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 anaerobic 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::IslacZ/hah, narH-B04::ISphoA/hah, narG-C11::IslacZ/hah, narH-B04::ISphoA/hah.

Received 15 April 2013 Returned for modification 2 July 2013 Accepted 11 August 2013 Published ahead of print 19 August 2013

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Supplemental material for this article may be found at http://dx.doi.org/10.1128/AAC.00750-13.

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nark₁-E08::IslacZ/hah, nark₁-H03::IphoA/hah, phnA-D11::IslacZ/hah, 
phnB-H01::IslacZ/hah, zwf-B12::IslacZ/hah, gpt-G02::IslacZ/hah, 
and muaA-B03::IphoA/hah mutants (University of Washington transposon 
mutant library) (13); PA14 and PA14 las::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 treat-
ment of pulmonary exacerbations.

Anaerobic conditions. For all experiments investigating anaerobic 
growth, anaerobic conditions were achieved using an anaerobic worksta-
(Whitley A35 anaerobic workstation; Don Whitley Scientific, Shi-
pley, United Kingdom). The presence of anaerobic conditions was con-
tinuously 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. Microa-
array 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 sup-
plemental material]) of fosfomycin, tobramycin, and F:T under both aer-
obic 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 (OD₅₅₀) of 0.15 (approximately 
1 × 10⁶ CFU/ml). This culture was diluted 1:50 into 24 flasks, each con-
taining 100 ml MHB plus 1% (100 mM) potassium nitrate (KNO₃); 1-
cultures were then grown with shaking under aerobic conditions (n = 12 flasks) or anaerobic (n = 12 flasks) conditions until early exponential 
phase (OD₅₅₀ = 0.4). One milliliter of culture was then withdrawn, and 1 ml of 
solution containing the appropriate concentration of antibiotic (fosfomy-
cin, 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 (Invit-
rogen, United Kingdom) with subsequent DNase I 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 experi-
ments were carried out using Affymetrix GeneChip P. aeruginosa ge-
ome 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 compara-
tive threshold cycle (Ct) method (ΔΔCt). Full details of quantitative real-
time PCR (qRT-PCR) are included in the supplemental material.

Nitrile utilization assay. Nitrile utilization was determined for PA14 
and derived mutant PA14 las::Gm, for PAO1 and derived narg-C11:: 
IslacZ/hah, narg-H04::IphoA/hah, nark₁-E08::IslacZ/hah, and nark₂- 
H03::IphoA/hah transposon mutants, and for 3 clinical isolates (CM6, 
CF31, and AN2) using a nitrile/nitrite colorimetric assay kit (Cayman 
Chemical, USA) according to the manufacturer’s instructions. Isolates 
were grown overnight and adjusted to an OD₅₅₀ of 0.15 (approximately 
1 × 10⁶ CFU/ml), and 100 μl was added to 10 ml MHB plus 1% KNO₃ in 
duplicate, with one 10-ml volume incubated under aerobic and one incu-
bated 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 superna-
tant 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 nitrile in each sample. The concentrations were 
normalized to nitrate levels in MHB plus 1% KNO₃ which had been 
inecubated 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-sided Student t test using 
GraphPad Prism software (version 5.00 for Windows; GraphPad 
Software, San Diego, CA, USA). Nitrile utilization was also assessed for 
6 strains (AN2, CM6, PAO1, PA14, PA14 las::Gm, and PAO1 
narg-C11::IslacZ/hah) following growth in subinhibitory concentra-
tions 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. Suscepti-
ilitiy to fosfomycin, tobramycin, and F:T was determined in duplicate by agar 
dilution for PA14 and its derived PA14 las::Gm mutant and for 
PAO1 and its derived gpt-G02::IslacZ/hah, narg-C11::IslacZ/hah, 
narg-H04::IphoA/hah, nark₁-E08::IslacZ/hah, nark₂-H03::IphoA/hah, 
and muaA-B03::IphoA/hah, zwf-B12::IslacZ/hah, phnA-D11::IphoA/hah, 
and phnB-H01::IslacZ/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 las::Gm and of PAO1 and PAO1 derived narg-C11:: 
IslacZ/hah, narg-H04::IphoA/hah, nark₁-E08::IslacZ/hah, nark₂- 
H03::IphoA/hah transposon mutants was determined in the presence 
and absence of 1% KNO₃ under aerobic and anaerobic conditions. Over-
night cultures were adjusted to an OD₅₅₀ of 0.15 (approximately 1 × 10⁶ 
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% KNO₃. Bacterial cultures were 
then incubated under both aerobic and anaerobic conditions and total 
visible counts determined after 0, 1, 2, 4, 6, and 24 h.

