Role for Ferredoxin:NAD(P)H Oxidoreductase (FprA) in Sulfate Assimilation and Siderophore Biosynthesis in Pseudomonads

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Pyridine-2,6-bis(thiocarboxylate) (PDTC), produced by certain pseudomonads, is a sulfur-containing siderophore that binds iron, as well as a wide range of transition metals, and it affects the net hydrolysis of the environmental contaminant carbon tetrachloride. The pathway of PDTC biosynthesis has not been defined. Here, we performed a transposon screen of *Pseudomonas putida* DSM 3601 to identify genes necessary for PDTC production (Pdt phenotype). Transposon insertions within genes for sulfate assimilation (*cysD*, *cysNC*, and *cysG* [*cobA2*]) dominated the collection of Pdt mutations. In addition, two insertions were within the gene for the LysR-type transcriptional activator FinR (PP1637). Phenotypic characterization indicated that *finR* mutants were cysteine bradytrophs. The Pdt phenotype of *finR* mutants could be complemented by the known target of FinR regulation, *fprA* (encoding ferredoxin:NADP$^+$ oxidoreductase), or by *Escherichia coli cysJI* (encoding sulfite reductase). These data indicate that *fprA* is necessary for effective sulfate assimilation by *P. putida* and that the effect of *finR* mutation on PDTC production was due to deficient expression of *fprA* and sulfite reduction. *fprA* expression in both *P. putida* and *P. aeruginosa* was found to be regulated by FinR, but in a manner dependent upon reduced sulfur sources, implicating FinR in sulfur regulatory physiology. The genes and phenotypes identified in this study indicated a strong dependence upon intracellular reduced sulfur/cysteine for PDTC biosynthesis and that pseudomonads utilize sulfite reduction enzymology distinct from that of *E. coli* and possibly similar to that of chloroplasts and other proteobacteria.

Pyridine-2,6-bis(thiocarboxylate) (PDTC) is a novel siderophore produced by certain strains of bacteria of the genus *Pseudomonas* (1, 2). Its novelty lies in the ability to form stable complexes with a wide range of transition metals in addition to iron (3), a role in zinc nutrition (4), and unique reactivity with a toxic pollutant (carbon tetrachloride [CCl$_4$]) (5). Its novel characteristics are imparted by the constituent ligands thiocarboxylate sulfur and pyridine nitrogen atoms, which coordinate both hard and soft metal ions. Evidence that PDTC is a siderophore includes the high stability constant of the ferric complex (6), iron-repressible production (7, 8), and receptor-mediated uptake of the ferric-PDTC complex (9). This has yielded insights into how PDTC production is regulated (10), but questions remain as to its biosynthesis and how that may relate to global regulatory circuits.

Individual organisms may be capable of producing more than one siderophore (11, 12). How an organism makes the metabolic "decision" to produce one siderophore as opposed to an alternative in its repertoire is not completely understood. Regulatory features reflecting the unique demands of specific siderophore biosynthetic pathways may also be present. Siderophore biosynthetic genes may thus have been selected for appropriate regulation by global regulators that prevent the export of a limiting nutrient. There are few data to support this assumption in the current literature on siderophore regulation, however. Studies have identified global regulatory systems that affect siderophore production such as quorum sensing (13–15). Studies that have identified nutritional cues other than iron are limited. Farmer and Thomas (16) identified a connection between sulfur assimilatory processes and production of a sulfur-containing siderophore. In that case, *Burkholderia cenocepacia* was found to curtail pyochelin production when sulfate starvation conditions were imposed. More recently, Matthijs et al. (17) have shown that production of the only other characterized thiocarboxylate siderophore, thioquinolobactin, requires effective sulfate assimilation or provision of a suitable sulfur source. It is not known how sulfur sources affect production of the respective secondary metabolites; regulation could be somewhat passive, e.g., due to parameters such as intracellular cysteine concentrations and the relative affinities of primary metabolic enzymes versus secondary metabolism, or more active such as via transcriptional or posttranscriptional regulatory processes.

Although the PDTC biosynthetic pathway has not yet been established biochemically, genetic sequence data and limited isotopic tracer studies have informed speculation as to how it may proceed (8, 18). Genes necessary for PDTC production by *P. stutzeri* KC and *P. putida* DSM 3601 (*pdt* gene clusters) have been described (9, 18) (GenBank accession no, AY319946). Homology of some of the respective gene products with proteins known to function in forming protein thiocarboxylates indicated that cysteine desulfurase, sulfur transferase, and acyl-adenylate ligase activities are involved (Fig. 1) (19–21). An obvious candidate for the carboxylic acid substrate is dipicolinate (DPA) (Fig. 1). Isotopically labeled DPA ([3-^{2}H]DPA) was found to be incorporated into PDTC when provided to cells of *P. putida* DSM 3601 (22). Based on the above-mentioned homology the precursor molecule providing sulfur for PDTC biosynthesis is presumed to be cysteine
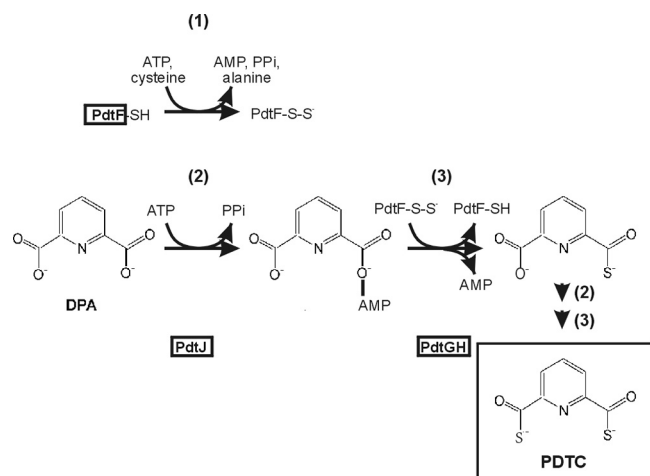
FIG 1 Predicted sulfur transfer steps in PDTC biosynthesis. The three steps depicted are (i) cysteine desulfurylase activity attributed to PdtF, an autosulfurylation forming persulfide-containing, modified PdtF; (ii) acyl activation; and (iii) transulfurylation activities, giving 6-(monothiocarboxylic acid)-picolinic acid. A second cycle of activation/transulfurylation of that product would give PDTC. Gene products (PdtF, PdtJ, PdtG, and PdtH) catalyzing the respective steps are denoted by boxes within or below each reaction.

(Fig. 1). It should be pointed out that a pathway for PDTC biosynthesis that includes a sulfenic acid as an intermediate has been proposed (23). That pathway predicts a cleavage (apparently monooxygenase-dependent) of a thioester intermediate, and reduction of the resulting sulfenic acid to form the thiocarboxylate. That pathway would require genes (e.g., for a monooxygenase system) that are not encoded within the described *pdt* gene clusters.

The hypothetical PDTC biosynthetic pathway described in Fig. 1 predicts PDTC production to be limited by factors affecting intracellular cysteine levels, as has been seen for pyochelin and thioquinolobactin (16, 17, 24). The biochemistry and genetics of cysteine biosynthesis has been extensively studied in Gram-negative bacteria in which it constitutes a major regulon (25). That system displays finely coordinated activities of serine activation and sulfur incorporation. Coordinate regulation is achieved through transcriptional, and posttranslational mechanisms. CysB is the master regulator of sulfur assimilation and is a LysR-type transcriptional activator. In the presence of its co-effector, *N*-acetylserine (NAS), CysB binds regulatory elements within promoters of sulfur assimilatory genes to allow their maximal expression. Production of inducer (NAS) is dependent upon the serine transacetylase activity of CysE. CysE is in turn (negatively) allosterically regulated by intracellular cysteine. Several gene products catalyzing steps of sulfate assimilation (e.g., CysD and CysN) display rapid turnover, requiring active transcription to maintain steady intracellular levels (25). The result is balance between the demand for reduced sulfur and the abundance of sulfur assimilatory enzymes. Although PDTC production is an example of secondary metabolism rather than assimilatory metabolism, its regulation may involve some overlap of regulatory elements in order to optimally allocate intracellular sulfur.

