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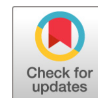

The Heterotrophic Bacterium *Cupriavidus pinatubonensis* JMP134 Oxidizes Sulfide to Sulfate with Thiosulfate as a Key Intermediate

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ABSTRACT Heterotrophic bacteria actively participate in the biogeochemical cycle of sulfur on Earth. The heterotrophic bacterium *Cupriavidus pinatubonensis* JMP134 contains several enzymes involved in sulfur oxidation, but how these enzymes work together to oxidize sulfide in the bacterium has not been studied. Using gene-deletion and whole-cell assays, we determined that the bacterium uses sulfide:quinone oxidoreductase to oxidize sulfide to polysulfide, which is further oxidized to sulfite by persulfide dioxygenase. Sulfite spontaneously reacts with polysulfide to produce thiosulfate. The sulfur-oxidizing (Sox) system oxidizes thiosulfate to sulfate. Flavocytochrome *c* sulfide dehydrogenase enhances thiosulfate oxidation by the Sox system but couples with the Sox system for sulfide oxidation to sulfate in the absence of sulfide:quinone oxidoreductase. Thus, *C. pinatubonensis* JMP134 contains a main pathway and a contingent pathway for sulfide oxidation.

IMPORTANCE We establish a new pathway of sulfide oxidation with thiosulfate as a key intermediate in *Cupriavidus pinatubonensis* JMP134. The bacterium mainly oxidizes sulfide by using sulfide:quinone oxidoreductase, persulfide dioxygenase, and the Sox system with thiosulfate as a key intermediate. Although the purified and reconstituted Sox system oxidizes sulfide, its rate of sulfide oxidation in *C. pinatubonensis* JMP134 is too low to be physiologically relevant. The findings reveal how these sulfur-oxidizing enzymes participate in sulfide oxidation in a single bacterium.

KEYWORDS sulfide, sulfane sulfur, thiosulfate, sulfate, heterotrophic bacteria, sulfur oxidation pathway, sulfate reduction

Sulfur oxidation is a key step in the biogeochemical cycling of sulfur on Earth. Under anoxic conditions, sulfur-reducing bacteria use sulfate as the terminal electron acceptor for the oxidation of organic compounds, generating hydrogen sulfide (H_2S), which can be oxidized back to sulfate by chemolithoautotrophic bacteria under oxic conditions or used as the reducing power for photosynthesis by phototrophic bacteria under anoxic conditions (1, 2). Further, many heterotrophic bacteria are also capable of oxidizing H_2S (3, 4). H_2S is a common product of the microbial metabolism of L-cysteine, and heterotrophic bacteria carrying sulfide:quinone oxidoreductase (SQR) and persulfide dioxygenase (PDO) oxidize self-produced H_2S or exogenously introduced H_2S to sulfite and thiosulfate (3, 5, 6). *Pseudomonas putida* oxidizes thiosulfate to tetrathionate (7, 8), *Hyphomicrobium denitrificans* oxidizes thiosulfate to tetrathionate and sulfate (9), and other bacteria, including bacteria isolated from marine sediments and hydrothermal vents, oxidize thiosulfate to sulfate (10). These reports suggest that different heterotrophic bacteria may work together to oxidize sulfide to sulfate.

Citation Xin Y, Gao R, Cui F, Lü C, Liu H, Liu H, Xia Y, Xun L. 2020. The heterotrophic bacterium *Cupriavidus pinatubonensis* JMP134 oxidizes sulfide to sulfate with thiosulfate as a key intermediate. Appl Environ Microbiol 86:e01835-20. https://doi.org/10.1128/AEM.01835-20.

Editor Ning-Yi Zhou, Shanghai Jiao Tong University

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Received 27 July 2020
Accepted 2 September 2020
Accepted manuscript posted online 11 September 2020
Published 28 October 2020

The soil bacterium *Cupriavidus pinatubonensis* (formerly *Ralstonia eutropha* and *Alcaligenes eutrophus*) JMP134 has been widely used to study the microbial degradation of aromatic compounds (11, 12). It also oxidizes H_2S during normal growth on organic compounds (3), offering an opportunity to investigate how the heterotrophic bacterium oxidizes H_2S. When its SQR and PDO genes are introduced into *Escherichia coli*, the recombinant cells oxidize H_2S to sulfite with polysulfide as an intermediate, and the sulfite spontaneously reacts with polysulfide to produce thiosulfate (5). From genome sequence analysis, *C. pinatubonensis* JMP134 also contains genes coding for the flavocytochrome *c* sulfide dehydrogenase (FCSD) system, the sulfur-oxidizing (Sox) system, and sulfite:cytochrome *c* oxidoreductase (SOR).

The FCSD system consists of a flavin adenine dinucleotide-containing protein (FccB) and a cytochrome *c* (FccA), which are soluble proteins in the periplasmic space. FccB and FccA are also called SoxF and SoxE, respectively, in chemolithoautotrophic bacteria, as their genes are often clustered with other *sox* genes (13). FCSD uses cytochrome *c* as the electronic acceptor, while SQR uses ubiquinone. FCSD was first identified in the purple photosynthetic bacterium *Allochromatium vinosum* (14), and it has subsequently been found to be widely present in autotrophic bacteria (15) and in heterotrophic bacteria (16). FCSD from *Cupriavidus necator* H16 oxidizes sulfide to polysulfide, which is further oxidized by PDO to sulfite and thiosulfate when both are cloned in *Pseudomonas aeruginosa* (16). Since *C. pinatubonensis* JMP134 contains both SQR and FCSD, it is unclear which one is preferentially used to oxidize H_2S.

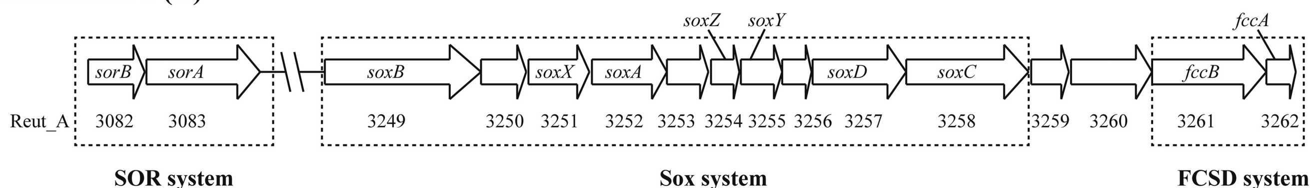
The Sox system is a versatile enzyme system for the oxidation of reduced sulfur species, and the Sox proteins are soluble in the periplasmic space (17). The most extensively studied Sox system is from *Paracoccus pantotrophus* GB17 (ATCC 35512^T or DSM 2944), a facultative chemolithotroph able to growth on thiosulfate (17). The core enzyme of the Sox system consists of four enzymes, SoxYZ (a heterodimer of SoxY and SoxZ), SoxXA, SoxCD, and SoxB, encoded by seven *sox* genes. How the Sox system oxidizes thiosulfate has been characterized. SoxYZ is a carrier protein with the sulfur molecule to be oxidized being covalently linked to a conserved cysteine residue of SoxY (18, 19). SoxXA catalyzes the loading of thiosulfate to SoxYZ-SH, producing SoxYZ-S-thiosulfate (20). SoxB hydrolytically removes the terminal sulfonate group, producing SoxYZ-SSH and sulfate (21). SoxYZ-SSH is oxidized by SoxCD to SoxYZ-S-SO_3^- (22, 23), and the sulfonate group is again released by SoxB. The electrons from sulfur oxidation enter the electron transfer chain via a cytochrome *c*-type cytochrome (24). The purified and reconstituted Sox system also oxidizes sulfide, elemental sulfur, and sulfite to sulfate (24), but it has not been confirmed whether *P. pantotrophus* or any other bacteria use the Sox system to oxidize these sulfur species.