Microarray data accession number. The Array Express accession num-
ber for the microarray experiments presented in this study is E-MEXP-3764.
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 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 *narK₁* (3.57-fold) and *narK₂* (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
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 $\text{narG}$ and $\text{narH}$ under anaerobic conditions in response to F:T, as found in microarray experiments. $P. \text{aeruginosa}$ 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 $\text{narG}$C11::IS$^\text{lacZ}$/hah, $\text{narH}$-B04::IS$^\text{phoA}$/hah, $\text{narK}$1-E08::IS$^\text{lacZ}$/hah, and $\text{narK}$2-H03::IS$^\text{phoA}$/hah transposon mutants, and 3 clinical isolates (CM6, CF31, and AN2) were determined under both aerobic and anaerobic conditions (Fig. 3). The $\text{narG}$C11::IS$^\text{lacZ}$/hah and $\text{narH}$-B04::IS$^\text{phoA}$/hah nitrate reductase transposon mutants did not utilize any nitrate under either aerobic or anaerobic conditions. In contrast, the $\text{narK}$1-E08::IS$^\text{lacZ}$/hah ($P$/$H11005$0.0064) and $\text{narK}$2-H03::IS$^\text{phoA}$/hah

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.

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

<table>
<thead>
<tr>
<th>Category</th>
<th>Gene</th>
<th>PseudoCAP class</th>
<th>Function</th>
<th>Aerobic conditions with:</th>
<th>Anaerobic vs aerobic conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nitrate reduction/anaerobic growth</td>
<td>$\text{narG}$</td>
<td>Energy metabolism</td>
<td>Nitrate reductase</td>
<td>$-2.74$</td>
<td>$-1.67$</td>
</tr>
<tr>
<td></td>
<td>$\text{narH}$</td>
<td>Energy metabolism</td>
<td>Nitrate reductase</td>
<td>$-3.57$</td>
<td></td>
</tr>
<tr>
<td></td>
<td>$\text{narK}_1$</td>
<td>Membrane/transport</td>
<td>Nitrate extrusion</td>
<td>$-3.54$</td>
<td></td>
</tr>
<tr>
<td></td>
<td>$\text{narK}_2$</td>
<td>Membrane/transport</td>
<td>Nitrate extrusion</td>
<td>$-2.54$</td>
<td></td>
</tr>
<tr>
<td>Fosfomycin target/uptake</td>
<td>$\text{marA}$</td>
<td>Cell wall/LPS/capsule</td>
<td>Cell wall synthesis</td>
<td>$-1.25$</td>
<td>$-1.90$</td>
</tr>
<tr>
<td></td>
<td>$\text{zwf}$</td>
<td>Energy/carbon compound</td>
<td>G6P dehydrogenase</td>
<td>$-1.73$</td>
<td>$-1.70$</td>
</tr>
<tr>
<td></td>
<td>$\text{glpT}$</td>
<td>Membrane/transport</td>
<td>G3P transporter</td>
<td>$-1.66$</td>
<td>$-1.44$</td>
</tr>
<tr>
<td>Tobramycin target/uptake</td>
<td>$\text{phmA}$</td>
<td>Adaption/protection</td>
<td>Electron transport chain</td>
<td>$1.82$</td>
<td>$2.71$</td>
</tr>
<tr>
<td></td>
<td>$\text{phmB}$</td>
<td>Adaption/protection</td>
<td>Electron transport chain</td>
<td>$1.55$</td>
<td>$1.99$</td>
</tr>
<tr>
<td></td>
<td>$\text{hcnA}$</td>
<td>Central intermediary</td>
<td>Hydrogen cyanide synthase</td>
<td>$-1.72$</td>
<td>$-1.63$</td>
</tr>
</tbody>
</table>

