Pseudomonas aeruginosa fosfomycin resistance mechanisms affect non-inherited fluoroquinolone tolerance

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Pseudomonas aeruginosa is an opportunistic pathogen that poses a threat in clinical settings due to its intrinsic and acquired resistance to a wide spectrum of antibiotics. Additionally, the presence of a subpopulation of cells surviving high concentrations of antibiotics, called persisters, makes it virtually impossible to eradicate a chronic infection. The mechanism underlying persistence is still unclear, partly due to the fact that it is a non-inherited phenotype. Based on our findings from a previously performed screening effort for *P. aeruginosa* persistence genes, we hypothesize that crosstalk can occur between two clinically relevant mechanisms: the persistence phenomenon and antibiotic resistance. This was tested by determining the persistence phenotype of *P. aeruginosa* strains that are resistant to the antibiotic fosfomycin due to either of two unrelated fosfomycin resistance mechanisms. Overexpression of *fosA* (PA1129) confers fosfomycin resistance by enzymic modification of the antibiotic, and in addition causes a decrease in the number of persister cells surviving ofloxacin treatment. Both phenotypes require the enzymic function of FosA, as mutation of the Arg¹¹⁹ residue abolishes fosfomycin resistance as well as low persistence. The role for fosfomycin resistance mechanisms in persistence is corroborated by demonstrating a similar phenotype in a strain with a mutation in *glpT* (PA5235), which encodes a glycerol-3-phosphate transporter essential for fosfomycin uptake. These results indicate that fosfomycin resistance, conferred by *glpT* mutation or by overexpression of *fosA*, results in a decrease in the number of persister cells after treatment with ofloxacin and additionally stress that further research into the interplay between fosfomycin resistance and persistence is warranted.

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INTRODUCTION

Persister cells are specialized dormant cells that constitute a subfraction of planktonic cultures and biofilm populations of numerous pathogenic bacteria. They survive elevated levels of antibiotics even though their offspring exhibit the same antibiotic sensitivity as the original population, demonstrating that the appearance of persisters is not a consequence of stable resistance-conferring mutations (Bigger, 1944). Persistence will be defined throughout this paper as previously defined by Dörr *et al.* (2009): 'Persistence is the ability of a subpopulation of susceptible bacteria to survive lethal doses of antibiotics. It is a transient and non-hereditary phenotype unlike resistance, which is due to genetic modification'. Even though persister cells were discovered more than 60 years ago when analysing the action of penicillin on *Staphylococcus*

aureus cultures (Bigger, 1944), the mechanism by which bacteria switch to this persister state is still unclear. Persistence has been best studied at the genetic level in *Escherichia coli* (Dörr *et al.*, 2010; Harrison *et al.*, 2009; Kim & Wood, 2010; Kim *et al.*, 2010; Schumacher *et al.*, 2009; Shah *et al.*, 2006; Spoering *et al.*, 2006; Vázquez-Laslop *et al.*, 2006) and in the opportunistic pathogen *Pseudomonas aeruginosa* (De Groote *et al.*, 2009; Harrison *et al.*, 2005; Keren *et al.*, 2004; Möker *et al.*, 2010; Spoering & Lewis, 2001). *P. aeruginosa* is a source of nosocomial infection outbreaks and causes life-threatening infections in people suffering from cystic fibrosis (Lyczak *et al.*, 2002). In addition to the intrinsic resistance to antibiotics and genetically acquired resistance mechanisms of *P. aeruginosa*, it is now believed that persister cells contribute significantly to treatment failure by their presence in infection-related biofilm populations (Brooun *et al.*, 2000; LaFleur *et al.*, 2010; Mulcahy *et al.*, 2010; Spoering & Lewis, 2001).

Abbreviations: MDR, multidrug resistance; RT-qPCR, reverse transcription quantitative real-time PCR.

The presence of persister cells has been confirmed in numerous other bacterial species, such as the human pathogens *Mycobacterium tuberculosis* (Dhar & McKinney, 2007) and *Salmonella enterica* serovar Typhimurium, and in the eukaryotic pathogen *Candida albicans* (Harrison *et al.*, 2007; LaFleur *et al.*, 2006, 2010). Many of these species are involved in chronic infection outbreaks and the presence of persister cells has been proposed to be the main reason for treatment failures in the combat of these chronic diseases. Indeed, clinical isolates of *P. aeruginosa* from cystic fibrosis patients show an increase in high persistence mutants the longer that these isolates remain in the host (Mulcahy *et al.*, 2010). This indicates that persistence plays a major role in the failure to remove these bacterial populations from the cystic fibrosis lung. A similar observation was made when analysing *C. albicans* clinical isolates from chronically ill patients: high persistence mutants are more abundantly present in late isolates than in isolates from early on in the chronic infection (LaFleur *et al.*, 2010).