SOR consists of two proteins, SorA and SorB. SorA is a large protein containing molybdopterin, and SorB is a small protein containing cytochrome *c*. SOR, located in the periplasmic space, oxidizes sulfite to sulfate (25). It is unknown if SOR oxidizes the sulfite generated by PDO to sulfate in *C. pinatubonensis* JMP134.

In this study, we investigated how *C. pinatubonensis* JMP134 uses SQR/PDO, FCSD, the Sox system, and SOR to oxidize sulfide to sulfate. On the basis of genomic analysis and our experimental data, we identified a new pathway of H_2S oxidation in which SQR and PDO collectively oxidize sulfide to thiosulfate and then the Sox system oxidizes thiosulfate to sulfate. FCSD's main function was to enhance thiosulfate oxidation by the Sox system; however, FCSD oxidized H_2S to zero-valent sulfur, part of which was directly oxidized by the Sox system when *sqr* was deleted in *C. pinatubonensis* JMP134.

RESULTS

Sulfur-oxidizing genes in *C. pinatubonensis* JMP134. The genome of *C. pinatubonensis* JMP134 consists of two chromosomes, chromosome A (3.8 Mb) and chromosome B (2.72 Mb), and two large plasmids (11). The *sqr-pdo2* gene cluster, located on chromosome B (Fig. 1; see also Table S1 in the supplemental material), codes for four possible proteins, SQR, PDO, a possible sulfite exporter protein (TauE), and a gene regulator (FisR) (3, 26). Another *pdo* gene (*pdo1*) is located on chromosome A (Table S1)



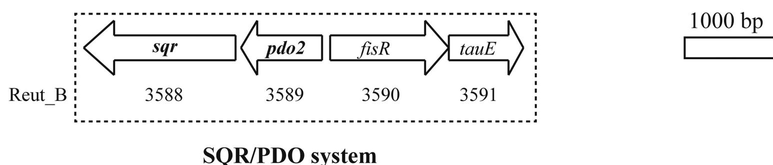


FIG 1 Schematic overview of the sulfur-oxidizing genes in *C. pinatubonensis* JMP134. The genome of *C. pinatubonensis* JMP134 includes two chromosomes, A and B. Genes encoding the SOR, Sox, and FCSD systems are located on chromosome A, and the *sqr-pdo* operon is on chromosome B. The SOR system is encoded by *sorA* (GenBank accession number AAZ62443.1) and *sorB* (GenBank accession number AAZ62442.1). The Sox system genes include *soxB* (GenBank accession number AAZ62608.1), *soxX* (GenBank accession number AAZ62610.1), *soxA* (GenBank accession number AAZ62611.1), *soxZ* (GenBank accession number AAZ62613.1), *soxY* (GenBank accession number AAZ62614.1), *soxD* (GenBank accession number AAZ62616.1), and *soxC* (GenBank accession number AAZ62617.1). The FCSD system is encoded by *fccB* (GenBank accession number AAZ62620.1) and *fccA* (a possible cytochrome *c*; GenBank accession number AAZ62621.1). An operon coding for the SQR/PDO system genes consists of *sqr* (GenBank accession number AAZ62946.1), *pdo2* (GenBank accession number AAZ62947.1), *fisR* (GenBank accession number AAZ62948.1), and *tauE* (GenBank accession number AAZ62949.1). The locus tag of each gene is given below the gene (e.g., Reut_A3252 is the tag for *soxA*).

and encodes a type I PDO (27). The *sox* genes on chromosome A consist of seven *sox* genes (*soxB*, *soxX*, *soxA*, *soxZ*, *soxY*, *soxD*, *soxC*), encoding the core components of the Sox system (Fig. 1; Table S1). Downstream (locus tags, Reut_A3261 and Reut_A3262) of the *sox* genes are *soxF* and *soxE*, which are also known as the *fccB* and *fccA* genes, respectively, coding for FccB and FccA of the FCSD system. The sequence similarities of FccB and FccA in *C. pinatubonensis* JMP134 to FccB (GenBank accession number CAJ94633.1) and FccA (GenBank accession number CAJ94634.1) in *C. necator* H16 were 85% (query cover, 99%) and 79% (query cover, 84%), respectively. In *C. necator* H16, which does not have SQR, the FCSD system oxidizes H_2S (16). SOR has two subunits, SorA and SorB, whose genes are also located on chromosome A (Fig. 1; Table S1). Genes coding for thiosulfate dehydrogenases, oxidizing thiosulfate to tetrathionate (28, 29), and tetrathionate hydrolase, converting tetrathionate to sulfate, sulfur, and thiosulfate (30), were not found in *C. pinatubonensis* JMP134. Genes involved in dissimilatory sulfur reduction, such as *qmoABC*, *aprAB*, and *sat* (31), were not found in the bacterium. The gene coding for a ribulose bisphosphate carboxylase, necessary for carbon fixation and chemolithoautotrophic growth, was not found in the bacterium either.

Deletion of genes involved in sulfur oxidation. Selected genes were deleted via homologous recombination by using a suicide plasmid carrying the DNA fragments before and after the gene. All the deletions were in frame, with about 0 to 10 amino acid residues at the N terminus and about 0 to 36 residues at the C terminus remaining in the mutants to avoid affecting downstream and upstream genes. All mutants grew similarly to the wild type when growing in Luria-Bertani (LB) medium or in a mineral medium (MM) with sulfate, sulfite, or thiosulfate as the sole sulfur source (data not shown), suggesting that these genes associated with sulfur oxidation are not essential for the bacterium during heterotrophic growth.

SQR was the primary enzyme responsible for sulfide oxidation. To investigate whether SQR, FCSD, or the Sox system was mainly responsible for sulfide oxidation in *C. pinatubonensis* JMP134, we tested the rate of sulfide oxidation by sulfide-induced cells of the wild type and the *sqr*, *fccB*, or *soxY-soxZ* deletion mutants. The Δ*soxYZ* mutant oxidized sulfide at a rate similar to that for the wild type (Table 1; Fig. 2), the Δ*fccB*, Δ*sqr*, Δ*sqr* Δ*fccB*, and Δ*sqr* Δ*fccB* Δ*soxYZ* strains oxidized sulfide at 98%, 27%, 11%, and 8% of the wild-type rate, respectively (Table 1; Fig. 2). The slow sulfide oxidation by

TABLE 1 Rates of sulfur oxidation by JMP134 and its mutant cells[a]

Strain	Oxidation rate (nmol/mg/min)[b]		
	HS^-	HS_n^-	$S_2O_3^{2-}$
JMP134	6.2 ± 0.1	11.8 ± 0.4	1.7 ± 0.1
Δ*sqr*	1.7 ± 0.4**	—	—
Δ*fccB*	6.1 ± 0.2	—	0.4 ± 0.1*
Δ*fccB*/*fccB*	6.1 ± 0.5	—	0.9 ± 0.1*
Δ*sqr* Δ*pdo1* Δ*pdo2*	1.2 ± 0.2**	—	—
Δ*sqr* Δ*pdo1* Δ*pdo2*/*sqr pdo2*	6.1 ± 1.0	—	—
Δ*sqr* Δ*pdo1* Δ*pdo2* Δ*soxYZ*	1.6 ± 0.7**	—	—
Δ*sqr* Δ*fccB*	0.7 ± 0.1**	—	—
Δ*sqr* Δ*fccB* Δ*soxYZ*	0.5 ± 0.4**	—	—
Δ*soxYZ*	6.3 ± 1.0	10.8 ± 1.3	0.2 ± 0.1*
Δ*soxYZ*/*soxYZ*	6.4 ± 0.2	—	1.2 ± 0.3
Δ*pdo1*	6.1 ± 0.5	11.0 ± 0.6	—
Δ*pdo2*	3.5 ± 0.2**	2.6 ± 0.5**	—
Δ*pdo1* Δ*pdo2*	2.2 ± 0.4**	1.3 ± 0.2**	—
Δ*pdo1* Δ*pdo2*/*pdo2*	5.7 ± 0.2	8.1 ± 0.4*	—
Δ*pdo1* Δ*pdo2* Δ*soxYZ*	1.9 ± 0.3**	0.7 ± 0.1**	—
Δ*sorA*	6.0 ± 0.9	—	—
Δ*tauE*	6.1 ± 0.2	—	—
Buffer	0.6 ± 0.3**	—	—

[a]Cells were induced, harvested, and resuspended in 100 mM HEPES buffer (pH 7.4) at an OD of 2. The cell suspensions were used to oxidize 500 μM sulfide, 800 μM polysulfide, or 600 μM thiosulfate at 30°C. The induction was with the corresponding substrate before harvesting.

