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Fosfomycin and Tobramycin in Combination Downregulate Nitrate Reductase Genes *narG* and *narH*, Resulting in Increased Activity against *Pseudomonas aeruginosa* under Anaerobic Conditions

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The activity of aminoglycosides, which are used to treat *Pseudomonas aeruginosa* respiratory infection in cystic fibrosis (CF) patients, is reduced under the anaerobic conditions that reflect the CF lung *in vivo*. In contrast, a 4:1 (wt/wt) combination of fosfomycin and tobramycin (F:T), which is under investigation for use in the treatment of CF lung infection, has increased activity against *P. aeruginosa* under anaerobic conditions. The aim of this study was to elucidate the mechanisms underlying the increased activity of F:T under anaerobic conditions. Microarray analysis was used to identify the transcriptional basis of increased F:T activity under anaerobic conditions, and key findings were confirmed by microbiological tests, including nitrate utilization assays, growth curves, and susceptibility testing. Notably, growth in subinhibitory concentrations of F:T, but not tobramycin or fosfomycin alone, significantly downregulated ($P < 0.05$) nitrate reductase genes *narG* and *narH*, which are essential for normal anaerobic growth of *P. aeruginosa*. Under anaerobic conditions, F:T significantly decreased ($P < 0.001$) nitrate utilization in *P. aeruginosa* strains PAO1, PA14, and PA14 *lasR*::Gm, a mutant known to exhibit increased nitrate utilization. A similar effect was observed with two clinical *P. aeruginosa* isolates. Growth curves indicate that nitrate reductase transposon mutants had reduced growth under anaerobic conditions, with these mutants also having increased susceptibility to F:T compared to the wild type under similar conditions. The results of this study suggest that downregulation of nitrate reductase genes resulting in reduced nitrate utilization is the mechanism underlying the increased activity of F:T under anaerobic conditions.

The lungs of cystic fibrosis (CF) patients contain aerobic, microaerophilic, and anoxic regions, with pathogens such as *Pseudomonas aeruginosa* and strict anaerobic bacterial species growing in diverse polymicrobial communities within these niches (1, 2). *P. aeruginosa*, which is regarded as the principal causative pathogen of respiratory infection in CF patients (3), is well adapted to proliferate under anaerobic conditions by the use of nitrate as the terminal electron acceptor in respiration (4). Nitrate is present in CF sputum at sufficient levels (average of approximately 400 μM) to support the anaerobic growth of *P. aeruginosa* (5). Moreover, anaerobiosis is known to affect the activity of some classes of antimicrobials, with previous studies showing that tobramycin, amikacin, aztreonam, colistin, and ciprofloxacin have reduced bactericidal activity against *P. aeruginosa* under these conditions (6–8).

P. aeruginosa has the ability to become rapidly multiply antibiotic resistant, via either the acquisition of resistance elements or mutation; current reports show that it is becoming progressively more resistant to many currently available antimicrobials (9–11). Therefore, there is a need for new agents with activity against this pathogen, and it would be particularly beneficial if such agents were active under anaerobic conditions.

A 4:1 (wt/wt) combination of fosfomycin and tobramycin (F:T) is under investigation as a potential inhalation therapy for use in CF patients. We have previously shown that F:T or fosfomycin alone has good *in vitro* activity against *P. aeruginosa* and, importantly, increased activity under anaerobic conditions, reflecting the CF lung environment *in vivo* (12). In addition, we have shown that F:T was bactericidal against *P. aeruginosa* grown in biofilms under both aerobic and anaerobic conditions (12).

We hypothesized that the increased activity of F:T under anaerobic conditions may be mediated through either fosfomycin or tobramycin alone or could be due to effects apparent only when fosfomycin and tobramycin were combined in F:T. For example, altered expression of the fosfomycin target *murA*, the gene encoding the fosfomycin uptake protein (*glpT*), or genes involved in tobramycin uptake under anaerobic conditions may be responsible for the increased activity of F:T. Therefore, the aim of this study was to characterize the transcriptional response of *P. aeruginosa* to fosfomycin, tobramycin alone, or F:T, to elucidate the molecular mechanisms underpinning the increased activity of F:T under anaerobic conditions.

MATERIALS AND METHODS

Bacterial isolates. The following *P. aeruginosa* isolates were used in this study: PAO1 and its *narG*-C11::IS*lacZ*/hah, *narH*-B04::IS*phoA*/hah,

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narK$_1$-E08::IS*lacZ*/hah, *narK*$_2$-H03::IS*phoA*/hah, *phnA*-D11::IS*lacZ*/hah, *phnB*-H01::IS*lacZ*/hah, *zwf*-B12::IS*lacZ*/hah, *glpT*-G02::IS*lacZ*/hah, and *murA*-B03::IS*phoA*/hah mutants (University of Washington transposon mutant library) (13); PA14 and PA14 *lasR*::Gm (14) (Seattle Children's Hospital, Seattle, WA); and CM6, CF31, and AN2 (Adult CF Center, Belfast, United Kingdom). Clinical *P. aeruginosa* isolates CM6 and CF31 were cultured from CF sputum when patients were clinically stable, and AN2 was cultured from a sputum sample collected prior to antibiotic treatment of an acute infective exacerbation. Isolates were cultured from chronically colonized patients aged 19 (CF31), 22 (CM6), and 41 (AN2) years, all of whom had received multiple courses of antibiotics for treatment of pulmonary exacerbations.

Anaerobic conditions. For all experiments investigating anaerobic growth, anaerobic conditions were achieved using an anaerobic workstation (Whitley A35 anaerobic workstation; Don Whitley Scientific, Shipley, United Kingdom). The presence of anaerobic conditions was continually monitored using an anaerobic indicator solution (Don Whitley Scientific, Shipley, United Kingdom). All media used for anaerobic growth experiments were preincubated in the anaerobic cabinet for at least 24 h prior to use, to ensure the elimination of oxygen.

