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Cupriavidus necator H16 Uses Flavocytochrome *c* Sulfide Dehydrogenase To Oxidize Self-Produced and Added Sulfide

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ABSTRACT Production of sulfide (H_2S, HS^-, and S^{2-}) by heterotrophic bacteria during aerobic growth is a common phenomenon. Some bacteria with sulfide:quinone oxidoreductase (SQR) and persulfide dioxygenase (PDO) can oxidize self-produced sulfide to sulfite and thiosulfate, but other bacteria without these enzymes release sulfide into the medium, from which H_2S can volatilize into the gas phase. Here, we report that *Cupriavidus necator* H16, with the *fccA* and *fccB* genes encoding flavocytochrome *c* sulfide dehydrogenases (FCSDs), also oxidized self-produced H_2S. A mutant in which *fccA* and *fccB* were deleted accumulated and released H_2S. When *fccA* and *fccB* were expressed in *Pseudomonas aeruginosa* strain Pa3K with deletions of its *sqr* and *pdo* genes, the recombinant rapidly oxidized sulfide to sulfane sulfur. When PDO was also cloned into the recombinant, the recombinant with both FCSD and PDO oxidized sulfide to sulfite and thiosulfate. Thus, the proposed pathway is similar to the pathway catalyzed by SQR and PDO, in which FCSD oxidizes sulfide to polysulfide, polysulfide spontaneously reacts with reduced glutathione (GSH) to produce glutathione persulfide (GSSH), and PDO oxidizes GSSH to sulfite, which chemically reacts with polysulfide to produce thiosulfate. About 20.6% of sequenced bacterial genomes contain SQR, and only 3.9% contain FCSD. This is not a surprise, since SQR is more efficient in conserving energy because it passes electrons from sulfide oxidation into the electron transport chain at the quinone level, while FCSD passes electrons to cytochrome *c*. The transport of electrons from the latter to O_2 conserves less energy. FCSDs are grouped into three subgroups, well conserved at the taxonomic level. Thus, our data show the diversity in sulfide oxidation by heterotrophic bacteria.

IMPORTANCE Heterotrophic bacteria with SQR and PDO can oxidize self-produced sulfide and do not release H_2S into the gas phase. *C. necator* H16 has FCSD but not SQR, and it does not release H_2S. We confirmed that the bacterium used FCSD for the oxidation of self-produced sulfide. The bacterium also oxidized added sulfide. The common presence of SQRs, FCSDs, and PDOs in heterotrophic bacteria suggests the significant role of heterotrophic bacteria in sulfide oxidation, participating in sulfur biogeochemical cycling. Further, FCSDs have been identified in anaerobic photosynthetic bacteria and chemolithotrophic bacteria, but their physiological roles are unknown. We showed that heterotrophic bacteria use FCSDs to oxidize self-produced sulfide and extraneous sulfide, and they may be used for H_2S bioremediation.

KEYWORDS *Cupriavidus necator* H16, sulfide, flavocytochrome *c*, persulfide dioxygenase, sulfide dehydrogenase

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Sulfide (H_2S, HS^-, and S^{2-}) is the most reduced form of sulfur in the biogeochemical cycle, and it is mainly produced by sulfur-reducing bacteria under anaerobic conditions, such as in marine sediment (1). When sulfide diffuses from the anaerobic sediment into the water interface, it is oxidized by chemolithotrophic sulfur oxidizers that use O_2 or nitrate as the electron acceptor; the process conserves energy for bacterial growth (2–4). Anaerobic photosynthetic bacteria can also use the reducing power of sulfide for photosynthesis (5, 6). However, these bacteria normally grow in environments where H_2S is abundant.

Animals, plants, and bacteria can also generate sulfide from cysteine metabolism under aerobic conditions (7–9). The sulfide produced has recently been reported as a new signaling molecule in mammals (10–15), and it can also protect bacteria against antibiotics by minimizing the production of hydroxyl radical, the most damaging reactive oxygen species, inside bacterial cells (16–18). Most heterotrophic bacteria produce sulfide from sulfur-containing amino acids during growth; however, some heterotrophic bacteria harbor sulfide:quinone oxidoreductase (SQR) and persulfide dioxygenase (PDO) (formerly termed sulfur dioxygenase) and oxidize self-produced sulfide (19). Bacteria without these enzymes produce sulfide and, when accumulated, sulfide is released as H_2S into the gas phase (19). The SQR-PDO pathway was first discovered in human mitochondria because mutations in PDO can lead to a human disease, ethylmalonic encephalopathy (20, 21). Subsequently, the presence of PDOs as well as SQRs in heterotrophic bacteria was demonstrated (22–25); the sulfide oxidation pathway is slightly different from that reported in humans (26, 27). In bacteria, SQR oxidizes sulfide to polysulfide, which spontaneously reacts with reduced glutathione (GSH) to produce glutathione persulfide (GSSH); PDO oxidizes GSSH to sulfite, and sulfite spontaneously reacts with polysulfide to produce thiosulfate. Rhodanese, which is almost universal in bacteria, can accelerate the reaction between polysulfide and GSH (28). In *Cupriavidus pinatubonensis* JMP134, SQR is a fusion protein with a rhodanese domain and an SQR domain (28).

SQRs, grouped into six types, have been extensively investigated in chemolithotrophic bacteria and anaerobic photosynthetic bacteria, and their presence in heterotrophic bacteria has recently become known (29). SQRs oxidize sulfide to zero-valence sulfur, likely polysulfide (28), and pass the electrons to the electron transport system via ubiquinone (29). Similar to SQRs, flavocytochrome *c* sulfide dehydrogenases (FCSDs) also oxidize sulfide to zero-valence sulfur; however, the electrons enter the electron transport system at the level of cytochrome *c* (30). The sulfide dehydrogenase is a flavocytochrome *c* (FccAB) system, consisting of a large sulfide-binding flavoprotein (FccB) and a small cytochrome *c* (FccA) (5, 6). FCSDs are widely distributed in purple and green phototrophic bacteria and in chemolithotrophic sulfur-oxidizing bacteria (31, 32). However, the physiological role of FCSDs is still debatable. A FCSD isolated from *Allochromatium* (formerly *Chromatium*) *vinosum* is active in sulfide oxidation (33), but the FCSD-inactive mutant of *A. vinosum* does not have any apparent decrease in sulfur oxidation and sulfur-supported phototrophic growth, suggesting that SQR is the main enzyme for sulfide oxidation in the bacterium (34, 35). In chemolithotrophic sulfur-oxidizing bacteria, FccA and FccB are often referred as SoxE and SoxF, respectively, because their genes are clustered with other Sox genes involved in thiosulfate oxidation (5). In a reconstituted Sox system, however, SoxF did not stimulate sulfide oxidation (36). When *soxE* is not next to *soxF*, the gene coding for the SoxF homolog is referred to as *soxJ* (5), and the protein has two functions *in vitro*, i.e., oxidizing sulfide and enhancing the Sox system for thiosulfate oxidation (37). Sulfide oxidation by FCSDs *in vivo* has yet to be shown, and the physiological roles remain to be discovered.

