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Sulfide production and oxidation by heterotrophic bacteria under aerobic conditions

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Sulfide (H_2S, HS^- and S^{2-}) oxidation to sulfite and thiosulfate by heterotrophic bacteria, using sulfide: quinone oxidoreductase (SQR) and persulfide dioxygenase (PDO), has recently been reported as a possible detoxification mechanism for sulfide at high levels. Bioinformatic analysis revealed that the *sqr* and *pdo* genes were common in sequenced bacterial genomes, implying the sulfide oxidation may have other physiological functions. SQRs have previously been classified into six types. Here we grouped PDOs into three types and showed that some heterotrophic bacteria produced and released H_2S from organic sulfur into the headspace during aerobic growth, and others, for example, *Pseudomonas aeruginosa* PAO1, with *sqr* and *pdo* did not release H_2S. When the *sqr* and *pdo* genes were deleted, the mutants also released H_2S. Both sulfide-oxidizing and non-oxidizing heterotrophic bacteria were readily isolated from various environmental samples. The *sqr* and *pdo* genes were also common in the published marine metagenomic and metatranscriptomic data, indicating that the genes are present and expressed. Thus, heterotrophic bacteria actively produce and consume sulfide when growing on organic compounds under aerobic conditions. Given their abundance on Earth, their contribution to the sulfur cycle should not be overlooked.
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Introduction

Microorganisms are able to metabolize organic sulfur and release H_2S under aerobic conditions (Clarke, 1953). Cysteine desulfhydrase and 3-mercaptopyruvate sulfurtransferase are two key enzymes involved in releasing H_2S from cysteine catabolism (Morra and Dick, 1991; Oguri *et al.*, 2012). Cystathionine β-synthase and cystathionine γ-lyase, involved in converting methionine to cysteine, catalyze side reactions that release H_2S (Shatalin *et al.*, 2011). The self-produced sulfide is known to function as a signaling molecule in mammals (Wagner, 2009) and to act as a general defense against oxidative stress to confer bacteria with resistance to antibiotics (Shatalin *et al.*, 2011; Oguri *et al.*, 2012). However, the prevalence of sulfide production by heterotrophic bacteria during aerobic growth has not been documented.

The produced sulfide may accumulate and inhibit aerobic respiration (Sohn *et al.*, 2000). Mammalian cells prevent the accumulation of sulfide through oxidation by the concerted actions of three enzymes: sulfide:quinone oxidoreductase (SQR), persulfide dioxygenase (PDO), and rhodanese that is a type of sulfurtransferases (Hildebrandt and Grieshaber, 2008). Recently, we have reported that heterotrophic bacteria also possess SQR, PDO and rhodanese, oxidizing sulfide to sulfite and thiosulfate (Liu *et al.*, 2014; Xin *et al.*, 2016). Gram-negative bacteria contain at least two types of PDOs: the type I PDOs include the human PDO (ETHE1), plant PDOs, and bacterial homologs, and the type II PDOs consist of several reported proteins from Proteobacteria (Liu *et al.*, 2014; Xin *et al.*, 2016). Although both types of PDOs use glutathione persulfide (GSSH) as the substrate, they share limited sequence similarity and the substrate binding sites are different between them (Sattler *et al.*, 2015). Further, a PDO (CstB) from the Gram-positive bacterium *Staphylococcus aureus* uses low-molecular-weight persulfides (RSSH and RSS^-), rather than GSSH, as the substrates (Shen *et al.*, 2015). Bacterial SQRs are grouped into six types, I to VI (Marcia *et al.*, 2010). Some PDO-containing heterotrophic bacteria harbor *sqr* and *pdo* in a gene cluster (Liu *et al.*, 2014; Shen *et al.*, 2015; Xin *et al.*, 2016). Rhodaneses are universally present in microorganisms, plants, and animals, and a rhodanese domain is often fused to SQR or PDO in heterotrophic bacteria (Guimaraes *et al.*, 2011;

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Liu *et al.*, 2014; Shen *et al.*, 2015; Xin *et al.*, 2016). In Gram-negative bacteria, SQR oxidizes sulfide to polysulfide, which spontaneously reacts with GSH to produce GSSH, and PDO oxidizes GSSH to sulfite; sulfite spontaneously reacts with polysulfide to produce thiosulfate. Rhodanese speeds up the reaction of polysulfide with GSH to produce GSSH, but the reaction can occur spontaneously (Xin *et al.*, 2016).