Microarray studies. (i) Antibiotic treatment and sampling. Microarray experiments were conducted in triplicate for the clinical isolate *P. aeruginosa* CM6 exposed to subinhibitory concentrations (defined as the highest concentration that did not affect growth [see Fig. S1 in the supplemental material]) of fosfomycin, tobramycin, and F:T under both aerobic and anaerobic conditions (24 arrays in total). CM6 was inoculated into Mueller-Hinton broth (MHB), incubated overnight at 37°C, and adjusted to an optical density at 550 nm (OD_{550}) of 0.15 (approximately 1×10^8 CFU/ml). This culture was diluted 1:50 into 24 flasks, each containing 100 ml MHB plus 1% (100 mM) potassium nitrate (KNO_3); cultures were then grown with shaking under either aerobic ($n = 12$ flasks) or anaerobic ($n = 12$ flasks) conditions until early exponential phase ($OD_{550} = 0.4$). One milliliter of culture was then withdrawn, and 1 ml of solution containing the appropriate concentration of antibiotic (fosfomycin, 1 mg/liter; tobramycin, 0.25 mg/liter; F:T, 1.25 mg/liter [fosfomycin, 1 mg/liter; tobramycin, 0.25 mg/liter], and control) was added in triplicate under both aerobic and anaerobic conditions. Cultures were incubated with shaking for a further hour, after which 10 ml of culture was removed and centrifuged for 12 min at 3,220 × *g* at 4°C to harvest cells, before immediately proceeding with RNA extraction.

(ii) RNA extraction. RNA was isolated using TRIzol reagent (Invitrogen, United Kingdom) with subsequent DNase 1 digestion performed using the Turbo DNA-free DNase treatment kit (Ambion, United Kingdom). RNA cleanup was then performed with the Qiagen RNeasy kit (Qiagen, United Kingdom). RNA was eluted with 40 μl diethyl pyrocarbonate (DEPC)-water, split into aliquots, and immediately stored at −80°C. Full details of RNA extraction are included in the supplemental material.

(iii) Microarray hybridization and analysis. Microarray experiments were carried out using Affymetrix GeneChip *P. aeruginosa* genome arrays with hybridization performed by Source Bioscience Inc. (Berlin, Germany). RNA integrity was initially assessed on the Agilent Bioanalyzer 2100 system (Agilent Technologies, USA). Hybridization and processing were carried out according to the Affymetrix GeneChip Expression Analysis Technical Manual. Data were imported into the Partek Genomics suite (Partek Incorporated, USA) and normalized using robust multichip average (RMA). Pairwise comparisons were performed to identify genes that showed differential expression in response to fosfomycin, tobramycin, and F:T compared to control values under anaerobic and aerobic conditions. Analysis of variance (ANOVA) was used to assess significance, with a *P* value of ≤0.05 considered significant. Genes with a differential expression of ≥1.5-fold were considered for functional analysis.

(iv) Functional and pathway analysis. Genes showing a ≥1.5-fold expression change were classified according to PseudoCAP functional class using the *P. aeruginosa* genome database (http://www.pseudomonas.com/index.jsp). Where a gene had more than one functional class, it was included in all functional classes for the analysis.

(v) qRT-PCR. Validation of 10 genes with differential expression by microarray analysis was performed using quantitative PCR (qPCR) using the same RNA as that used in the microarray experiments (see Table S2 in the supplemental material). Reactions were carried out on an Applied Biosystems 7500 Fast real-time PCR detection system (Life Technologies, United Kingdom). Data were analyzed using the Applied Biosystems 7500 software suite, with differential expression determined by the comparative threshold cycle (C_T) method ($\Delta\Delta C_T$). Full details of quantitative real-time PCR (qRT-PCR) are included in the supplemental material.

Nitrate utilization assay. Nitrate utilization was determined for PA14 and derived mutant PA14 *lasR*::Gm, for PAO1 and derived *narG*-C11::IS*lacZ*/hah, *narH*-B04::IS*phoA*/hah, *narK*$_1$-E08::IS*lacZ*/hah, and *narK*$_2$-H03::IS*phoA*/hah transposon mutants, and for 3 clinical isolates (CM6, CF31, and AN2) using a nitrate/nitrite colorimetric assay kit (Cayman Chemical, USA) according to the manufacturer's instructions. Isolates were grown overnight and adjusted to an OD_{550} of 0.15 (approximately 1×10^8 CFU/ml), and 100 μl was added to 10 ml MHB plus 1% KNO_3 in duplicate, with one 10-ml volume incubated under aerobic and one incubated under anaerobic conditions with shaking. After 18 h, total viable counts were determined by serially diluting the bacterial suspension 10-fold in quarter-strength Ringer's solution and plating five 10-μl drops of each dilution onto Mueller-Hinton agar (MHA) plates for enumeration after 24 h. Bacterial suspensions were then centrifuged at 3,220 × *g* for 10 min. The supernatant was removed and vortexed, and 100 μl of supernatant was diluted 1:2,000 in nitrate assay buffer. Absorbance was then measured in triplicate at 540 nm and a standard curve used to calculate the concentration of nitrate in each sample. The concentrations were normalized to nitrate levels in MHB plus 1% KNO_3 which had been incubated without inoculation. Finally, nitrate concentrations were normalized by cell density in order to correct for the effect of varying growth rates. Differences in nitrate utilization under anaerobic versus aerobic conditions were determined by a two-tailed Student *t* test using GraphPad Prism software (version 5.00 for Windows; GraphPad Software, San Diego, CA, USA). Nitrate utilization was also assessed for 6 strains (AN2, CM6, PAO1, PA14, PA14 *lasR*::Gm, and PAO1 *narG*-C11::IS*lacZ*/hah) following growth in subinhibitory concentrations of fosfomycin, tobramycin, and F:T under anaerobic conditions. Significant differences in nitrate utilization by the 6 isolates when exposed to each antibiotic preparation compared to relevant controls were determined with the GraphPad Prism software using a two-way ANOVA, with Bonferroni *post hoc* tests.