We recently reported that most bacteria produce sulfide during heterotrophic growth under aerobic conditions (19). H_2S is produced from sulfur-containing amino acids via cysteine desulfhydrase, cystathionine β-synthase (CBS), cystathionine γ-lyase (CSE), and 3-mercaptopyruvate sulfurtransferase (3MST) (7, 38). Bacteria with SQR and PDO, such as *Cupriavidus pinatubonensis* JMP134 and *Pseudomonas aeruginosa* PAO1,

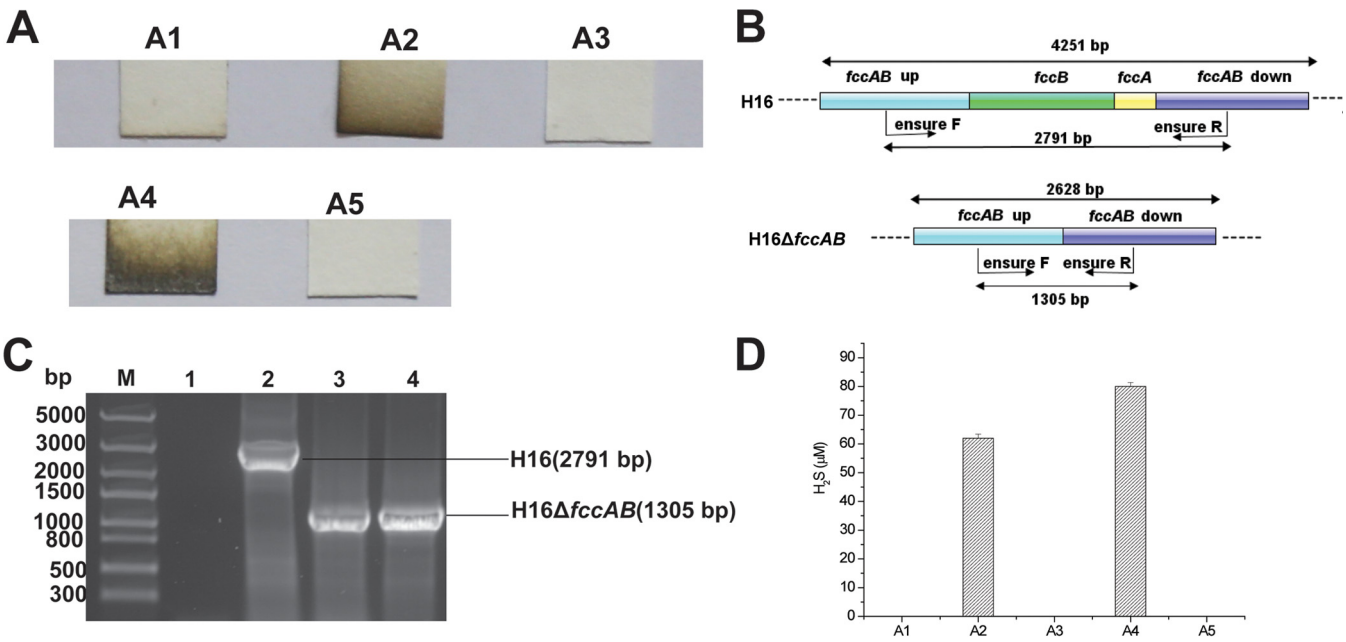

FIG 1 Genetic analysis of *fccAB* in *C. necator* H16. (A) H_2S production abilities of different bacteria. A1, *C. necator* H16; A2, *C. necator* H16 Δ*fccAB*; A3, *C. necator* H16 Δ*fccAB*/*fccAB*; A4, Pa3K; A5, Pa3K(pBBR5-*fccAB*). (B) Process for construction of *C. necator* H16 Δ*fccAB*. (C) Analysis of PCR fragments to confirm *fccAB* disruption. Lane M, molecular size standards; lane 1, product amplified with water as the template (negative control); lane 2, product amplified with *C. necator* H16 genomic DNA as the template; lane 3, product amplified with *C. necator* H16 Δ*fccAB* genomic DNA as the template; lane 4, product amplified with *C. necator* H16 Δ*fccAB*/*fccAB* genomic DNA as the template. The PCRs were performed with primers ensure F and ensure R. (D) Relative production of H_2S in different strains. The data were generated by scanning and comparison to standards. The detection limit with the lead-acetate paper strips was about 5 μM.

produce and oxidize sulfide, while bacteria without SQR and PDO produce and release H_2S into the gas phase in pure cultures. *Cupriavidus necator* H16 is best known for its ability to produce and to store large amounts of poly[*R*-(−)-3-hydroxybutyrate], which can be used to make biodegradable plastics (39). We report here that *C. necator* H16 without SQR uses FCSD to oxidize self-produced sulfide and FCSD couples with PDO to oxidize sulfide to sulfite and thiosulfate, via a pathway similar to the SQR-PDO pathway.

RESULTS

***C. necator* H16 oxidizes self-produced H_2S with FCSD.** Since bacteria without SQR often release H_2S during heterotrophic growth (19), *C. necator* H16, without SQR, was expected to produce and to release H_2S during aerobic growth in LB medium, but it did not (Fig. 1A). Sequence analysis of the *C. necator* H16 genome revealed that the bacterium has two PDOs (NCBI accession numbers WP_011616222.1 and WP_010809980) and one FCSD, consisting of FccA (NCBI accession number WP_010812125.1) and FccB (NCBI accession number WP_011616200.1). To analyze whether the FCSD in *C. necator* H16 was responsible for the oxidation of self-produced H_2S, the *fccAB* gene cluster was deleted by using homologous recombination, and the deletion was confirmed by PCR analysis (Fig. 1B and C). The *C. necator* H16 Δ*fccAB* mutant accumulated and released about 60 μM H_2S (Fig. 1A and D). The estimation was performed with sulfide added to LB medium, and lead-acetate paper strips detect sulfide levels as low as 5 μM added to LB medium (19). The *C. necator* H16 Δ*fccAB* mutant with *fccAB* complementation consumed sulfide and did not release H_2S into the gas phase (Fig. 1A and D). *P. aeruginosa* Pa3K (19), a mutant of *P. aeruginosa* PAO1 with its two *sqr* genes and one *pdo* gene deleted, produced detectable H_2S. Pa3K expressing *fccAB* did not release H_2S (Fig. 1A and D), indicating that FCSD oxidizes self-produced H_2S in both *C. necator* H16 and the recombinant *P. aeruginosa* strain. The FCSD of *C. necator* H16 did not function in *Escherichia coli* BL21(DE3)(pBBR5-*fccAB*) (data not shown). The nonfunctional FCSD is likely due to the lack of maturation proteins for *c*-type cytochromes in *E. coli* under aerobic conditions (40, 41).