Resistance is the result of heritable genetic changes that allow the cells to grow in the presence of the antibiotic (Lewis, 2010). In the past decades, the increasing prevalence of resistance mechanisms in numerous pathogens such as *Staphylococcus aureus*, *Acinetobacter baumannii*, *Neisseria gonorrhoeae* and *P. aeruginosa* has become problematic, especially for nosocomial infections (Livermore, 2009). The ability of bacteria to rapidly evolve and develop resistance as well as the lack of novel antibiotics have dramatically increased the need to search for alternative means to combat these pathogens. Development of coadministration therapies in which drugs targeting non-essential cellular functions are combined with antibiotic administration are expected to significantly improve the efficacy of existing antibiotic treatments (Smith & Romesberg, 2007). A potentially non-essential mechanism in bacteria to be targeted in such a coadministration therapy is persistence. Unlike resistance, persistence is a non-inherited physiological state acting on a phenotypic level and it has been argued that drugs targeting persistence are less likely to cause selection pressure that would drive multidrug resistance (MDR) development (del Pozo & Patel, 2007; Smith & Romesberg, 2007). While the idea of simultaneously targeting both susceptible and persistent cells in a single therapy is immensely appealing, little is known about possible crosstalk between the molecular processes underlying these respective cellular responses. As the danger of inadvertently stimulating either resistance or persistence looms large, research into this matter is clearly needed.

Fosfomycin is an antibiotic discovered over 40 years ago and is structurally unrelated to any other known class of antimicrobial agents (Hendlin *et al.*, 1969). Because of its broad activity spectrum and interesting pharmacokinetic properties (low toxicity, easy administration and high *in vitro* activity) it is still used today in the clinical setting, especially for uncomplicated urinary tract infections of *E.*

coli and *P. aeruginosa* (Rigsby *et al.*, 2005). Surprisingly, its use has been limited and has not been explored for complicated infections with MDR strains (Falagas *et al.*, 2009). Even though plasmid-mediated resistance developed shortly after the introduction of this antibiotic in clinical settings, the emergence of resistant strains has been limited in comparison to other antibiotics (Nilsson *et al.*, 2003). Resistance to fosfomycin in *P. aeruginosa* results from the activity of the antibiotic-altering enzyme FosA (PA1129) (Bernat *et al.*, 1997) or is caused by inactivation of the fosfomycin transport protein GlpT (PA5235) (Castañeda-García *et al.*, 2009). Due to its limited use and the relatively low level of reported resistance, fosfomycin has now been revisited for its possible effectiveness against MDR strains (Falagas *et al.*, 2009).

In this study, we report that fosfomycin resistance mechanisms affect persistence of *P. aeruginosa* upon treatment with the fluoroquinolone antibiotic ofloxacin. Crosstalk between resistance and persistence has not been described before and may have strong implications for combination therapy and the future development of possible coadministration therapies.

METHODS

Bacteria and culture conditions. *P. aeruginosa* strains were cultured in Trypticase Soy Broth (TSB) or on solidified medium (1.5 % agar) at 37 °C. The following antibiotics were used: fosfomycin (39–20 000 µg ml⁻¹), ofloxacin (5 µg ml⁻¹), piperacillin (15 µg ml⁻¹) and tetracycline (200 µg ml⁻¹). *E. coli* strains were grown in Luria–Bertani broth or on solidified medium (1.5 % agar) at 37 °C. The following antibiotics were used: ampicillin (100 µg ml⁻¹) and tetracycline (10 µg ml⁻¹). All restriction enzymes were purchased from Westburg. Strains and plasmids used in this study are summarized in Table 1.