[b]For HS^- and HS_n^-, the rates were calculated with data obtained at 0.5 h by using the cell dry weight. For $S_2O_3^{2-}$, the rates were calculated with data obtained at 2 h by using the cell dry weight. When calculating all rates, the rate for the heat-inactivated cell as a control was deducted. The rates of sulfide, polysulfide (HS_n^-), and thiosulfate oxidation of heat-inactivated cells were 6.0 ± 0.1, 0.7 ± 0.1, and 0 nmol/mg/min, respectively. —, not measured; *, the result for the mutant was different ($P < 0.05$) from that for the wild type; **, the result for the mutant was significantly different ($P < 0.01$) from that for the wild type. The data are the averages for at least three samples with standard deviations.

the Δ*sqr* Δ*fccB* Δ*soxYZ* mutant is likely due to nonspecific oxidation by other enzymes, as superoxide dismutase and catalase are able to oxidize sulfide (32, 33). The reduced rates of sulfide oxidation in the mutants were recovered when the deleted genes were complemented in *trans* on a plasmid (Table 1; Fig. S1). These results indicate that for sulfide oxidation, SQR is the main enzyme, FCSD plays a supplemental role, and the Sox system is only marginally active.

***C. pinatubonensis* JMP134 oxidizes sulfide to sulfate with sulfane sulfur and thiosulfate as detectable intermediates.** To understand the pathway, we monitored the intermediates and products during sulfide oxidation by *C. pinatubonensis* JMP134. When 500 μM NaHS was added to a suspension of sulfide-induced *C. pinatubonensis* JMP134 cells at an optical density at 600 nm (OD_{600}) of 2, about 100 μM sulfide remained at 1 h (Fig. 2A) and the cells produced about 32 μM sulfane sulfur (S^0) (including polysulfide and elemental sulfur), 120 μM thiosulfate, and 45 μM sulfate (Fig. 2B to D). Sulfane and thiosulfate were further consumed and gradually decreased to almost zero (Fig. 2B and C), and the cell suspension produced 362 μM sulfate at 7 h (Table 2; Fig. 2D). Sulfite was not detected during sulfide oxidation by the bacterium. The rate of sulfide oxidation was lower than that of polysulfide oxidation but higher than that of thiosulfate oxidation by *C. pinatubonensis* JMP134 (Table 1), reflecting the increased accumulation of thiosulfate during sulfide oxidation (Fig. 2).

SQR, PDO, and the Sox system sequentially oxidized sulfide to sulfate. Whether *C. pinatubonensis* JMP134 used PDO or the Sox system to oxidize the sulfane sulfur produced by SQR was further investigated by the construction of mutants with deletions of the corresponding genes. The deletion of *pdo1* did not affect sulfide oxidation, but the deletion of *pdo2* significantly slowed sulfide oxidation (Table 1). The Δ*pdo1* Δ*pdo2* mutant showed a further reduction in the sulfide oxidation rate (Table 1; Fig. 2A), with the elevated production of sulfane sulfur, the decreased production of thiosulfate, as well as the decreased production of sulfate (Table 2; Fig. 2B to D). This observation implies product inhibition: SQR oxidizes sulfide to sulfane sulfur, which

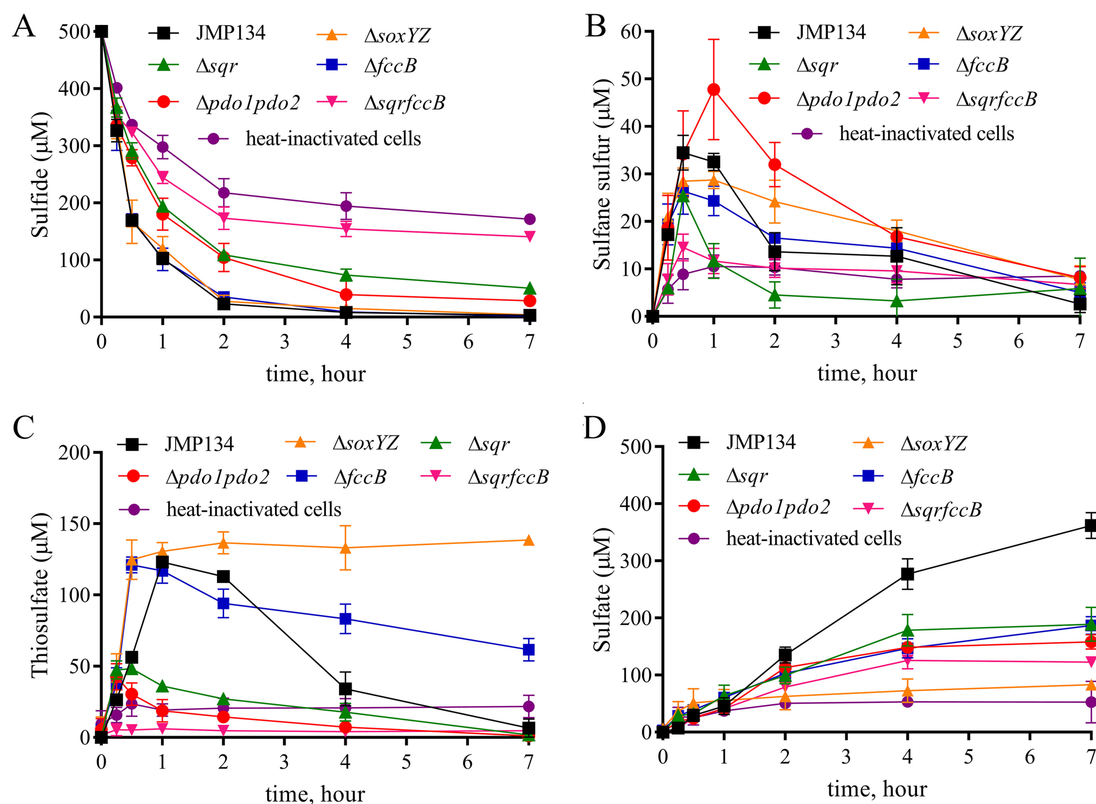


FIG 2 The function of SQR, PDO, the Sox system, and FccAB in *C. pinatubonensis* JMP134. Cells were harvested, washed, and resuspended in 100 mM HEPES buffer, pH 7.4, at an OD_{600} of 2.0. Sulfide was added to 500 μM to initiate the reaction. Sulfide (A), sulfane sulfur (B), thiosulfate (C), and sulfate (D) concentrations were determined. There was no apparent difference in the decrease in sulfide levels in HEPES buffer with or without heat-inactivated cells, but the heat-inactivated cells produced more sulfate (53 $\pm$ 13 μM) than the buffer (5 $\pm$ 3 μM) at 7 h (see Fig. S1 in the supplemental material). The apparent decrease in the buffer with heat-inactivated cells was likely due to evaporation via shaking and autoxidation. All data are averages for at least three samples with standard deviations (error bars).

accumulates and inhibits SQR activity in the absence of PDO, which oxidizes sulfane sulfur (5). The resting state of cells of the Δ*soxYZ* mutant did not affect sulfide oxidation or the transitory accumulation of sulfane sulfur but largely abolished thiosulfate oxidation and sulfate production (Table 2; Fig. 2A to D); complementation of *soxYZ* partially restored thiosulfate oxidation and sulfate production (Table 2; Fig. S1). The partial recovery seen after complementation could have been due to the level of gene expression or an imbalanced ratio of the proteins that make up the Sox system. A downstream effect is also possible, as it is not clear whether there are internal promoters within the deleted gene, albeit the deletion was in frame.