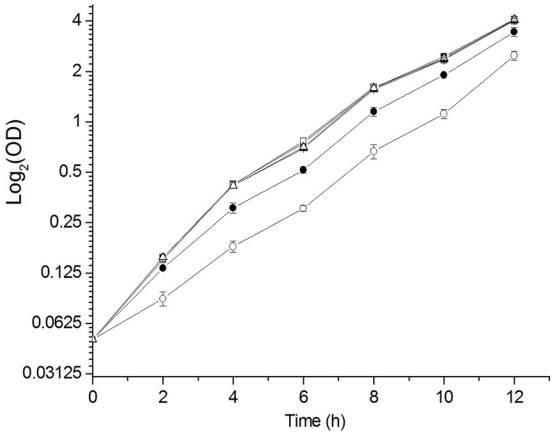

FIG 2 Growth curves in LB medium and LB medium with 200 μM NaHS. Overnight cultures were inoculated into LB medium or LB medium containing 200 μM NaHS, at an initial OD_{600} of 0.05. Cell turbidity was assessed every 2 h. ■, *C. necator* H16 in LB medium; ●, *C. necator* H16 in LB medium with 200 μM NaHS; □, *C. necator* H16 Δ*fccAB* in LB medium; ○, *C. necator* H16 Δ*fccAB* in LB medium with 200 μM NaHS; ▲, *C. necator* H16 Δ*fccAB*/*fccAB* in LB medium; △, *C. necator* H16 Δ*fccAB*/*fccAB* in LB medium with 200 μM NaHS. All data are averages of three samples with standard deviations (error bars). All cultures reached final OD_{600} values of about 6.1 after 24 h of incubation.

FCSD plays a detoxification role in *C. necator* H16. Although *C. necator* H16 Δ*fccAB* could not oxidize self-produced H_2S, the mutation did not affect growth in LB medium, and the wild-type and mutant strains had similar initial growth rates of about 0.8 h^{-1} (Fig. 2). When 200 μM sulfide was added to the LB medium, *C. necator* H16 was slightly inhibited (initial growth rate of 0.71 $\pm$ 0.06 h^{-1}) and the mutant was severely inhibited (initial growth rate of 0.41 $\pm$ 0.15 h^{-1}). The complemented strain *C. necator* H16 Δ*fccAB*/*fccAB* recovered its growth rate in LB medium containing 200 μM sulfide (initial growth rate of 0.8 $\pm$ 0.13 h^{-1}). Resting cells of *C. necator* H16 (3.49 $\pm$ 0.7 nmol min^{-1} mg [dry weight] $cells^{-1}$) oxidized sulfide faster than the mutant H16 Δ*fccAB* (2.5 $\pm$ 0.5 nmol min^{-1} mg [dry weight] $cells^{-1}$), and the complemented mutant H16 Δ*fccAB*/*fccAB* (6.7 $\pm$ 0.4 nmol min^{-1} mg [dry weight] $cells^{-1}$) oxidized sulfide even faster, possibly due to the increased production of FccAB from the introduced plasmid (Fig. 3). The data confirm that FCSD oxidizes sulfide and plays a role in the detoxification of sulfide.

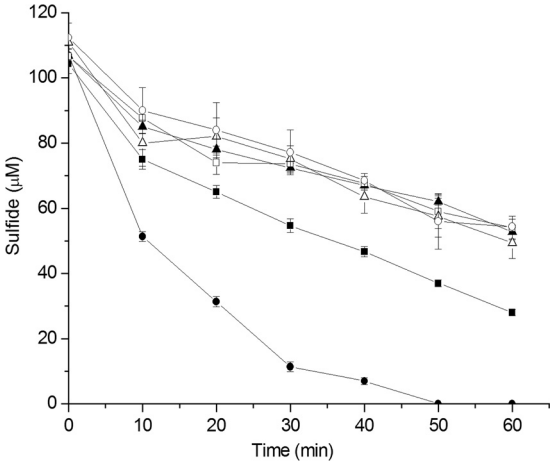

FIG 3 Sulfide oxidation by *C. necator* H16 and its mutants. Cells were suspended in 100 mM Tris buffer (pH 8.0) at an OD_{600} of 2, and NaHS (100 μM) was added to initiate the reaction. Controls used the same buffer without bacterial cells. ■, *C. necator* H16; ▲, *C. necator* H16 Δ*fccAB*; ●, *C. necator* H16 Δ*fccAB*/*fccAB*; □, buffer; △, heat-killed *C. necator* H16; ○, heat-killed *C. necator* H16 Δ*fccAB*/*fccAB*. All data are averages of three samples with standard deviations (error bars).

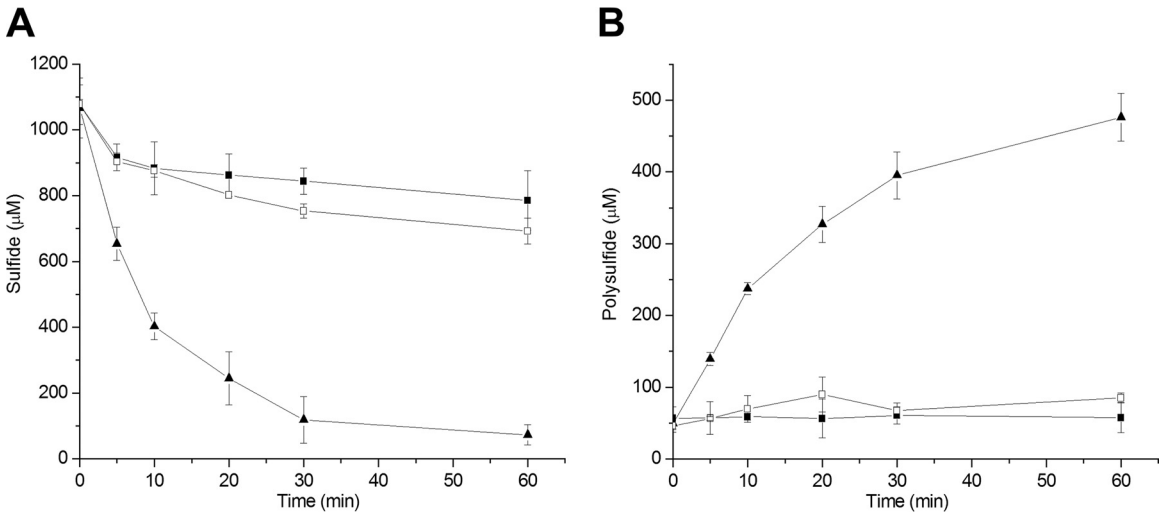

FIG 4 Effect of FccAB expression in *P. aeruginosa* Pa3K on H_2S oxidization. The cell suspension in 100 mM Tris buffer (pH 8.0), at an OD_{600} of 2, oxidized 1 mM sulfide. Sulfide (A) and polysulfide (B) were consumed or produced by Pa3K (■), Pa3K(pBBR5-*fccAB*) (▲), and heat-killed Pa3K(pBBR5-*fccAB*) (□). All data are averages of at least three samples with standard deviations (error bars).

