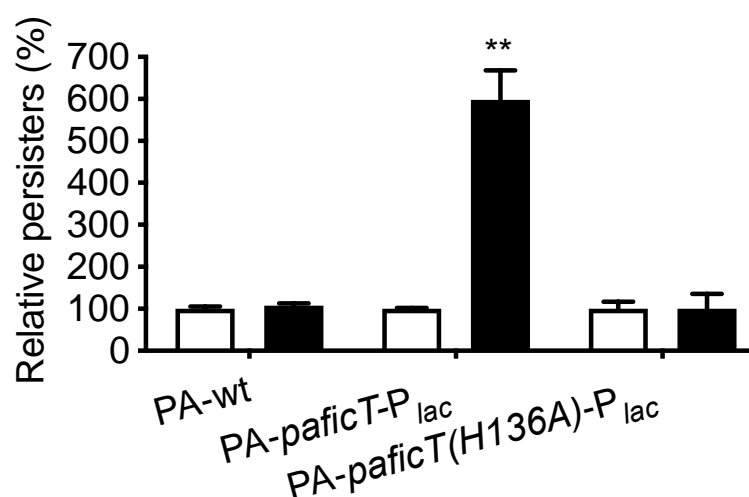


Fig. 4. PaFicT induces persister cell formation and requires a functional FIC catalytic loop motif. Relative persister cells isolated from PA-wt, PA-paficT-P_{lac}, and PA-paficT(H136A)-P_{lac} cultures. Strains were grown to mid-log phase, and persister cells were enumerated following 2 h incubation in inducing (LB + 0.1 mM IPTG) or non-inducing (LB) conditions. White and black bars indicate persister cells from uninduced and induced samples, respectively. Data represents the mean of three independent replicates (± SEM) expressed as a percentage of the uninduced treatment. Significant differences between the means of uninduced and induced populations for each strain were calculated using an unpaired *t*-test and indicated by asterisks (** $P \leq 0.01$).

PaFicT-induced persister cells are multidrug tolerant

It is known that bacterial which enter a dormant, persistent state can survive exposure to various antibiotics(3). It was therefore relevant to test the tolerance of growing and PaFicT-induced persister populations to clinically relevant antibiotics at 10×MIC. Results showed that the survival of PaFicT-induced persister cells was unaffected by treatment with meropenem(Figure 5).

In contrast, under the same conditions, an 85% reduction in survival was observed in the parental, mostly non-persister, population. While tobramycin killed 75% of PaFicT-induced persister cells, this surviving fraction was 6.2×10^5 -fold higher than that of the parental, mostly non-persister treated sample (Figure 5). Finally, amikacin treatment was found highly effective against both growing and persister *Pa* cells (Figure 5). Overall, these data demonstrate that in *Pa*, the formation of persister