Cells in the polysulfide-induced resting state were also used to determine the rates of polysulfide oxidation by *C. pinatubonensis* JMP134 and its mutants (Table 1). The Δ*soxYZ*, Δ*pdo1* Δ*pdo2*, and Δ*pdo1* Δ*pdo2* Δ*soxYZ* mutants oxidized polysulfide at rates of 91%, 11%, and 6% of the wild-type rate, respectively, suggesting that the two PDOs are the primary enzymes for the oxidation of added polysulfide, which is a specific form of sulfane sulfur and which is likely the direct product of SQR (5, 34). The Δ*pdo1* mutant and the Δ*pdo2* mutant oxidized polysulfide at rates of 93% and 22% of the rate of the wild type, respectively (Table 1), suggesting that PDO2 is the main PDO in the bacterium. The complementation of the Δ*pdo1* Δ*pdo2* mutant with *pdo2* led to recovery of the rate of polysulfide oxidation to 69% of the wild-type rate (Table 1).

Cells in the thiosulfate-induced resting state were used to determine the rates of thiosulfate oxidation by *C. pinatubonensis* JMP134 and its mutants (Table 1), as thiosulfate induces the expression of *sox* genes in *P. pantotrophus* GB17 (17). The Δ*soxYZ* cells oxidized thiosulfate at a rate of 12% of the wild-type rate. Complementation with

TABLE 2 Substrates and products at 7 h of sulfide oxidation by whole cells[a]

Strain	Concn (μM)				Recovery of sulfur (%)
	HS^-	Sulfane sulfur	$S_2O_3^{2-}$	SO_4^{2-}	
JMP134	3 ± 1	3 ± 1	7 ± 5	362 ± 16	76
Δ*sqr*	51 ± 6	6 ± 5	2 ± 0	189 ± 21	44
Δ*fccB*	2 ± 2	5 ± 2	62 ± 6	187 ± 11	63
Δ*fccB/fccB*	4 ± 1	2 ± 1	1 ± 0	252 ± 17	52
Δ*sqr* Δ*pdo1* Δ*pdo2*	92 ± 8	6 ± 3	1 ± 0	200 ± 11	51
Δ*sqr* Δ*pdo1* Δ*pdo2/sqr pdo2*	5 ± 2	3 ± 1	0 ± 0	436 ± 38	89
Δ*sqr* Δ*pdo1* Δ*pdo2* Δ*soxYZ*	100 ± 5	24 ± 1	40 ± 1	33 ± 2	34
Δ*sqr* Δ*fccB*	141 ± 5	7 ± 1	5 ± 2	123 ± 4	39
Δ*sqr* Δ*fccB* Δ*soxYZ*	143 ± 15	10 ± 1	69 ± 3	28 ± 1	49
Δ*soxYZ*	4 ± 2	8 ± 2	139 ± 2	83 ± 6	74
Δ*soxYZ/soxYZ*	20 ± 4	3 ± 3	1 ± 0	448 ± 17	94
Δ*pdo1* Δ*pdo2*	29 ± 7	8 ± 2	1 ± 0	158 ± 9	36
Δ*pdo1* Δ*pdo2/pdo2*	7 ± 2	3 ± 1	8 ± 4	327 ± 18	70
Δ*pdo1* Δ*pdo2* Δ*soxYZ*	65 ± 9	4 ± 1	38 ± 4	30 ± 1	25
Δ*sorA*	3 ± 1	3 ± 0	0 ± 0	357 ± 32	72
Δ*tauE*	7 ± 1	5 ± 3	7 ± 4	363 ± 5	77
Buffer	176 ± 10	3 ± 2	16 ± 5	5 ± 3	12
Heat-inactivated cell	172 ± 7	8 ± 2	22 ± 6	53 ± 13	32

[a]The oxidation of 500 μM sulfide by cell suspensions at an OD of 2 in 100 mM HEPES buffer, pH 7.4, at 30°C was used. Sulfur compounds were analyzed at 7 h. All data are the averages for at least three samples with standard deviations.

soxYZ led to the partial recovery of the rate of thiosulfate oxidation to 71% of the wild-type rate (Table 1).

Collectively, the data suggest that SQR oxidizes sulfide to sulfane sulfur; PDO oxidizes sulfane sulfur to sulfite, which reacts with sulfane sulfur to generate thiosulfate in *C. pinatubonensis* JMP134 (5); and the Sox system further oxidizes thiosulfate to sulfate.

SQR did not couple with the Sox system for sulfide oxidation. The direct coupling of SQR and the Sox system was not apparent, as the Δ*pdo1* Δ*pdo2* and Δ*pdo1* Δ*pdo2* Δ*soxYZ* mutants oxidized sulfide at similar rates (Table 1; Fig. 3A), suggesting that the product (sulfane sulfur) inhibition in SQR was not alleviated by the Sox system in the Δ*pdo1* Δ*pdo2* mutant. The level of sulfane sulfur was higher for both the Δ*pdo1* Δ*pdo2* and the Δ*pdo1* Δ*pdo2* Δ*soxYZ* mutants than for the wild type (Fig. 2B and 3B). Thiosulfate was also produced by the Δ*pdo1* Δ*pdo2* and Δ*pdo1* Δ*pdo2* Δ*soxYZ* mutants (Fig. 2C and 3C), suggesting that sulfane sulfur is slowly oxidized to thiosulfate by other enzymes, like catalase (33), or by autoxidation (35). The Δ*pdo1* Δ*pdo2* mutant with the Sox system further oxidized thiosulfate to sulfate, while the Δ*pdo1* Δ*pdo2* Δ*soxYZ* mutant accumulated thiosulfate (Fig. 3C and D). However, the Δ*pdo1* Δ*pdo2* Δ*soxYZ* mutant also produced 30 μM sulfate (Table 2; Fig. 3D), suggesting nonspecific oxidation of either sulfane sulfur or thiosulfate at a very low rate by the mutant. Thus, the sulfane sulfur produced by SQR is mainly oxidized by PDO to thiosulfate, which is oxidized by the Sox system to sulfate.

FccAB coupled with the Sox system for sulfide oxidation. The Δ*sqr* Δ*pdo1* Δ*pdo2* mutant produced some thiosulfate during sulfide oxidation, suggesting that the sulfane sulfur produced by FccAB is oxidized to thiosulfate by other enzymes, as catalase slowly oxidizes polysulfide (33). Further, three lines of evidence supported the suggestion that the sulfane sulfur produced by FCSD was directly oxidized to sulfate by the Sox system in the absence of SQR. First, the Δ*pdo1* Δ*pdo2* mutant produced less sulfate than the Δ*sqr* Δ*pdo1* Δ*pdo2* mutant did (Table 2; Fig. 3D), even though it oxidized sulfide faster (Table 1; Fig. 3A) and transitorily accumulated more sulfane sulfur and thiosulfate (Fig. 3C). Here (Fig. 3C), the reduced production of thiosulfate by the Δ*sqr* Δ*pdo1* Δ*pdo2* mutant in comparison with that by the Δ*pdo1* Δ*pdo2* mutant suggests the direct coupling of FCSD with the Sox system, in which thiosulfate is not produced. Second, the rates of sulfide oxidation and the final levels of production of sulfate by the Δ*sqr* and the Δ*sqr* Δ*pdo1 pdo2* mutants were similar (Tables 1 and 2), indicating that PDO is not