To more fully address questions of how PDTC production is integrated into central metabolism, a more comprehensive analysis of functions associated with PDTC production, and encoded outside the *pdt* cluster was undertaken. We used a genetic approach to search for potential accessory functions within the genome of a PDTC-producing pseudomonad. We used the genomic background of *P. putida* DSM 3601, owing to the advantages afforded by the availability of a published genome sequence for that species (26), and a well-characterized alternative siderophore system (pyoverdine). Using a pyoverdine-deficient genetic background, a transposon insertion library was screened for strains with altered PDTC production by exploiting the iron-containing dye chrome azurol S (CAS) (12, 27). That screening procedure yielded only a single *pdt* insertion (*pdtI*) that has been described elsewhere (24). In the present study, we describe other insertions that led to the complete loss of or to a reduction in PDTC production. Identification of the affected genes and characterization of the resulting phenotypes yielded insights into sulfate assimilation by pseudomonads and support a role for cysteine as the immediate sulfur donor for PDTC biosynthesis.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The strains used in the present study are listed in Table 1. *P. putida* strains were routinely maintained on tryptic soy medium. Minimal media used for studies of siderophore production in *Pseudomonas* were PM and M9. PM is a PIPES-buffered minimal medium and was prepared as described previously (24). PIPES (>99.0%) was from Research Organics (Cleveland, OH). M9 is a phosphate-buffered minimal medium and was prepared as described by Maniatis et al. (24a). M9 was made without sulfur by replacing ammonium sulfate with ammonium chloride and magnesium sulfate with magnesium chloride to give an equivalent amount of ammonium and magnesium. M9 minimal medium with 0.2% sodium citrate was used for selection of *Pseudomonas* transconjugants. CAS plates were prepared by the method of Schwyn and Neilands (27). *P. aeruginosa* and *E. coli* strains were maintained on Luria-Bertani (LB) medium. Antibiotics were used at the following concentrations: kanamycin (Km), 50 μg/ml; tetracycline (Tc), 15 μg/ml; gentamicin (Gm), 15 μg/ml for *E. coli* and 30 μg/ml for *Pseudomonas*; ampicillin (Ap), 100 μg/ml; and chloramphenicol (Cm), 25 μg/ml. *P. putida* cultures were grown at 30°C, and *P. aeruginosa* and *E. coli* cultures were grown at 37°C. Cultures used for siderophore quantitation were 5 ml in 17-by-125-mm test tubes grown with constant shaking (*P. putida*) or 25 ml in 125-ml baffled flasks with constant shaking (*P. aeruginosa*). For the growth and luminescence measurements, 96-well plates were used with a final medium volume of 250 μl per well using a BioTek Synergy H4 plate reader and constant agitation. Optical density (OD) readings were adjusted to a 1-cm path-length value. Clear plates (Thermo/Nunc, catalog no. 266120) were used when growth was measured alone, and white plates with clear optical bottoms (Thermo/Nunc, catalog no. 165306) were used whenever luminescence measurement was included (200 μl of medium volume per well). Luminescence was measured using the extended range setting of the instrument.

Transposon mutagenesis and insertion characterization. *P. putida* strain BK8 was mutagenized with Mini-TnKm*xylE* using the filter-mating technique (28). Several independent matings were performed on each of two separate occasions. Kanamycin-resistant clones were replicated onto CAS Km plates with 0.3% Casamino Acids (first screen) or without Casamino Acids (second screen). Sites of transposon insertion were determined by sequencing. Strains LL1, LL3, PP1, TL1, LLBr1, and SEMBr1 were characterized by inverse PCR using a nested set of primers (xylE with KMR, followed by KmlacZRV with KmlacZFW). The remaining transposon insertions were characterized using arbitrary PCR with primers ARB6 and ARB2 (29) in combination with individual, nested transposon-derived primers given above.

Strains obtained from the University of Washington Transposon Mutant Collection were screened by PCR and sequencing as described by Bailey and Manoil (30).

TABLE 1 Bacterial strains and plasmids examined in this study

Strain or plasmid	Description or genotype[a]	Source or reference[b]
Strains		
P. putida		
DSM 3601	Wild-type PDTC producer	DSMZ (2)
BK8	DSM 3601 *pfrI*::Tc[r]; Pvd[−]	Laboratory collection (24)
SO3B9	BK8 *finR*::mini-Tn5*xyl*E Km[r]	This work
TA690	BK8 Δ*finR*::Gm[r] (+ orientation)	This study
TA691	BK8 Δ*finR*::Gm[r] (− orientation)	This study
P. aeruginosa		
MPA01	Wild type	UWGSD
UWID#1789	MPA01 PA4130::Tn*phoA*	UWGSD (54)
UWID#1631	MPA01 PA4130::Tn*phoA*	UWGSD (54)
UWID#33115	MPA01 PA4513::Tn*phoA*	UWGSD (54)
UWID#33735	MPA01 PA4513::Tn*phoA*	UWGSD (54)
Δ3398	PA01 Δ*finR*	This study
TA791	MPA01 Δ*pvdF*	This study
TA975	MPA01 Δ*pvdF* Δ*finR*	This study
E. coli		
JM109	*recA endA* host	Promega, Madison, WI
DH5α λ*pir*	*pir*[+] *recA endA* host	K. Mintz, University of Vermont
BW20767	*pir*[+] *mob*[+] donor	32
Plasmids		
pUTKm mini-Tn5*xyl*E	Mini-transposon vehicle	28
pGEM-Teasy	Ap[r]; *ori*colE1 TA cloning vector used for direct cloning of PCR products	Promega
pBluescript SK(−)	Ap[r]; *ori*colE1 cloning vector	Stratagene, La Jolla, CA
pBsdelSal	pBluescript SK(−) with XhoI-SalI deletion	This study
pUCGM	Source of Gm[r] cassette	55
pJB3Tc20	Tc[r] broad-host-range vector	56
pJB3Km1	Km[r] broad-host-range vector	56
pJB3TcGm	pJB3Tc20 with Gm[r] cassette inserted at BamHI	This study
pJB3*cys*DNC	pJB3TcGm with *P. putida* DSM 3601 *cysDNC* and flanking DNA inserted at EcoRI	This study
pJB3PP1637	pJB3TcGm with *P. putida* mt-2 *finR* gene and flanking DNA inserted at EcoRI	This study
pVT1460	*ori*R6K, Ap[r], *mob*RP4 suicide vector, Km[r] derivative of pGP704	K. Mintz (57)
pJBKm1	Km[r] broad-host-range vector	56
pJBKm*finR*	pJB3Km1 with *P. putida* DSM 3601 *finR*	This study
pJBKm*fprA*	pJB3Km1 with *P. putida* DSM 3601 *fprA*	This study
pJN105	GmR broad-host-range expression vector with P$_{araBAD}$ promoter	58
pJN105GW	pJN105 with Gateway recombination site inserted at SmaI	This study
pJN105GW::*fpr*2-3	pJN105GW with MPA01 *fprA*	This study
pJN105GW::*cys*JI	pJN105GW with *E. coli cysJI*	This study
pEX18T	*ori* ColE1 Ap[r], *sacB* suicide vector	59
pEX18T::Δ3398	pEX18T with PA *finR* deletion allele, Gm[r] cassette	This study
pEX18T::Δ*pvd*F	pEX18T with PA *pvdF* deletion allele, Gm[r] cassette	This study
pMF54	Ap[r]/Cb[r] broad-host-range vector	60
pMF418	pMF54 with PA *finR*	This study
pUC18-mini-Tn7T-Gm-lux	Mini-Tn7lux vector	31
pAG4lux	pUC18-mini-Tn7T-Gm-lux with a 1048-1114 deletion and a 2036-2503 deletion for reduced-background *lux* expression	A. Glassing and T. A. Lewis, unpublished data
pAG4lux::P$_{fprA}$	pAG4lux with *P. aeruginosa fprA* promoter	This study

[a] Gm[r], gentamicin resistance; Tet[r], tetracycline resistance; Ap[r], ampicillin resistance; Cb[r], carbenicillin resistance; Km[r], kanamycin resistance.

[b] DSMZ, Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany; UWGSD, University of Washington, Genome Sciences Department.