Construction of *P. aeruginosa* *fosA*⁺ and *fosA*_{RI19A} over-expression strains. Wild-type *fosA*⁺ (PA1129) was PCR-amplified with *Pfx* polymerase (Invitrogen) using isolated genomic DNA of *P. aeruginosa* PA14 as template. The mutant *fosA*_{RI19A} allele was amplified using pTP123 (kindly provided by Timothy Palzkill, Baylor College of Medicine, TX, USA) as a template (Beharry & Palzkill, 2005). PCR amplifications were performed using primers SPI-1559 and SPI-1713 (Table 2). The resulting 460 bp fragments were digested with *Xba*I and *Eco*RI, cloned into pUC18, confirmed by sequencing and subcloned into pFAJ1708 (Daniels *et al.*, 2006), resulting in pCMPG13402 (referred to as pFosA) and pCMPG13403 (referred to as pFosA_{RI19A}), constitutively expressing *fosA*⁺ and *fosA*_{RI19A}, respectively. Plasmids were introduced into *P. aeruginosa* PAO1 by electroporation (Choi *et al.*, 2006) to obtain strains CMPG13411 and CMPG13435, referred to as PAO(pFosA) and PAO(pFosA_{RI19A}), respectively. Vector pFAJ1708 alone was electroporated similarly to obtain strain CMPG13410.

Complementation of PW2039 with PA1128. A 1.5 kb fragment was obtained by PCR amplification with *Pfx* polymerase (Invitrogen) with primers SPI-683 and SPI-684 (Table 2) using genomic DNA of *P. aeruginosa* PA14 as template. The *Eco*RI/*Hind*III fragment containing PA1128 was subcloned into pUCP19 (Schweizer, 1991) to obtain plasmid pCMPG13405 (referred to as pUCP-PA1128). pUCP-PA1128 was electroporated (Choi *et al.*, 2006) to PAO1 wild-type and PW2039 (referred to as PAO1128), resulting in strains

Table 1. Strains and plasmids used in this study

Strain or plasmid	Name throughout text	Description*	Source or reference
Strains			
<i>Pseudomonas aeruginosa</i>			
PAO1	PAO1	Wild-type	Jacobs <i>et al.</i> (2003)
PW2039	PAO1128	PAO1 <i>PA1128</i> -C12::IS <i>lacZ</i> /hah-Tet ^r	Jacobs <i>et al.</i> (2003)
PA14	PA14	Wild-type	Liberati <i>et al.</i> (2006)
<i>glpT</i> ::MAR2xT7	<i>glpT</i> ::MAR2xT7	PA14 <i>glpT</i> ::MAR2xT7; Gen ^r	Liberati <i>et al.</i> (2006)
CMPG13410	CMPG13410	PAO1 wild-type with pFAJ1708; Tet ^r , Pip ^r	This study
CMPG13411	PAO(pFosA)	PAO1 wild-type with pFosA; Tet ^r , Pip ^r	This study
CMPG13428	PAO(pUCP)	PAO1 wild-type with pUCP19; Pip ^r	This study
CMPG13430	PAO1128(pUCP)	PW2039 PA1128-C12::IS <i>lacZ</i> /hah-Tet ^r with pUCP19; Pip ^r	This study
CMPG13431	PAO(pUCP-PA1128)	PAO1 wild-type with pCMPG13405; Pip ^r	This study
CMPG13432	PAO1128(pUCP-PA1128)	PW2039 PA1128-C12::IS <i>lacZ</i> /hah-Tet ^r with pUCP-PA1128; Pip ^r	This study
CMPG13435	PAO(pFosA _{R119A})	PAO1 wild-type with pFosA _{R119A} ; Tet ^r , Pip ^r	This study
<i>Escherichia coli</i>			
TOP10	TOP10	F ⁻ <i>mcrA</i> Δ(<i>mrr</i> – <i>hsdRMS</i> – <i>mcrBC</i>) Φ80 <i>lacZ</i> Δ <i>M15</i> Δ <i>lacX74</i> <i>nupG</i> <i>recA1</i> <i>araD139</i> Δ(<i>ara</i> – <i>leu</i>)7697 <i>galE15</i> <i>galK16</i> <i>rpsL</i> (Str ^r) <i>endA1</i> λ ⁻	Invitrogen
Plasmids			
pUC18	pUC18	Broad-host-range shuttle vector; Amp ^r	Yanisch-Perron <i>et al.</i> (1985)
pUCP19	pUCP19	Broad-host-range shuttle vector; Amp ^r	Schweizer (1991)
pFAJ1708	pFAJ1708	Broad-host-range plasmid with <i>nptII</i> promoter used for overexpression; Tet ^r	Daniels <i>et al.</i> (2006)
pCMPG13402	pFosA	<i>fosA</i> cloned into pFAJ1708 downstream from <i>nptII</i> promoter; Tet ^r	This study
pCMPG13403	pFosA _{R119A}	<i>fosA</i> (R119A) cloned into pFAJ1708 downstream from <i>nptII</i> promoter; Tet ^r	This study
pCMPG13405	pUCP-PA1128	<i>PA1128</i> cloned into pUCP19; Amp ^r	This study