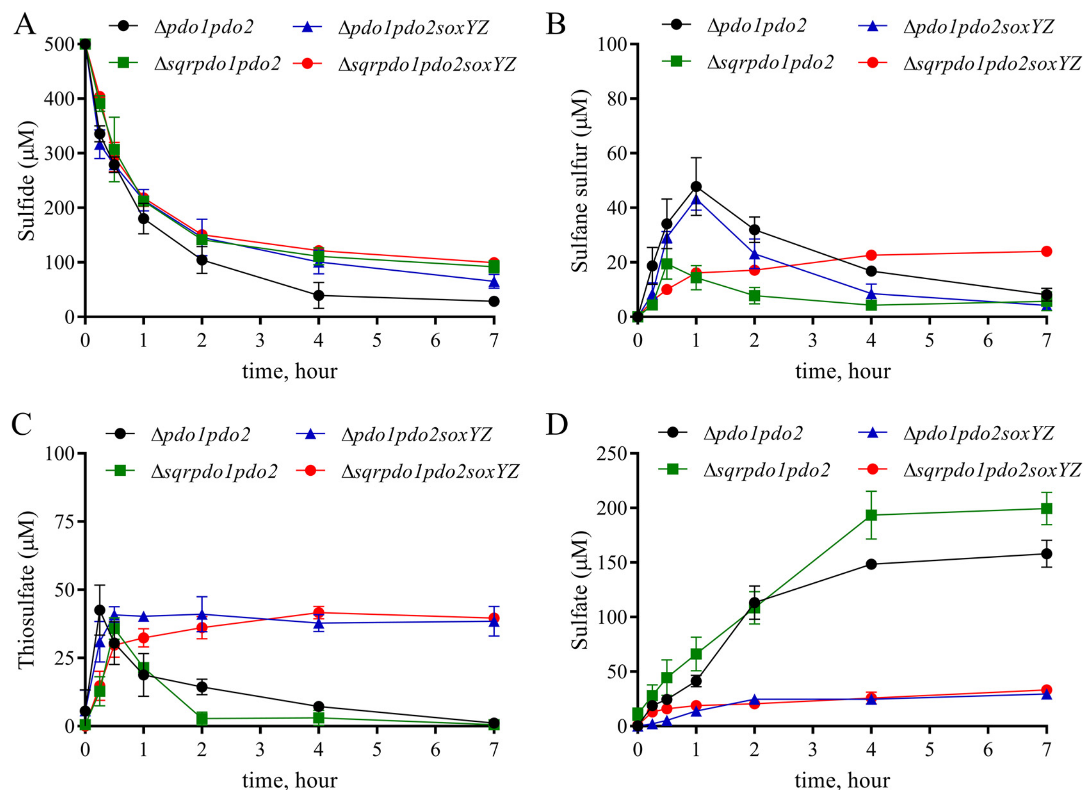


FIG 3 Sulfide oxidation by the Δ*pdo1* Δ*pdo2* mutant and its derivatives. Cells were harvested, washed, and resuspended at an OD_{600} of 2.0 in 100 mM HEPES buffer, pH 7.4. Sulfide was added to 500 μM to initiate the reaction. Sulfide (A), polysulfides (B), thiosulfate (C), and sulfate (D) levels were determined at different times. All data are averages for at least three samples with standard deviations (error bars).

necessary during sulfide oxidation in that Δ*sqr* mutant and that FCSD can couple with the Sox system for sulfide oxidation. Third, the Δ*sqr* Δ*pdo1* Δ*pdo2* Δ*soxYZ* mutant accumulated more sulfane sulfur than the Δ*sqr* Δ*pdo1* Δ*pdo2* mutant did during sulfide oxidation (Table 2; Fig. 3B), suggesting that the Sox system oxidizes sulfane sulfur in the Δ*sqr* Δ*pdo1* Δ*pdo2* mutant. Thus, in the *sqr* deletion mutants, the sulfane sulfur produced by FCSD is oxidized either by nonspecific enzymes, like catalase, or by the Sox system. When FCSD couples with the Sox system, thiosulfate is not an intermediate during sulfide oxidation to sulfate.

The sulfite produced during sulfide oxidation is not released into the medium. Recombinant *E. coli* with cloned *sqr* and *pdo2* from *C. pinatubonensis* JMP134 oxidizes sulfide to sulfite and thiosulfate (5), but we did not detect a transitory accumulation of sulfite in *C. pinatubonensis* JMP134 or its mutants during sulfide oxidation. *C. pinatubonensis* JMP134 contains a SOR system, encoded by *sorA* and *sorB*; however, the Δ*sorA* mutant metabolized sulfide in a similar way as the wild type did (Fig. 2; Fig. S2), and sulfite was still undetectable. The results indicate that *C. pinatubonensis* JMP134 produces sulfite during sulfide oxidation and that sulfite reacts with sulfane sulfur to generate thiosulfate inside the cell without being released.

Sulfite was oxidized to sulfate by the SOR system and the Sox system. The sulfite-induced resting cells of *C. pinatubonensis* JMP134 quickly consumed 500 μM sulfite within 2 h, producing about 500 μM sulfate; the Δ*sorA* mutant completely lost the ability to oxidize sulfite; sulfite oxidation was restored in the Δ*sorA*/*sorA* strain. The Δ*soxYZ* mutant oxidized sulfite as fast as the wild type did (Fig. S3), suggesting that the Sox system is not involved in sulfite oxidation in the wild type. When the cells were induced with thiosulfate and then used to oxidize sulfite, the Δ*sorA* mutant oxidized sulfite at a significantly lower rate (0.6 $\pm$ 0.3 nmol/mg [dry weight]/min) than the wild type did (5.4 $\pm$ 0.2 nmol/mg [dry weight]/min), and the Δ*sorA* Δ*soxYZ* double mutant

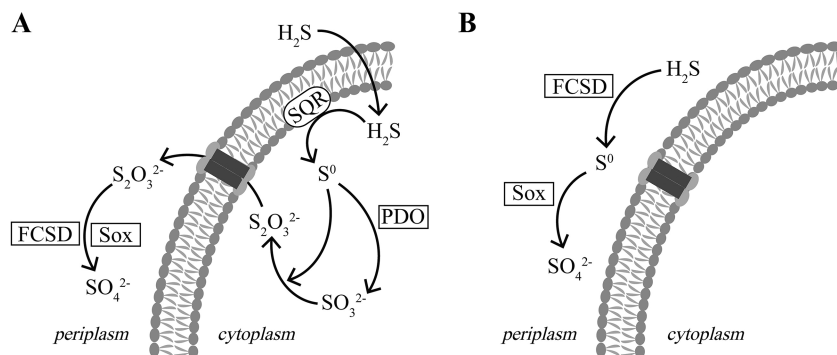


FIG 4 Proposed pathways of sulfide oxidation in *C. pinatubonensis* JMP134 and its Δ*sqr* Δ*pdo1* Δ*pdo2* mutant. (A) Sulfide oxidation in the wild type. Sulfide is oxidized to sulfane sulfur (S^0) by SQR; PDO oxidizes S^0 to sulfite, which spontaneously reacts with S^0 to generate thiosulfate in the cytoplasm. Thiosulfate is transported to the periplasmic space and is oxidized by the Sox system to sulfate. (B) Sulfide oxidation by the Δ*sqr* Δ*pdo1* Δ*pdo2* mutant without SQR and PDO. FCSD oxidizes sulfide to S^0, which is then oxidized by the Sox system to sulfate. This pathway is marginal in the wild type.

completely lost the ability to oxidize sulfite (Fig. S4). Thus, the Sox system also oxidized sulfite but did so at a rate of 11% of the rate that SOR did in *C. pinatubonensis* JMP134.