P. putida finR deletion construction. *P. putida* deletion alleles were constructed by individually amplifying upstream and downstream *finR*-flanking sequences using the primer pairs dgkFWSal/dgkRVXba, and fprAFWXba/fprARVSal, respectively. The two products were assembled using engineered XbaI sites and ligated products were amplified using the corresponding outside primers to give the fused deletion allele. That product was cloned into pGEM-T Easy and prepared for insertion of the gentamicin resistance cassette from pUCGM by digestion with XbaI. The *aacC1* gene was removed from pUCGM by digestion with XbaI and gel purification. Alleles with both orientations of *aacC1* were identified by PCR with combinations of either primer GmF1311 or GmRV1269 in combination with primers dgkFWSal or fprARVSal. Each respective allele was then ligated into the vector pVT1460 using the SalI site and the resulting plasmid vehicles were used in binary matings from *E. coli* host BW20767 into *P. putida* BK8. Transconjugants that underwent double recombination events, resulting in replacement of the wild-type allele were selected on M9 citrate with Gm (TA690) or tryptic soy agar with Tc and Gm (TA691). Replacements were resolved from cointegrants by PCR

with primers PP1635FW2 and fprARV805 in combination with either GmF1311 or GmRV1269. The resulting products obtained from strains TA690 and TA691 were cloned and sequenced to verify that no other mutations were introduced in the affected locus.

P. aeruginosa deletion alleles. Overlap-extension PCR was used to generate *P. aeruginosa* deletion alleles as described by Choi and Schweizer (31). Gm-F and Gm-R primers designed by those authors were used to produce the excisable gentamicin resistance cassette. The primers PA3398-EcoRI3′Dn, PA3398-DnF-Gm, PA3398-UpR-Gm, and PA3398-EcoRI5′Up were used to generate the Δ3398 strain, and pvdF-UpF2-GWL, pvdF-UpR2-Gm, pvdF-DnF2-Gm, and pvdF-DnR2-GWR were used for the Δ*pvdF* strain. Products were cloned into the EcoRI site of pEX18T and transferred into PA01 by conjugation. Genomic replacements were selected on LB medium lacking sodium chloride and containing 10% sucrose. The gentamicin resistance cassette was removed by FLP-mediated recombination as described by Choi and Schweizer (31). Replacements were verified by PCR. For the Δ3398 strain, these were PA33985′Up and PA33983′Dn. For Δ*pvdF* primers that annealed outside the cloned region were used in combination with primers annealing within the Gm^r cassette; pvdFUpdiag, pvdFDndiag, GmRV1269, and GmF1311.

P. aeruginosa transposon mutants. Strains were obtained from the University of Washington Genomes Sciences Department *P. aeruginosa* two-allele library and positions of the transposon insertions verified by the methods of Bailey and Manoil (30).

Functional gene and promoter cloning. Cloning for complementation testing was carried out by designing PCR primers from the *P. putida* KT2440 genome, the DSM 3601 region encompassing *finR-fprA* region, the *P. aeruginosa* PA01 genome, or the *E. coli* W3110 genome. The corresponding genes were amplified using genomic DNA or colony suspension as a template. PCR products for CysDNC$_{DSM3601}$, and finR$_{KT2440}$, were ligated into the pGEM-T Easy vector (Promega, Madison, WI) according to the manufacturer's protocol. Cloned inserts were then excised and recloned into broad-host-range vectors with appropriate selective markers (vectors are listed in Table 1). For complementation of SO3B9 by *E. coli cysJI*, and *P. aeruginosa fprA*, the vector pJN105GW was used. The respective genes amplified with *att* sequence-containing primers and cloned into pDONR221 (Invitrogen, Carlsbad, CA) and the pDONR clone used for cloning into the destination vector as recommended by the supplier. Clones were mobilized from *E. coli* BW20767 into *Pseudomonas* strains by binary matings (32). Primer sequences are given in Table 2 and were used in the following combinations: for *cysDNC* amplification (mt-2 genomic DNA as a template), cysDF2 and cysNCRv2; for *finR* (mt-2 genomic DNA as a template), PP1637F and PP1637R; for *finR* (DSM 3601), PP1637FWXba and 1637RVXba; for *fprA* (DSM 3601) 1637W4Xba and fprARV810Xba; for *P. aeruginosa finR*, PA3398 NcoI 5′, PA3398 Xba 3′; for *P. aeruginosa fprA*, PA fprFW GWL, PAfprRV GWR; for *E. coli cysJI*, Ec cysJFW GWL2, Ec cysJRV GWR; and for the *P. aeruginosa fprA* promoter, Pfpr Bam L2, Pfpr EcoR.

Analytical procedures. PDTC was assayed from culture supernatants as described previously (24). The detection limit for PDTC in culture supernatants was ~2.5 μM. CAS medium was used to assess pyochelin production.

Northern analysis. To measure *fpr* expression in response to sulfur sources by *P. putida* strains, overnight cultures grown in M9 succinate medium with 0.5 mM L-cystine were used to inoculate fresh cultures in the same medium and grown to an optical density at 600 nm (OD$_{600}$) of 0.8. The cultures were washed in M9 succinate medium without sulfur and resuspended in 10 ml of either M9 succinate 0.5 mM L-cystine or M9 succinate 1 mM sulfate to an OD$_{600}$ of 0.2 to 0.3. Cells were incubated in 50-ml baffle flasks with shaking for 2 h before harvesting for RNA extraction. For comparison of gene expression in response to paraquat, cells were grown in M9 succinate 1 mM sulfate, resuspended in 10 ml of the same medium to an OD$_{600}$ of 0.3 (*P. aeruginosa*) or 0.1 to 0.3 (*P. putida*), and incubated in 50-ml flasks with shaking until an OD$_{600}$ of 0.6 to 0.8 was

reached. Those cultures were then washed once in M9 succinate without sulfur, before resuspending to an OD$_{600}$ of 0.10 (*P. aeruginosa*) or 0.15 (*P. putida*) in 10-ml aliquots of M9 succinate 1 mM sulfate to which either no additives were included (control), or 0.5 mM L-cysteine, L-cystine, or D-cystine, or the same treatments plus 1 mM paraquat (*N,N*′-dimethyl-4,4′-bipyridinium dichloride; Sigma-Aldrich, Milwaukee, WI). Cells were incubated under those conditions for 30 min (*P. putida*) or for 10 min and 30 min (*P. aeruginosa*) before RNA extraction. RNA was extracted using the hot phenol method. Northern analysis was carried out using RNA (amounts of indicated in figure legends) run on 1% agarose morpholinepropanesulfonic acid denaturing gels (6.5% formaldehyde). Probes were generated by PCR using primers dsm3601fprfor, and dsm3601fprrev for *fprA*$_{Pp}$ (*P. putida* DSM 3601 template) or PAO1fprfor1 and PAO1fprrev1 for *fprA*$_{Pa}$ (*P. aeruginosa* MPA01 template). Probes were labeled using the BrightStar Psoralen-Biotin kit from Ambion (Austin, TX) and detected using the BrightStar BioDetect kit (Ambion) and a Kodak IS440 Imaging system.

RESULTS

Mutations generating Pdt phenotypes. The approach taken to obtain mutants with altered PDTC production relied on PDTC-dependent bleaching of the iron-containing dye CAS (27) to produce "halos" surrounding siderophore-producing colonies. Strain BK8, defective in production of pyoverdine, was used for random transposon mutagenesis. Two screening media were used, one minimal medium formulation and one utilizing Casamino Acid supplementation. Approximately 9,000 transposon mutants were screened. Strains lacking halos were further characterized by quantitation of PDTC in a liquid minimal test medium (PM) that allowed normalization to growth yields. Two phenotypic categories were distinguished: class I mutants (nine isolates) gave no detectable PDTC, and class II mutants (two isolates) showed PDTC production significantly lower than the parental strain (Table 3). The locations of transposon insertions were determined by inverse PCR and sequencing of DNA flanking the various transposon insertion sites and are shown in Fig. 2. To confirm that the PDTC production phenotypes could be ascribed to the respective insertions, complementation experiments were undertaken with each class of mutant. These used either single genes or intact operons (Fig. 2) from DSM 3601 or *P. putida* mt-2, provided in multicopy. PDTC production data indicated successful *trans* complementation and therefore that the Pdt phenotypes observed were due to loss of the indicated gene products (Table 3).