***P. aeruginosa* Pa3K with FCSD oxidizes sulfide to sulfane sulfur.** *P. aeruginosa* strains Pa3K and Pa3K(pBBR5-*fccAB*) were cultured, harvested, and resuspended at an optical density at 600 nm (OD_{600}) of 2 in 100 mM Tris buffer (pH 8.0). A cell suspension of Pa3K(pBBR5-*fccAB*) oxidized 1 mM sulfide in 60 min, with an initial rate of 81.9 ± 2 nmol min^{-1} mg (dry weight) $cells^{-1}$, and the control PA3K strain had a minimal initial rate of sulfide oxidation of 2.3 ± 1.6 nmol min^{-1} mg (dry weight) $cells^{-1}$ (Fig. 4). Sulfane sulfur, including polysulfide, was produced. If the sulfane sulfur produced is in the form of disulfide (HSSH), then 1 mM sulfide is expected to produce 0.5 mM sulfane sulfur. Disulfide and trisulfide were detectable during sulfide oxidation by Pa3K(pBBR5-*fccAB*) (data not shown), similar to levels produced by recombinant *E. coli* with cloned *C. pinatubonensis sqr* (28). Sulfide levels were also decreased in the control experiment with *P. aeruginosa* Pa3K, but the small loss could be due to volatilization, autoxidation (42), and fortuitous activities of certain enzymes, such as superoxide dismutase (43). Polysulfide production was not detectable for the control *P. aeruginosa* Pa3K strain during incubation with 1 mM sulfide (Fig. 4). Sulfite and thiosulfate levels remained low, at less than 50 μM and 25 μM, respectively, during the course of analysis for *P. aeruginosa* Pa3K and the recombinant strains with *fccAB* or *sqr*.

FCSD and PDO collectively oxidize H_2S to sulfite and thiosulfate in *P. aeruginosa* Pa3K. *C. necator* H16 has two *pdo* genes, *Cnpdo*1 and *Cnpdo*2. The genes were separately cloned with FccAB in *P. aeruginosa* Pa3K. Resting Pa3K(pBBR5-*fccAB-Cnpdo1*) and Pa3K(pBBR5-*fccAB-Cnpdo2*) cells, containing FCSD and PDO, oxidized sulfide to sulfane sulfur, sulfite, and thiosulfate in 60 min, with initial rates of sulfide oxidation of 81.9 ± 2 nmol min^{-1} mg (dry weight) $cells^{-1}$ and 81.0 ± 3.9 nmol min^{-1} mg (dry weight) $cells^{-1}$, respectively (Fig. 5). The bacteria with different PDOs showed similar catalytic activities during sulfide oxidation. The bacteria with PDOs accumulated polysulfide more slowly than did Pa3K(pBBR5-*fccAB*), without PDO, and they produced 250 to 275 μM polysulfide after 60 min (Fig. 5). Interestingly, sulfite was rapidly produced to about 200 μM at 10 min and then levels gradually decreased to about 36 ± 10 μM at 60 min (Fig. 5C). During the same period, thiosulfate levels increased to 203 ± 11 μM (Fig. 5D), due the spontaneous reaction of sulfite with polysulfide (28). When the loss of sulfide from the control was subtracted, 824 μM sulfide was consumed by the bacteria with FccAB and PDO. Given that thiosulfate contains two sulfur atoms and polysulfide has two or more sulfur atoms, the sulfur balance is close.

Distribution of FccAB in bacteria. Of the 46 reported FccBs (5, 29), 34 were reported with accession numbers and were retrieved from GenBank. After duplicate

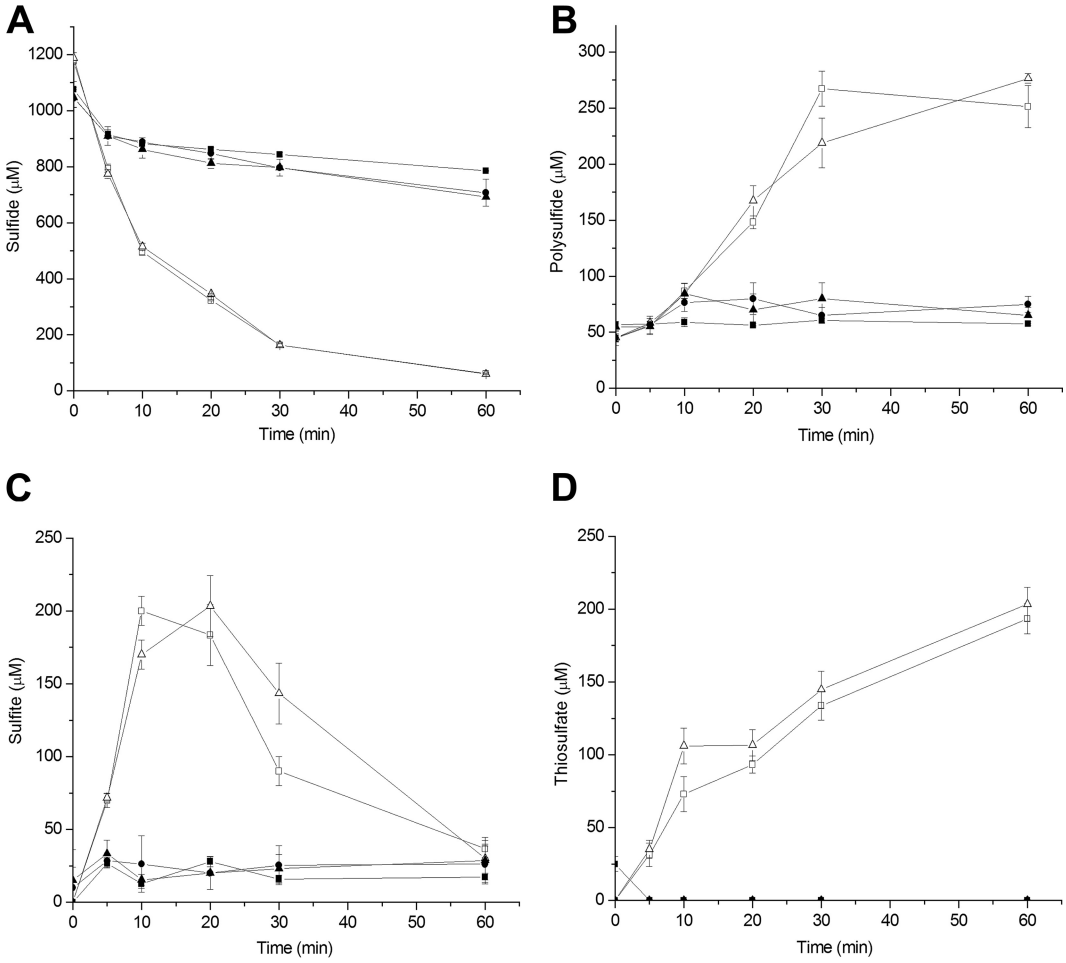

FIG 5 Effect of FccAB and PDO coexpression in *P. aeruginosa* Pa3K on H_2S oxidization. Sulfide was added at 1 mM to initiate the reaction. Sulfide (A), polysulfide (B), sulfite (C), and thiosulfate (D) were consumed or produced by Pa3K (■), Pa3K(pBBR5-*fccAB-Cnpdo1*) (□), Pa3K(pBBR5-*fccAB-Cnpdo2*) (△), heat-killed Pa3K(pBBR5-*fccAB-Cnpdo1*) (▲), and heat-killed Pa3K(pBBR5-*fccAB-Cnpdo2*) (●). All data are averages of at least three samples with standard deviations (error bars).

sequences were removed, 32 sequences were used for a BLAST search of a microbial genomic protein sequence set of 4,929 bacterial genomes (NCBI database, updated to 15 April 2016). A total of 351 candidates were identified, and phylogenetic tree analysis narrowed the list to 257 proteins. Since all of the seed FccBs contained the FCSD flavin-binding domain (pfam09242) and FadH2 domain (COG0446), these domains were also used to check the candidates. Finally, 240 candidates were identified as FccBs, and all were from Gram-negative bacteria. In contrast, SQRs are also present in Gram-positive bacteria or archaea (19). The 240 proteins were from 190 Gram-negative bacterial genomes, representing 3.85% of the 4,929 sequenced bacterial genomes, including 160 *Proteobacteria*, 10 *Chlorobia*, and 10 *Deinococcus* genomes and genomes from a few *Aquificales*, *Cytophagia*, and *Deferribacterales* species (Table 1). Of the 190 bacteria with FccBs, 34 contained more than one FccB and 121 (63.7%) also carried SQR. The frequent presence of both FccB and SQR within single bacteria is a surprise, as both are involved in sulfide oxidation.