DISCUSSION

Our results indicate that *C. pinatubonensis* JMP134 oxidizes sulfide to sulfate via a new pathway (Fig. 4A). The pathway has not been previously observed in any bacteria. First, H_2S enters the cytoplasm via diffusion, as membrane transporters are not required for the process (36). Second, SQR oxidizes H_2S to sulfane sulfur (S^0) in the cytoplasm (37). Third, PDO oxidizes sulfane sulfur to sulfite, which spontaneously reacts with sulfane sulfur to generate thiosulfate (5). The reaction between sulfane sulfur and sulfite likely occurs inside the cytoplasm, as both reactants are produced in the cytoplasm (37). Fourth, the produced thiosulfate is transported to the periplasmic space by an unknown transporter. Although *tauE*, encoding a hypothetical sulfite exporter (see Table S1 in the supplemental material), is next to the *sqr-pdo2* operon, its role is not essential, as its deletion did not have detectable effects on sulfide oxidation and sulfate production (Tables 1 and 2). Fifth, the Sox system oxidizes thiosulfate to sulfate in the periplasmic space (Fig. 4A). In *C. pinatubonensis* JMP134, FCSD does not play a significant role in sulfide oxidation but enhances thiosulfate oxidation by the Sox system, as the Δ*fccB* mutant oxidized sulfide at a rate similar to that for the wild type but oxidized thiosulfate at a significantly reduced rate (Table 1). The role of FCSD in enhancing thiosulfate oxidation by the Sox system also occurs in *P. pantotrophus* GB17 (38). In *sqr* deletion mutants, such as the Δ*sqr* and the Δ*sqr* Δ*pdo1* Δ*pdo2* mutants, FCSD is likely able to direct couple with the Sox system for sulfide oxidation to sulfate (Fig. 4B), but at a significantly reduced rate (Table 1). Thus, *C. pinatubonensis* JMP134 contains a main pathway (Fig. 4A) and a contingent pathway (Fig. 4B) for sulfide oxidation.

The main pathway is different from other documented pathways in bacteria. The anoxygenic purple sulfur bacterium *Allochromatium vinosum* uses H_2S as the electron donor for photosynthesis, producing sulfate. It oxidizes sulfide to polysulfide by SQR and then to sulfite by reverse dissimilatory sulfite reductase (34). Sulfite is mainly oxidized to sulfate by a polysulfide reductase-like iron–sulfur molybdoprotein (SoeABC) (39).

The dimethylsulfide-degrading bacterium *Hyphomicrobium denitrificans* degrades dimethylsulfide to sulfide and then further oxidizes sulfide to sulfate under oxic conditions. Interestingly, the bacterium also produces thiosulfate as a metabolic intermediate. Although it contains PDO, its role in sulfane sulfur oxidation during dimethylsulfide oxidation is not observed. It employs heterodisulfide reductase to oxidize sulfane sulfur to sulfite, which reacts with sulfane sulfur to generate thiosulfate (9). Since the bacterium contains the incomplete Sox system without SoxCD, the system

cannot completely oxidize thiosulfate to sulfate. The incomplete Sox system works with heterodisulfide reductase to oxidize thiosulfate to sulfate and sulfite (9). Sulfite reacts with sulfane sulfur to generate thiosulfate. The net process leads to the oxidation of reduced sulfur compounds to sulfate (40).

The chemolithotroph *Acidithiobacillus caldus*, a bioleaching agent, and members of the genus *Thioalkalivibrio*, a genus of haloalkaliphilic sulfur-oxidizing bacteria, contain genes similar to those seen in *H. denitrificans* (41, 42), and they may produce thiosulfate as a key intermediate during sulfur oxidation. However, *Acidithiobacillus* spp. and *Thioalkalivibrio* spp. also possess sulfite:quinone oxidoreductase, which may directly oxidize sulfite to sulfate (41, 42).

Both SQR and FCSD oxidize sulfide to sulfane sulfur (Fig. 2 and 3). Sequence analysis of 4,929 bacterial genomes showed that 1,014 bacteria contain SQR (3) and 190 bacteria contain FccB (16). The popularity of SQR may suggest its significance in H_2S oxidation. Among the 190 bacteria with FccB, 121 bacteria (63.7%) also carried SQR (16). The frequent co-occurrence of FccB and SQR within a single bacterium indicates that they may have different physiological roles. In *C. pinatubonensis* JMP134, SQR is the primary sulfide oxidase, and the deletion of *fccB* did not reduce the sulfide oxidation rate but significantly reduced the rate of thiosulfate oxidation (Table 1). This finding is similar to that for *A. vinosum*, which contains both *sqr* and *fccAB*, and the inactivation of *fccAB* does not affect its rate of sulfide oxidation (43). The enhancement of the Sox activity by FCSD has previously been reported (38). Further, FCSD oxidizes sulfide at a lower rate in the Δ*sqr* mutant (Table 1). Thus, FCSD oxidizes sulfide in bacteria that do not contain SQR, as previously reported (16). The choice of SQR over FCSD is likely due to energy conservation, as SQR uses ubiquinone as its electron acceptor (44), producing more membrane potential than FCSD, which uses cytochrome *c* as its electron acceptor (16).

The Sox system was extensively studied in *P. pantotrophus*, which is able to grow on organic compounds as well as on thiosulfate (45). Because the purified and reconstituted Sox system from *P. pantotrophus* oxidizes sulfide, thiosulfate, sulfur, and sulfite, the Sox system has been proposed to be a general sulfur-oxidizing system (24, 46). Early genetic analysis further supported the involvement of the Sox system in the oxidation of sulfide and thiosulfate in the anaerobic phototrophic bacterium *Rhodovulum sulfidophilum* (47). However, the role of Sox in sulfide oxidation should be revisited, as it should be confirmed whether *R. sulfidophilum* contains SQR and FCSD. Our data support the suggestion that the physiological substrate of the Sox system is thiosulfate in *C. pinatubonensis* JMP134 (Table 1). In the Δ*sqr* Δ*pdo1* Δ*pdo2* mutant (Fig. 3) and the Δ*sorA* mutant (Fig. S2), the Sox system oxidizes S^0 and sulfite at significantly reduced rates, suggesting that the Sox system can oxidize S^0 and sulfite but that the efficiency is much lower than that for PDO and SOR in *C. pinatubonensis* JMP134.

The Sox system oxidized the sulfane sulfur generated by FCSD, but not by SQR or added polysulfide (Table 1). Since both FCSD and the Sox system are in the periplasmic space, it is possible that FccB directly transfers sulfane sulfur to SoxY, producing SoxYZ-SSH, which can be further oxidized by the Sox system to sulfate (Fig. 4B). When polysulfide is added to neutral solutions, it is rapidly converted to elemental sulfur in the form of S_8 (48); perhaps the reaction of S_8 with SoxYZ-SH is kinetically slow or needs additional reducing power. Since the active site of SQR in *C. pinatubonensis* JMP134 is in the cytoplasm (37), the membrane separation of SQR-produced sulfane sulfur and the Sox system may contribute to the uncoupling of SQR and the Sox system.

Our evidence does not support the suggestion that the bacterium uses the Sox system for the efficient oxidation of sulfide. First, the Δ*soxYZ* mutant oxidized sulfide at the same rate as the wild type (Table 1; Fig. 2). *A. vinosum* also does not use the Sox system for sulfide oxidation, as the deletion of the *sox* genes in *A. vinosum* does not affect its sulfide oxidation (29). Second, the Δ*sqr* Δ*fccB* mutant with the Sox system did not show any meaningful rate of sulfide oxidation (Table 1; Fig. 2), suggesting that *C. pinatubonensis* JMP134 does not use the Sox system for H_2S oxidation. Since *P. pantotrophus* contains SQR (GenBank accession number RKS43125.1) and FCSD (49), it should be investigated whether it uses the Sox system to oxidize sulfide.