Class I mutants. Sulfate assimilation defects affect PDTC production by altering the mode of sulfur acquisition. With the single exception of the *pdtI* insertion described previously (9), all class I mutants obtained had defects in known sulfate assimilation genes. Both auxotrophs and prototrophs were found, defined by whether the strains could grow without preformed amino acid supplements.

Auxotrophic mutants. The *cobA2* gene, also denoted *cysG* in the literature regarding sulfate assimilation by enteric bacteria (25), is necessary for synthesis of the siroheme cofactor of sulfite reductase (CysI) and was identified as an auxotroph among the class I mutants. A hypermutator locus (*mutL*) was also retrieved in this screen. That strain (TL1) showed a Cys⁻ phenotype, being unable to utilize sulfate or sulfite but able to utilize sulfide or cysteine as sources of sulfur (data not shown). The hypermutator effect of *mutL* defects makes it likely that a secondary mutation was responsible for the Cys phenotype, and consequently this mutant was not examined further.

TABLE 2 PCR primers used in this study

Primer	Sequence (5′-3′)^a
cysDF2	ATTCTAGACACCTGTTCATCGATTGCC
cysNCRv2	GGATCCTTACTGACGCAGTACGTCCAACAC
PP1637F	ATTCTAGAAAAATGCCAAGGACATGGG
PP1637R	ATGGATCCGAACTGACCGTTCTCGAAGC
dgkFWSal	ATAGCTGTCGACATGACATCGCCATTCAAGG
dgkRVXba	TCTAGATCGCTTAAAGCAGGATCACC
fprAFWXba	TCTAGAGATATTGTCGCTGCCCCTAA
fprARVSal	ATAGCTGTCGACATGTCGCTGAACAGCTTGC
GmF1311	GGCTCAAGTATGGGCATCAT
GmRV1269	CAAGCGCGATGAATGTCTTA
PP1635FW2	GCTGCTGGACTTGAACATGC
fprARV805	TCGCCTTCTTCGTACCTACC
PP1637FWXba	ACCATCTAGAGATGGGGCTCCTGAAGAAA
1637RVXba	ACCATCTAGAGTCGAAAAACGCCAAGGAC
1637W4Xba	ACCATCTAGAACGAAGACTTGCAGTTGACG
fprARV810Xba	ACCATCTAGATACCTGCGCCTTATTTCTCG
KMR	TCAGCAACACCTTCTTCAG
KmlacZFW	GCCGCACTTGTGTATAAG
KmlacZRV	GGCCAGATCTGATCAAGA
PAfprFW GWL	TACAAAAAAGCAGGCTCCGCGTTTTCCTAGGAGTCT
PAfprRV GWR	TACAAGAAAGCTGGGTGGGCCGGAAAGCAGAAAG
Ec cysJFW GWL2	TACAAAAAAGCAGGCTAACATAACGACGCATGACGA
Ec cysJIRV GWR	TACAAGAAAGCTGGGTCGCGTTCTTATCAGGCCTAC
PA3398-EcoRI3′Dn	GAATTCTACATGGCCGGCTACAGCTGG
PA3398-DnF-Gm	AGGAACTTCAAGATCCCCAATTCGTGACTCAGTTGGCCAGGGACAGGT
PA3398-UpR-Gm	TCAGACGCTTTTGAAGCTAATTCGCATCCAGGCTTCCTCGTCTAGAGC
PA3398-EcoRI5′Up	GAATTCAGGTGCTGCAGGCGCGAGGTC
Pfpr Bam L2	ATGGATCCTGAATTTCATCCAGGCTTCC
Pfpr EcoR	ATGAATTCACACCAACAGCAGCAGAC
pvdF-UpF2-GWL	TACAAAAAAGCAGGCTCGCTTGGGATTGGTCATAGT
pvdF-UpR2-Gm	TCAGAGCGCTTTTGAAGCTGCGACACCTCTTCCTGATCT
pvdF-DnF2-Gm	AGGAACTTCAAGATCCCCAATTCGCTCCGGCCTTCTTCATTCT
pvdF-DnR2-GWR	TACAAGAAAGCTGGGTAAGACCGGCAAACGCTAC
pvdFUpdiag	GAGTGCAAGGCGTTGTTGAT
pvdFDndiag	GGTATGCGTCGACTACAACG
PA3398 NcoI 5′	CTCCATGGAATTCACCCTCCGCCAGCTCG
PA3398 Xba 3′	CGTCTAGACCGGATCGCCGGTGGCGCCG
PAO1fprfor1	CCTGGAGTTCTTCAGCATCAA
PAO1fprrev1	CTCGTAGCGCTCGTAGGTTTC
dsm3601fprfor	CTGAAGGAAGGCGATGAGA
dsm3601fprrev	CAGGTGCTCGGTGATGAA

^{a} Underlined letters indicate engineered restriction sites.

Prototrophic mutants. Insertions into the *cysDNC* operon, encoding subunits of adenosine phosphosulfate synthase, were the most frequently obtained class I mutations (Fig. 2). The *cysDNC* mutants were not capable of growth in minimal liquid medium with sulfate as sole sulfur source. Since the mutants grew on the screening medium lacking preformed amino acids, they were obviously capable of assimilating sulfur by some means. The only sulfur source present in CAS medium, other than sulfate (normally present in agar), was the pH buffer ingredient PIPES which contains sulfur in sulfonic acid groups. Pseudomonads are well known for their ability to utilize alkanesulfonates as sulfur sources (33, 34). The fact that strains limited to utilizing PIPES did not produce PDTC, whereas those capable of utilizing sulfate did indicated that the mode of sulfur assimilation can determine PDTC production. Utilization of alkanesulfonates as S sources requires induction of enzymes regulated as part of the sulfate starvation response; hence, their assignment as *ssi* (sulfate starvation-in-

duced) genes (35). The wild-type and parental strains showed little or no detectable PDTC when provided with PIPES or methionine as sole sulfur sources (see Fig. S1 in the supplemental material and unpublished results), suggesting mutual exclusivity between the sulfate starvation response and PDTC production.

The reduced sulfur sources sulfite, sulfide, thiosulfate, or cysteine were also tested for their ability to support PDTC production by the wild type. These reduced sulfur sources were less effective than sulfate; however, the Pdt⁻ phenotype of the *cysNC* and *cobA2* mutants could be suppressed by those that supported growth (see Fig. S1 in the supplemental material). This eliminated APS (adenosine 5′-phosphosulfate), PAPS (2′-phosphoadenosine-5′-phosphosulfate), or sulfite as essential intermediates for PDTC biosynthesis since, for example, none of these compounds would be present in the *cysNC* or *cobA2* mutants grown on sulfide or cysteine.

Class II mutants. The FinR transcriptional regulator is re-

TABLE 3 PDTC production by wild type, Pdt mutants obtained by CAS screening, and *trans* complements on PM medium

Strain	Genotype	Phenotype[a]	Avg PDTC production (μM/OD$_{600}$) $\pm$ SD[b]
Wild type			
DSM 3601	Wild type	Pdt$^+$	47.3 $\pm$ 9.0
DSM 3601/pJB3Tc20	Wild type/vector	Tc^r control	19.0 $\pm$ 0.9
BK8	*pfrI* (*pvdS*)	Pvd$^-$	40.8 $\pm$ 14.9
Class I mutants			
SEM1	*pfrI pdtI*	Pvd$^-$, Pdt$^-$	BD
LL1	*pfrI cysG* (*cobA2*)	Pvd$^-$, Pdt$^-$, Cys$^-$	BD
SEM10D10	*pfrI cysD*	Pvd$^-$, Pdt$^-$, Cys$^-$ (aux)	BD
LL3	*pfrI cysNC*	Pvd$^-$, Pdt$^-$, Cys$^-$	BD
LL3/pJB3TcGm	LL3/vector control	Pvd$^-$, Pdt$^-$, Cys$^-$	BD
LL3/pJB3TcGm::*cysDNC*	LL3/*cysDNC*	Pvd$^-$	27.4 $\pm$ 1.6
TL1	*pfrI mutL*	Pvd$^-$, Pdt$^-$, Cys$^-$	BD
Class II mutants			
SO3B9	*pfrI finR*	Pvd$^-$, Pdt$^-$, Cys (brad)	4.7 $\pm$ 0.5
SO3B9/pJB3TcGm	SO3B9/vector control	Pvd$^-$, Pdt$^-$, Cys (brad)	4.4 $\pm$ 2.9
SO3B9/pJB3TcGm::PP1637	SO3B9/*finR*	Pvd$^-$	26.4 $\pm$ 3.4