Antibiotic susceptibilities of selected transposon mutants. Susceptibility to fosfomycin, tobramycin, and F:T was determined in duplicate by agar dilution for PA14 and its derived PA14 *lasR*::Gm mutant and for PAO1 and its derived *glpT*-G02::IS*lacZ*/hah, *narG*-C11::IS*lacZ*/hah, *narH*-B04::IS*phoA*/hah, *narK*$_1$-E08::IS*lacZ*/hah, *narK*$_2$-H03::IS*phoA*/hah, *murA*-B03::IS*phoA*/hah, *zwf*-B12::IS*lacZ*/hah, *phnA*-D11::IS*lacZ*/hah, and *phnB*-H01::IS*lacZ*/hah transposon mutants under aerobic and anaerobic conditions as described previously (12).

Growth of selected transposon mutants. Growth of PA14 and PA14 derived mutant PA14 *lasR*::Gm and of PAO1 and PAO1 derived *narG*-C11::IS*lacZ*/hah, *narH*-B04::IS*phoA*/hah, *narK*$_1$-E08::IS*lacZ*/hah, *narK*$_2$-H03::IS*phoA*/hah transposon mutants was determined in the presence and absence of 1% KNO_3 under aerobic and anaerobic conditions. Overnight cultures were adjusted to an OD_{550} of 0.15 (approximately 1×10^8 CFU/ml) in MHB and further diluted 1:10, and 50 μl was added to a total volume of 20 ml MHB with or without 1% KNO_3. Bacterial cultures were then incubated under both aerobic and anaerobic conditions and total viable counts determined after 0, 1, 2, 4, 6, and 24 h.

Microarray data accession number. The Array Express accession number for the microarray experiments presented in this study is E-MEXP-3764.

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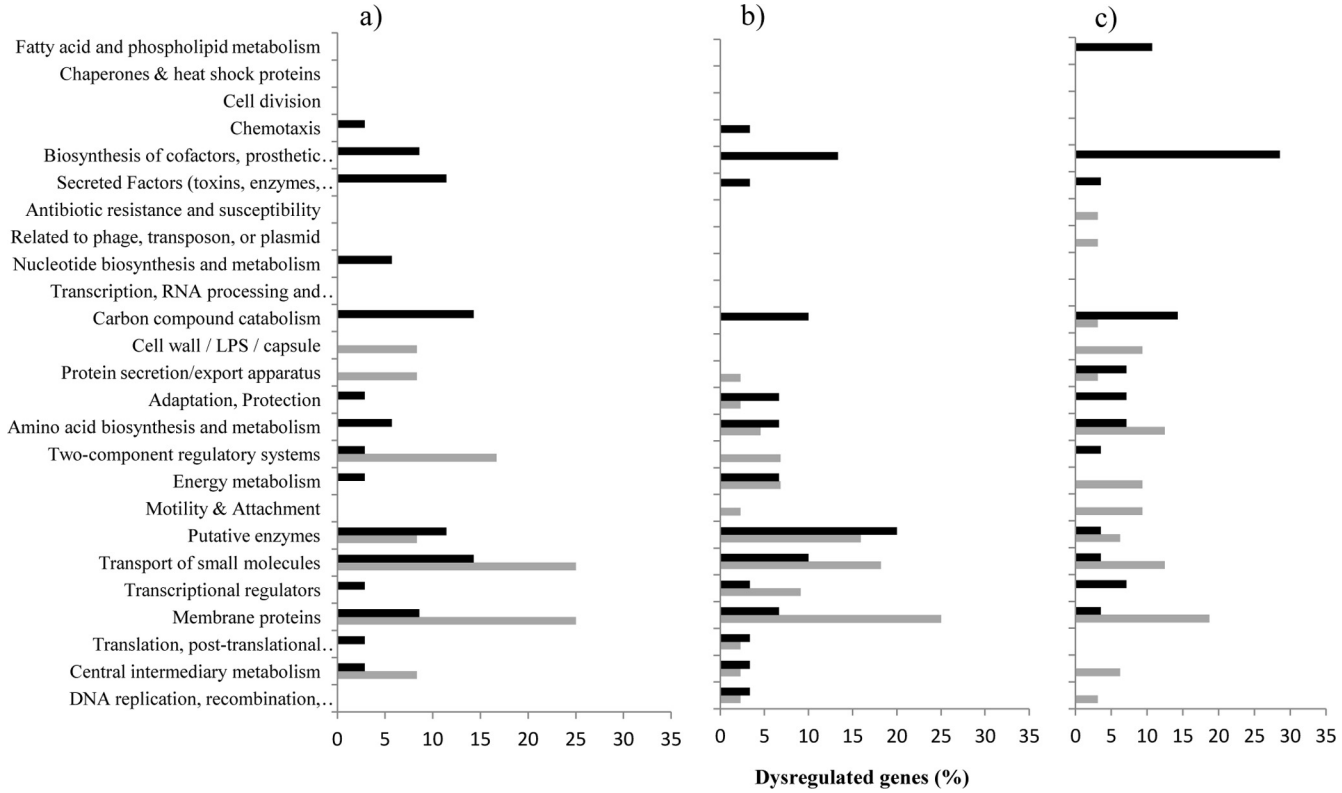

FIG 1 PseudoCAP functional class distribution of genes with significantly increased expression ($P \leq 0.05$) in response to fosfomycin (a), tobramycin (b), and F:T (c) compared to control values under aerobic (gray bars) and anaerobic (black bars) conditions (excluding genes classified as hypothetical) as identified by microarray analysis. LPS, lipopolysaccharide.