TABLE 3 Strains and plasmids used in this study

Strain or plasmid	Characteristic or target protein	Source
Strains		
E. coli S17-1	*recA pro thi hsdS*, RP4 *tra* functions, *supE44*	Invitrogen
C. pinatubonensis JMP134	Wild type	Our labs
Plasmids	Characteristics	Source
pBBR1MCS2	Kanamycin resistance, mob$^+$, pBBR1 replicon, cloning vector	Qi Qingsheng
pK18mobsacB	Widely used gene-knockout vector, kanamycin resistance	Our labs
pBBR-*soxYZ*	pBBR1MCS2 containing *soxY* and *soxZ*	This study
pBBR-*pdo2-sqr*	pBBR1MCS2 containing *pdo2* and *sqr*	This study
pBBR-*fccB*	pBBR1MCS2 containing *fccB*	This study
pBBR-*pdo2*	pBBR1MCS2 containing *pdo2*	This study
pBBR-*sorA*	pBBR1MCS2 containing *sorA*	This study
Mutants of JMP134		
Δ*sqr*	*sqr* deleted	This study
Δ*fccB*	*fccB* deleted	This study
Δ*sqr* Δ*pdo1* Δ*pdo2*	*sqr*, *pdo2*, and *pdo1* deleted	This study
Δ*sqr* Δ*pdo1* Δ*pdo2* Δ*soxYZ*	*sqr*, *pdo2*, *pdo1*, *soxY*, and *soxZ* deleted	This study
Δ*sqr* Δ*fccB*	*sqr* and *fccB* deleted	This study
Δ*sqr* Δ*fccB* Δ*soxYZ*	*sqr*, *fccB*, *soxY*, and *soxZ* deleted	This study
Δ*soxYZ*	*soxY* and *soxZ* deleted	This study
Δ*pdo1*	*pdo1* deleted	This study
Δ*pdo2*	*pdo2* deleted	This study
Δ*pdo1* Δ*pdo2*	*pdo1* and *pdo2* deleted	This study
Δ*pdo1* Δ*pdo2* Δ*soxYZ*	*pdo1*, *pdo2*, *soxY*, and *soxZ* deleted	This study
Δ*sorA*	*sorA* deleted	This study
Δ*tauE*	*tauE* deleted	This study
Complemented strains		
Δ*fccB*/*fccB*	Δ*fccB* mutant with plasmid pBBR-*fccB*	This study
Δ*sqr* Δ*pdo1* Δ*pdo2*/*sqr pdo2*	Δ*sqr* Δ*pdo1* Δ*pdo2* mutant with plasmid pBBR-*pdo2-sqr*	This study
Δ*soxYZ*/*soxYZ*	Δ*soxYZ* mutant with plasmid pBBR-*soxYZ*	This study
Δ*pdo1* Δ*pdo2*/*pdo2*	Δ*pdo1* Δ*pdo2* mutant with plasmid pBBR-*pdo2*	This study
Δ*sorA*/*sorA*	Δ*sorA* mutant with plasmid pBBR-*sorA*	This study
Δ*sqr* Δ*pdo1* Δ*pdo2* Δ*soxYZ*/*soxYZ*	Δ*sqr* Δ*pdo1* Δ*pdo2* Δ*soxYZ* mutant with plasmid pBBR-*soxYZ*	This study

In *C. pinatubonensis* JMP134, SQR prefers to work with PDO, and the Sox system may oxidize the sulfane sulfur produced by FCSD. The preference may be related to the subcellular localization of the enzymes (Fig. 4). SQR is a membrane protein with its active site in the cytoplasm, and PDO is also in the cytoplasm (37), whereas FCSD and the Sox system are soluble proteins in the periplasmic space (16, 46, 50). The preference is also reflected in the linkages of these genes on the chromosome, with *sqr* and *pdo2* being organized in an operon on chromosome B and the *sox* genes and *fccAB* being adjacently located on chromosome A (Fig. 1).

The H_2S oxidation network of SQR-PDO, FCSD, and the Sox system of *C. pinatubonensis* JMP134 may also be present in other bacteria, including *P. pantotrophus* GB17, *Roseobacter denitrificans* OCh 114, and *Ruegeria pomeroyi* DSS-3, as they all contain these genes (Table S2). These bacteria may use the same pathway to oxidize H_2S. *C. pinatubonensis* JMP134 is a soil bacterium (12); *P. pantotrophus* GB17 is from a denitrifying, sulfide-oxidizing effluent treatment plant in the Netherlands (51); *R. denitrificans* OCh 114 and *R. pomeroyi* DSS-3 are the model bacteria of the *Roseobacter* clade, dominant in coastal seawater and surface sediments (52). These bacteria all belong to the *Proteobacteria* and are capable of heterotrophic growth. Their wide distribution in soil, wastewater treatment plants, and marine waters implies the potential role of these bacteria in H_2S oxidation in the natural environment.

MATERIALS AND METHODS

Strains, plasmids, and primers. The strains and plasmids used in this study are listed in Table 3. All the primers are listed in Table 4.

Culture conditions. *C. pinatubonensis* JMP134 and its mutants were grown at 30°C in Luria-Bertani (LB) medium containing 10 g of NaCl per liter or in a mineral medium (MM) (53). MM consisted of 0.58

TABLE 4 Primers used in this study

Target gene	Primer[a]	Sequence (5′–3′)[b]
Deletion		
soxY and *soxZ*	Up-f	CAGGAAACAGCTATGACATGATTACGAATTCCGTCATTTTCGGATATCGC
	Up-r	TGGTCGCTTCCCGCAGTACTTCTCTTCGTTTC
	Down-f	AGTACTGCGGGAAGCGACCATCGCCTGATT
	Down-r	TTCAGGATCCCCGGGTACCGAGCTCGAATTCGCTGTTCGAGATCCATGAC
	V-f	GATGGTAGGGTGGATTCTTGAGGC
	V-r	CTACGGGGCCTTCAAAGGTGTT
fccB	UP-f	CAGGAAACAGCTATGACATGATTACGAATTGTCAACGAACTGGAAATCACGTC
	UP-r	TAGCTCAGCACTTCAGAAAGTTGCGTCGTTGC
	Down-f	CTTTCTGAAGTGCTGAGCTAGCCGTTCCGCAT
	Down-r	TTCAGGATCCCCGGGTACCGAGCTCGAATTAACCGGCCCCACATGGTGTAG
	V-f	GATCAAGCCTCCACCTCGCAGAAC
	V-r	CGCAAAGCGTCAACAGAAACCCG
sqr	Up-f	CAGGAAACAGCTATGACATGATTACGAATTGACGGGGGCCTTGAACTTTTATC
	Up-r	GACCATGCAATTGCAGGGATAACACCCGAAG
	Down-f	ATCCCTGCAATTGCATGGTCGTCTGTTCCTTG
	Down-r	TTCAGGATCCCCGGGTACCGAGCTCGAATTAATCGCAACGCTCTGCTAACC
	V-f	GCACCGGTGCCTTTGTATTG
	V-r	TCCTGTACATGTGCCACGAC
pdo1	Up-f	CAGGAAACAGCTATGACATGATTACGAATTAGACGATTACCTGGTCTACACCTTC
	Up-r	CAGCTGTTCGTACAGGCGCGTCAAATCCTTCTAT
	Down-f	CGCGCCTGTACGAACAGCTGATAGAAGGTTTGCAT
	Down-r	TTCAGGATCCCCGGGTACCGAGCTCGAATTGGCTGATGATGGAGAACGAAC
	V-f	TATTGGCTGCCATCTGCT
	V-r	GCTCTACAAGCTCAATGCG
pdo2	Up-f	CAGGAAACAGCTATGACATGATTACGAATTCGAGGTCGTAGCGGTAGTTG
	Up-r	ACACACATGAGCTATCTGAAGATTCCCCTCAAC
	Down-f	TTCAGATAGCTCATGTGTGTCTATCCGTGGTTAGC
	Down-r	TTCAGGATCCCCGGGTACCGAGCTCGAATTCCATTTCATCGAGGAATAGCGT
	V-f	ATGGCGTCCCAATCCAGCTT
	V-r	TTGCCTGGAGAGTGGCTTTG
sorA	Up-f	CAGGAAACAGCTATGACATGATTACGAATTATCCGTCGGCAGGTAACAGC
	Up-r	CATATAGCCGATTGTCAGGCGCATTCATGG
	Down-f	GCCTGACAATCGGCTATATGCGCAACGTGG
	Down-r	TTCAGGATCCCCGGGTACCGAGCTCGAATTGCGCGTGGAGGAAGTGATCC
	V-f	TCTTTCGCTTGCGTCTGATG
	V-r	CAGCGGATCTTGCGATCTAC
tauE	Up-f	CAGGAAACAGCTATGACATGATTACGAATTGCTGTCGATGCAGGTCAAATT
	Up-r	ACGTTCAGCTCAATGTCTTGCATCTGTCATTCC
	Down-f	CAAGACATTGAGCTGAACGTCAGGGTGAAATC
	Down-r	TTCAGGATCCCCGGGTACCGAGCTCGAATTCACCTCTGCGAGTGTTTCATTAG
	V-f	TGATGGTCAGTCAAGGGCTGTT
	V-r	CTGTTGAAGGGACAGGGATAGG
Complementation		
soxY and *soxZ*	Forward	CACACAGGAAACAGCTATGAATTCGAAACGAAGAGAAG
	Reverse	TTCCATTCGCCATTCATCAGGCGATGGTCGCT
fccB	Forward	CACACAGGAAACAGCTATGCAACGACGCAACTTTCTGAAG
	Reverse	TAACAAAATATTAACGCTTAGCTCAGCATGTCGGC
sorA	Forward	CACACAGGAAACAGCTATGAATGCGCCTGACAATCCCC
	Reverse	TTCCATTCGCCATTCAACTGCGGTAACGCGCGTGGA
sqr and *pdo2*	Forward	CACACAGGAAACAGCTATGACACCGACCATGCCAAGC
	Reverse	TTCCATTCGCCATTCATTATCCCTGCAACTCGGGTGTCG
pdo2	Forward	CACACAGGAAACAGCTATGACACCGACCATGCCAAG
	Reverse	TTCCATTCGCCATTCATCAGAGGGCGTTGAGGGG
Linearization of pBBR1MCS2	Forward	TGAATGGCGAATGGAAATTGTAAG
	Reverse	AGCTGTTTCCTGTGTGAAATTGTTATC