FccB and SQR are evolutionarily related, and both contain flavin adenine dinucleotide (29). However, SQR transfers the electrons from sulfide oxidation to quinone, whereas FccB transfers the electrons to cytochrome *c*. Since FccBs are often used as outgroups for SQR phylogenetic analysis (5, 29), SQRs were used as outgroups for FccB phylogenetic analysis in this study. The 240 sequences were further assembled into 34 unique groups by using the CD-HIT program, with 50% identity as the cutoff value (44).

TABLE 1 Distribution of FccA and FccB in different bacterial phyla

Taxon	No. of genomes		
	fccB	Linked *fccA* and *fccB*	More than one *fccB*
Alphaproteobacteria	56	49	11
Betaproteobacteria	72	64	5
Gammaproteobacteria	27	23	7
Epsilonproteobacteria	5	1	0
Aquificales	7	1	4
Chlorobia	10	10	6
Cytophagia	1	1	0
Deferribacterales	2	2	0
Deinococcus	10	0	1

With the same strategy, the 32 FccB seed sequences and the 102 SQR sequences were clustered into 7 and 22 unique groups, respectively. One representative sequence from each unique group was selected. Thus, 41 FccBs together with FccBs from *C. necator* H16 and *C. pinatubonensis* JMP134 were used to build a phylogenetic tree with 22 SQRs as outgroups (MEGA 7.0) (Fig. 6). FccBs were divided into 3 subgroups on the phylogenetic tree.

The distribution of FccA near FccB was checked; 114 of the 138 FccBs in subgroup 1 and 68 of the 72 FccBs in subgroup 2 had *fccA* located within 5 loci of the corresponding *fccB* gene within the chromosome. However, only 6 of the 30 FccBs in subgroup 3 had their genes next to *fccA* within the chromosome.

The association of *fccB* with the *sox* genes coding for the SOX enzyme system was also investigated. Thirty strains contained the full set of *sox* genes, including *soxA*, *soxB*, *soxC*, *soxD*, *soxX*, *soxY*, and *soxZ*. Twenty-one strains had the *sox* genes without *soxC* and *soxD*, and the Sox system without SoxC and SoxD also oxidizes thiosulfate (45). An additional 67 strains had *fccB* near *soxYZ*. Thus, a total of 118 of 190 strains had *soxYZ* near *fccB*.

The association of *fccB* with the *pdo* genes was analyzed. Of the 190 strains, 137 contained PDOs. All of the green sulfur bacteria and most of the purple sulfur bacteria did not have PDOs. Most of the heterotrophic bacteria possessed PDOs.

DISCUSSION

We reported previously that significant proportions of heterotrophic bacteria with SQR and PDO are able to oxidize self-produced H_2S. These bacteria are rather common in nature, such as *Bacillus* spp. and *Pseudomonas* spp. in soil and *Roseobacter* spp. in marine environments (19). Here we demonstrated that bacteria with FCSD and PDO also have the ability to oxidize self-produced H_2S. These heterotrophic bacteria are likely to oxidize sulfide in the environment, especially where organic compounds are seasonally abundant, such as in garden soil or during algal blooms. The oxidation is significant, facilitating the geochemical cycling of sulfur, as chemical oxidation of sulfide is relatively slow, with a half-life of 26 h in seawater at 25°C (46).

Although the sulfide oxidation activity of FCSD *in vitro* has been shown, its *in vivo* activity and physiological functions in sulfide oxidation have not been demonstrated (33). Our data show that FCSD also oxidizes sulfide *in vivo*. In *C. necator* H16, FCSD oxidizes self-produced and extraneous sulfide, preventing volatilization of H_2S (Fig. 1A). It also plays a detoxification role, as the *fccAB* mutant grows slowly in the presence of sulfide levels as low as 200 μM (Fig. 2).

FCSD and SQR both oxidize sulfide to polysulfide in bacteria (Fig. 4) (19), but SQR is more common. A total of 1,014 of 4,929 sequenced bacterial genomes contain SQR, while only 190 possess FccB. One possible reason for its abundance is that SQR is more efficient in conserving energy, as it passes the electrons from sulfide oxidation to the electron transport chain at the level of quinone, while FCSD transfers electrons to the electron transport chain at the level of cytochrome *c*. The transport of electrons from ubiquinones to O_2 pumps more protons across the membrane than does that from