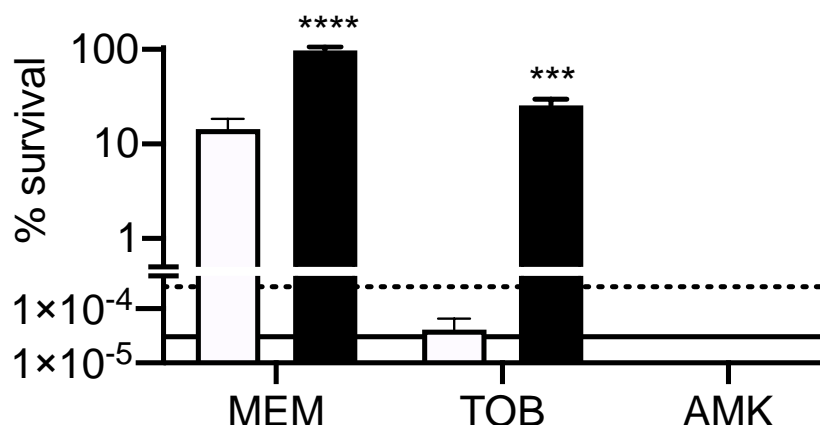


Fig. 5. Antibiotic tolerance of PaFicT-induced *Pa* persister cells. Viable *Pa* cells after 4 h treatment $10 \times$ MIC of clinically relevant antibiotics. White bars indicate the survival of parental populations, at mid-log phase, containing mostly non-persister cells. Black bars indicate the survival of persister cells isolated from PaFicT-induced cultures. Solid and dashed horizontal lines indicate the limit of detection for parental and persister populations, respectively. Data represent the mean of three independent replicates (\pm SEM) expressed as a percentage of survival compared to time 0 (100% survival). MEM: meropenem; TOB: tobramycin; AMK: amikacin. Significant differences between the survival of parental and persister populations were calculated using an unpaired *t*-test and indicated by asterisks (*** $P \leq 0.001$, **** $P \leq 0.0001$).

cells caused by the expression of PaFicT can effectively protect cells from some clinically relevant antibiotics.

Discussion

In the present study multiple experimental approaches provide strong evidence that the biological function of PaFicT includes the production of persister cells. Specifically, it was found that the unmarked deletion of *paficT* in the PAO1 genome resulted in a significant 7.2-fold reduction in persister cell formation, suggesting that PaFicT is involved in the formation of these variants. This observation was confirmed by single copy chromosomal insertion of the gene back into the genome under the native promoter, which fully complemented persister cell formation to wild-type levels. Furthermore, the overexpression of PaFicT significantly increases persister cell formation and requires the functional FIC toxin active site.

Upon establishing the involvement of PaFicT in *Pa* persister cell formation, the potential relevance of these dormant variants to antibiotic tolerance was investigated. Results showed that compared to cells in growing populations, PaFicT-induced persister cells were significantly more

tolerant to treatment with high concentrations of norfloxacin and the clinically relevant antibiotics, meropenem and tobramycin. Although it was found that amikacin could eradicate all detectable persister and non-persister cells, the concentration used was approximately $8\text{--}40 \times$ higher than the concentration achieved in the sputum of patients receiving intravenous amikacin treatment (24). Clearly, some antibiotics are better than others at killing PaFicT-induced persister cells, and this phenomenon could be due to the molecular mechanism of PaFicT-induced persistence.

FIC toxin family proteins, including PaFicT, have been shown to inactivate type IIA bacterial topoisomerases (10). Specifically, FIC toxins enzymatically inactivate DNA gyrase and topoisomerase IV through the AMPylation of DNA gyrase subunit B (GyrB) and DNA topoisomerase IV subunit B (ParE) ATP binding sites (10). Therefore, the topoisomerases natural function in controlling DNA topology through supercoiling and decatenation is lost and results in the cell entering a non-growing dormant state. This effect can be reversed by increasing expression of the GyrB and ParE targets, which allows the cell to re-enter a growing state (10). Extending this model to

Pa persister cell formation is consistent with results reported here and can be used to help explain the different antibiotic tolerances of PaFicT-induced persister cells. For example, the fluoroquinolone class antibiotic norfloxacin, used in the selection of persister cells, is known to inhibit DNA gyrase activity through binding to the substrate DNA (17). In contrast, PaFicT and other FIC toxin proteins directly inhibit DNA gyrase activity by enzymatically modifying the topoisomerase (10). As a result, cells in the PaFicT-induced persister state would already have inactivated DNA gyrase, and therefore norfloxacin would provide no additional antibacterial effects. In addition to norfloxacin, PaFicT-induced persister cells were highly tolerant to the β -lactam class antibiotic meropenem. Interestingly meropenem exerts its antibacterial activity through binding to penicillin-binding proteins (PBPs) which results in the inhibition of bacterial cell wall crosslinking (25). Since PaFicT-induced persister cells maintain a dormant cell state, inhibition of cell wall synthesis by meropenem would not be detrimental to survival. Moreover, upon the removal of meropenem the surviving dormant cells could resume growth by increasing expression of GyrB and ParE while utilizing newly synthesized PBPs with unhindered transpeptidase activity for cell wall synthesis. Notably, compared to norfloxacin and meropenem, PaFicT-induced persister cells were more sensitive to killing by tobramycin and amikacin. This observation may be explained by the fact that both amikacin and tobramycin are aminoglycoside class antibiotics which inhibit protein synthesis by binding to the 30S ribosome (26, 27). As a result of blocking protein synthesis, PaFicT-induced persister cells would be unable to generate DNA gyrase and topoisomerase IV needed to re-enter a growing state. Additionally, both tobramycin and amikacin bind to their targets with dissociation constants in the micromolar range (28, 29). This tight binding may help explain why even