RESULTS

Transcriptional response of *P. aeruginosa* to subinhibitory concentrations of fosfomycin, tobramycin, and F:T. The gene expression of clinical *P. aeruginosa* isolate CM6 was assessed after 1 h of treatment with subinhibitory concentrations of fosfomycin, tobramycin, and F:T under aerobic and anaerobic conditions (see Table S3 in the supplemental material). Under aerobic conditions, there were 48 (12 upregulated and 36 downregulated), 287 (62 upregulated and 225 downregulated), and 180 (34 upregulated and 146 downregulated) genes with significant expression changes of ≥1.5-fold in response to growth in subinhibitory fosfomycin, tobramycin, and F:T, respectively, compared to controls. Under anaerobic conditions, there were 131 (39 upregulated and 92 downregulated), 227 (32 upregulated and 195 downregulated), and 81 (33 upregulated and 48 downregulated) genes with significant expression changes of ≥1.5-fold in response to subinhibitory fosfomycin, tobramycin, and F:T, respectively. In general, the patterns of altered gene expression as assessed by functional class distribution for both fosfomycin (Fig. 1a and 2a) and F:T (Fig. 1c and 2c) were different under aerobic compared with anaerobic conditions. In contrast, the pattern of altered gene expression for tobramycin was similar under aerobic and anaerobic conditions (Fig. 1b and 2b). Furthermore, there were 1,039 genes (531 upregulated and 508 downregulated) with ≥1.5-fold expression changes in response to growth under anaerobic compared to aerobic conditions (see Table S3 and Fig. S2 in the supplemental material).

F:T downregulates nitrate reductase genes essential for the growth of *P. aeruginosa* under anaerobic conditions. The nitrate reductase gene *narG* was downregulated most (2.74-fold) by F:T under anaerobic conditions, with another nitrate reductase gene, *narH* (1.67-fold), also downregulated in comparison to the control (Table 1). The *nar* genes, which normally are essential for anaerobic growth of *P. aeruginosa* (5), did not show altered expression when exposed to either fosfomycin or tobramycin alone under either aerobic or anaerobic conditions. Furthermore, other genes in the *nar* operon did not show altered expression under any of the experimental conditions, except for $narK_1$ (3.57-fold) and $narK_2$ (2.54-fold), encoding nitrate extrusion proteins, which were significantly downregulated under anaerobic compared with aerobic conditions.

The gene *murA*, which encodes the fosfomycin target enzyme, was downregulated by both tobramycin (1.90-fold) and F:T (1.63-fold) under aerobic conditions. Furthermore, this gene was also upregulated (1.92-fold) under anaerobic compared to aerobic conditions. *zwf*, encoding glucose-6-phosphate dehydrogenase, which is potentially involved in fosfomycin uptake, was downregulated by fosfomycin (1.25-fold), tobramycin (1.73-fold), and F:T (1.70-fold) under aerobic conditions and also by F:T under anaerobic conditions (1.44-fold).

Genes potentially involved in tobramycin uptake, *phnA* (1.82-fold) and *phnB* (1.55-fold), were upregulated in response to F:T under anaerobic conditions. In contrast, *phnB* was downregulated in response to both tobramycin (1.66-fold) and F:T (1.44-fold) under aerobic conditions. Fosfomycin alone did not affect the expression of these genes under either condition.

Microarray validation. The results of microarray validation by

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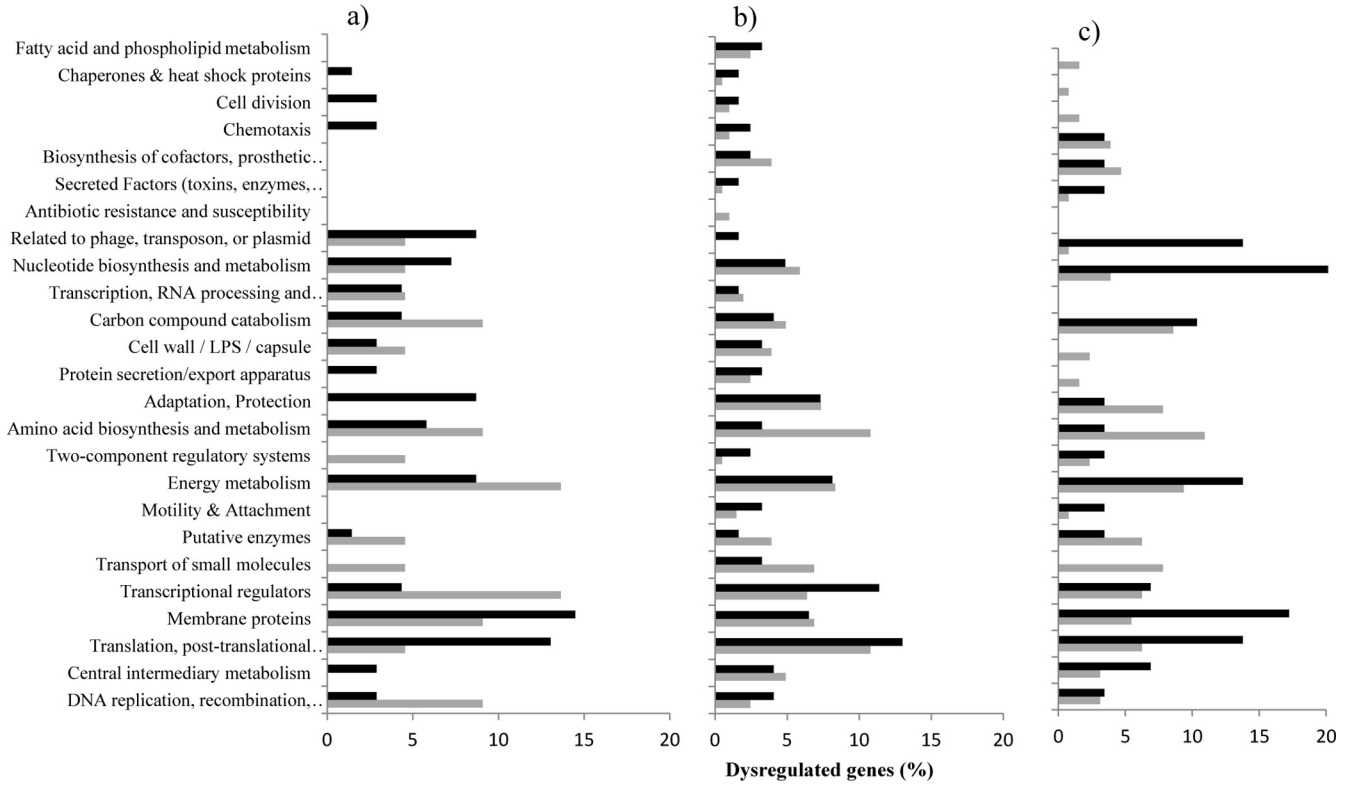

FIG 2 PseudoCAP functional class distribution of genes with significantly decreased expression ($P \leq 0.05$) in response to fosfomycin (a), tobramycin (b), and F:T (c) compared to control values under aerobic (gray bars) and anaerobic (black bars) conditions (excluding genes classified as hypothetical) as identified by microarray analysis.

qRT-PCR are shown in Fig. S3 to S5 in the supplemental material. Notably, the qRT-PCR results validated the downregulation of nitrate reductase genes *narG* and *narH* under anaerobic conditions in response to F:T, as found in microarray experiments.