[a] aux, cysteine auxotroph; brad, cysteine bradytroph.
[b] BD, below detection. Averages and standard deviations of at least three independent experiments are shown.

quired for wild-type PDTC production. The two class II mutants obtained contained disruptions of a conserved protein sharing homology with members of the LysR-type transcriptional regulator family FinR (PP1637). An ortholog of this gene was also found in a search for mutants defective in thioquinolobactin production by *P. fluorescens* ATCC 17400 (36). In *P. putida* FinR (fpr-inducing regulator) has been shown to regulate transcription of its upstream neighboring gene, *fprA* (ferredoxin:NADP$^+$ oxidoreductase) in response to paraquat-induced oxidative stress (37). FinR-independent expression was also seen; FinR apparently being responsible for the bulk of the stress response but appreciable *fprA* expression was also observed in a *finR* truncation mutant (37). FprA is a redox mediator, shown to facilitate transfer of electrons between NADPH and ferredoxin (38). Mutants with defects in *finR* or *fprA* showed increased sensitivity to oxidative stress, and a growth defect in a minimal medium that could be suppressed by Casamino Acids (37). Sulfate assimilation is a reducing equivalent-intensive process. In enteric bacteria, sulfite reductase is a hetero-oligomeric enzyme, requiring the NADPH oxidoreductase activity of the CysJ flavoprotein, and the siroheme-containing CysI which directly reduces sulfite by six electrons to produce sulfide (25). The *cysJIH* genes form an operon on the *E. coli* chromosome. The 12 annotated *Pseudomonas* sp. genomes include *cysI* homologs that are not clustered with other *cys* genes (39). No *cysJ* homolog was identified in those genomes. Two genes encoding proteins with a high degree of sequence similarity to the *E. coli* CysJ (PA4513 and PA4130 [39]) were tested for roles in sulfate assimilation. Two mutants containing unique transposon insertions in each gene were found to be capable of growth on minimal media with sulfate as sole sulfur source (data not shown), indicating that neither PA4513 nor PA4130 are essential for sulfate assimilation by *P. aeruginosa*.

Plant CysI proteins have been shown to use reduced ferredoxin as a source of reducing power rather than a flavoprotein subunit such as CysJ (40). It is therefore likely that *Pseudomonas* Fpr proteins serve as components of an alternative CysI-reducing system.
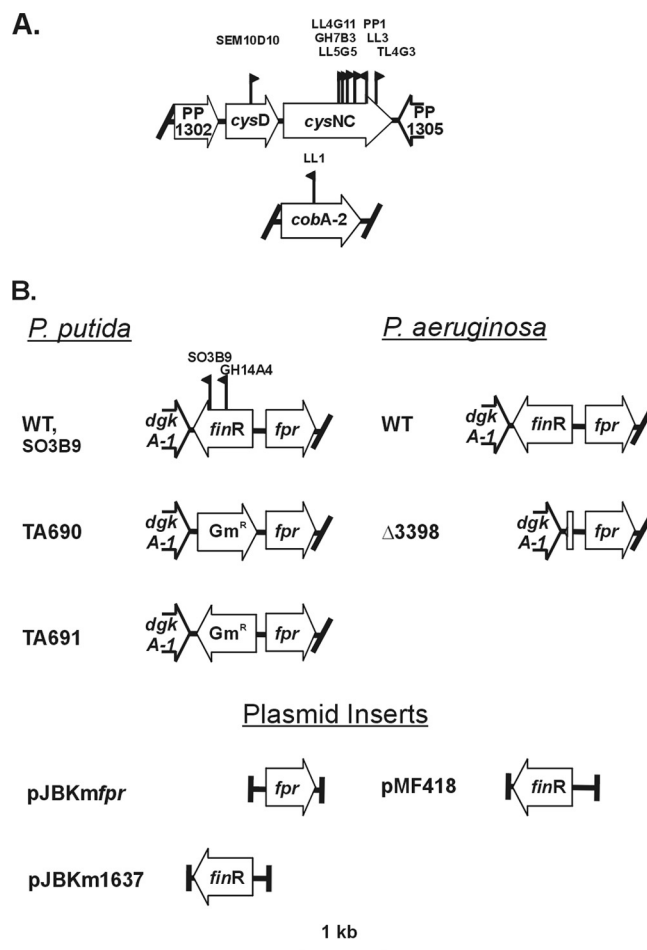

FIG 2 Genome segments and transposon insertions characterized. (A) Class I mutants; (B) class II mutants and *P. putida* and *P. aeruginosa* genomic segments analyzed involving the *finR-fprA* locus. Flag symbols indicate positions and orientations of mini-TnXylEKm insertions in respective mutants, with strain designations listed above. Half arrows above genes indicate the positions of PCR primers used to amplify genome segments for cloning/complementation.

DSM 3601   BK8   SO3B9   TA690   TA691
C   S   C   S   C   S   C   S   C   S
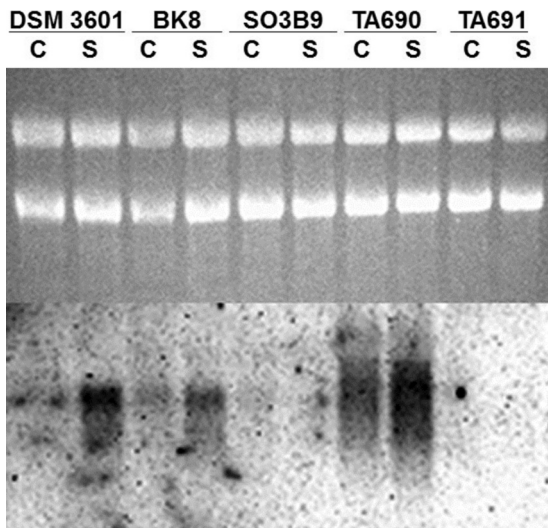
FIG 3 *P. putida* DSM 3601 *fprA* expression is dependent on sulfur source, as well as *finR* expression. Northern analysis of strains grown on M9 succinate medium with cystine (C) or sulfate (S) as sole sulfur sources. Strain genotypes are: DSM 3601, wild type; BK8, Δ*pvdS* (Δ*pfrI*) (pyoverdine-negative); SO3B9, *finR*::mini-Tn*5*; TA690, Δ*finR*::Gm^r (gentamicin resistance cassette oriented toward *fprA*); TA691, Δ*finR*::Gm^r (gentamicin resistance cassette oriented opposing *fprA*). Upper portion, ethidium-stained gel. Lower portion, Northern blot hybridized with *fprA* probe. 4.5 μg of RNA was loaded per lane.

Based on the described properties of FinR, at least two alternative models can be invoked to explain how a defect in FinR may affect PDTC production; either indirectly due to a requirement for FprA to supply reducing equivalents for cysteine biosynthesis or directly via transcriptional regulation of *pdt* genes.