*Tet^r, tetracycline resistant; Gen^r, gentamicin resistant; Pip^r, piperacillin resistant; Str^r, streptomycin resistant; Amp^r, ampicillin resistant.

CMPG13431 and CMPG13432, referred to as PAO(pUCP-PA1128) and PAO1128(pUCP-PA1128), respectively. Vector pUCP19 alone was introduced as a control in strains CMPG13428 and CMPG13430, referred to as PAO(pUCP) and PAO1128(pUCP), respectively.

Persistence assay. The persistence assay was performed essentially as described previously (De Groote *et al.*, 2009). Briefly, 1 ml of a stationary phase culture was treated with 10 μl ofloxacin at a final concentration of 5 μg ml⁻¹; a control treatment was performed with sterile water. Both treatments were performed at 37 °C, shaking at 200 r.p.m., for 5 h, after which the number of c.f.u. was determined by plate counts. The persister fraction is defined as the number of surviving cells after treatment with ofloxacin, divided by the number of cells after the control treatment. The relative persister fraction for

each strain is the persister fraction of the strain divided by the persister fraction of the wild-type or of the wild-type carrying the appropriate vector alone. The mean relative persister fraction is calculated as the inverse logarithm of the mean of the logarithmic values of these relative persister fractions of separate experiments. Each experiment was independently repeated at least four times. In Fig. 1, the mean relative persister fractions are displayed with the bars representing the 25th and 75th percentiles.

Selection of persistence mutants. Selection of persistence mutants was performed as described previously (De Groote *et al.*, 2009). Briefly, after ofloxacin treatment, the cultures were diluted 100-fold and incubated in an automated OD plate reader (Bioscreen C; Oy Growth Curves). Based on the lag phase of the growth curves

Table 2. Primer sequences

Primer name	Sequence	Source
SPI-1559	5'-CACCTCTAGAAGGAGGAAGCCCCCATGCTTACCGGTC-3'	This study
SPI-1713	5'-CACCGAATTCTAGTGGTGGTGGTGGTGGTGGTCGGCGAAA CGCATTCCCG-3'	This study
SPI-683	5'-AGTCAAGCTTAAGGCCTAGTCGGCGAAACG-3'	This study
SPI-684	5'-CACCGAATTCCGTGTCGATACCTCGATGC-3'	This study

Fig. 1. Mean relative persister fractions. The persister fraction is defined as the number of surviving cells after treatment with ofloxacin, divided by the number of cells of the control condition. The relative persister fraction for each strain is the persister fraction of the strain divided by that of the corresponding wild-type (value of 1 in the y-axis). Data points correspond to mean relative persister fractions for the strains indicated in the x-axis. Each experiment was repeated independently at least four times. The bars represent the 25th and 75th percentiles of the data series.

generated by the plate reader, mutants with an altered number of persister cells were selected.

MIC₅₀ determination. The MIC₅₀ was defined as the minimal antibiotic concentration needed to inhibit growth by 50 % and was determined with a broth macrodilution procedure. Ofloxacin concentrations ranged from 10 µg ml⁻¹ to 0.02 µg ml⁻¹ in a twofold dilution series and fosfomycin concentrations ranged from 20 000 µg ml⁻¹ to 39 µg ml⁻¹. The test was carried out in TSB broth at 37 °C, shaking at 200 r.p.m.

Expression analysis. The expression of the *fosA* gene was verified by reverse transcription quantitative real-time PCR (RT-qPCR) using the StepOnePlus System and Power SYBR Green PCR Master Mix containing AmpliTaq Gold DNA Polymerase (Applied Biosystems) as described previously (Vercruysse *et al.*, 2010).