***P. aeruginosa* nitrate utilization is increased under anaerobic conditions.** As microarray analysis indicated downregulation of nitrate reductase genes as a possible mechanism for the increased activity of F:T under anaerobic conditions, the nitrate utilization of PA14, PA14 *lasR*::Gm, PAO1, PAO1 derived *narG*-C11::IS*lacZ*/hah, *narH*-B04::IS*phoA*/hah, *narK*$_1$-E08::IS*lacZ*/hah, and *narK*$_2$-H03::IS*phoA*/hah transposon mutants, and 3 clinical isolates (CM6, CF31, and AN2) were determined under both aerobic and anaerobic conditions (Fig. 3). The *narG*-C11::IS*lacZ*/hah and *narH*-B04::IS*phoA*/hah nitrate reductase transposon mutants did not utilize any nitrate under either aerobic or anaerobic conditions. In contrast, the *narK*$_1$-E08::IS*lacZ*/hah ($P = 0.0064$) and

TABLE 1 Fold expression change in selected genes that were significantly differentially expressed ($P \leq 0.05$) in response to subinhibitory concentrations of fosfomycin, tobramycin, and F:T compared to control values under aerobic and anaerobic conditions as identified by microarray analysis[a]

				Fold expression change						
				Aerobic conditions with:			Anaerobic conditions with:			Anaerobic vs aerobic conditions
Category	Gene	PseudoCAP class	Function	Fosfomycin	Tobramycin	F:T	Fosfomycin	Tobramycin	F:T	
Nitrate reduction/ anaerobic growth	*narG*	Energy metabolism	Nitrate reductase						−2.74	
	narH	Energy metabolism	Nitrate reductase						−1.67	
	narK$_1$	Membrane/transport	Nitrate extrusion							−3.57
	narK$_2$	Membrane/transport	Nitrate extrusion							−2.54
Fosfomycin target/uptake	*murA*	Cell wall/LPS/capsule	Cell wall synthesis		−1.90	−1.63				1.92
	zwf	Energy/carbon compound	G6P dehydrogenase	−1.25	−1.73	−1.70			−1.44	−2.17
	glpT	Membrane/transport	G3P transporter							
Tobramycin target/ uptake	*phnA*	Adaption/protection	Electron transport chain						1.82	2.71
	phnB	Adaption/protection	Electron transport chain		−1.66	−1.44			1.55	1.99
	hcnA	Central intermediary	Hydrogen cyanide synthase		−1.80	−1.68			−1.72	−1.63

[a] The full lists of significantly differentially expressed genes can be found in Table S2 in the supplemental material.

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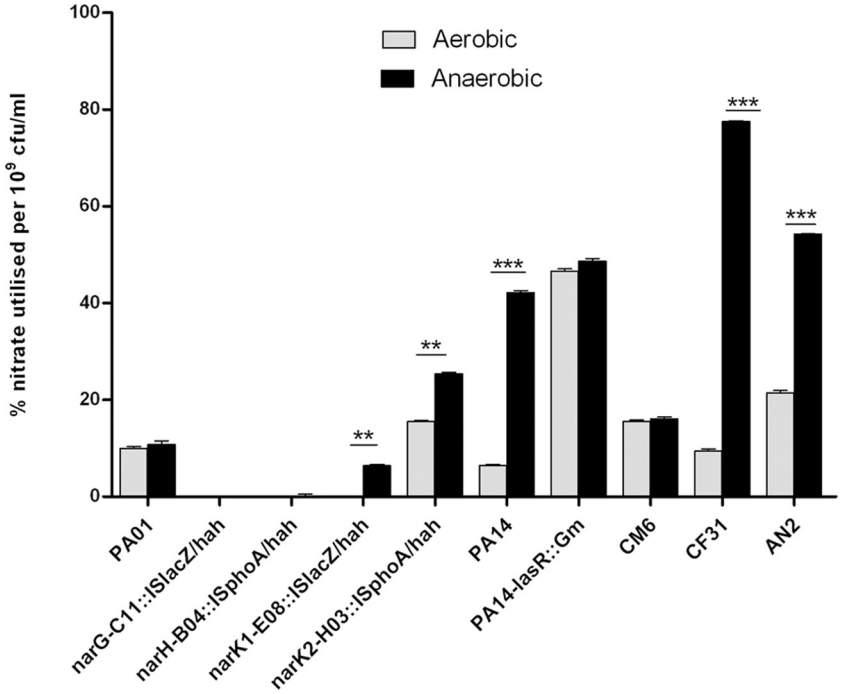

FIG 3 Average nitrate utilization (+ standard deviation [SD]; $n = 3$) of *P. aeruginosa* isolates grown in MHB plus 1% KNO_3 under aerobic and anaerobic conditions. Significant differences in nitrate utilization are denoted by asterisks (***, $P \leq 0.001$; **, $P \leq 0.01$; *, $P \leq 0.05$).

narK$_2$-H03::IS*phoA*/hah ($P = 0.0016$) nitrate extrusion transposon mutants and isolates PA14, CF31, and AN2 (all $P < 0.001$) all utilized significantly greater proportions of nitrate under anaerobic than under aerobic conditions.