FprA is required for assimilatory sulfite reduction and PDTC biosynthesis. We sought to resolve the effects due to *fprA* expression from other possible effects of *finR* mutation by constructing null mutants that varied in their potential for *fprA* expression (Fig. 2) and by complementation with *fprA* or with the *E. coli cysJI* genes. We were unsuccessful in constructing a complete deletion of *fprA*. Both Δ*finR* alleles constructed in *P. putida* had identical chromosomal deletions encompassing the entire *finR* coding sequence plus upstream sequences that included the predicted *fprA* −35 promoter element (37) (Fig. 2). The resulting *fprA* gene lacked its native promoter, but was fused to an *aacC1* gentamicin-resistance (Gm^r) cassette which lacked a transcriptional terminator. In strain TA690, the Gm^r cassette was oriented toward *fprA*

(Δ*finR*::Gm^r→). In strain TA691 it was oriented oppositely (Δ*finR*::Gm^r←). Thus, the Δ*finR*::Gm^r→ strain was expected to show *fprA* expression under the control of the *aac*C1 promoter, whereas the Δ*finR*::Gm^r← strain was expected to have severely reduced *fprA* expression. Strains TA690 and TA691were compared to the originally isolated *finR* transposon insertion mutant, SO3B9 (*finR*::mini-Tn*5*), for *fprA* mRNA abundance and PDTC production. Since the *finR*::mini-Tn*5* strain retained an intact *fprA* promoter, it should have retained FinR-independent *fprA* expression. Northern analysis of those strains confirmed robust expression of *fpr*-hybridizing RNA of a larger size than the wild-type transcript the Δ*finR*::Gm^r→ strain, and no detectable hybridization with RNA from the Δ*finR*::Gm^r← or *finR*::mini-Tn*5* strains (Fig. 3). PDTC production was not impaired in the Δ*finR*::Gm^r→ strain, but no PDTC was detected from cultures of the Δ*finR*::Gm^r← strain (Table 4). Low but detectable levels of PDTC were seen with the *finR*::mini-Tn*5* strain (Table 3). Those results recapitulate those which originally established FinR as a transcriptional regulator of *fprA* (37) and show that FprA is required for PDTC production, possibly through the assimilation of sulfate.

Comparisons of the growth of Δ*finR*::Gm^r→, Δ*finR*::Gm^r←, *finR*::mini-Tn*5* and parental strains in minimal media are shown in Fig. 4. Δ*finR*::Gm^r→ showed growth that was indistinguishable from the parental strain, whereas Δ*finR*::Gm^r← and *finR*::mini-Tn*5* strains showed decreased growth rates and an extended lag period on sulfate as sole sulfur source, with the Δ*finR*::Gm^r← strain showing the more severe impairment. The growth defects of the Δ*finR*::Gm^r← and *finR*::mini-Tn*5* strains were suppressed by addition of cystine (Fig. 4) or thiosulfate, but not sulfite (data not shown).

The PDTC production defect and the cysteine bradytrophic phenotype of the Δ*finR*::Gm^r← strain could be complemented by *fprA* cloned in multicopy. However, *finR* cloned in the same vector could not complement either defect (Table 4 and data not shown). Those results are consistent with FinR acting as a transcriptional activator of *fprA* since the Δ*finR*::Gm^r← strain contains a truncation of the *fprA* promoter. The *finR*::mini-Tn*5* strain was complemented for PDTC production by *finR* in *trans* (Table 3). The *finR*::mini-Tn*5* strain was also complemented by either *P. aeruginosa fprA* or by *E. coli cysJI* cloned under the control of the *ara*BAD promoter (Table 4; see also Fig. S2 in the supplemental material). Interestingly, while complementing the PDTC defect, *cysJI* only modestly improved the growth of the *finR*::mini-Tn*5* strain and induction had a detrimental effect on growth by the mutant and wild-type strain with sulfate as sole sulfur source under the iron-

TABLE 4 PDTC production by *finR*-null mutants: complementation by cloned *P. putida fprA* and *E. coli cysJI*

Strain	Genotype	Presence of arabinose	Avg PDTC production (μM/OD$_{600}$) ± SD[a]
TA690	*pfrI* Δ*finR*::GmFW	−	34.2 ± 8.5
TA691	*pfrI* Δ*finR*::GmRV	−	BD
TA691/pJBKm1	TA691/vector control	−	BD
TA691/pJBKm::*finR*	TA691/*finR*$_{Pp}$	−	BD
TA691/pJBKm::*fprA*	TA691/*fprA*$_{Pp}$	−	28.1 ± 1.6
SO3B9/pJN105	*pfrI finR*/vector control	−	5.9 ± 1.2
SO3B9/pJN105	*pfrI finR*/vector control	+	10.0 ± 0.5
SO3B9/pJN105::*cysJI*	SO3B9/arabinose-inducible *cysJI*$_{Ec}$	−	31.0 ± 0.3
SO3B9/pJN105::*cysJI*	SO3B9/arabinose-inducible *cysJI*$_{Ec}$	+	41.5 ± 1.1

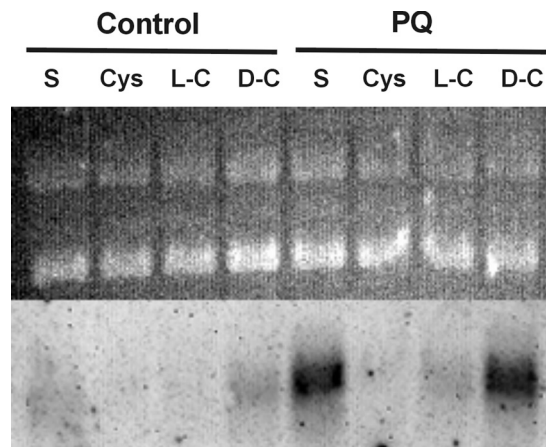[a] BD, below detection. Averages and standard deviations of triplicate assays are shown.
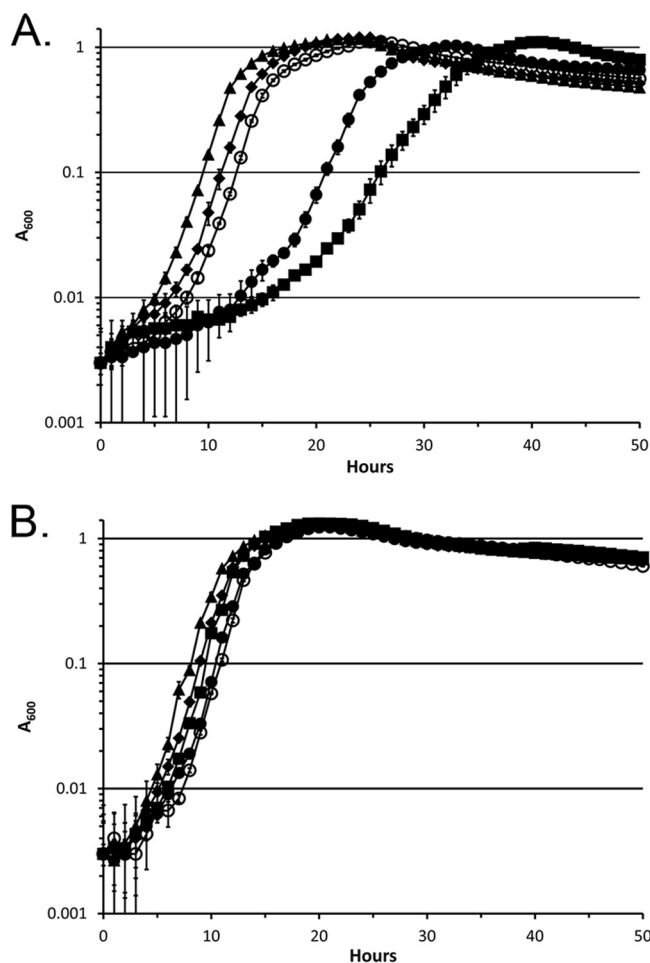
FIG 4 Growth of *finR* mutants with sulfate or cystine as sole sulfur sources. (A) M9 succinate medium with 1 mM sulfate as sulfur source; (B) M9 succinate medium with 0.5 mM cystine as sulfur source. Symbols: ○, DSM 3601 (WT); ◆, BK8 (Pvd⁻ parental); ▲, TA690 (Δ*finR*::Gmʳ-forward); ■, TA691 (Δ*finR*::Gmʳ-reverse); ●, SO3B9 (*finR*::mini-Tn5Km*xyl*E). The data are means of triplicate cultures. Error bars represent standard deviations.