RESULTS AND DISCUSSION

Low persistence phenotype of PAO1128

Persistence was discovered more than 60 years ago, but its clinical relevance was ignored at the time (Bigger, 1944). Over the past decades, it has become clear that persister cells contribute significantly to treatment failure in biofilm-related infections (Brooun *et al.*, 2000). Only a limited number of persistence genes have been identified to date, and the mechanism underlying this phenomenon still remains unclear (Levin & Rozen, 2006; Lewis, 2007).

Recently, we were able to expand upon this number of persistence genes by screening a non-exhaustive mutant library of *P. aeruginosa* for mutants displaying an altered number of persister cells after treatment with the fluoroquinolone antibiotic ofloxacin (De Groote *et al.*, 2009). In this screening, a mutant with a plasmid insertion in the gene *PA14_49790* was selected with a persister fraction below wild-type level. The inactivated gene encodes a predicted transcriptional regulator that possesses a LysR-type regulatory helix–turn–helix domain and a LysR-type substrate-binding domain, and is therefore a predicted member of the LysR-type transcriptional regulator protein family (Maddocks & Oyston, 2008). This mutant was not selected for further analysis due to a variable persistence phenotype in confirmatory experiments.

The corresponding PAO1-derived mutant, PW2039 (further referred to as PAO1128), with a transposon (*ISlacZ/hah*) insertion in gene *PA1128*, orthologous to *PA14_49790*, was obtained from the Manoil mutant library (Jacobs *et al.*, 2003). PAO1128 displayed a reproducible low persistence phenotype (see Fig. 1). MIC₅₀ values for ofloxacin were determined and were found to be in the same range as for the wild-type strain, ensuring that the low persistence phenotype was not caused by a lowered resistance to ofloxacin (data not shown). We attempted to complement the PAO1128 mutant phenotype by providing *PA1128 in trans*, but this did not restore persistence to wild-type levels (see Fig. 1). Upon taking a closer look at the PAO1128 strain, we noticed that the *ISlacZ/hah* transposon that was used to construct the mutant possesses an outward reading *nptII* promoter to reduce possible polar effects of the mutation (Jacobs *et al.*, 2003). The presence of this promoter, however, might also lead to a gain-of-function phenotype caused by overexpression of the downstream genes, in this case *fosA* (*PA1129*), the fosfomycin resistance gene. The overexpression of *fosA*, caused by the *nptII* promoter, was confirmed by RT-qPCR and a 15-fold increase in *fosA* expression was detected in the PAO1128 insertion mutant compared to *fosA* expression levels in the wild-type PAO1 strain.

fosA is the first fosfomycin resistance gene identified and encodes a Mn(II)-dependent metalloenzyme that catalyses the conjugation of glutathione to the epoxide ring of fosfomycin, inactivating the antibiotic (Suárez & Mendoza, 1991). *FosA* is well characterized and is conserved among other *Pseudomonas* species, namely *Pseudomonas entomophila* and *Pseudomonas fluorescens*. Two other fosfomycin resistance proteins have been discovered that are more distantly related to the *FosA* protein, *FosB* and *FosX* (Fillgrove *et al.*, 2003). When determining the MIC₅₀ of fosfomycin for mutant PAO1128, we found that it is indeed significantly increased (see Table 3). The MIC₅₀ of fosfomycin in the complemented strain CMPG13432 [further referred to as PAO1128(pUCP-PA1128)] was unchanged compared to the MIC₅₀ of fosfomycin for PAO1128, showing that the high resistance to fosfomycin is

not caused by the mutation in *PA1128*. This is supported by results from a recent screening effort for fosfomycin resistance genes in *P. aeruginosa*, in which *PA1128* tested negative (Castañeda-García *et al.*, 2009). Hence we conclude that the high MIC₅₀ of fosfomycin in PAO1128 is caused by *fosA* overexpression and not by the *PA1128* mutation.

***fosA* overexpression leads to low persistence and requires enzymic activity**

To determine whether *fosA* overexpression is sufficient to cause low persistence, we transformed wild-type *P. aeruginosa* PAO1 with a vector in which *fosA*⁺ is under the control of a constitutively active *nptII* promoter, giving rise to strain CMPG13411 [further referred to as PAO(pFosA)]. The MIC₅₀ value of fosfomycin increased as expected (see Table 3), overexpression of *fosA* was confirmed by RT-qPCR (data not shown) and the persister fraction was found to decrease by a factor of two compared to wild-type persistence (see Fig. 1), similar to, though less pronounced than, what was observed for PAO1128. The decreased number of persister cells that we observed in PAO(pFosA) compared to the wild-type was lower than that caused by single-copy genomic *fosA* overexpression (PAO1128). This also corresponds to a smaller increase of *fosA* expression (determined by RT-qPCR) and of the MIC₅₀ of fosfomycin for PAO(pFosA) than for PAO1128 (see Table 3).