We report here that most bacteria produced sulfide from rich media during aerobic growth and bacteria with SQR and PDO oxidized the self-produced sulfide. Further, the oxidizers also oxidized sulfide produced by bacteria without the enzymes in mixed cultures. Some bacteria contained only SQR or PDO, and they were able to cooperate to oxidize sulfide to thiosulfate. Bioinformatics analysis showed that *sqr* and *pdo* genes are common in sequenced bacterial genomes from GenBank, marine metagenomes and metatranscriptomes.

Materials and methods

Bacterial strains and culture conditions
The bacterial strains and plasmids used in this study are listed in Supplementary Table 1. Lysogeny broth (LB) was used for culturing most bacteria. *Zymomonas mobilis* ATCC 31821 and *Gluconobacter oxydans* 621H were grown in the Rich Medium (Goodman *et al.*, 1982) and D-sorbitol medium (Yang *et al.*, 2008), respectively. The sulfur contents of yeast extract and tryptone (Oxoid, Thermo, Beijing, China) were measured by using an elemental analyzer (The Vario EL Cube, Elementar Trading, Shanghai, China).

Cloning and gene knockout
The *pdo* and *sqr* genes were PCR-amplified from genomic DNA with primers (Supplementary Table 2) and cloned into pBBR1MCS2 or pBBR1MCS5 for expression (Kovach *et al.*, 1995). The vector pK18mobsacB$_{tet}$ was used to generate deletions in *Cupriavidus pinatubonensis* JMP134 and *Pseudomonas aeruginosa* PAO1 (Harighi, 2009). Details are given in Supplementary Methods.

Testing bacteria for H_2S production
Selected bacteria were used to test for H_2S production. Bacteria were transferred into 2 ml of LB or the specified media in a 15-ml glass tube, a paper strip with lead(II)-acetate was affixed at the top of the tube with a rubber stopper. The culture was incubated with shaking. After incubation, the paper strip was photographed to detect any lead(II)-sulfide black precipitates, as a measure of H_2S production. LB spiked with known concentrations of NaHS was used to generate dark stains on lead(II)-acetate paper strips for the estimation of H_2S production from bacteria cultures. The estimation was done by visually matching the darkness of the paper strips.

The sulfide spiking test with selected strains
Resting cells of *C. pinatubonensis* JMP134, *C. pinatubonensis* 2 K (the *pdo2* and *sqr* deletion mutant), *P. aeruginosa* PAO1, *P. aeruginosa* 3 K (the *pdo*, *sqr1* and *sqr2* deletion mutant), *E. coli* BL21 (DE3), *E. coli* BL21(DE3)(Pa*pdo*-Pa*sqr*2), *Bacillus subtilis* 168, *Agrobacterium tumefaciens* C58, *Klebsiella pneumonia* DSM30104, *Serratia fonticola* DSM4576 and *Corynebacterium vitaeruminis* DSM20294 were tested to oxidize spiked sulfide. The selected bacteria were cultured in LB at 30 °C with shaking and harvested at OD_{600nm} of about 1. If the bacteria were induced with isopropyl-β-D-thiogalactopyranoside (IPTG) or sulfide, they were first cultured to OD_{600nm} of about 0.5. *E. coli* was induced with 0.4 mM IPTG; *Serratia fonticola* DSM4576, *C. vitaeruminis* DSM20294, and *Sinorhizobium meliloti* 1021 were induced with 200 μM NaHS for 2 h before harvesting. The harvested cells were washed with 50 mM Tris buffer (pH 8.0) and suspended in the Tris buffer with 50 μM diethylenetriaminepentaacetic acid (DTPA) at OD_{600nm} of 1. DTPA was added to minimize sulfide oxidation catalyzed by trace transition metals in the buffer (Shen *et al.*, 2012). Then, 100 μM NaHS was added into the cell suspension to initiate sulfide oxidation, and sulfide was determined at various time intervals. Three replicates were done at the same time. Sulfide was analyzed by a colorimetric method (Kamyshny, 2009).

Sulfide oxidation by combined resting cells containing sqr or pdo
The resting were induced and prepared as above. In combined tests, *C. vitaeruminis* DSM20294 and *S. meliloti* 1021 were equally mixed, and *E. coli* BL21 (DE3)(Cp*sqr*) and *E. coli* BL21 (DE3)(Cp*pdo*2) were equally mixed at individual OD_{600nm} of 2 and combined OD_{600nm} of 4. NaHS (1 mM) was added into the cell suspension to initiate sulfide oxidation; sulfide, polysulfide, sulfite and thiosulfate were determined at various time points as we previously reported (Xin *et al.*, 2016).