***P. aeruginosa* nitrate utilization is decreased by subinhibitory concentrations of F:T under anaerobic conditions.** We also measured nitrate utilization for some of the strains shown in Fig. 3 under anaerobic conditions in the absence and presence of subinhibitory concentrations of fosfomycin, tobramycin, and F:T, as shown in Fig. 4. Both antibiotic treatment and strain had a significant effect on nitrate utilization. There was also a significant interaction between treatment and isolate ($P < 0.001$), indicating that the effect of treatment was dependent on the isolate tested. Bonferroni *post hoc* tests showed that growth in subinhibitory concentrations of F:T under anaerobic conditions significantly decreased ($P < 0.001$) nitrate utilization by strains AN2, CM6, PAO1, PA14, and PA14 *lasR*::Gm compared to the control, with no difference apparent for the *narG*-C11::IS*lacZ*/hah mutant (Fig. 4). Growth in subinhibitory tobramycin resulted in a significant decrease ($P < 0.001$) in nitrate utilization for strains AN2, CM6, and PAO1 ($P < 0.001$) and a significant increase ($P < 0.001$) for the *narG*-C11::IS*lacZ*/hah mutant ($P < 0.001$), with no difference apparent for PA14 and PA14 *lasR*::Gm. Nitrate utilization by these 6 strains grown in subinhibitory concentrations of F:T and tobramycin was also measured, demonstrating that nitrate utilization by PA14, PA14 *lasR*::Gm, and the *narG*-C11::IS*lacZ*/hah mutant significantly decreased ($P < 0.001$) when grown in subinhibitory F:T, with no difference apparent for the other 3 strains.

Nitrate reductase transposon mutants have increased susceptibility to F:T under anaerobic compared to aerobic conditions. The *narG*-C11::IS*lacZ*/hah, *narH*-B04::IS*phoA*/hah, *narK*$_1$-E08::IS*lacZ*/hah, and *narK*$_2$-H03::IS*phoA*/hah mutants each had 2-fold-lower F:T MICs than the parent strain PAO1 under anaerobic conditions and also had 2-fold-lower F:T MICs under anaerobic than under aerobic conditions (results identical for two independent experiments) (Table 2). All other PAO1 transposon

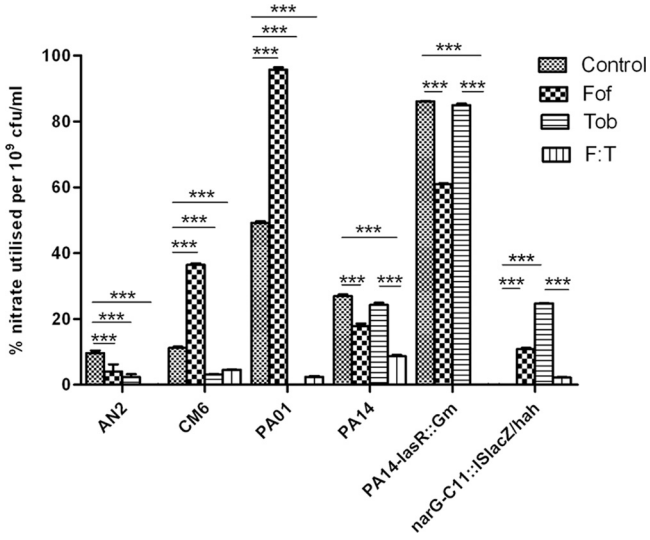

FIG 4 Average nitrate utilization (+SD; $n = 3$) by *P. aeruginosa* isolates when exposed to subinhibitory concentrations of fosfomycin (Fof), tobramycin (Tob), or F:T or to control treatment (no antibiotic added) under anaerobic conditions. Significant differences in nitrate utilization when exposed to fosfomycin, tobramycin, and F:T compared to control values and for F:T compared to tobramycin are denoted by asterisks (***, $P \leq 0.001$; **, $P \leq 0.01$; *, $P \leq 0.05$). F:T significantly decreased the nitrate utilization of all isolates tested, including a *lasR* mutant isolate known to exhibit increased nitrate utilization.

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TABLE 2 Fosfomycin, tobramycin, and F:T MICs of *P. aeruginosa* transposon mutants and clinical strains under aerobic and anaerobic conditions

Strain	MIC (mg/liter)						Fold change in F:T MIC under anaerobic conditions[b]
	Aerobic conditions			Anaerobic conditions			
	Fosfomycin	Tobramycin	F:T	Fosfomycin	Tobramycin	F:T	
PAO1	32	1	10	16	8	10	
PAO1 *narG*-C11::IS*lacZ*/hah	32	1	10	16	2	5	−2
PAO1 *narH*-B04::IS*phoA*/hah	32	1	10	16	4	5	−2
PAO1 $narK_1$-E08::IS*lacZ*/hah	32	1	10	16	4	5	−2
PAO1 $narK_2$-H03::IS*phoA*/hah	32	1	10	16	4	5	−2
PAO1 *phnA*-D11::IS*lacZ*/hah	32	1	5	16	4	5	
PAO1 *phnB*-H01::IS*lacZ*/hah	64	1	5	16	4	5	
PAO1 *zwf*-B12::IS*lacZ*/hah	64	1	5	512	4	5	
PAO1 *glpT*-G02::IS*lacZ*/hah	>,2048	1	5	>2,048	4	5	
PAO1 *murA*-B03::IS*phoA*/hah	32	1	5	64	2	5	
PA14	16	1	2.5	4	8	10	+4
PA14 *lasR*::Gm	32	2	10	16	8	10	
CM6[a]	16	128	40	32	8	10	−4
CF31[a]	4	32	20	16	32	10	−2
AN2[a]	4	64	20	16	16	10	−2

[a] Susceptibility data taken from previously published data (12).
[b] Negative values indicate a reduction in MIC under anaerobic conditions, and positive values indicate an increase in MIC under anaerobic conditions. Nitrate reductase transposon mutants had 2-fold-lower F:T MICs under anaerobic than under aerobic conditions.

mutants had 2-fold-lower F:T MICs than wild-type PAO1 under both aerobic and anaerobic conditions.

