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The Glycerol-3-Phosphate Permease GlpT Is the Only Fosfomycin Transporter in *Pseudomonas aeruginosa*▽

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Fosfomycin is transported into *Escherichia coli* via both glycerol-3-phosphate (GlpT) and a hexose phosphate transporter (UhpT). Consequently, the inactivation of either *glpT* or *uhpT* confers increased fosfomycin resistance in this species. The inactivation of other genes, including *ptsI* and *cyaA*, also confers significant fosfomycin resistance. It has been assumed that identical mechanisms are responsible for fosfomycin transport into *Pseudomonas aeruginosa* cells. The study of an ordered library of insertion mutants in *P. aeruginosa* PA14 demonstrated that only insertions in *glpT* confer significant resistance. To explore the uniqueness of this resistance target in *P. aeruginosa*, the linkage between fosfomycin resistance and the use of glycerol-3-phosphate was tested. Fosfomycin-resistant (Fos-R) mutants were obtained in LB and minimal medium containing glycerol as the sole carbon source at a frequency of 10^{-6}. However, no Fos-R mutants grew on plates containing fosfomycin and glycerol-3-phosphate instead of glycerol (mutant frequency, $\leq 5 \times 10^{-11}$). In addition, 10 out of 10 independent spontaneous Fos-R mutants, obtained on LB-fosfomycin, harbored mutations in *glpT*, and in all cases the sensitivity to fosfomycin was recovered upon complementation with the wild-type *glpT* gene. The analysis of these mutants provides additional insights into the structure-function relationship of glycerol-3-phosphate the transporter in *P. aeruginosa*. Studies with glucose-6-phosphate and different mutant derivatives strongly suggest that *P. aeruginosa* lacks a specific transport system for this sugar. Thus, *glpT* seems to be the only fosfomycin resistance mutational target in *P. aeruginosa*. The high frequency of Fos-R mutations and their apparent lack of fitness cost suggest that Fos-R variants will be obtained easily in vivo upon the fosfomycin treatment of *P. aeruginosa* infections.

Pseudomonas aeruginosa is an opportunistic, life-threatening bacterial pathogen that especially affects critically ill patients in intensive care units or those suffering from chronic respiratory diseases such as cystic fibrosis (19, 40). Its 6.3-Mb genome supports its enormous metabolic versatility and, consequently, its adaptability to almost any challenging environment. One of the consequences of this versatility is the rapid adaptation to stressful environmental conditions, including starvation, desiccation, and antibiotic treatments (14, 40). Mutants resistant to one or several antibiotics will evolve during sufficiently prolonged treatments, this being a process facilitated by the presence of hypermutable alleles (31, 32). After years of treating cystic fibrosis patients with antibiotics, *P. aeruginosa* became unavoidably resistant to many or all of them (5). Multidrug-resistant strains of *P. aeruginosa* are an important problem for the treatment of nosocomial outbreaks and cystic fibrosis patients (27, 37). Currently, the treatment of multidrug-resistant *P. aeruginosa* requires the combination of various antimicrobial agents. Fosfomycin (Fos) has been reported to be effective in combination with other antipseudomonal agents (6, 29, 42, 44). The proportion of Fos-resistant (Fos-R) strains in clinical isolates of *P. aeruginosa* currently is not well known, and even the mechanisms that support Fos resistance in *P. aeruginosa* are not clear. Thus, the knowledge of the molecular bases involved in the development of spontaneous Fos resistance in *P. aeruginosa* is of particular interest.

Fos is a unique broad-spectrum bactericidal antibiotic that is chemically unrelated to any other known antimicrobial agent used to treat urinary tract and gastrointestinal infections in humans (9, 35). It binds UDP-GlcNAc enol-pyruvyltransferase (MurA), acting as a phosphoenolpyruvate analogue and avoiding the formation of UDP-*N*-acetylglucosamine-3-O-enolpyruvate from UDP-*N*-acetylglucosamine and phosphoenolpyruvate (12, 33). Fos is taken up actively into bacterial cells via transport systems. In *Escherichia coli*, Fos is imported through two nutrient transport systems, the glycerol-3-phosphate (glycerol-3-P) transporter (GlpT) and glucose-6-phosphate (glucose-6-P) transporter (UhpT), to achieve its target and inhibits the initial step in cell wall synthesis (12, 17). The expression of these transport systems is induced by their substrates (glycerol-3-P and glucose-6P) and requires the presence of the cyclic AMP receptor protein (cAMP-CRP) complex (23, 30). Additionally, the high-level expression of UhpT requires the regulatory genes *uhpA*, *uhpB*, and *uhpC* (12, 30). Therefore, Fos-R strains isolated in *E. coli* contain mutations that prevent Fos transport using GlpT or UhpT (23, 30). Plasmid-encoded resistance also has been described previously (4, 41).

In this paper, we describe the screening and analysis of Fos-R clones in a *P. aeruginosa* PA14 ordered insertional library (18). In addition, we studied the mutations responsible for the spontaneous resistance to Fos in *P. aeruginosa* PA14, the effect of these mutations on the in vitro growth rate, and the uniqueness of the mutational target.

MATERIALS AND METHODS

Bacteria and media. Table 1 shows the *E. coli* and *P. aeruginosa* strains used in this work. The *P. aeruginosa* mutant library PA14 *glpT*::*MAR2xT7* and its

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TABLE 1. Fos MICs for mutants of *E. coli* K-12 BW25113 and *P. aeruginosa* PA14 in LB alone or with 0.05% glucose-6-phosphate[a] (G6P)

Strain	Fos MIC (μg/ml) for *E. coli* BW25113 with:		Strain	Fos MIC (μg/ml) for *P. aeruginosa* PA14 with:		Identity[b] (%)
	LB	G6P		LB	G6P	
Wild type	2	2	Wild type	8	8	
glpT	32	8	*glpT*	1,024	1,024	74.76
uhpT	8	8	*hexP1*(PA14_49610)	8	8	23.04
			hexP2(PA14_44190)	8	8	24.01
			hexP3(PA14_38560)	8	8	26.49
uhpA	8	8				
uhpB	8	8				
uhpC	4	4				
cyaA	16	8	*cyaA*	8	8	36.94
ptsI	4	4	*ptsI*	8	8	39.72

[a] *E. coli* strains are deletion mutants with a kanamycin resistance cassette. *P. aeruginosa* are insertional mutants harboring an *MAR2xT7* gentamicin resistance transposon.