Determine the end products of sulfide oxidation by resting cells
E. coli BL21(DE3) and its recombinant cells were suspended in 100 mM Tris buffer (pH 8) at OD_{600nm} of 2. One mililiter of the cell suspension was transferred into a 15-ml glass tube. Freshly prepared NaHS (500 μM) was added to initiate the reaction. The tube was capped with a rubber stopper and incubated at 25 °C without shaking to minimize autoxidation and volatilization. The sulfide, sulfane sulfur that includes polysulfide and persulfide, sulfite and thiosulfate were analyzed at various time intervals. For details, see Supplementary Methods.

Heterotrophic sulfide oxidizers from various environmental samples
Samples were collected in the summer of 2015. Soil samples were from a wheat field and forest, and freshwater samples were from a lake, around Jinan, Shandong, China; near-shore seawater samples were collected from Qingdao, Shandong, China. To collect soil samples, the top layer of 2 centimeter (cm) was removed and soil from 2–10 cm depth was transferred into a sterile bottle. Water samples were directly collected into sterile bottles. All samples were immediately transported back to lab and processed in the same day.

Two grams of soil were added to a 50-ml centrifugation tube containing 20 ml of 10 mM phosphate buffered saline solution and three glass beads (5 mm in diameter). The samples were vigorously vortexed to disperse bacterial colonies and left on a bench for 2 min. Five ml of the soil leachate, freshwater, or seawater was centrifuged at 8000 × *g* for 10 min to precipitate bacteria and the pellets were re-suspended in 2 ml of LB and transferred into a 15-ml tube, incubated with shaking at 30 °C for 24 h. H_2S was detected with the paper strip containing Lead(II)-acetate in the gas phase. Simultaneously, the soil leachates and water samples were diluted and spread onto LB plates, incubated at 25 °C for 48 h. A circle with twenty colonies on a LB plate was randomly drawn, and the colonies were individually transferred into 2 ml of LB in a 15-ml tube to test for H_2S production. The isolated pure bacterial cultures of sulfide-oxidizing and non-oxidizing bacteria were also mixed to various ratios according OD_{600nm} in a fixed volume of 200 microliter (μl) and then transferred into 2 ml of LB medium to test for H_2S production with the paper strip method. Seawater samples and bacteria isolated from seawater samples were incubated in LB containing 2% of NaCl and 10 mM $MgSO_4$.

Bioinformatic analysis
The genes coding for PDOs and SQRs were first identified from the sequenced bacterial and archaeal genomes of GenBank by using BLAST, and only the *sqr* and *pdo* genes that are located next to each other in a gene cluster were selected to construct phylogenetic trees and establish SQR or PDO groups. These selected SQRs and PDOs were then used as seeds to find SQR and PDO homologs, whose genes are not located next to each other, from the sequenced bacterial and archaeal genomes, and the identified homologs were further confirmed if they were mapped into the established SQR or PDO groups in the phylogenetic trees. Similar strategies were used to identify the genes from marine metagenomes of the Global Ocean Sampling (GOS) Expedition and marine metatranscriptomic data sets (Supplementary Methods).

Results

The identification and distribution of pdo and sqr genes in sequenced bacterial and archaeal genomes
The reports that *sqr* and *pdo* are often present in a gene cluster (Liu *et al.*, 2014; Shen *et al.*, 2015; Xin *et al.*, 2016) prompted us to search for adjacent *pdo* and *sqr* genes in the 4929 genomes from GenBank (updated until 15 April 2016). 454 *pdo* and 455 *sqr* genes were adjacently located in 441 genomes (Supplementary Tables 3–4). After the reconstruction of phylogenetic tree with known SQRs and PDOs, our data supported the topology of six types of SQRs (Marcia *et al.*, 2010; Supplementary Figure 1) and yielded three types of PDOs (Figure 1). The types I and II PDOs are well characterized and present only in Gram-negative bacteria (Liu *et al.*, 2014; Sattler *et al.*, 2015; Xin *et al.*, 2016). The type III PDOs are present in both Gram-negative an positive bacteria, of which only the PDO from *Staphylococcus aureus* ATCC 6538 P has been characterized to use small organic persulfides, rather than GSSH, as substrates (Shen *et al.*, 2015). As the type III PDOs were diverse and shared about 30% sequence identity between the PDOs from Gram-negative bacteria and Gram-positive bacteria, we tested their activity. When the type III PDOs of *Bacillus cereus* ATCC10876, *S. aureus* ATCC 6538 P, and the Gram-negative bacterium *Zunongwangia profunda* SM-A87 were cloned together with Pa*sqr*2, the recombinant *E. coli* oxidized sulfide to thiosulfate (Figures 2a–c). *E. coli* with only SQR oxidized sulfide to sulfane sulfur, for example, polysulfide and persulfide (Figure 2d). Sulfide oxidation by *E. coli* with the vector or the cloned PDOs was slow with no apparent production of sulfane sulfur or thiosulfate (Figure 2d). The results demonstrate that the type III PDOs are functional in the *E. coli* host.