FIG 5 Reduced source of sulfur suppresses paraquat-induced *fprA* expression by *P. putida* DSM 3601. Northern analysis of control cultures grown on M9 succinate medium with sulfate as the sole sulfur source (S) or sulfate plus L-cysteine (Cys), L-cystine (L-C), or D-cystine (D-C). Paraquat-treated cultures (PQ) were exposed to 1 mM paraquat for 20 min with the respective sulfur supplements prior to RNA extraction. Upper portion, ethidium-stained gel. Lower portion, Northern blot hybridized with *fprA*_{Pp} probe. A total of 6 μg of RNA was loaded per lane.

limited conditions used (see Fig. S2 in the supplemental material). The data are consistent with a model in which FinR serves a role in PDTC production solely as a component of sulfite reduction/cysteine production. Transcriptional activation of *fprA* is critical but can be replaced by ectopic expression of *E. coli cysJI*. FinR is not necessary for *pdt* gene expression since the Δ*finR*::Gmʳ→ strain showed significant PDTC production (Table 4).

Regulation of *fpr* in response to sulfur nutrition and oxidative stress. The only previous description of the regulation of *fprA* expression in pseudomonads demonstrated *finR*-dependent induction in response to paraquat-induced oxidative stress (37). However, our observations indicated a role in sulfur metabolism and some effect of sulfur sources was seen in Northern analysis (Fig. 3). Those data indicated that *fprA* transcript abundance was higher in cultures grown with sulfate than with a reduced sulfur source (cystine; here we refer to sulfur at a formal oxidation state of 0 or less as 'reduced'). We tested whether reduced sulfur sources had an effect on the robust, paraquat-induced stress response described by Lee et al. (37). This was done by exposing cells to paraquat in the presence of sulfate as sole sulfur source, or with added

supplements of L-cysteine, L-cystine, or D-cystine. The results are shown in Fig. 5 in which a striking suppression of *fprA* induction was affected by L-cysteine or L-cystine supplementation, but not by D-cystine. The data indicated that intracellular reduced sulfur/cysteine levels can suppress paraquat-dependent *fprA* induction.

To determine whether our observations of *fprA* transcription in *P. putida* were also relevant to other pseudomonads, we constructed a deletion of the *P. aeruginosa* PA01 *finR* gene (PA3398) and examined *fprA* expression in that species. *P. aeruginosa* also showed *finR*-dependent *fprA* expression that was repressed by reduced sulfur sources as shown by time course experiments with a chromosomally integrated reporter (*fprA* promoter fused to *lux-CDABE*), and Northern analysis (Fig. 6). Comparison of a *P. aeruginosa* pyoverdine-defective mutant (i.e., the Δ*pvdF* mutant) and a Δ*pvdF* Δ*finR* double mutant on CAS was used to assess affects of *finR* deletion on pyochelin production. Halo size was not significantly different between the two strains, i.e., *finR* mutants would not have been identified using the same methodology used to obtain the *P. putida finR* transposon mutants described in this work.

DISCUSSION

Sulfur donors for PDTC biosynthesis. The approach taken here and elsewhere (17), although aimed at further characterizing siderophore physiology, proved to be a rather effective means of identifying defects in sulfate assimilation since the majority of mutants isolated had Cys phenotypes. Although surprising, the screen for *B. cepacia* mutants defective in pyochelin production conducted by Farmer and Thomas (16) retrieved only a *cysW* (sulfate/thiosulfate transporter) mutant. That result and ours point out that transposon mutagenesis/screening protocols may not simply yield mutations based on target size alone; the *pdt* operon contains several genes that produce Pdt⁻ phenotypes (41; T. Lewis, unpublished data) but only one insertion was retrieved.

Our collective data support a role for cysteine, rather than an intermediate of sulfate assimilation, as the sulfur donor for thio-
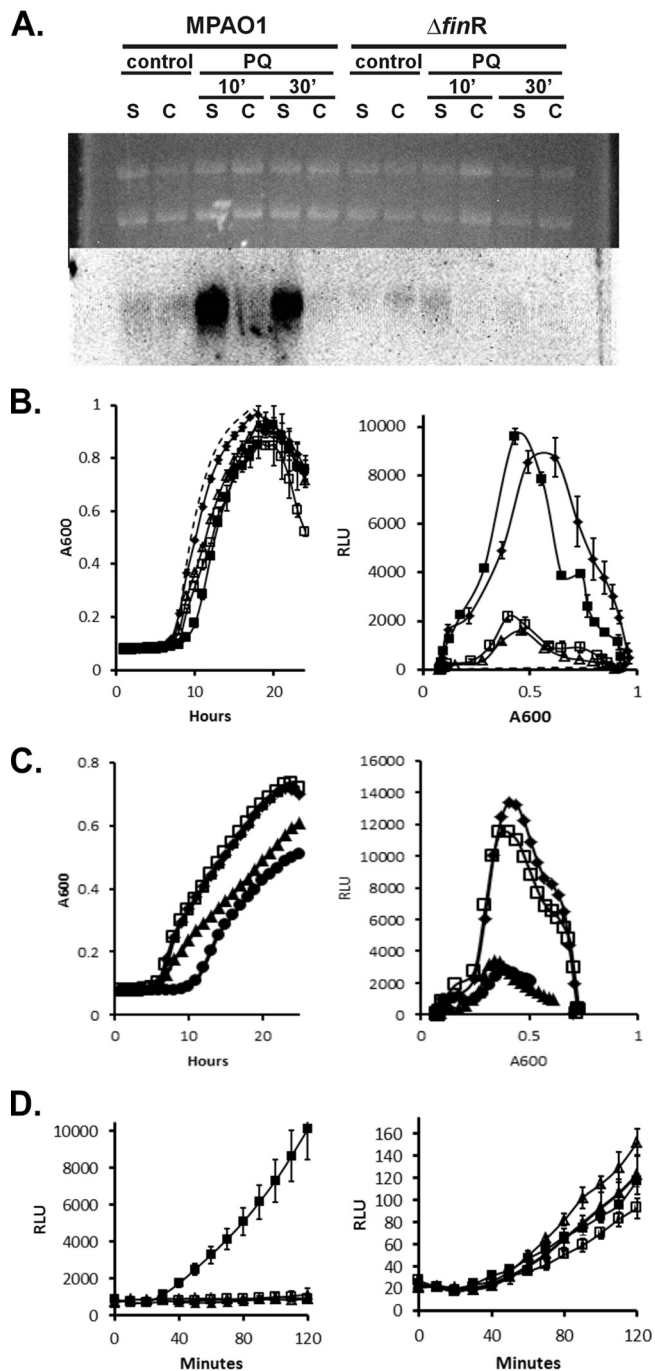
FIG 6 *P. aeruginosa* shows *finR*-dependent *fprA* expression which is responsive to reduced sulfur sources. (A) Northern analysis of cultures of wild type (MPAO1) or *finR* deletion mutant (Δ3398) grown with sulfate as the sole sulfur source (S), or sulfate plus L-cystine (C). Paraquat-treated cultures (PQ) were exposed to 1 mM paraquat for 10 or 30 min with the respective sulfur supplements prior to RNA extraction. Upper portion, ethidium-stained gel; lower portion, Northern blot hybridized with an *fprA*$_{Pa}$ probe. A total of 10 μg of RNA was loaded per lane. (B) Complementation analysis of the *finR* deletion strain Δ3398. Cultures were grown with sulfate as the sole sulfur source. The expression of a chromosomal *luxCDABE* operon fused to the P*fprA*$_{Pa}$ promoter was monitored by luminescence. Left, growth; right, luminescence versus cell density (RLU, relative luminescence units). Symbols: ◆, MPAO1; ▲, Δ3398; □, Δ3398/pMF54 (vector control); ■, 3398/pMF418 (plasmid-borne *finR*$_{Pa}$); dashed line, MPA01::Tn7*lux* control (no promoter). (C) Expression of P$_{fprA}$-*lux* reporter by wild-type *P. aeruginosa* during growth with various sole

carboxylate siderophore biosynthesis. The results obtained using mutants unable to produce intermediates of sulfate assimilation, or using media that circumvented their production by wild-type strains, eliminated all but sulfide as necessary substrates for PDTC production. It is not possible to resolve sulfide and cysteine for this role by the methods used since sulfide can be produced from cysteine through the activities of many enzymes *in vivo*. In fact, either may be chemically sufficient as sulfur donors for thiocarboxylate synthesis; however, the steady-state concentration of intracellular sulfide would be expected to be maintained at a relatively low level by the activities of the two known cysteine synthases (CysK and CysM) (25).