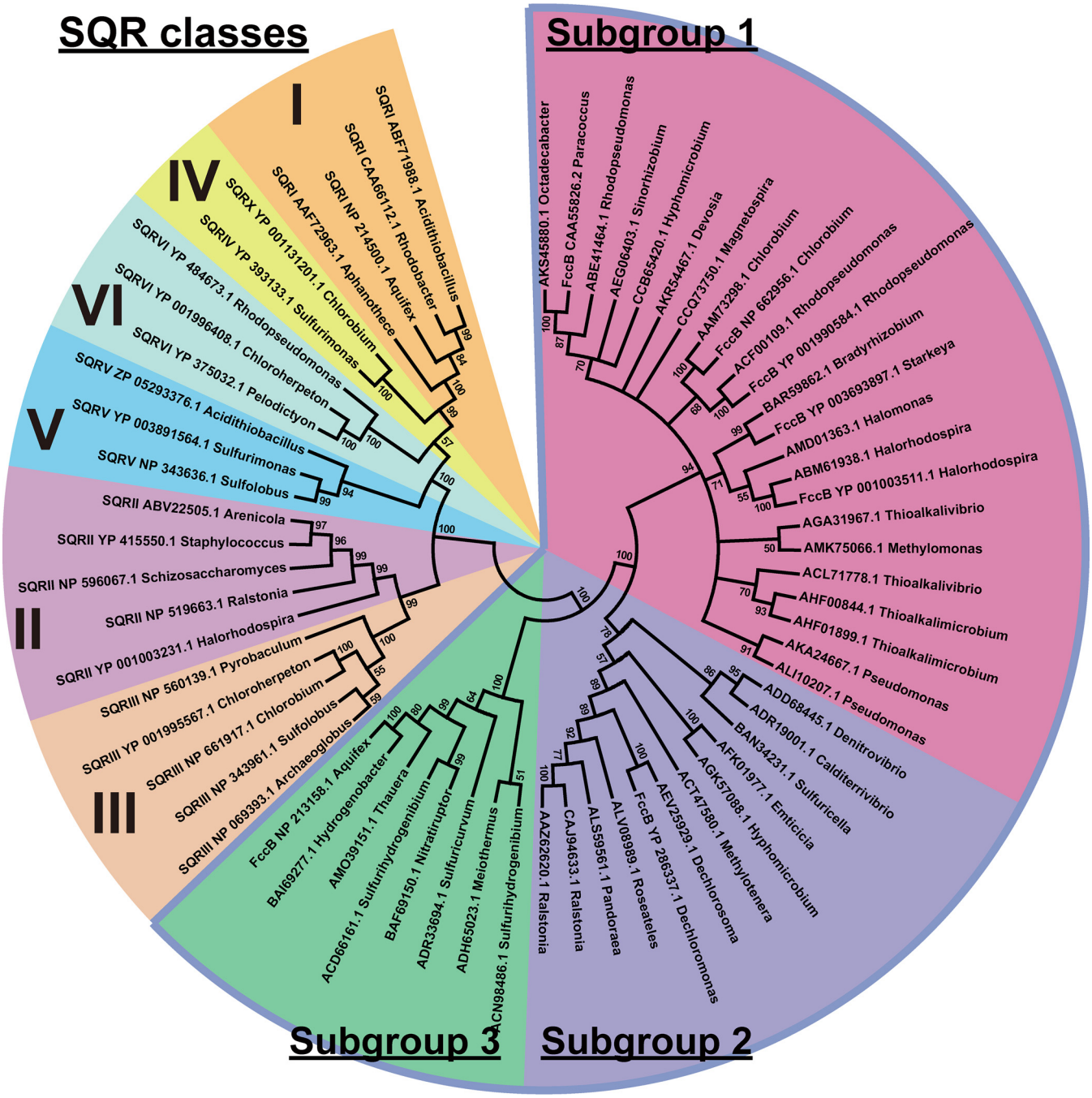

FIG 6 Phylogenetic analysis of FccBs and SQRs. The tree was generated with a neighbor-joining method by using MEGA 7 software. Proteins are listed with their accession numbers and organism origins.

cytochrome *c* to O_2. Thus, it has been reported that SQR is the primary enzyme for sulfide oxidation in *A. vinosum*, which contains both SQR and FCSD (5, 34, 35).

All reported FCSDs are in the periplasm (5, 34). FccBs containing flavin adenine diphosphate are thought to be folded and assembled with the flavin in the cytoplasm and then translocated to the periplasm by the TAT transport system (47). Indeed, FccB of *C. necator* H16 has a typical TAT signal peptide with a twin arginine motif at the N terminus, suggesting that it is transported by the TAT system. SignalP analysis identified a typical Sec-dependent signal peptide at the N terminus of FccA (48), suggesting that apo-FccA is transported by the Sec system into the periplasm, where FccA is assembled

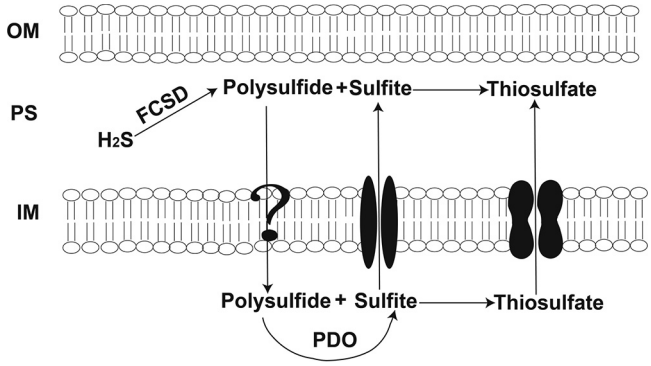

FIG 7 Proposed model for sulfide oxidation by FCSD and PDO in *C. necator* H16. Sulfide is oxidized by FCSD to polysulfide in the periplasmic space (PS). Polysulfide moves into the cytoplasm in an unknown way, and it is oxidized by PDO to produce sulfite in the cytoplasm. Sulfite is transported into the periplasmic space by a transport protein. Polysulfide and sulfite react to produce thiosulfate in both the periplasmic space and the cytoplasm. OM, outer membrane; IM, inner membrane.

with heme to generate the cytochrome *c* (49). A cytoplasmic location of PDOs has been proposed because PDOs usually do not have the signal peptide for protein trafficking across the cytoplasmic membrane into the periplasm and their activity requires Fe^{2+}, which is readily oxidized to Fe^{3+} outside the reducing cytoplasm (23). Thus, the scarce Fe^{2+} in the periplasm will likely not satisfy the Fe^{2+} requirement of PDOs unless Fe^{2+} is tightly bound to the enzyme, and it must be regenerated when oxidized to Fe^{3+}. On the basis of our data, we propose that the pathway of sulfide oxidation by the concerted actions of FCSD and PDO in *P. aeruginosa* Pa3K is similar to the SQR-PDO pathway (28). FCSD oxidizes sulfide to polysulfide in the periplasmic space (Fig. 7), polysulfide is transported into the cytoplasm and spontaneously reacts with GSH to produce GSSH, which is oxidized by PDO to sulfite, and sulfite chemically reacts with polysulfide to produce thiosulfate. Due to the lack of a rhodanese domain in FCSD, more thiosulfate was produced, similar to the *C. pinatubonensis* SQR-PDO system when the rhodanese activity is inactivated (28). It is currently unclear whether polysulfide simply diffuses into the cells or is transported by membrane proteins.

The slow movement of polysulfide from the periplasm into the cytoplasm is implied by the transitory accumulation of sulfite during the initial sulfide oxidation (Fig. 5C). Sulfite is produced by PDO in the cytoplasm and exported into the periplasm, which has a pH value lower than that of the cytoplasm. The chemical reaction between sulfite and polysulfide is slower at low pH, because the protonated forms of the species are less reactive. Thus, the slow reaction and rapid diffusion out of the periplasm through porins on the outer membrane allow the escape of sulfite into the medium. However, sulfite can diffuse back into the periplasm or even be transported into the cytoplasm; in both spaces, sulfite reacts with polysulfide to produce thiosulfate, with subsequent decreases in sulfite levels and increases in thiosulfate levels (Fig. 5C and D). Thus, a model of sulfide oxidation in Gram-negative bacteria with FCSD in the periplasm and PDO in the cytoplasm is proposed (Fig. 7).