[b] Identity indicates a comparison between *E. coli* and *P. aeruginosa* protein sequences encoded by homologous genes.

wild-type strain PA14 were kindly provided by N. T. Liberati (18). All *P. aeruginosa* strains were cultured at 37°C in Luria-Bertani (LB) or M9 medium (38) containing gentamicin (15 μg/ml) or tetracycline (final concentration of 150 μg/ml for plasmid selection) when appropriate. *E. coli* K-12 mutant strains were obtained from the NARA institute (3) and were cultured routinely at 37°C in LB supplemented with (40 μg/ml) when appropriate.

Screening of the *P. aeruginosa* PA14 insertional library. The PA14 *MAR2xT7* nonredundant insertional library (18) was screened for Fos resistance. The library contains 5,459 mutants, each in a well of 96-well plates. The library was replicated in 96-well plates containing LB plus gentamicin (15 μg/ml) and incubated overnight at 37°C. Aliquots of 5 μl from each well were spotted onto LB plates containing either 128 μg/ml Fos or no antibiotic. Plates were incubated for 24 h at 37°C and immediately submitted to screening for the resistance phenotype. Mutants showing resistance or producing a much higher number of colonies than the wild-type strain on Fos-containing plates were isolated from the frozen 96-well plates and submitted to a second round of verification. To retest candidate strains, three independent colonies from each mutant were grown in LB broth, and appropriate dilutions were plated onto LB and LB-Fos to verify resistance.

Estimation of spontaneous Fos-R mutant frequencies. For spontaneous mutation frequency measurements of PA14, *mutS* and *mutT* mutant derivatives, and *E. coli* BW25113, approximately 10^2 cells from overnight cultures were inoculated into five tubes, each containing 1 ml of LB, and the tubes were incubated at 37°C with strong shaking for 24 h. Aliquots from successive dilutions were plated onto LB plates with Fos (final concentration, 128 μg/ml) or without any antibiotic to estimate viability. The numbers of colonies growing after 24 h of incubation were determined, and the mutation frequency was estimated as the number of Fos-R colonies divided by the number of viable cells.

Generation and analysis of spontaneous Fos-R mutants. To test the linkage between glycerol-3-P use and Fos resistance, 20 flasks containing M9-glucose were inoculated with about 10^3 wild-type PA14 cells. Flasks were incubated overnight with shaking at 37°C. About 10^9 cells from each culture were plated on plates of M9 agar containing glycerol-3-P plus Fos (128 μg/ml) and on M9 agar plates containing glycerol, instead of glycerol-3-P, plus Fos. Appropriate dilutions from these flasks also were plated on M9 agar-glycerol-3-P and M9 agar-glycerol plates without Fos to estimate the number of viable cells.

For the isolation and characterization of Fos-R mutants, about 10^3 cells of PA14 were inoculated in 10 25-ml flasks containing 5 ml of LB and were incubated overnight at 37°C with shaking. One hundred microliters of each of the 10 independent overnight cultures was plated on petri dishes containing LB agar supplemented with Fos (128 μg/ml) and incubated at 37°C for 24 h. From each plate, a single colony was isolated and purified on LB agar containing Fos at the same concentration. Chromosomal DNA was isolated from each of them and the wild-type strain. The corresponding *glpT* genes were PCR amplified and sequenced using the oligonucleotides glpT-P14-F1 (5′-AGCGGAGCTCGCGATGTTC-3′) and glpT-P14-R1 (5′-TCAGCCGGCTTGCTGCGG-3′) as forward and reverse primers, respectively.

Construction of plasmid pBBR-glpT and complementation of Fos-R mutants. A DNA fragment containing the *glpT* gene from the wild-type PA14 genomic DNA was generated by PCR using the oligonucleotides glpT-P14-F1 and glpT-P14-R1. The wild-type *glpT* gene was cloned directly into the vector pBBR1MCS-3 (15). For this purpose, the plasmid was digested with SmaI and converted in a T-vector as previously described (36). The resulting plasmid was named pBBR-glpT. Finally, pBBR-glpT was introduced by electroporation into the wild-type PA14, the *glpT* insertional mutant (PA14 *glpT*::*MAR2xT7*), and all of the isolated Fos-R mutants. Transformants were selected on LB agar supplemented with 150 μg/ml tetracycline.

Antibiotic susceptibility testing. MICs were determined by the broth microdilution method as recommended by the CLSI (7), except that LB was used instead of Mueller-Hinton medium. To stimulate hexose phosphate transporter expression, 0.05 mM glucose-6-phosphate was added, as an inducer, to the LB broth when necessary.

Growth curves. Growth curves of wild-type PA14 and *glpT* mutant strains were determined in rich medium (LB broth) and in minimal medium (M9). Strains were grown overnight at 37°C with shaking in LB or M9-glucose, respectively. These cultures were diluted 100-fold in the same medium and incubated for 3 h until they reached exponential-phase growth. Approximately 10^4 cells of each strain then were inoculated in quadruplicate into the wells of a 96-well microplate containing 100 μl of LB or M9 broth, using 0.1% glucose, 0.5% glucose-6-phosphate, 0.1% glycerol, or 0.5% glycerol-3-phospate as the sole carbon source. Cultures were incubated at 37°C with shaking in an Infinite M200 multiwell fluorimeter (Tecan, Switzerland). The absorbance at 595 nm was monitored every 15 min. The growth rates were calculated with values from the exponential part of each one of the growth curves (the regression coefficient was >0.95 in all cases).