FosA inactivates fosfomycin by conjugating glutathione to the epoxide ring of the antibiotic. To elucidate whether the persistence phenotype is associated with this enzymic activity, we performed the persistence assay on PAO1 overexpressing the mutant *fosA*_{R119A} allele. In this allele, Arg¹¹⁹ is replaced by Ala¹¹⁹ and prevents the gene product

from conferring resistance to fosfomycin in *E. coli* (Beharry & Palzkill, 2005). Arg¹¹⁹ is located in the active site of FosA and is assumed to be involved in direct binding of the substrate (Bernat *et al.*, 1999; Smoukov *et al.*, 2002). PAO(pFosA_{R119A}) has a MIC₅₀ value lower than both the strain overexpressing wild-type *fosA*⁺ (see Table 3) and, surprisingly, the wild-type (vector alone) PAO1 strain. This clearly indicates that the resistance function of FosA in *P. aeruginosa* is impaired by the point mutation. Because FosA acts as a dimer (Pakhomova *et al.*, 2004), the decreased MIC₅₀ that we observed compared to wild-type could be due to non-functional heterodimer formation between the mutant FosA_{R119A} protein and the genomically encoded wild-type FosA. Importantly, we also found that strain CMPG13435 [further referred to as PAO(pFosA_{R119A})] no longer displays the low persistence phenotype that was present in strain PAO(pFosA), demonstrating that the enzymic function of the FosA enzyme is essential for the low persistence phenotype and involves the Arg¹¹⁹ residue. PAO(pFosA_{R119A}) even displays slightly increased persistence, which offers further support for the observed inverse correlation between the two phenotypes.

In addition to fosfomycin, FosA has been shown to bind several other small phosphonates that act as inhibitors of the enzyme, and Arg¹¹⁹ plays a key role in the binding with these inhibitors (Rigsby *et al.*, 2004). This residue is also conserved in other fosfomycin resistance proteins such as FosB and FosX and it has been demonstrated that it is indeed involved in substrate binding in FosX, and likely has the same function in FosB (Fillgrove *et al.*, 2007). This illustrates the essential role that Arg¹¹⁹ plays in binding with different substrates. Since no fosfomycin was present in the persistence assay, we propose that other FosA substrates might be involved in regulating persistence (see further below).

Table 3. Mean relative persister fraction and MIC₅₀ values of the *P. aeruginosa* strains

Strain	Strain properties	Mean relative persister fraction*	MIC ₅₀ fosfomycin (µg ml ⁻¹)
PA14	Wild-type	1.00	62.5
<i>glpT</i> ::MAR2xT7	<i>glpT</i> mutant of PA14	0.43	5000
PAO1	Wild-type	1.00	5000
PAO1128	PAO1 <i>PA1128</i> ::ISlacZ	0.20	>20 000
CMPG13410	PAO1 wild-type carrying pFAJ1708	1.00	2500
PAO(pFosA)	PAO1 wild-type with pFosA	0.47	10 000
PAO(pFosA _{R119A})	PAO1 wild-type with pFosA _{R119A}	1.53	313
PAO(pUCP)	PAO1 wild-type carrying pUCP19	1.00	2500
PAO1128(pUCP-PA1128)	PAO1128 with pUCP19-PA1128	0.21	>20 000

*The persister fraction is defined as the number of surviving cells after treatment with ofloxacin, divided by the number of cells of the control condition. The relative persister fraction for each mutant strain is the persister fraction of the mutant divided by that of the corresponding wild-type. Mutant strains are listed subsequent to their corresponding wild-type (with a mean persister fraction of 1). Each value in the table is calculated as the inverse logarithm of the mean of the logarithmic values of these relative persister fractions of separate experiments (each experiment was independently repeated at least four times).