Phylogenetic analyses divided FccBs into three subgroups and the SQR outgroups into six subgroups (Fig. 6). The six classes of SQRs were the same as reported previously (29). The three subgroups of FccBs were also well conserved within taxonomic classes (Table 2). The subgroup 1 FccBs were mainly distributed in *Alphaproteobacteria*, *Chlorobia*, and *Gammaproteobacteria*. Most *Chlorobia* and *Gammaproteobacteria* members are green or purple sulfur bacteria that use H_2S for anaerobic photosynthesis (5). Some *Alphaproteobacteria* members are common heterotrophs, such as *Rhodopseudomonas* spp., *Bradyrhizobium* spp., and *Sinorhizobium* spp. *Rhodopseudomonas* spp. are purple nonsulfur bacteria that are also able to grow phototrophically under anaerobic conditions (50). The subgroup 2 FccBs were primarily present in *Betaproteobacteria*, most of which are heterotrophs, including *C. necator* H16. The subgroup 3 FccBs were well

TABLE 2 Taxonomic distribution of FccB subgroups

Taxon	No. of FccB proteins	FccB subgroup
Alphaproteobacteria	68	1
Aquificales	1	1
Betaproteobacteria	9	1
Chlorobia	16	1
Epsilonproteobacteria	1	1
Gammaproteobacteria	43	1
Alphaproteobacteria	1	2
Betaproteobacteria	68	2
Cytophagia	1	2
Gammaproteobacteria	2	2
Aquificales	12	3
Betaproteobacteria	1	3
Deinococci	11	3
Epsilonproteobacteria	4	3

conserved in autotrophic bacteria of *Aquificales*, *Epsilonproteobacteria*, and *Deferribacterales*, which can oxidize H_2 or H_2S, and they were also present in *Thermus* spp. of the *Deinococcus-Thermus* phylum, which are often isolated from hot springs or hot composts where H_2S is abundant (51). FccBs were previously named SoxJ, SoxF, and FccB, according to whether their genes are associated with the *sox* genes or FccA is present (5). Here, we established a phylogenetic tree by using the FccBs identified from the whole GenBank database, and the previously reported SoxJ, SoxF, and FccB were randomly distributed in the subgroups (Fig. 6). Thus, the previously reported SoxJ and SoxF proteins are all FccBs. FccBs in each of the phylogenetic subgroups are well conserved at the taxonomic level (Table 2).

As reported, the *fccA* and *sox* genes frequently appeared near the *fccB* genes (5). We found that the *fccA* gene was often associated with *fccB* genes coding for subgroup 1 and 2 FccBs but not subgroup 3 FccBs. Since FccB is known to reduce other cytochrome *c* forms *in vivo* (31, 32), it may use alternative cytochrome *c* for electron transfer, reflecting the divergent evolution of FccBs. In contrast, the *sox* genes were less commonly associated with *fccB*, especially the full set of the Sox system; interestingly, *fccB* is often near *soxYZ*. The common presence of *soxYZ* with *fccB* (in 118 of 190 strains) suggests that SoxYZ may serve as a carrier for the sulfane sulfur produced by FccB. This possibility needs further investigation.

In summary, *C. necator* H16 without SQR actively oxidizes self-produced sulfide, and it uses FCSD to oxidize sulfide to polysulfide in the periplasm. The polysulfide produced is either transported or diffused into the cytoplasm, where it reacts with GSH to produce GSSH and PDO oxidizes GSSH to sulfite (28). Sulfite is exported to the periplasm, where it spontaneously reacts with polysulfide to generate thiosulfate (Fig. 7). Most of the heterotrophic bacteria containing FCSD also had PDOs, and the two enzymes may function for sulfide oxidation in other heterotrophic bacteria. Heterotrophic bacteria with FCSD may have the potential to be used for bioremediation of H_2S.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The bacterial strains and plasmids used in this study are listed in Table 3. *C. pinatubonensis* JMP134 and *C. necator* H16 were cultivated in LB medium at 30°C. *E. coli* and *P. aeruginosa* were cultured in LB medium at 37°C or as stated. *C. necator* H16 was also grown in minimal salt medium (MM) supplemented with 1% sodium gluconate during the process of gene inactivation (52). Gentamicin, tetracycline, and kanamycin were added as needed. The culture growth was monitored with OD_{600} measurements, and the growth rate was calculated via the change in $\log_2 OD_{600}$ per hour.

Testing of H_2S production. The method used for the detection of H_2S production was the same as described previously (19). Briefly, bacteria were transferred into 2 ml of LB medium in a 15-ml glass tube, and a paper strip with lead acetate was affixed at the top of the tube with a rubber stopper. The culture was incubated with shaking for 48 h, and then the paper strip was photographed to detect any lead(II)-sulfide black precipitates, as a measure of H_2S production. NaHS added to LB medium was used to generate black precipitates on the paper strips to estimate the amount of H_2S produced.

TABLE 3 Bacteria and plasmids used in this study

Strain or plasmid	Genotype and/or description[a]	Source or reference
Strain		
E. coli DH5α	Cloning strain	Novagen
E. coli S17-1	RP4 derivative integrated in chromosome	56
E. coli BL21(DE3)	Cloning strain	Novagen
C. pinatubonensis JMP134	Wild type	Ron L. Crawford
C. necator H16	Wild type	ATCC 17699
P. aeruginosa PAO1	Wild type	ATCC 15692
Pa3K	Mutant of *P. aeruginosa* PAO1 in which *sqr1*, *sqr2*, and *pdo* were deleted	This study
Plasmid		
pBBR1MCS-3	Tet^r, P_{lac}; expression vector	57
pBBR1MCS-5	Gm^r, P_{lac}; expression vector	57
pK18mobsacBtet	Km^r and Tet^r, *sacB*, RP4 oriT, ColE1 ori; suicide vector	This study
pBBR5-*fccAB*	pBBR1MCS-5 containing *fccAB* from *C. necator* H16	This study
pBBR5-*fccAB-pdo1*	pBBR1MCS-5 containing *fccAB* and *pdo1* from *C. necator* H16	This study
pBBR5-*fccAB-pdo2*	pBBR1MCS-5 containing *fccAB* and *pdo2* from *C. necator* H16	This study
pBBR3-*fccAB*	pBBR1MCS-3 containing *fccAB* from *C. necator* H16	This study
pBBR3-*fccAB-pdo1*	pBBR1MCS-3 containing *fccAB* and *pdo1* from *C. necator* H16	This study
pBBR3-*fccAB-pdo2*	pBBR1MCS-3 containing *fccAB* and *pdo2* from *C. necator* H16	This study
pK18mobsacBtet-Δ*fccAB*	Tet^r; suicide vector for deletion of *fccAB* of *C. necator* H16	This study

[a]Tet^r, tetracycline resistance; Gm^r, gentamycin resistance; Km^r, kanamycin resistance.

Deletion of *fccAB* from *C. necator* H16. The method used for the knockout of *fccAB* was the same as described previously (19, 53). Briefly, 1-kb regions immediately upstream and downstream of *fccAB* were amplified from genomic DNA of *C. necator* H16 via PCR with the primer sets fccuf/fccur and fccdf/fccdr (Table 4). Then the two fragments were joined and cloned into pK18mobsacB_{tet} at the EcoRI site by using the In-fusion kit (TaKaRa, Beijing, China), to produce pK18mobsacB_{tet}-ΔH16*fccAB* in *E. coli* S17-1. The plasmid pK18mobsacB_{tet}-ΔH16*fccAB* was transferred into *C. necator* H16 by conjugation. The plasmid pK18mobsacB_{tet}-ΔH16*fccAB* was integrated into the chromosome of *C. necator* H16 by the first crossover and selected on MM plates with sodium gluconate as the sole carbon source. Cells with the second crossover to generate the deletion were selected by culture on low-salt LB medium plates supplemented with 12% sucrose. The deletions were validated by PCR and DNA sequencing.