Biofilms. An abiotic solid-surface biofilm formation assay was performed in 96-well polystyrene microtiter plates after 20 h of incubation at 37°C, as described previously (36). After crystal violet staining, the absorbance was measured at 595 nm. Forty independent replicas were carried out for each strain.

RESULTS

Screening of the PA14 insertional library of mutants for Fos resistance. The 5,459 mutants of the *P. aeruginosa* PA14 *MAR2xT7* insertional library (18) were screened to identify genes whose inactivation conferred resistance to Fos (128 μg/ml), as described in Materials and Methods. Putative resistance mutants were retested, and those able to grow on Fos-containing LB agar plates were submitted to further analysis. Several mutants showed an increased number of colonies compared to the that of wild-type strain PA14 due to a hypermutator phenotype (32). Only two additional mutants, 30348 and 39942 (mutant identification numbers are from the PA14 collection) (18), grew clearly on this concentration of Fos. Both mutants carried insertions that interrupted the same gene, *glpT* (PA14_69130), and we thus conclude that *glpT* inactivation plays an essential role in resistance to the antibiotic Fos in *P. aeruginosa*.

In *P. aeruginosa*, the *glpT* gene encodes a putative glycerol-3-P permease protein that belongs to the organophosphate: phosphate antiporter (OPA) family of the major facilitator superfamily (MFS). Hydrophobicity sequence analysis revealed that the GlpT protein of *P. aeruginosa* contained the 12 highly conserved regions typical of all glycerol-3-P permeases, and they were arranged as putative transmembrane α-helices (named TM1 to TM12 in this study). Proteins within the OPA family identified in different bacterial species conserve significant sequence homology. In this sense, the *P. aeruginosa* PA14 GlpT protein shares the same sequence with the *P. aeruginosa* PAO1 ortholog, and it shows a high degree of homology to GlpT of *E. coli* K-12 (75% identity) and *B. subtilis* (62% iden-

TABLE 2. Genotypic and phenotypic characteristics of Fos-R mutants and their positions in the *glpT* gene in 10 independent Fos-R mutants[a]

Strain	Fos MIC (μg/ml) for strain harboring plasmid:		Resistance mutation
	pBBR1MCS-3	pBBR-glpT	
PA14	8	8	
glpT::*MAR2xT7*	1,024	8	Insertional inactivation
FosR-7	1,024	8	A_{59} deletion, 1 bp (frameshift); stop TGA_{318}
FosR-9	1,024	8	$C_{219}ATCGC_{225}$ deletion, 6 bp; Δ (Ile_{74}-Ala_{75}); TM2
FosR-1	1,024	8	$A_{220}TCGCC_{226}$ deletion, 6 bp; Δ (Ile_{74}-Ala_{75}); TM2
FosR-2	1,024	8	$T_{365}CATGTT_{371}$ deletion, 7 bp (frameshift); stop TGA_{507}
FosR-3	1,024	8	G to A; Gly_{137} ($GG_{410}C$)→ Asp (GAC); TM4
FosR-5	1,024	8	G_{596} deletion, 1 bp (frameshift); stop TAG_{600}
FosR-10	512	8	$C_{975}GG$ insertion, 3 bp; +Gly_{326}; TM9
FosR-4	1,024	8	A to C; Thr_{336} ($A_{1006}CC$)→ Pro (CCC); TM9
FosR-8	1,024	8	C to G; Tyr_{362} (TAC_{1086})→ stop TAG; TM10
FosR-6	512	8	G to A; Met $_{366}$ (ATG_{1098})→ Ile (ATA); TM10

[a] The levels of resistance when harboring the empty vector pBBR1MCS-3 or the complementing plasmid pBBR-glpT also are shown. The mutants are ordered in the table according to the position of their mutations in the *glpT* gene.

tity). In *E. coli*, GlpT acts as a secondary active transporter for glycerol-3-P uptake that exchanges a phosphate ion for a glycerol-3-P molecule (8, 11). This transporter also provides an entrance mechanism for Fos inside cells owing to this antibiotic mimicking glycerol-3-P. Thus, the impaired function of GlpT produces Fos resistance in *E. coli* (12).

Effect of inactivation in *P. aeruginosa* of known mutational targets that confer Fos resistance in *E. coli*. In *E. coli*, Fos can enter cells via the GlpT or UhpT transporter; thus, defects blocking either one of these transport systems decrease Fos susceptibility (11). UhpT is involved in the transport of glucose-6-P into the cell (2), and the expression of this protein is regulated by *uhpA*, *uhpB*, and *uhpC* (10, 26). The expression of both UhpT and GlpT is positively regulated by cAMP; thus, mutations in *cyaA* and *ptsI* that lower the cAMP levels will produce decreased Fos sensitivity in this species (1, 43). Therefore, apart from those in *glpT*, mutations in the genes *uhpA*, *uhpB*, *uhpC*, *uhpT*, *cyaA*, or *ptsI* confer Fos resistance in *E. coli* (26).

To study the effect of the inactivation of these genes on Fos resistance in *P. aeruginosa*, we first performed a BLAST search to identify their homologues in the PA14 genomic sequence database (http://www.pseudomonas.com). We detected proteins that maintain a high degree of identity to *E. coli* CyaA and PtsI (Table 2), but we failed to identify clear Uhp homologues encoded by the PA14 genome sequence, indicating that *P. aeruginosa* probably lacks a specific glucose-6-P transport system. When we compared the *E. coli* UhpT sequence to the PA14 genome database, the most similar protein was *P. aeruginosa* GlpT. Nevertheless, three different predicted MFS proteins (named here as HexP1, HexP2, and HexP3, for putative hexose phosphate transport) retain a certain homology with the *E. coli* UhpT protein (Table 2) (E value, $\leq 1.0 \times 10^{-5}$), reaching the highest levels of similarity among all of the transporters encoded in the *P. aeruginosa* PA14 genome (23 to 26% identity).