Fosfomycin resistance caused by *glpT* mutation likewise induces low persistence

GlpT was originally identified as a glycerol-3-phosphate transporter in *E. coli* (Boos *et al.*, 1977), but is also able to transport glycerol 2-phosphate, fosfomycin, arsenate and inorganic phosphate (P_i) into the bacterial cell (Elvin *et al.*, 1985), which illustrates its relatively low substrate specificity (Law *et al.*, 2009). A recent report by Castañeda-García *et al.* (2009) suggests that GlpT is the only fosfomycin transporter present in *P. aeruginosa*. Similar to *fosA* overexpression, a *glpT* mutation causes resistance to fosfomycin. This prompted us to include a strain mutated in *glpT* in the current study. The high resistance to fosfomycin for the *glpT*::MAR2xT7 (ID39942) mutant strain (Liberati *et al.*, 2006) was confirmed (Table 3). Importantly, we could establish that the *glpT* mutation caused the persister fraction to be lowered by a factor of 2 compared to the wild-type, as illustrated in Fig. 1. This low persistence phenotype is reminiscent of the phenotype of a *fosA* overexpression strain, showing the same correlation between fosfomycin resistance and ofloxacin persistence. As in our experiments with PAO(pFosA), no fosfomycin was present in the persistence assays performed on the *glpT* mutant strain. We therefore propose that one or more other substrates transported by GlpT are likely to be involved in persistence. Glycerol 2-phosphate, glycerol 3-phosphate or P_i are possible candidates, but considering the broad substrate specificity of GlpT, other phosphates or

phosphonates could also be possible inducers of persistence.

Model for persistence regulation and possible *in vivo* consequences

Based on the results obtained in this work, we suggest a model for a hypothetical pathway leading to persistence (illustrated in Fig. 2). An unknown substrate is transported by GlpT into the cell and induces persister formation. Upon expression of *fosA*, the substrate is broken down and the persister fraction decreases. The same effect can be seen when GlpT is inactivated, and so can no longer transport the substrate. It has already been established that GlpT accepts both fosfomycin and organic phosphates (glycerol 2-phosphate, glycerol 3-phosphate) as substrates for transport into the bacterial cell. Furthermore, there are several small phosphonates known to bind FosA with varying affinities, e.g. phosphonoformate (Rigsby *et al.*, 2004). We therefore propose that the unknown compound is either an organic phosphate or a phosphonate, serving as a substrate for both GlpT and FosA. Interestingly, Spoering *et al.* (2006) found several genes of the glycerol-3-phosphate metabolic pathway, such as *glpD* and *plsB*, to be involved in persistence in *E. coli*, lending further support to our hypothesis.

The results that we have reported above imply that the occurrence of fosfomycin resistance may reduce the

Fig. 2. Model for crosstalk between fosfomycin resistance and fluoroquinolone persistence. GlpT transports fosfomycin (FOF) into the bacterial cell cytoplasm, causing cell death. *fosA* overexpression induces resistance against fosfomycin by enzymic addition of glutathione, rendering the antibiotic inactive. Based on the results obtained in this work, we suggest that GlpT additionally transports an unknown substrate (X), structurally related to fosfomycin (e.g. a phosphate or phosphonate), into the bacterial cell, inducing persistence. Upon *fosA* overexpression, it will enzymically inactivate substrate X, causing a low persistence phenotype. Solid arrows indicate transport routes, dashed arrows indicate the cell fate induced by the substrates FOF and X, and white block arrows indicate enzymic modification. GS/GSH, Glutathione bound/unbound, respectively.

survival of persister cells. Because fosfomycin, overlooked until recently, is now being considered for treating complicated infections with MDR strains, further investigation of interactions between fosfomycin resistance and persistence phenotypes is crucial. Most therapies currently used for treating these MDR bacteria are based on a combination therapy of two antibiotics of different classes, for example an aminoglycoside and a fluoroquinolone. The efficacy of fosfomycin combined with ofloxacin to treat a *P. aeruginosa* biofilm has already yielded promising results both *in vitro* and *in vivo* (Mikuniya *et al.*, 2005, 2007), indicating that this strategy might be a realistic option in clinical settings. In addition to combining two antibiotics, other possible routes for fighting these infections are being pursued. One of these routes is the inhibition of a non-essential bacterial process, such as persistence, combined with the use of an antibiotic (Smith & Romesberg, 2007). While certainly an attractive option, our results underline the important need to further investigate crosstalk between resistance and persistence before embarking upon the development of such co-therapies.

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