Complementation of *fccAB*. All primers are listed in Table 4. The *fccA and fccB* genes were amplified by using PCR with the *C. necator* H16 genome as the template and fccf and fccr as the primers. Linearized pBBR1MCS-3 was obtained via PCR with pBBR1MCS-3 as the template and pBBRf and pBBRr as the primers; the PCR product was treated with DpnI to degrade the template plasmid and was gel purified. Finally, the *fccAB* fragment and pBBR1MCS-3 were linked by using the In-fusion kit (TaKaRa). The resulting plasmid was cloned into *E. coli* and then transferred into *C. necator* H16 Δ*fccAB* via electroporation. For overexpression, pBBR1MCS-5-*fccAB* was constructed using the same method as described above.

Expression of *fccAB* and *pdo* of *C. necator* H16 in *P. aeruginosa*. The fragments *Cnpdo1* and *Cnpdo2* were PCR amplified with the primer pairs pdo1f/pdo1r and pdo2f/pdo2r, containing 15- to 20-bp extensions overlapping the adjacent fragment or the cloning site. For coexpression of *fccAB* and *Cnpdo1*, the primers pBBRf and fccr2 were used to amplify pBBR1MCS-5-*fccAB*(pBBR5-*fccAB*). The fragment *Cnpdo1* was cloned into pBBR5-*fccAB* by using the In-fusion kit, to generate pBBR5-*fccAB-Cnpdo1*. Using the same approach, pBBR5-*fccAB-Cnpdo2* was constructed. The resulting plasmids pBBR5-*fccAB*, pBBR5-*fccAB-Cnpdo1*, and pBBR5-*fccAB-Cnpdo2* were transferred into Pa3K via electroporation.

TABLE 4 Primers used in this study

Primer name	Sequence	Source
Fccf	CACACAGGAAACAGCTATGACAATCTTCCCCCGGCC	This study
fccr	TTCCATTCGCCATTCACTACCCCAGCATCTCCGCCC	This study
Pdo1f	GGGCGGAGATGCTGGGGTAGACGAAAAACAAGAGGGCAGCC	This study
Pdo1r	TTCCATTCGCCATTCATCAGGCGCCGTGCGGCACG	This study
Pdo2f	GGGCGGAGATGCTGGGGTAGGCATCCAAGGAGGCGAGCATG	This study
Pdo2r	TTCCATTCGCCATTCATCAGATGACGTCCAGAGGGATT	This study
Fccr2	CTACCCCAGCATCTCCGCCC	This study
pBBRf	TGAATGGCGAATGGAAATTGTAAG	This study
pBBRr	AGCTGTTTCCTGTGTGAAATTGTTATC	This study
fccuf	CAGGAAACAGCTATGACATGATTACGAATTGCAATTCTGCCAACGTGATCGCCGC	This study
fccur	CCCAGCATCTCCGCTGTCATGGTCTCGCTCCGTGTAGGG	This study
fccdf	GCGAGACCATGACAGCGGAGATGCTGGGGTAGGTGCAGG	This study
fccdr	TTCAGGATCCCCGGGTACCGAGCTCGAATTACAGCTCGATCACGCTTTCGTCCGC	This study
Ensure F	GGTCGACCGGGCCGATCTTC	This study
Ensure R	GACCCTGCTGATGACCGCCG	This study

Whole-cell analysis of sulfide oxidation. Cells of Pa3K and recombinant Pa3K(pBBR5-*fccAB*), Pa3K(pBBR5-*fccAB-Cnpdo1*), and Pa3K(pBBR5-*fccAB-Cnpdo2*) strains were grown in LB medium without induction to an OD_{600} of 3, harvested by centrifugation (6,000 × *g* for 10 min), and suspended in 100 mM Tris buffer (pH 8) at an OD_{600} of 2. For the heat-killed control, the cell suspension was heated in boiling water for 10 min and cooled to room temperature. Two milliliters of the cell suspension was transferred to a 15-ml capped centrifuge tube, and freshly prepared NaHS was added to initiate the reaction. The tube was incubated at 30°C, with shaking at 200 rpm. The pH was selected to minimize H_2S from rapid valorization, as the dominant species of sulfide is HS^- at pH 8 (54). The sulfide, polysulfide, sulfite, and thiosulfate levels were analyzed at various times.

Analytical procedures. The sulfide, polysulfide, sulfite, and thiosulfate levels were analyzed as described previously (28). Briefly, sulfide levels were analyzed by a colorimetric method; sulfite and thiosulfate levels were determined by ion chromatography as described previously, with minor modifications. Specific polysulfide species from sulfide oxidation by whole cells were also detected, after monobromobimane derivatization, by using high-performance liquid chromatography (HPLC) (28).

Analysis of FccB in sequenced bacterial genomes. A microbial genomic protein sequence set from NCBI (updated to 15 April 2016) was downloaded for the FccB search. The query sequences for FccBs were reported FccBs (5, 29) and were used to search the database with the standalone BLASTP algorithm, using conventional criteria (E value of $\leq 1e^{-10}$, coverage of $\geq$50%, and identity of $\geq$30%), to obtain FccB candidates from a total of 4,929 bacterial genomes. The candidates were combined with the seed FccBs for phylogenetic tree analysis using ClustalW for alignment and MEGA 7.0 for neighbor-joining tree building, with pairwise deletion, *p*-distance distribution, and bootstrap analysis of 1,000 repeats as parameters (55). The candidates that were in the same clade as the seed FccBs were picked for further analysis. The seed FccBs were searched for in the Conserved Domain Database (CDD) at the NCBI website. All seed FccBs contained the FCSD flavin-binding domain (pfam09242) and FadH2 domain (COG0446); therefore, these two motifs were used as standard features for further filtration of FccB candidates. The identified FccB sequences and seed sequences were separately grouped into unique groups by using the CD-HIT program, with identity of >50% as the threshold (44). Published SQR sequences were also collected (5, 29) and grouped into unique groups to be used as outgroups. One representative sequence from each unique group was selected and used for phylogenetic analysis (MEGA 7.0).

The 190 strains containing these 240 FccB proteins were manually checked for their heterotrophy with respect to the representative strains at the genus level, as described previously (19). Whether *fccA* appeared within 5 loci of the 240 *fccB* genes was also checked; to do so, the accession numbers of five genes around *fccB* were collected and used for the CDD search to check for cytochrome c_{553} (COG5863), using default parameters. If the transcription directions were the same and the intergenic region was smaller than 500 bp, then the cytochrome *c* gene was considered to be *fccA*.

Whether genes encoding the Sox enzyme system appeared within 10 loci of the 240 *fccB* genes in the genome was also checked. To find the conserved domains of these proteins, we collected all of the sequences of the Sox proteins from the KEGG gene database and used them to search the CDD with default parameters. All of the proteins in the Sox system had the conserved domain feature; SoxA (TIGR04484), SoxB (TIGR04486), SoxC (TIGR04555), SoxX (TIGR04485), SoxY (pfam13501), SoxZ (pfam08770), and SoxD (COG3474, COG4654, or COG3258) were used to check the corresponding proteins. The accession numbers of 10 genes around *fccB* were collected and used to search the CDD with default parameters. The nearby genes coding for proteins with the conserved Sox domains were identified. Genes coding for SQRs and PDOs within the same genome were detected as described previously (19).

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We declare we have no conflicts of interest regarding the contents of this article.

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