The level of Fos resistance conferred by the inactivation of these genes in *P. aeruginosa* PA14 was studied and compared to that conferred by their inactivation in *E. coli* K-12 BW25113 (Table 1). *P. aeruginosa* and *E. coli* mutants were obtained from the available libraries (PA14 mutant collections from Harvard Medical School and the NARA institute, respectively) (3, 18). Interestingly, the inactivation of *glpT* produced a dramatic increase in Fos resistance in *P. aeruginosa* with respect to the wild type (MICs of 1,024 and 8 μg/ml, respectively), whereas the inactivation of this gene produced only a moderate increase in Fos resistance in *E. coli* (MICs of 32 and 2 μg/ml, respectively). The inactivation of *uhpT* conferred a moderate Fos resistance in *E. coli* (MIC of 8 μg/ml). However, the inactivation of any of the three genes that encoded MFS proteins related to UhpT in *P. aeruginosa* did not produce any effect on Fos resistance. Also, the inactivation of *cyaA* and *ptsI* decreased susceptibility in *E. coli* but had no effect in *P. aeruginosa* (Table 2).

As stated before, UhpT expression is induced by its substrate, glucose-6-P. Thus, it is expected that, in the presence of glucose-6-P, resistance to Fos conferred by *glpT* mutations will be suppressed or attenuated due to an increase in Fos transport via UhpT permease. Table 2 shows that the level of Fos resistance conferred by the deletion of *glpT* decreased in *E. coli* when glucose-6-P was present. However, glucose-6-P had no effect on resistance in *P. aeruginosa*. These results strongly suggest that Fos enters *P. aeruginosa* cells using solely the GlpT permease, which also is essential for glycerol-3-P uptake, whereas in *E. coli* Fos can use both UhpT and GlpT permeases.

Fos-R spontaneous mutants do not grow in minimal medium with glycerol-3-P as the sole carbon source. To test if mutations in genes other than *glpT* can confer Fos resistance in *P. aeruginosa*, 20 flasks containing M9-glucose medium were inoculated with about 10^3 wild-type PA14 cells and incubated overnight at 37°C with shaking. About 10^9 cells from each culture were plated on M9 agar plates containing Fos (128 μg/ml) and glycerol-3-P as the sole carbon source and on M9 agar plates containing Fos plus glycerol instead of glycerol-3-P. Appropriate dilutions from these flasks also were plated on these media without Fos to estimate the number of viable cells. While about 1,000 Fos-R colonies grew on glycerol-Fos plates (frequency of Fos-R mutants, 1×10^{-6}), none grew on glycerol-3-P plus Fos (frequency, $\leq 5 \times 10^{-11}$). This result demonstrates that all mutations conferring Fos resistance affected the capacity to use glycerol-3-P as the sole carbon source.

Therefore, it seems that Fos is imported into *P. aeruginosa* cells exclusively via the glycerol-3-P transport system. Thus, the spontaneous acquisition of Fos resistance requires the inactivation of GlpT permease, leading to a glycerol-3-phosphate-defective growth phenotype in all mutant isolates.

Analysis of spontaneous PA14 Fos-R mutants. The inability to select Fos-R mutants in minimal medium with glycerol-3-P as the sole carbon source prompted us to analyze the *glpT* sequence carried by spontaneous PA14 Fos-R mutants to know whether the *glpT* gene is the only target for Fos resistance in *P. aeruginosa*.

Ten independent PA14 Fos-R mutants were obtained in LB-Fos as described in Materials and Methods. Chromosomal DNA was extracted from each resistant mutant, and their *glpT* sequences were PCR amplified and sequenced. The analysis of their respective sequences showed that all of them contained mutations in the *glpT* gene sequence and putatively were able to impair GlpT function. This strongly suggests that *glpT* inactivation is essential for acquiring Fos resistance in *P. aeruginosa*. Table 2 shows the type of mutations, their positions in the *glpT* gene, and the level of resistance conferred.

A detailed analysis of the type of mutations identified in these isolates reveals that five mutants contained deletions in the *glpT* gene, four mutants contained point mutations, and one harbored an insertion. Two mutants, 1 and 9, contained 6-bp deletions in the 6-bp repeated sequence CATCGCCAT CGC, yet each displaced 1 bp (the deletion of ATCGCC and CATCGC in mutants 1 and 9, respectively). These deletions lead, in both cases, to the loss of amino acids Ile-74 and Ala-75 in TM2, conferring a highly Fos-R phenotype and a MIC of 1,024 μg/ml (Table 2). Three other mutants also carried deletions (1 bp in mutants 5 and 7 and 7 bp in mutant 2) producing frameshifts that led to premature stop codons. Truncated proteins putatively are nonfunctional and reach a high level of Fos resistance (MIC of 1,024 μg/ml in all three cases) (Table 2). Four mutants contained point mutations, two transitions (mutants 3 and 6), and two transversions (mutants 4 and 8). Mutant 3 contained the mutation G to A, leading to a Gly-137-Asp change in the middle of the TM4 region. The appearance of a negatively charged amino acid may drastically distort the conformation of this transmembrane helix. Mutant 4 contained an A-to-C mutation originating the change Thr-336-Pro inside TM9, probably producing a great structural distortion in this region. Mutant 8 carried a C-to-G mutation, creating a stop codon at position 362, which is located in the middle of TM10. This produces the loss of the last two α-helices of the protein. These three mutants also are highly resistant to Fos, which has a MIC of 1,024 μg/ml for all three. Mutant 6 contained a G-to-A mutation, originating the conservative change Met-366-Ile, which, in principle, maintains the hydrophobic nature in the TM10 region. This change, however, produces high Fos resistance (MIC of 512 μg/ml), although slightly less than that of the previous mutants. This suggests that methionine in this position of TM10 is important for the function of the protein. Finally, mutant 10 contained an in-frame insertion of the triplet CGG after another CGG codon, originating the insertion of a Gly at position 326 (inside TM9). The level of Fos resistance is similar to that of mutant 6 (MIC of 512 μg/ml). Mutations carried by the last two isolates demonstrate that even small changes in the GlpT amino acid sequence can produce considerable increases in Fos resistance levels in *P. aeruginosa*.