Nitrate reductase transposon mutants are at a growth disadvantage under anaerobic conditions. Under aerobic conditions, all PAO1 transposon mutants showed growth rates similar to that of the PAO1 wild-type strain when grown in either the presence or absence of nitrate (see Fig. S6 in the supplemental material). Under anaerobic conditions, the PAO1 *narG*-C11::IS*lacZ*/hah, *narH*-B04::IS*phoA*/hah, $narK_1$-E08::IS*lacZ*/hah, and $narK_2$-H03::IS*phoA*/hah nitrate reductase transposon mutants were at a growth disadvantage compared to wild-type PAO1 when grown in the absence of 1% KNO_3 (Fig. 5). Similarly, when these 4 strains were grown under anaerobic conditions in the presence of 1% KNO_3, all were still at a growth disadvantage compared to wild-type PAO1. This growth disadvantage was relatively less pronounced for the $narK_2$-H03::IS*phoA*/hah mutant. PA14 and PA14 *lasR*::Gm demonstrated similar growth under anaerobic conditions in both the absence and presence of nitrate.

DISCUSSION

Previous work has showed that F:T, a 4:1 combination of fosfomycin and tobramycin for treatment of CF respiratory infection, has enhanced activity under anaerobic conditions (12). In this study, we used DNA microarrays to analyze the transcriptional response of a clinical *P. aeruginosa* isolate (CM6) to subinhibitory F:T, fosfomycin, and tobramycin under both aerobic and anaerobic conditions. The results of microarray studies were confirmed by laboratory-based experiments using both clinical *P. aeruginosa* isolates and transposon mutants of laboratory strains of PAO1 and PA14, as the clinical isolate employed in the microarray study was multidrug resistant and not suitable for genetic manipulation.

Our results show that F:T elicited a transcriptional response distinct from that of either of its constituents alone under both aerobic and anaerobic conditions. Importantly, we report that F:T, but not fosfomycin or tobramycin alone, can downregulate the expression of membrane-bound nitrate reductase genes *narG* and *narH* under anaerobic conditions. This was confirmed by nitrate utilization assays, which demonstrated that subinhibitory concentrations of F:T significantly reduced nitrate utilization in *P. aeruginosa*. Previously, it has been shown that *narG* and *narH* are required for anaerobic growth of *P. aeruginosa*, with knockout mutants unable to grow under these conditions (4). Furthermore, a *narG* mutant had a severe growth defect under anaerobic conditions in media containing nitrate at concentrations found in CF

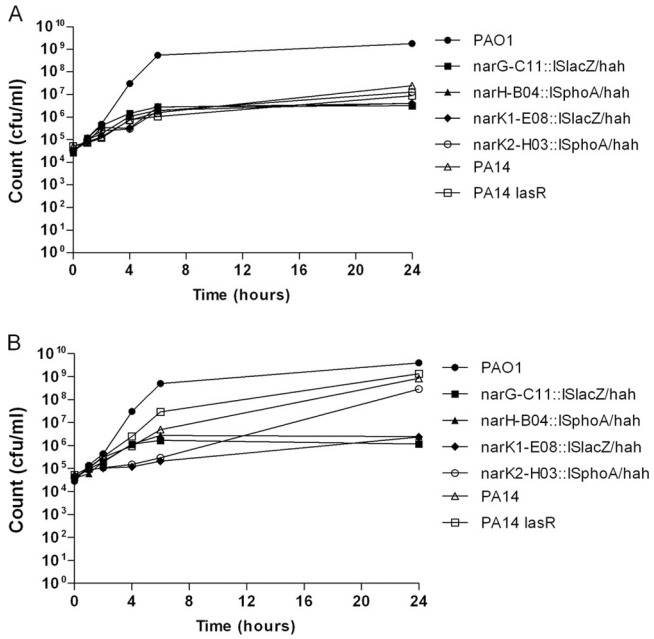

FIG 5 Growth of nitrate reductase transposon mutants in MHB under anaerobic conditions without KNO_3 (A) and with 1% KNO_3 added to the growth medium (B). Nitrate reductase transposon mutants had a growth defect compared to the parent PAO1 strain in both the presence and absence of KNO_3.

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sputum (4, 5). These results are consistent with the findings of the current study, which found that *narG*, *narH*, $narK_1$, and $narK_2$ transposon mutants had significant growth defects compared with their parent strain under anaerobic conditions. We also showed that *narG* and *narH* transposon mutants were unable to reduce nitrate under either condition. While the *nar* transposon mutants are unable to reduce nitrate, it is possible that their limited growth under anaerobic conditions is supported by other pathways involved in anaerobic growth, such as the nitric oxide reductase (*nor*) and the periplasmic nitrate reductase (*nap*) pathways (15, 16). In addition, pyruvate fermentation may contribute to the survival of these strains under anaerobic conditions (17). The nitrate reductase transposon mutants had a 2-fold reduction in F:T MIC under anaerobic compared to aerobic conditions, in agreement with results of a previous study using clinical *P. aeruginosa* isolates with F:T (12). Therefore, it is highly likely that the effect of F:T on the expression of nitrate reductase genes explains the increased activity of F:T against *P. aeruginosa* under anaerobic conditions.

Given the importance of nitrate reduction to the anaerobic growth of *P. aeruginosa* in the CF lung, the ability of F:T to reverse nitrate utilization by *P. aeruginosa* isolates, including a *lasR* mutant known to exhibit increased nitrate utilization, was investigated. *lasR* mutants are associated with poor prognostic outcome in CF, have increased antimicrobial resistance, and have a growth advantage in the nitrate-rich CF lung (14, 18, 19). Furthermore, there is selective pressure in favor of nitrate utilization in the CF lung, suggesting that mutations that increase the ability of *P. aeruginosa* to respire nitrate confer a selective advantage in this environment (20, 21). F:T significantly reduced nitrate utilization in *P. aeruginosa* isolates, including the *lasR* mutant. Tobramycin alone also significantly reduced nitrate utilization in some of the strains; however, importantly, it was unable to reverse nitrate utilization by the *lasR* mutant.