after removal of the antibiotics, many PaFicT-induced persisters were still unable to re-enter a growing cell state. However, it is unclear why amikacin treatment was more effective compared to tobramycin at killing both parental and PaFicT-induced persister cells. The apparent increased killing by 10 x MIC amikacin, compared to tobramycin may have resulted from the 2-fold limitation on precision achievable when calculating initial MIC by 2-fold dilution methods.

In summary, clear evidence is shown here for the involvement of PaFicT in *Pa* persister cell formation. Further characterization revealed that PaFicT-induced persister cell formation occurs through FIC specific enzymatic activity. Finally, these specialized dormant cells have various, antibiotic specific, degrees of tolerance. This multidrug tolerance of PaFicT-induced persister cells may help explain the current difficulty in treating chronic *Pa* infections and could implicate the use of select antibiotics in the aggressive treatments targeting dormant cells. Future research investigating the regulation of PaFicT could help unravel the role of PaFicT in persister cell formation.

Acknowledgements

Special thanks are due to Dr. Yun Heacock-Kang, Dr. Ian McMillan, Darlene Cabanas, Sun Zhenxin, Jan Zarzycki-Siek, Andrew Bluhm, Hung Vo, and Charly Boyd for instructive discussions on the experiments results. D.F acknowledges Dr. Tung Hoang in part for providing funding through the US National Institutes of Health (NIH)/National Institute of General Medical Sciences (NIGMS) grant number R01GM103580.

Conflict of Interest

The author declares no conflict of interest.

References

1. Ciofu O, Hansen CR, Hoiby N. Respiratory bacterial infections in cystic fibrosis. *Curr Opin Pulm Med* 2013; 19:251-8.