TABLE 3. Effect of Fos-R mutations on growth rates and biofilm formation[a]

Strain	Relative growth rate (%)	SD	Relative biofilm formation (%)	SD
Wild type	100	4.84	100	7.13
glpT::*MAR2xT7*	106.79	6.47	95.34	9.21
Fos-R7	99.92	8.45	103.79	7.23
Fos-R9	97.20	4.78	95.37	11.34
Fos-R1	105.93	4.80	94.78	9.00
Fos-R2	106.47	5.05	97.84	12.20
Fos-R3	106.31	5.05	91.84	9.12
Fos-R5	100.40	3.18	94.24	6.10
Fos-R10	96.77	7.43	101.79	2.05
Fos-R4	104.10	6.53	98.32	13.40
Fos-R8	98.24	5.46	116.13	12.20
Fos-R6	100.17	5.55	92.85	8.70

[a] All values are relative to that for the wild-type strain (100%). In all cases, the Student's *t* test indicates no differences between wild-type and mutant strains ($P > 0.05$). The mutants are ordered in the table according to the position of their mutations in the *glpT* gene. SD, standard deviations.

Finally, none of the Fos-R mutants, including both insertional *glpT*::*MAR2xT7* and spontaneous mutants, was able to grow in glycerol-3-P as the sole carbon source, even after prolonged incubation for 4 days in either solid or liquid medium (data not shown).

Only *glpT* mutations are responsible for the Fos-R phenotype and the inability to grow in glycerol-3-P as the sole carbon source. To further prove that only the mutations found in the *glpT* gene are responsible for the Fos-R phenotype, we introduced the plasmid pBBR-glpT, containing the wild-type *glpT* gene, into the 10 spontaneous Fos-R mutants and also into both the insertion mutant *glpT*::*MAR2xT7* and the wild-type PA14. In all cases, the wild-type level of Fos susceptibility was recovered upon complementation with the wild-type *glpT* gene, with no effect on the MIC of Fos for the wild-type strain (Table 2). The ability to grow in glycerol-3-P was recovered after the introduction of the wild-type *glpT* gene in these isolates (data not shown).

On the other hand, glucose-6-P did not have any effect on the level of the Fos resistance of the spontaneous Fos-R isolates (Table 2), suggesting again the lack of a glucose-6-P-specific transport in *P. aeruginosa*.

Effect of Fos-R mutations on growth and biofilm formation. To know the possible effect of Fos-R mutations on the growth rate of *P. aeruginosa*, we compared the growth curves of the Fos-R mutants, including that of the insertion mutant *glpT*::*MAR2xT7*, to that of the wild-type PA14 strain. Fos-R mutations seem to have no apparent effect on growth rates in LB (Table 3) and in M9 minimal medium with glucose or glycerol as the sole carbon sources compared to those for the wild type (data not shown), although more accurate experiments should be performed. In addition, neither wild-type nor mutant derivatives were able to grow in minimal medium with glucose-6-P as the sole carbon source (data not shown). These results suggest that, unlike *E. coli* Fos-R mutants (26), mutations conferring Fos resistance in *P. aeruginosa* do not have an effect on fitness in vitro. On the other hand, we studied the effect of Fos-R mutations on the ability of *P. aeruginosa* to

TABLE 4. Frequency of mutants resistant to Fos (128 μg/ml) of the wild-type strain PA14 and its hypermutable variants *mutS* and *mutT*

Strain	Mutant frequency	Increase (fold)
PA14	1.4×10^{-6}	1
mutS::*MAR2xT7*	1.2×10^{-4}	86
mutT::*MAR2xT7*	2.8×10^{-5}	20

form biofilm on an abiotic surface. Our results demonstrate that, under our conditions, the Fos-R mutants produce biofilm at a level similar to that of the wild type (Table 3).

Frequency of mutants conferring resistance to Fos in hypermutator strains. The frequency of Fos-R mutants was calculated for wild-type and hypermutator *P. aeruginosa* strains with deficiency in *mutS* and *mutT*. The *mutS* allele is more frequently found in isolates from cystic fibrosis patients (20, 31). A *P. aeruginosa* hypermutator strain with deficiency in *mutT* recently was isolated in a cystic fibrosis patient (22). Table 4 shows that the frequency of *P. aeruginosa* Fos-R mutants was considerably increased in the *mutS* strain (close to two orders of magnitude). The frequency also was increased, although moderately (20-fold), in the *mutT* strain. Thus, the acquisition of Fos resistance is a very easy task for *P. aeruginosa* hypermutator strains, suggesting that even very small populations, such as those that probably colonize the lungs of cystic fibrosis patients at the beginning of the natural history of *P. aeruginosa* infections, contain Fos-R mutants. On the other hand, the mutant frequency for Fos-R (128 μg/ml) was higher in the *P. aeruginosa* wild-type strain (1.4×10^{-6}) (Table 4) than in *E. coli* BW25113 ($\leq 2.6 \times 10^{-10}$) (not shown). Thus, it seems very difficult to reach a high level of Fos resistance (128 μg/ml) by single spontaneous mutations in *E. coli*, as it has two different Fos import systems.

DISCUSSION

Fos is active against both gram-positive and gram-negative bacteria and is structurally unrelated to any other antibiotic, minimizing the possibility of cross-resistance with them (35). Interestingly, despite its long-term use in *E. coli* urinary tract infections, the frequency of Fos resistance in uropathogenic *E. coli* strains remains very low (13). Resistance mutations have been demonstrated to confer a fitness cost that prevents resistant strains from being established in the bladder, suggesting that this cost is responsible for the low frequency of resistance (26).

In cystic fibrosis patients, after continuous exposure to antibiotics, *P. aeruginosa* develops resistance to many or all of them (5). Prolonged therapy courses also lead to the selection of hypermutator strains that, indirectly, favor the acquisition of resistance (31, 32). Therefore, the treatment of multidrug-resistant *P. aeruginosa* infections requires the combination of various antimicrobial agents (7, 29, 42, 44). Most antibiotic resistance mechanisms in cystic fibrosis isolates of *P. aeruginosa* are based on mutation (31); therefore, it is expected that Fos resistance follows the same principle. In this sense, the molecular bases of Fos resistance in this species has been extrapolated from *E. coli*, suggesting that Fos could be transported into *P. aeruginosa* through the same permeases, i.e., via glucose-6-P and glycerol-3-P transport systems (10, 24, 28, 39). Thus, a systematic study to unveil the molecular bases involved in the development of Fos resistance in *P. aeruginosa* was of particular interest.