This is the first report of an antimicrobial combination reducing the expression of nitrate reductase genes and reducing the ability of *P. aeruginosa* to respire using nitrate, a feature essential for normal anaerobic growth. This finding could be of clinical importance, as reversal of nitrate utilization may affect the ability of *P. aeruginosa* to chronically colonize the nitrate-rich CF lung and may reverse the selective advantage of *lasR* strains in this environment. Our results also suggest that nitrate utilization is tractable and may potentially be manipulated in the CF lung. The *nar* operon is conserved in a broad range of bacteria (22) and also allows for hypoxic or anaerobic respiration in clinically relevant pathogens, including *Escherichia coli*, *Mycobacterium tuberculosis*, and *Moraxella catarrhalis* (23–26). Furthermore, it has recently been shown that nitrate generated as a by-product of the host inflammatory response conferred a fitness advantage to *E. coli* in the gut (27). It is possible that a similar mechanism could generate nitrate in the CF lung, where excessive inflammation is present. This, together with the link between increased nitrate utilization and antimicrobial resistance, suggests nitrate metabolism and the *nar* operon as attractive targets for the development of antimicrobial adjuvants for use in chronic infections.

P. aeruginosa uses nitrate as an alternative terminal electron acceptor during anaerobic respiration and can also perform aerobic denitrification (28–31). In the present study, a significant difference in the expression of the majority of nitrate reductase genes under anaerobic as opposed to aerobic conditions was not found. While this finding was surprising, other studies have reported similar results. For example, Wagner et al. found that $narK_1$ and $narK_2$, nitrate extrusion protein genes, were downregulated under anaerobic conditions (32). Similarly, Filiatrault et al. (33) found that genes subsequently identified as essential for growth under anaerobic conditions were downregulated under anaerobic conditions (15). In contrast, Platt et al. reported that genes involved in nitrate reduction are upregulated under anaerobic compared to aerobic conditions (34). Interestingly, CM6 utilized similar amounts of nitrate under both aerobic and anaerobic conditions, correlating with the similar expression levels of the *nar* genes under both conditions found in this study. It is also likely that many of these genes are constitutively expressed under both aerobic and anaerobic conditions, with low-level expression of the *nap* operon independent of oxygen tension having been previously observed (4).

We also hypothesized that differential expression of the fosfomycin target *murA* may have been responsible for the increased activity of fosfomycin and F:T under anaerobic conditions. *murA* encodes an enzyme involved in peptidoglycan synthesis and was downregulated by both tobramycin and F:T under aerobic conditions. Overexpression of this gene has previously been shown to confer fosfomycin resistance in *E. coli* (35). Interestingly, the *murA* transposon mutant had a 4-fold-higher fosfomycin MIC under anaerobic conditions but had lower F:T MICs than the wild type under aerobic and anaerobic conditions. The decreased susceptibility of the *murA* transposon mutant to fosfomycin is probably due to lack of a fosfomycin target, supporting a previous study that identified amino acid substitutions in *murA* that conferred fosfomycin resistance (36). Interestingly, Petek et al. did not find that fosfomycin caused significant differential expression of *murA*, in agreement with the current results (37). In addition, *murA* was upregulated under anaerobic compared to aerobic conditions, negating the possibility that decreased expression of the fosfomycin target under anaerobic conditions is responsible for the enhanced activity of fosfomycin or F:T.

Recently, it has been shown that the glycerol-3-phosphate transporter, GlpT, is the only transporter for fosfomycin in *P. aeruginosa* (38) and that fosfomycin is not taken up by a glucose-6-phosphate-induced transporter as in *E. coli* (39). Therefore, it was possible that the increased activity of fosfomycin and F:T under anaerobic conditions was due to an upregulation of GlpT; however, our results indicate that this transporter is not differentially regulated under anaerobic conditions. Thus, increased activity of fosfomycin under anaerobic conditions is unlikely to be as a result of increased expression of GlpT in *P. aeruginosa*.

The decreased activity of aminoglycosides under anaerobic conditions is frequently attributed to reduced uptake under these conditions (6–8). Macleod et al. used radiolabeled tobramycin to show that tobramycin uptake was energy dependent and was enhanced by fosfomycin under aerobic conditions (40). Therefore, we postulated that the increased activity of F:T may be due to fosfomycin-mediated increased uptake of tobramycin under anaerobic conditions. Previously, MacLeod et al. suggested a possible partial role in tobramycin uptake for the outer membrane porin OprB (40). However, in the current study, expression of the *oprB* gene was similar under all conditions tested and was not upregulated in the presence of fosfomycin or F:T. Two genes involved in the electron transport chain and in ubiquinone biosynthesis, both of which are required for aminoglycoside uptake (41),

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phnA and *phnB*, were upregulated in response to growth under anaerobic conditions; however, *phnA* and *phnB* transposon mutants did not have increased tobramycin MICs, and it is therefore unlikely that these genes encode products that are crucial for tobramycin uptake.

Both tobramycin and F:T were found to downregulate expression of the glucose-6-phosphate dehydrogenase gene, *zwf*, under aerobic conditions; F:T also downregulated this gene under anaerobic conditions. Previously, it has been shown that mutations that upregulate expression of this gene confer resistance to oxidative stress and paraquat (42, 43). Conversely, it would be expected that downregulation of this gene would increase susceptibility to oxidative stress, a potentially important effect, as aminoglycosides such as tobramycin are believed to exert their action at least in part by producing reactive oxygen species (44, 45). However, the *zwf* transposon mutant did not exhibit increased susceptibility to tobramycin under aerobic conditions. Interestingly, the *zwf* transposon mutant had an increased fosfomycin MIC under aerobic conditions, and the mutation conferred resistance under anaerobic conditions. This indicates that *zwf* has an as-yet-uncharacterized role in fosfomycin resistance.

In summary, this study showed that F:T downregulates the expression of nitrate reductase genes that are essential for the growth of *P. aeruginosa* under anaerobic conditions. This effect may explain the increased activity of F:T under anaerobic conditions. Expression of these genes was unchanged in response to F:T under aerobic conditions or in response to either fosfomycin or tobramycin alone. F:T also reduced nitrate utilization in CF *P. aeruginosa* isolates and in a *lasR* mutant strain, which is known to have increased nitrate utilization compared to that by the wild type. Therefore, F:T may be a particularly promising treatment option in patients chronically colonized with *lasR*-deficient isolates, which are associated with poorer clinical prognosis.

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