[a]Up, primers used to clone the upstream sequence of the target gene; Down, primers used to clone the downstream sequence of the target gene; V, primers used to verify the mutants.

[b]For the deletion mutants, underlining represents the sequences overlapping the plasmid pK18mobsacB sequence. For the complemented strains, underlining represents the sequences overlapping the plasmid pBBR1MCS2 sequence.

g of K_2HPO_4, 0.19 g of KH_2PO_4, 0.25 g of $NaNO_3$, 0.5 g of $MgCl_2$, and 1 ml of a trace element solution per liter of deionized water, and its pH was adjusted to 7.0. The carbon source was 0.5% (wt/vol) monosodium glutamate. $NaSO_4$, Na_2SO_3, or $Na_2S_2O_3$ (1 mM) was used as the sole sulfur source. The trace element solution contained 10 ml of concentrated HCl, 4.74 mg of $ZnCl_2$, 2.53 mg of $MnCl_2 \cdot 4H_2O$, 30 mg of H_3BO_3, 20 mg of $CoCl_2 \cdot 6H_2O$, 1 mg of $CuCl_2 \cdot 2H_2O$, 2 mg of $NiCl_2 \cdot 6H_2O$, 3.24 mg of $Na_2MoO_4 \cdot 2H_2O$ per liter.

Sulfur compound preparation. NaHS, Na_2SO_3, or $Na_2S_2O_3$ was freshly prepared in 100 mM HEPES buffer (pH 7.4). The polysulfide was prepared according to a published method by mixing sulfur power and sodium sulfide in anoxic distilled water under argon gas (5).

Gene deletion and complementation. The method used to delete the sulfur-related genes in *C. pinatubonensis* JMP134 is essentially similar to that previously reported (3). The primers used in the deletion process are shown in Table 4. After the upstream and downstream fragments of the target gene were obtained by PCR, these two fragments were ligated with the linearized plasmid pK18mobsacB by a modified In-Fusion method (54) to construct a deletion plasmid, and the deletion plasmid was transformed into *E. coli* S17-1 and then transferred to *C. pinatubonensis* JMP134 by conjugation. After two rounds of screening by using colony PCR, the correct deletion strain was obtained.

The complementation strain was generated by transforming a recombinant plasmid into the corresponding mutant. The recombinant plasmid was constructed by assembling the PCR-amplified gene in the broad-host-range plasmid pBBR1MCS2 (linearized via PCR) by using a modified In-Fusion method (54). The primers used to amplify the target gene and linearized plasmid pBBR1MCS2 are listed in Table 4.

Whole-cell assay. A single colony of the wild type, deletion strains, or complementation strains was inoculated in LB medium (with antibiotics, as necessary) and incubated at 30°C overnight (200 rpm). The culture was transferred to 100 ml fresh medium (1% inoculation) and incubated at 30°C with shaking (200 rpm) to an OD_{600} of 1.0 to 1.5, and then 20 μM NaHS, 20 μM polysulfides, 100 μM Na_2SO_3, or 100 μM $Na_2S_2O_3$ was added for induction with continuous incubation to an OD_{600} of 2.5 (about 1 h). Cells were collected by centrifugation (4,000 $\times$ *g*, 5 min) and resuspended in HEPES buffer (pH 7.4, 100 mM) at an OD_{600} of 2.0. The heat-inactivated cells were prepared by incubating 10 ml of cell suspension in a boiling water bath for 15 min.

Ten milliliters of the suspension was transferred to a 50-ml large plastic centrifuge tube (Labcon, USA); NaHS, polysulfide, Na_2SO_3, or $Na_2S_2O_3$ was added; and the tube was covered with a silicone stopper. The sample was mixed and incubated with shaking (30°C, 100 rpm). One milliliter of sample was taken at 15 min, 30 min, 1 h, 2 h, 4 h, and 7 h, and the concentrations of the sulfur compounds were measured. Suspensions were used directly for the detection of sulfide and sulfane sulfur. When sulfite, thiosulfate, and sulfate were tested, the suspension was centrifuged (13,000 $\times$ *g*, 3 min) and the supernatant was taken for detection.

Sulfur compound detection. Sulfide was detected by using the methylene blue method, and sulfane sulfur (including polysulfide, persulfide, and elemental sulfur) was detected by using the cyanide method (5). The detection of sulfite, thiosulfate, and sulfate in the supernatant was carried out by using an ion chromatograph system (ICS; model ICS-1100; Dionex, USA) in the anion detection mode with an Ion Pac AS19 column, an eluent automatic generation device (RFC-30), a column temperature of 30°C, an ASRS_4 mm suppressor, and an eluent of 22 mM KOH with isocratic elution (1 ml/min KOH). Under these conditions, the elution times of sulfite, sulfate, and thiosulfate were 7.9 min, 8.5 min, and 27.5 min, respectively. This method had a sulfite detection limit of 10 μM.

Bioinformatics. The basic sulfur metabolic pathway was derived from the analysis of *C. pinatubonensis* JMP134 at the KEGG website (55). The query sequences were from *A. vinosum* DSM 180^T (locus tag, Alvin_0091; GenBank accession number ADC61061) and *Cupriavidus metallidurans* CH34 (locus tag, Rmet_5347; GenBank accession number ABF12206) for thiosulfate dehydrogenases, from *Acidithiobacillus ferrooxidans* (GenBank accession number AAB93983) for tetrathionate hydrolase, and from *Thiobacillus denitrificans* ATCC 25259 (GenBank accession number Q60028.3) for ribulose bisphosphate carboxylase. Similar proteins of each sulfur-related enzyme of *C. pinatubonensis* JMP134 were searched for in selected microbial genomes by using the TBLASTN program (56).

Data availability. Data and NCBI accession numbers related to sulfide oxidation by additional mutants and complementation strains and sulfur-metabolizing enzymes in *C. pinatubonensis* JMP134 and other bacteria are given in the supplemental material.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 1 MB.

ACKNOWLEDGMENTS

The work was financially supported by grants from the National Natural Science Foundation of China (31870097 and 91951202).

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