In *E. coli*, spontaneous Fos resistance is caused by mutations in genes involved in glycerol-3-P transport (*glpT*) or glucose-6-P transport (*uhpA*, *uhpB*, *uhpC*, and *uhpT*) (23). However, *P. aeruginosa* does not appear to contain the UhpT system. Four different predicted proteins, including GlpT, share weak homology with the *E. coli* UhpT protein in *P. aeruginosa*. However, the inactivation of the genes encoding these additional MFS proteins did not produce any effect on Fos resistance. In addition, no *uhpA*, *uhpB*, or *uhpC* homologues were found in the PA14 genome sequence. Finally, although *cyaA* and *ptsI* mutants are resistant to Fos in *E. coli* (26), the inactivation of *cyaA* and *ptsI* homologues did not confer Fos resistance in *P. aeruginosa*. These results suggest that multiple components whose disruption leads to the acquisition of Fos resistance in *E. coli* are not conserved in *P. aeruginosa*.

We have demonstrated that only insertions and spontaneous mutations affecting *glpT* produce resistance to Fos in *P. aeruginosa* PA14. In addition, all Fos-R mutants are unable to use glycerol-3-P as the sole carbon source. Spontaneous Fos-R mutants appeared at a frequency of 10^{-6} on both rich and minimal medium with glycerol as the sole carbon source. However, no mutants were isolated on minimal medium with glycerol-3-P as the sole carbon source (frequency, $\leq 5 \times 10^{-11}$), which is consistent with a deficient transport of glycerol-3-P.

The analysis of the *glpT* sequence of 10 independent *P. aeruginosa* Fos-R mutants confirmed that all of them contained mutations in *glpT*. The identity of these mutations provides additional insight into the structure-function relationship of the GlpT permease (producing Fos-R mutants with different levels of resistance). For instance, it has become clear that even subtle changes in the GlpT amino acid sequence produce a considerable effect on the GlpT activity (impairing both growth in glycerol-3-P and Fos transport) in *P. aeruginosa*. In this sense, all changes in the amino acid sequence caused by point mutations always are located in any of the 12 TM domains of the GlpT permease, distorting the conformation of these α-helices. In *E. coli* GlpT, these 12 conserved TM domains are arranged in two compact bundles of six α-helices around a central pore, and the maintenance of the structure of these regions is essential for substrate translocation (11). Additionally, proteins within the OPA transporter family retain a high degree of sequence homology, with a considerable number of amino acids whose change impairs the transporter function (16).

On the other hand, generally it has been assumed that *P. aeruginosa* has a specific glucose-6-P transport system. Furthermore, it has been stated that glucose-6-P should be added to the growth medium for Fos sensitivity in in vitro testing to allow Fos efficacy (i.e., to induce the expression of glucose-6-P transport) (see, for instance, references 24 and 28) and references therein). However, four lines of evidence strongly suggest that, in contrast with *E. coli*, *P. aeruginosa* lacks a specific glucose-6-P transport system: (i) the absence of the detectable growth of wild-type PA14 and its mutant derivatives when cultured in minimal medium with glucose-6-P as the sole car-

bon source, (ii) the absence of *uhp* transport genes in the *P. aeruginosa* genome, (iii) the lack of an effect of the inactivation of the three putative hexose phosphate transporters, with low homology to the *E. coli* UhpT transporter, on Fos resistance, and (iv) the absence of an effect of glucose-6-P on the Fos MIC for the *P. aeruginosa glpT* Fos-R mutants.

Finally, we studied the effect of these mutations on the in vitro growth rate and the ability to form biofilms. Although all of the Fos-R mutants studied in this work showed a clear fitness cost when grown on glycerol-3-P as the sole carbon source, no apparent cost was observed when it was grown in rich medium or in minimal medium with glucose or glycerol, unlike the *E. coli* Fos-R mutants. Because of its metabolic versatility, *P. aeruginosa* can use a wider variety of carbon compounds than *E. coli* to obtain energy. Its ability to use a large number of compounds could allow *P. aeruginosa* to overcome the lack of glycerol-3-P supply, leading to the absence of a fitness cost. In contrast to *P. aeruginosa*, *E. coli* uses primarily sugars as energy and carbon sources through glycolysis. Thus, *E. coli* Fos-R mutants unable to provide glucose-6-P and/or glycerol-3-P could suffer an energy shortage, which might explain their reduced growth ability. However, competition experiments between Fos-R mutants and the wild-type strain should be performed to fully disprove a cost for these mutations in *P. aeruginosa*. In addition, these mutations did not affect the ability to form biofilm.

In summary, our results strongly suggest that *glpT* inactivation is the only mutational mechanism leading to Fos resistance in *P. aeruginosa*. Both the apparent lack of a fitness cost and the high frequency of the spontaneous Fos-R mutants suggest that Fos-R variants will be obtained easily in vivo upon the Fos treatment of *P. aeruginosa* infections. Furthermore, considering the frequency of Fos-R mutants in the *mutS* hypermutable strain (10^{-4}) found in this study and the reported frequency of mutants resistant to other antipseudomonal antibiotics (21, 25, 34), a dangerous frequency of spontaneous mutants with simultaneous resistance to the combination of Fos with other antibiotics can be expected even with combined treatments. Obviously, exploring both the frequency of *P. aeruginosa* Fos-R mutants in vivo and the ability of these mutants to colonize and survive in the lungs is essential.