2. Mulcahy LR, Burns JL, Lory S, et al. Emergence of *Pseudomonas aeruginosa* strains producing high levels of persister cells in patients with cystic fibrosis. *J Bacteriol* 2010;192:6191-9.
3. Lewis K. Persister cells, dormancy and infectious disease. *Nat Rev Microbiol* 2007;5:48-56.
4. Murakami K, Ono T, Viducic D, et al. Role for *rpoS* gene of *Pseudomonas aeruginosa* in antibiotic tolerance. *FEMS Microbiol Lett* 2005;242:161-7.
5. Viducic D, Ono T, Murakami K, et al. Functional analysis of *spoT*, *relA* and *dksA* genes on quinolone tolerance in *Pseudomonas aeruginosa* under nongrowing condition. *Microbiol Immunol* 2006;50:349-57.
6. Viducic D, Murakami K, Amoh T, et al. *RpoN* Promotes *Pseudomonas aeruginosa* Survival in the Presence of Tobramycin. *Front Microbiol* 2017;8:839.
7. Cameron DR, Shan Y, Zalis EA, et al. A Genetic Determinant of Persister Cell Formation in Bacterial Pathogens. *J Bacteriol* 2018;200.
8. Liebens V, Defraigne V, Van der Leyden A, et al. A putative de-N-acetylase of the PIG-L superfamily affects fluoroquinolone tolerance in *Pseudomonas aeruginosa*. *Pathog Dis* 2014;71:39-54.
9. Paul P, Sahu BR, Suar M. Plausible role of bacterial toxin-antitoxin system in persister cell formation and elimination. *Mol Oral Microbiol* 2019;34:97-107.
10. Harms A, Stanger FV, Scheu PD, et al. Adenylation of Gyrase and Topo IV by FicT Toxins Disrupts Bacterial DNA Topology. *Cell Rep* 2015;12:1497-507.
11. Stover CK, Pham XQ, Erwin AL, et al. Complete genome sequence of *Pseudomonas aeruginosa* PAO1, an opportunistic pathogen. *Nature* 2000;406:959-64.
12. Heacock-Kang Y, Sun Z, Zarzycki-Siek J, et al. Spatial transcriptomes within the *Pseudomonas aeruginosa* biofilm architecture. *Mol Microbiol* 2017;106:976-85.
13. Hoang TT, Karkhoff-Schweizer RR, Kutchma AJ, et al. A broad-host-range Flp-FRT recombination system for site-specific excision of chromosomally-located DNA sequences: application for isolation of unmarked *Pseudomonas aeruginosa* mutants. *Gene* 1998;212:77-86.
14. Choi KH, Schweizer HP. mini-Tn7 insertion in bacteria with single attTn7 sites: example *Pseudomonas aeruginosa*. *Nat Protoc* 2006;1:153-61.
15. Edelheit O, Hanukoglu A, Hanukoglu I. Simple and efficient site-directed mutagenesis using two single-primer reactions in parallel to generate mutants for protein structure-function studies. *BMC Biotechnol* 2009;9:61.
16. Golus J, Sawicki R, Widelski J, et al. The agar microdilution method - a new method for antimicrobial susceptibility testing for essential oils and plant extracts. *J Appl Microbiol* 2016;121:1291-9.
17. Shen LL, Pernet AG. Mechanism of inhibition of DNA gyrase by analogues of nalidixic acid: the target of the drugs is DNA. *Proc Natl Acad Sci U S A* 1985;82:307-11.
18. Naghili H, Tajik H, Mardani K, et al. Validation of drop plate technique for bacterial enumeration by parametric and nonparametric tests. *Vet Res Forum* 2013;4:179-83.
19. Winsor GL, Griffiths EJ, Lo R, et al. Enhanced annotations and features for comparing thousands of *Pseudomonas* genomes in the *Pseudomonas* genome database. *Nucleic Acids Res* 2016;44:D646-53.
20. Kelley LA, Mezulis S, Yates CM, et al. The Phyre2 web portal for protein modeling, prediction and analysis. *Nat Protoc* 2015;10:845-58.
21. Veyron S, Peyroche G, Cherfils J. FIC proteins: from bacteria to humans and back again. *Pathog Dis* 2018;76.
22. Maisonneuve E, Gerdes K. Molecular mechanisms underlying bacterial persisters. *Cell* 2014;157:539-48.
23. Vazquez-Laslop N, Lee H, Neyfakh AA. Increased persistence in *Escherichia coli* caused by controlled expression of toxins or other unrelated proteins. *J Bacteriol* 2006;188:3494-7.
24. Canis F, Husson MO, Turck D, et al. Pharmacokinetics and bronchial diffusion of single daily dose amikacin in cystic fibrosis patients. *J Antimicrob Chemother* 1997;39:431-3.
25. Sumita Y, Fukasawa M. Potent activity of meropenem against *Escherichia coli* arising from its simultaneous binding to penicillin-binding proteins 2 and 3. *J Antimicrob Chemother* 1995;36:53-64.
26. Kondo J, Francois B, Russell RJ, et al. Crystal structure of the bacterial ribosomal decoding site complexed with amikacin containing the gamma-amino-alpha-hydroxybutyryl (haba) group. *Biochimie* 2006;88:1027-31.
27. Yang G, Trylska J, Tor Y, et al. Binding of aminoglycosidic

antibiotics to the oligonucleotide A-site model and 30S ribosomal subunit: Poisson-Boltzmann model, thermal denaturation, and fluorescence studies. *J Med Chem* 2006;49:5478-90.

28. Dudek M, Romanowska J, Witula T, et al. Interactions of amikacin with the RNA model of the ribosomal A-site:

computational, spectroscopic and calorimetric studies. *Biochimie* 2014;102:188-202.

29. Griffey RH, Hofstadler SA, Sannes-Lowery KA, et al. Determinants of aminoglycoside-binding specificity for rRNA by using mass spectrometry. *Proc Natl Acad Sci U S A* 1999;96:10129-33.