Our observations of PDTC synthesis are consistent with observations of *B. cenocepacia* (16) and *P. aeruginosa* (T. Lewis, unpublished data) in which cultures grown with various alternative sulfur sources such as methionine or organosulfonates did not produce detectable amounts of pyochelin. It is still unclear how this regulation is achieved; however, greater pyochelin biosynthetic gene transcription was seen by others comparing sulfate-grown and sulfamate-grown *P. aeruginosa* (42). The results are complicated by the fact that a pyochelin-proficient strain was used, making it possible that pyochelin had accumulated in the sulfate-amended cultures at the time of RNA extraction (i.e., autoinduction occurred due to pyochelin acting as a potent effector of *pch* gene induction) (43). Aside from transcriptional control, passive regulation of PDTC or pyochelin production could occur by virtue of PDTC biosynthetic enzymes having a higher K_m for cysteine than cysteinyl tRNA synthase and/or other primary metabolic enzymes. The *ssi* sulfur sources likely do not allow the same rate of cysteine biosynthesis as sulfate, which would lead to decreased steady-state intracellular cysteine concentrations. It remains to be resolved whether active regulation or passive (kinetic) control are responsible for determining sulfur-containing siderophore production.

Implications for *Pseudomonas* sulfate assimilation. Our findings have suggested a new scheme for sulfate assimilation by pseudomonads distinct from that of *E. coli* and enteric bacteria (Fig. 7). The identification of FprA as a component of bacterial sulfate assimilation supports the assertion that *Pseudomonas* CysI is a distinct type of sulfite reductase which partners with reduced ferredoxin, or FprA directly, rather than being CysJ dependent. No *cysJ* ortholog has been identified among 12 annotated *Pseudomonas* genomes (39), and transposon insertions in genes encoding proteins sharing the greatest identity with the *E. coli* CysJ in the *P. aeruginosa* genome do not confer Cys phenotypes. Ferredoxin-dependent sulfite reductases are characteristic of chloroplasts, some cyanobacteria, and other proteobacteria (44). *fpr* genes have been discovered as part of gene clusters that include the genes of sulfate assimilation (*cysIHDN*), as well as a putative ferredoxin-like protein (*cysX*) in *Corynebacterium* genomes (45). The *fpr-2* gene of *C. glutamicum* was also shown to be coregulated with the sulfate assimilatory genes, and deletion of *fpr-2* caused an in-

sulfur sources. Symbols: ◆, sulfate; □, sulfite; ▲, cystine; ●, cysteine. (D) Expression of P$_{fprA}$-*lux* reporter by wild type and Δ*finR* mutant strains in response to paraquat and/or cysteine. Exponential-phase cells grown on minimal medium with sulfate as sulfur source were diluted into the same medium with or without additions of 1 mM paraquat or 0.5 mM cysteine. Left, wild type (MPA01); right, Δ*finR*. Symbols: □, no addition; ■, 1 mM paraquat; △, cysteine; ▲, cysteine plus 1 mM paraquat.
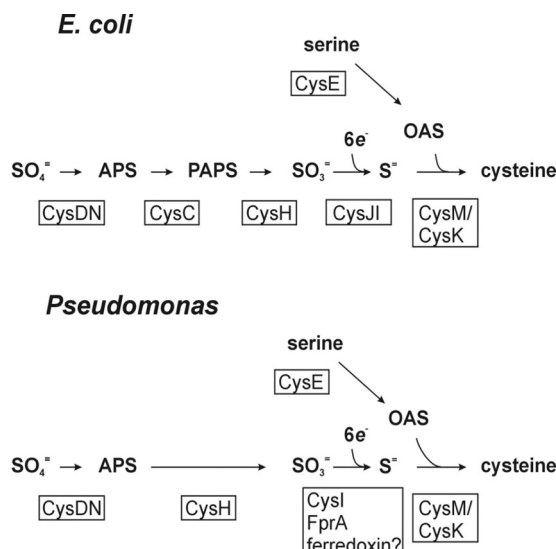
FIG 7 Pathway of sulfate assimilation/cysteine biosynthesis in enterics versus pseudomonads. Intermediates of cysteine biosynthesis are shown in bold. Gene products with relevant enzymatic activities (CysDN, CysC, etc.) are given below or beside the respective steps in boxes. Abbreviations: APS, adenosine phosphosulfate; PAPS, phosphoadenosine phosphosulfate; OAS, *O*-acetylserine. The negative allosteric regulation of CysE activity by intracellular cysteine, and the requirement for *N*-acetylserine (a product of spontaneous rearrangement of OAS) to induce sulfate assimilatory gene expression (via CysB), affords coordination between serine activation and sulfate activation/reduction activities in response to metabolic demand for cysteine (46).

creased lag time on minimal media. A ferredoxin-dependent sulfite reductase would not be the only aspect of *Pseudomonas* sulfate assimilation in common with chloroplasts: *Pseudomonas* sp. CysH are adenylylsulfate (APS) reductases (46) as opposed to phosphoadenylylsulfate (PAPS) reductase as is found in *E. coli* (25). However, it remains possible that the biochemistry of sulfite reduction in pseudomonads is unique. For example, it also remains possible that FprA transfers electrons directly to CysI. It has been shown that *P. aeruginosa* FprA efficiently transfers electrons from NADPH to heme oxygenase (47). Annotated conserved domains within bacterial and chloroplast CysI proteins include "ferredoxin-like" sequences (48).

Significance of FprA in pseudomonads. The fact that growth on sulfate is still observed in strains showing severe *fprA* deficiency (e.g., *P. putida* TA691 or the *P. putida* KT2440 *fprA* truncation/merodiploid strain described by Lee et al. [37]) may be explained by the presence of less-effective, alternative means of transfer of electrons to CysI. These may include other flavoproteins or ferredoxins. Pseudomonads possess a second annotated NADP(H):ferredoxin reductase, FprB. FprA and FprB of *P. putida* have been examined for their relative abilities to reduce cellular redox mediators such as ferredoxins (both [2Fe:2S] and [4Fe:4S]) and flavodoxin (49). It was found that FprA served most efficiently as a flavodoxin reductase and that FprB was most effective at reducing the product of *fdx-2* (referred to as FdA), and that FprB is also a ferric reductase (50). Sulfate assimilation by FprA⁻ strains may also be due to remnant FprA activity. In order to resolve these possibilities, we attempted to construct complete deletions of *fprA* in the *P. putida* DSM 3601 and *P. aeruginosa* MPA01 backgrounds using the same approaches used to generate *finR* deletion strains

but were unsuccessful. These results raise the possibility that some FprA activity must be present for viability. In addition, the alternatives to FprA may differ among pseudomonads. The *P. aeruginosa finR* mutant showed a strong defect in *fprA* expression but had a much milder growth phenotype (Fig. 7) and no significant siderophore phenotype (CAS halo size) compared to *P. putida finR* mutants.

Oxidative stress and sulfur assimilation. In the present study we also observed a linkage between an oxidative stress response (paraquat-induced *fpr* expression) and sulfur metabolism. Oxidative stress and sulfur assimilation could be expected to be linked due to turnover of iron-sulfur clusters and glutathione resulting from intracellular oxidative damage. Purely chemical linkage is also expected due to general reactivity between oxidants and cysteine (51, 52). This linkage has been observed in studies of transcriptional and proteomic responses of pseudomonads to various sulfur sources (33, 42). It is not yet clear whether *fprA* expression is integrated into cellular stress responses or exclusively responds to cysteine biosynthetic demands. *P. putida* FinR has been characterized in order to define its ability to respond to paraquat-induced oxidative stress (38). Although extensive site-directed mutagenesis was carried out, a model involving reversible disulfide bond formation was not supported. We have shown that *finR*-dependent, paraquat-induced *fprA* expression was suppressed by reduced sulfur sources (Fig. 6 and 7). It would be interesting to know whether FinR promoter-binding affinity responds to an effector or effectors derived from specific intracellular sulfur metabolites. Potential effectors could include oxidized sulfur species such as sulfoxides or sulfinates. Considering its importance for sulfate assimilation, it is also of interest to know whether *fprA* is part of the CysB regulon in pseudomonads.

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