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REFERENCES

1. **Alper, M. D., and B. N. Ames.** 1978. Transport of antibiotics and metabolite analogs by systems under cyclic AMP control: positive selection of *Salmonella typhimurium cya* and *crp* mutants. J. Bacteriol. **133:**149–157.
2. **Ambudkar, S. V., V. Anantharam, and P. C. Maloney.** 1990. UhpT, the sugar phosphate antiporter of *Escherichia coli*, functions as a monomer. J. Biol. Chem. **265:**12287–12292.
3. **Baba, T., T. Ara, M. Hasegawa, Y. Takai, Y. Okumura, M. Baba, K. A. Datsenko, M. Tomita, B. L. Wanner, and H. Mori.** 2006. Construction of *Escherichia coli* K-12 in-frame, single-gene knockout mutants: the Keio collection. Mol. Syst. Biol. **2:**2006–2008.
4. **Bernat, B. A., L. T. Laughlin, and R. N. Armstrong.** 1997. Fosfomycin resistance protein (FosA) is a manganese metalloglutathione transferase related to glyoxalase I and the extradiol dioxygenases. Biochemistry **36:**3050–3055.
5. **Ciofu, O., B. Giwercman, S. S. Pedersen, and N. Hoiby.** 1994. Development of antibiotic resistance in *Pseudomonas aeruginosa* during two decades of antipseudomonal treatment at the Danish CF Center. APMIS **102:**674–680.
6. **Chin, N. X., N. M. Neu, and H. C. Neu.** 1986. Synergy of fosfomycin with beta-lactam antibiotics against staphylococci and aerobic gram-negative bacilli. Drugs Exp. Clin. Res. **12:**943–947.
7. **CLSI.** 2006. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. Approved standard M7–A7. 7th ed. CLSI, Wayne, PA.
8. **Elvin, C. M., C. M. Hardy, and H. Rosenberg.** 1985. P_i exchange mediated by the GlpT-dependent *sn*-glycerol-3-phosphate transport system in *Escherichia coli*. J. Bacteriol. **161:**1054–1058.
9. **Falagas, M. E., A. C. Kastoris, D. E. Karageorgopoulos, and P. I. Rafailidis.** 2009. Fosfomycin for the treatment of infections caused by multidrug-resistant non-fermenting gram-negative bacilli: a systematic review of microbiological, animal and clinical studies. Int. J. Antimicrob. Agents. **34:**111–120.
10. **Hardisson, C., and J. Llaneza.** 1977. The action of fosfomycin on the growth of *Pseudomonas aeruginosa*. Chemotherapy **23**(Suppl. 1)**:**37–44.
11. **Huang, Y., M. J. Lemieux, J. Song, M. Auer, and D. N. Wang.** 2003. Structure and mechanism of the glycerol-3-phosphate transporter from *Escherichia coli*. Science **301:**616–620.
12. **Kahan, F. M., J. S. Kahan, P. J. Cassidy, and H. Kropp.** 1974. The mechanism of action of fosfomycin (phosphonomycin). Ann. N. Y. Acad. Sci. **235:**364–386.
13. **Kahlmeter, G.** 2000. The ECO.SENS Project: a prospective, multinational, multicentre epidemiological survey of the prevalence and antimicrobial susceptibility of urinary tract pathogens—interim report. J. Antimicrob. Chemother. **46**(Suppl. 1)**:**15–65.
14. **Kiewitz, C., and B. Tummler.** 2000. Sequence diversity of *Pseudomonas aeruginosa*: impact on population structure and genome evolution. J. Bacteriol. **182:**3125–3135.
15. **Kovach, M. E., P. H. Elzer, D. S. Hill, G. T. Robertson, M. A. Farris, R. M. Roop, Jr., and K. M. Peterson.** 1995. Four new derivatives of the broad-host-range cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes. Gene **166:**175–176.
16. **Lemieux, M. J., Y. Huang, and D. N. Wang.** 2004. Glycerol-3-phosphate transporter of *Escherichia coli*: structure, function and regulation. Res. Microbiol. **155:**623–629.
17. **Lemieux, M. J., Y. Huang, and D. N. Wang.** 2004. The structural basis of substrate translocation by the *Escherichia coli* glycerol-3-phosphate transporter: a member of the major facilitator superfamily. Curr. Opin. Struct. Biol. **14:**405–412.
18. **Liberati, N. T., J. M. Urbach, S. Miyata, D. G. Lee, E. Drenkard, G. Wu, J. Villanueva, T. Wei, and F. M. Ausubel.** 2006. An ordered, nonredundant library of *Pseudomonas aeruginosa* strain PA14 transposon insertion mutants. Proc. Natl. Acad. Sci. USA **103:**2833–2838.
19. **Lyczak, J. B., C. L. Cannon, and G. B. Pier.** 2002. Lung infections associated with cystic fibrosis. Clin. Microbiol. Rev. **15:**194–222.
20. **Maciá, M. D., D. Blanquer, B. Togores, J. Sauleda, J. L. Perez, and A. Oliver.** 2005. Hypermutation is a key factor in development of multiple-antimicrobial resistance in *Pseudomonas aeruginosa* strains causing chronic lung infections. Antimicrob. Agents Chemother. **49:**3382–3386.
21. **Maciá, M. D., N. Borrell, J. L. Perez, and A. Oliver.** 2004. Detection and susceptibility testing of hypermutable *Pseudomonas aeruginosa* strains with the Etest and disk diffusion. Antimicrob. Agents Chemother. **48:**2665–2672.
22. **Mandsberg, L. F., O. Ciofu, N. Kirkby, L. E. Christiansen, H. E. Poulsen, and N. Hoiby.** 2009. Antibiotic resistance in *Pseudomonas aeruginosa* strains with increased mutation frequency due to inactivation of the DNA oxidative repair system. Antimicrob. Agents Chemother. **53:**2483–2491.
23. **Merkel, T. J., J. L. Dahl, R. H. Ebright, and R. J. Kadner.** 1995. Transcription activation at the *Escherichia coli uhpT* promoter by the catabolite gene activator protein. J. Bacteriol. **177:**1712–1718.
24. **Mirakhur, A., M. J. Gallagher, M. J. Ledson, C. A. Hart, and M. J. Walshaw.** 2003. Fosfomycin therapy for multiresistant *Pseudomonas aeruginosa* in cystic fibrosis. J. Cyst. Fibros. **2:**19–24.
25. **Moya, B., A. Dotsch, C. Juan, J. Blazquez, L. Zamorano, S. Haussler, and A. Oliver.** 2009. Beta-lactam resistance response triggered by inactivation of a nonessential penicillin-binding protein. PLoS Pathog. **5:**e1000353.
26. **Nilsson, A. I., O. G. Berg, O. Aspevall, G. Kahlmeter, and D. I. Andersson.** 2003. Biological costs and mechanisms of fosfomycin resistance in *Escherichia coli*. Antimicrob. Agents Chemother. **47:**2850–2858.
27. **Obritsch, M. D., D. N. Fish, R. MacLaren, and R. Jung.** 2005. Nosocomial infections due to multidrug-resistant *Pseudomonas aeruginosa*: epidemiology and treatment options. Pharmacotherapy **25:**1353–1364.
28. **Okazaki, M., K. Suzuki, N. Asano, K. Araki, N. Shukuya, T. Egami, Y. Higurashi, K. Morita, H. Uchimura, and T. Watanabe.** 2002. Effectiveness of fosfomycin combined with other antimicrobial agents against multidrug-resistant *Pseudomonas aeruginosa* isolates using the efficacy time index assay. J. Infect. Chemother. **8:**37–42.
29. **Olay, T., A. Rodriguez, L. E. Oliver, M. V. Vicente, and M. C. Quecedo.** 1978. Interaction of fosfomycin with other antimicrobial agents: in vitro and in vivo studies. J. Antimicrob. Chemother. **4:**569–576.