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

November 2013 Volume 57 Number 11 aac.asm.org

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*narK*1-*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::IslacZ/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::IslacZ/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::IslacZ/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::IslacZ/hah, *narH*-B04::ISphoA/hah, *narK*1-E08::ISlacZ/hah, and *narK*2-*H03::ISphoA/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 3** Average nitrate utilization (+ standard deviation [SD]; n = 3) of *P. aeruginosa* isolates grown in MHB plus 1% KNO₃ under aerobic and anaerobic conditions. Significant differences in nitrate utilization are denoted by asterisks (***, P ≤ 0.001; **, P ≤ 0.01; *, P ≤ 0.05).**

**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 ≤ 0.001; **, P ≤ 0.01; *, P ≤ 0.05). F:T significantly decreased the nitrate utilization of all isolates tested, including a lasR mutant isolate known to exhibit increased nitrate utilization.
TABLE 2 Fosfomycin, tobramycin, and F:T MICs of *P. aeruginosa* transposon mutants and clinical strains under aerobic and anaerobic conditions

<table>
<thead>
<tr>
<th>Strain</th>
<th>MIC (mg/liter)</th>
<th>F:T MIC under anaerobic conditions</th>
<th>Fold change in F:T MIC under anaerobic conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Aerobic</td>
<td>Tobramycin</td>
<td>F:T</td>
</tr>
<tr>
<td>PAO1</td>
<td>32</td>
<td>1</td>
<td>10</td>
</tr>
<tr>
<td>PAO1 narG-C11::ISlacZ/hah</td>
<td>32</td>
<td>1</td>
<td>10</td>
</tr>
<tr>
<td>PAO1 narH-B04::ISphoA/hah</td>
<td>32</td>
<td>1</td>
<td>10</td>
</tr>
<tr>
<td>PAO1 narK_{1}-E08::ISphoA/hah</td>
<td>32</td>
<td>1</td>
<td>10</td>
</tr>
<tr>
<td>PAO1 narK_{2}-H03::ISphoA/hah</td>
<td>32</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>PAO1 phnA-D11::ISlacZ/hah</td>
<td>64</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>PAO1 zwf-B12::ISlacZ/hah</td>
<td>64</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>PAO1 gfp-T-G02::ISlacZ/hah</td>
<td>&gt;2,048</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>PAO1 mutA-B03::ISphoA/hah</td>
<td>32</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>PA14</td>
<td>16</td>
<td>1</td>
<td>2.5</td>
</tr>
<tr>
<td>PA14 lasR::Gm</td>
<td>32</td>
<td>2</td>
<td>10</td>
</tr>
<tr>
<td>CM6(^{a})</td>
<td>16</td>
<td>128</td>
<td>40</td>
</tr>
<tr>
<td>CF31(^{a})</td>
<td>4</td>
<td>32</td>
<td>20</td>
</tr>
<tr>
<td>AN2(^{a})</td>
<td>4</td>
<td>64</td>
<td>20</td>
</tr>
</tbody>
</table>

\(^{a}\) 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.

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::ISlacZ/hah, *narH*-B04::ISphoA/hah, *narK_{1}-E08::ISlacZ/hah, and *narK_{2}-H03::ISphoA/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::ISphoA/hah* mutant. PA14 and PA14 *lasR::Gm* demonstrated similar growth under anaerobic conditions in both the presence and absence of nitrate.

**DISCUSSION**

Previous work has shown 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.
sputum (4, 5). These results are consistent with the findings of the current study, which found that narG, narH, narK1, and narK2 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 narK1, and narK2 nitrate extrusion protein genes, were downregulated under anaerobic conditions (32). Similarly, Fillet 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 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),
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 upregulation 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 in 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.

ACKNOWLEDGMENTS

This work was supported by Gilead Sciences Inc. M.M.T was supported by a United Kingdom National Institute for Health Research Career Scientist Award funded by Health and Social Care Research and Development, Public Health Agency, Northern Ireland.

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