30. **Olekhnovich, I. N., J. L. Dahl, and R. J. Kadner.** 1999. Separate contributions of UhpA and CAP to activation of transcription of the *uhpT* promoter of *Escherichia coli*. J. Mol. Biol. **292:**973–986.
31. **Oliver, A., R. Canton, P. Campo, F. Baquero, and J. Blazquez.** 2000. High frequency of hypermutable *Pseudomonas aeruginosa* in cystic fibrosis lung infection. Science **288:**1251–1254.
32. **Oliver, A., B. R. Levin, C. Juan, F. Baquero, and J. Blazquez.** 2004. Hypermutation and the preexistence of antibiotic-resistant *Pseudomonas aeruginosa* mutants: implications for susceptibility testing and treatment of chronic infections. Antimicrob. Agents Chemother. **48:**4226–4233.
33. **Patel, S. S., J. A. Balfour, and H. M. Bryson.** 1997. Fosfomycin tromethamine. A review of its antibacterial activity, pharmacokinetic properties and therapeutic efficacy as a single-dose oral treatment for acute uncomplicated lower urinary tract infections. Drugs **53:**637–656.
34. **Plasencia, V., N. Borrell, M. D. Macia, B. Moya, J. L. Perez, and A. Oliver.** 2007. Influence of high mutation rates on the mechanisms and dynamics of in vitro and in vivo resistance development to single or combined antipseudomonal agents. Antimicrob. Agents Chemother. **51:**2574–2581.
35. **Reeves, D. S.** 1994. Fosfomycin trometamol. J. Antimicrob. Chemother. **34:**853–858.
36. **Rodríguez-Rojas, A., and J. Blazquez.** 2009. The *Pseudomonas aeruginosa pfpI* gene plays an antimutator role and provides general stress protection. J. Bacteriol. **191:**844–850.
37. **Saiman, L.** 2007. Clinical utility of synergy testing for multidrug-resistant *Pseudomonas aeruginosa* isolated from patients with cystic fibrosis: 'the motion for'. Paediatr. Respir. Rev. **8:**249–255.
38. **Sambrook, J., and D. W. Russell.** 2001. Molecular cloning: a laboratory manual, 3rd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
39. **Schweizer, H. P., and C. Po.** 1996. Regulation of glycerol metabolism in *Pseudomonas aeruginosa*: characterization of the *glpR* repressor gene. J. Bacteriol. **178:**5215–5221.
40. **Stover, C. K., X. Q. Pham, A. L. Erwin, S. D. Mizoguchi, P. Warrener, M. J. Hickey, F. S. Brinkman, W. O. Hufnagle, D. J. Kowalik, M. Lagrou, R. L. Garber, L. Goltry, E. Tolentino, S. Westbrock-Wadman, Y. Yuan, L. L. Brody, S. N. Coulter, K. R. Folger, A. Kas, K. Larbig, R. Lim, K. Smith, D. Spencer, G. K. Wong, Z. Wu, I. T. Paulsen, J. Reizer, M. H. Saier, R. E. Hancock, S. Lory, and M. V. Olson.** 2000. Complete genome sequence of *Pseudomonas aeruginosa* PA01, an opportunistic pathogen. Nature **406:** 959–964.
41. **Suárez, J. E., and M. C. Mendoza.** 1991. Plasmid-encoded fosfomycin resistance. Antimicrob. Agents Chemother. **35:**791–795.
42. **Tessier, F., and C. Quentin.** 1997. In vitro activity of fosfomycin combined with ceftazidime, imipenem, amikacin, and ciprofloxacin against *Pseudomonas aeruginosa*. Eur. J. Clin. Microbiol. Infect. Dis. **16:**159–162.
43. **Tsuruoka, T., A. Miyata, and Y. Yamada.** 1978. Two kinds of mutants defective in multiple carbohydrate utilization isolated from in vitro fosfomycin-resistant strains of *Escherichia coli* K-12. J. Antibiot. (Tokyo) **31:**192–201.
44. **Visalli, M. A., M. R. Jacobs, and P. C. Appelbaum.** 1998. Determination of activities of levofloxacin, alone and combined with gentamicin, ceftazidime, cefpirome, and meropenem, against 124 strains of *Pseudomonas aeruginosa* by checkerboard and time-kill methodology. Antimicrob. Agents Chemother. **42:**953–955.