The PDOs and SQRs encoded by adjacent genes were further used to search for potential PDOs and SQRs from the 4929 bacterial and 242 archaeal genomes. The potential PDOs and SQRs were then checked via phylogenetic analysis, and 1908 PDOs and 1310 SQRs were stably conserved on the phylogenetic trees (Supplementary Figures 2 and 3). The type II PDOs were the most abundant but limited to Proteobacteria; the type I PDOs had a wider distribution in Gram-negative bacteria; the type III PDOs had the widest distribution present in both Gram-positive and -negative bacteria (Table 1). SQRs were widely distributed among bacterial phyla, and the type II proteins were the most abundant (Table 1). Of the sequenced bacterial genomes, 646 genomes had only *pdo*, 208 genomes had only *sqr*, and 806 possessed both. The genomes carrying both *sqr* and *pdo* mainly distributed in the phyla of Proteobacteria, Cyanobacteria and Firmicutes (Supplementary Figure 4; Table 1). 733 of the 806 genomes with both *sqr* and *pdo* belonged to the genera of known aerobic heterotrophs. 122 of 208 genomes with only *sqr* belonged to the genera of known aerobic heterotrophs.

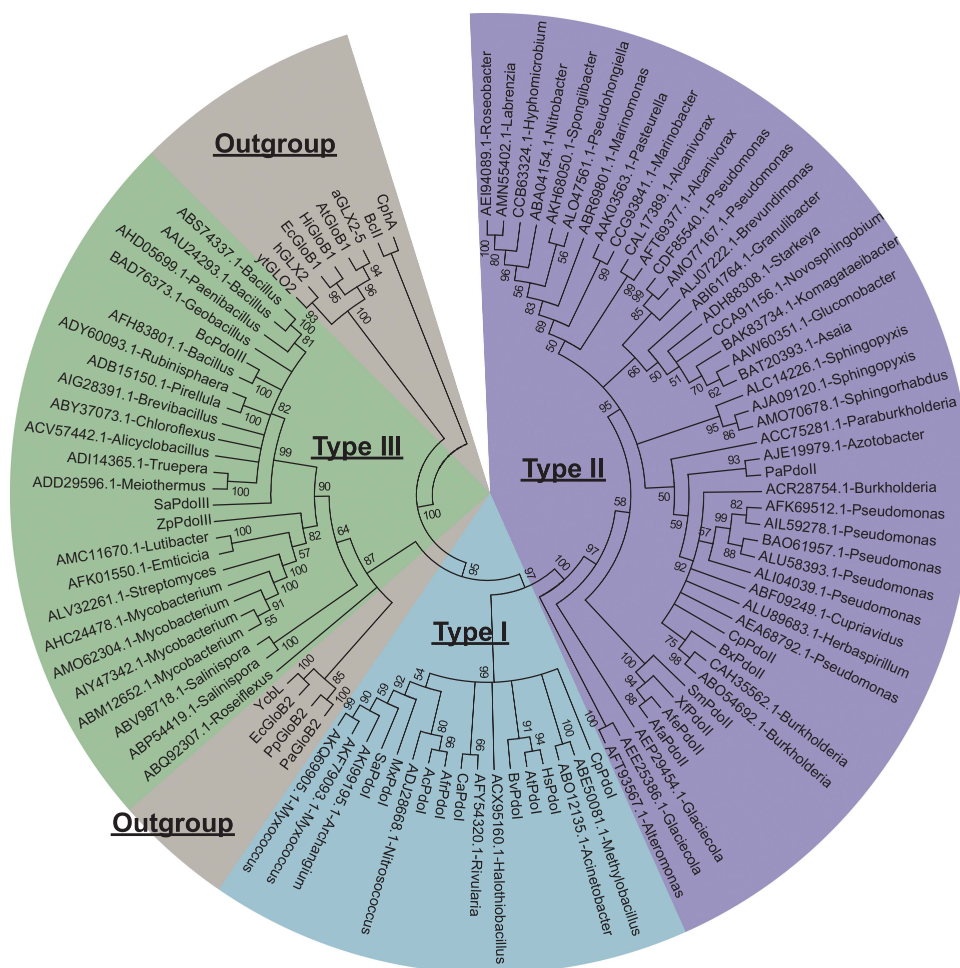

Figure 1 The phylogenetic tree of representative PDOs whose genes are physically linked to *sqr* in bacterial genomes. 69 representative PDOs were used for phylogenetic tree construction with reference sequences. PDOs belong to the metallo-beta-lactamase superfamily, and several related proteins, such as glyoxalase II (GloB) proteins, were also included as references. The representative proteins were labeled with their GenBank accession numbers and bacterial genera. These sequences were aligned by using ClustalW, and the tree was built by using MEGA6. Reference proteins with accession number were given below. Type I PDOs: SaPdoI (YP_003957083.1), CpPdoI (YP_297536.1), MxPdoI (YP_633997.1), HsPdoI (NP_055112.2), CaPdoI (YP_007162862.1), BvPdoI (ZP_00420127.1), AtPdoI (NP_974018.3), AcPdoI (AEK59246.1). Type II PDOs: XfPdoII (NP_298058.1), SmPdoII (NP_435818.1), CpPdoII (YP_297791.1), PaPdoII (NP_251605.1), BxPdoII (YP_554628.1), AfaPdoII (AAK89929.1), AfePdoII (ZP_11421028.1), AfrPdoII (YP_002424776.1). Type III PDOs: ZpPdoIII (ADF52140.1), SaPdoIII (WP_000465474.1), BcPdoIII (EEK49737.1). Glyoxalase II and related proteins: aGLX2-5 (NP_850166.1), AtGloB1 (NP_356997.2), BcII (AAA22276.1), CphA (CAA40386.1), EcGloB1 (NP_414748.1), EcGloB2 (NP_415447.1), hGLX2 (CAA62483.1), HiGloB1 (ADO96205.1), PaGloB2 (NP_249523.1), PpGloB2 (ABQ76961.1), YcbL (CAD05397.1) and ytGLO2 (CAA71335.1).

From the 242 archaeal genomes, 86 SQRs and 10 PDOs were identified. 10 Crenarchaeota genomes contained *pdo* and *sqr*, and 51 genomes of Crenarchaeota and Euryarchaeota had only *sqr*. Of the SQRs, 59 belonged to the type III, and the rest belonged to the type V. The 10 PDOs were all of the type III.

Heterotrophic bacteria with sqr and pdo oxidized self-produced and exogenous H_2S

A total of 24 heterotrophic bacteria with sequenced genomes were tested for releasing H_2S during growth in LB or the recommended rich medium for the bacterium. The sulfur contents of the yeast extract and tryptone were determined as 0.43 and 0.85%, and the calculated sulfur in LB was 3.3 mM. Nine bacteria with *sqr* and *pdo* did not release H_2S (Table 2), but the strains without *sqr* or *pdo* did. Of the 15 H_2S producers, *S. fonticola* DSM4576 and *C. vitaeruminis* DSM20294 possess only *sqr*; *A. tumefaciens* C58, *S. meliloti* 1021, and *Streptomyces griseus* NBRC13350 contain only *pdo* (Guimaraes *et al.*, 2011; Liu *et al.*, 2014); others do not have the genes.

The role of *sqr* and *pdo* was further tested with recombinants and mutants. During aerobic growth in LB, *E. coli* BL21(DE3) produced and released 50–100 μM H_2S into the headspace; however, *E. coli* BL21(DE3) with cloned *pdo-sqr* did not release H_2S (Figure 3a). *P. aeruginosa* PAO1 and *C. pinatubonensis* JMP134 (Supplementary Figure 5) did not release H_2S (Figures 3b and c), but the *P. aeruginosa* triple mutant Δ*sqr*1Δ*sqr*2Δ*pdo* (Pa3K) released 25–50 μM H_2S, and the *C. pinatubonensis* double mutant

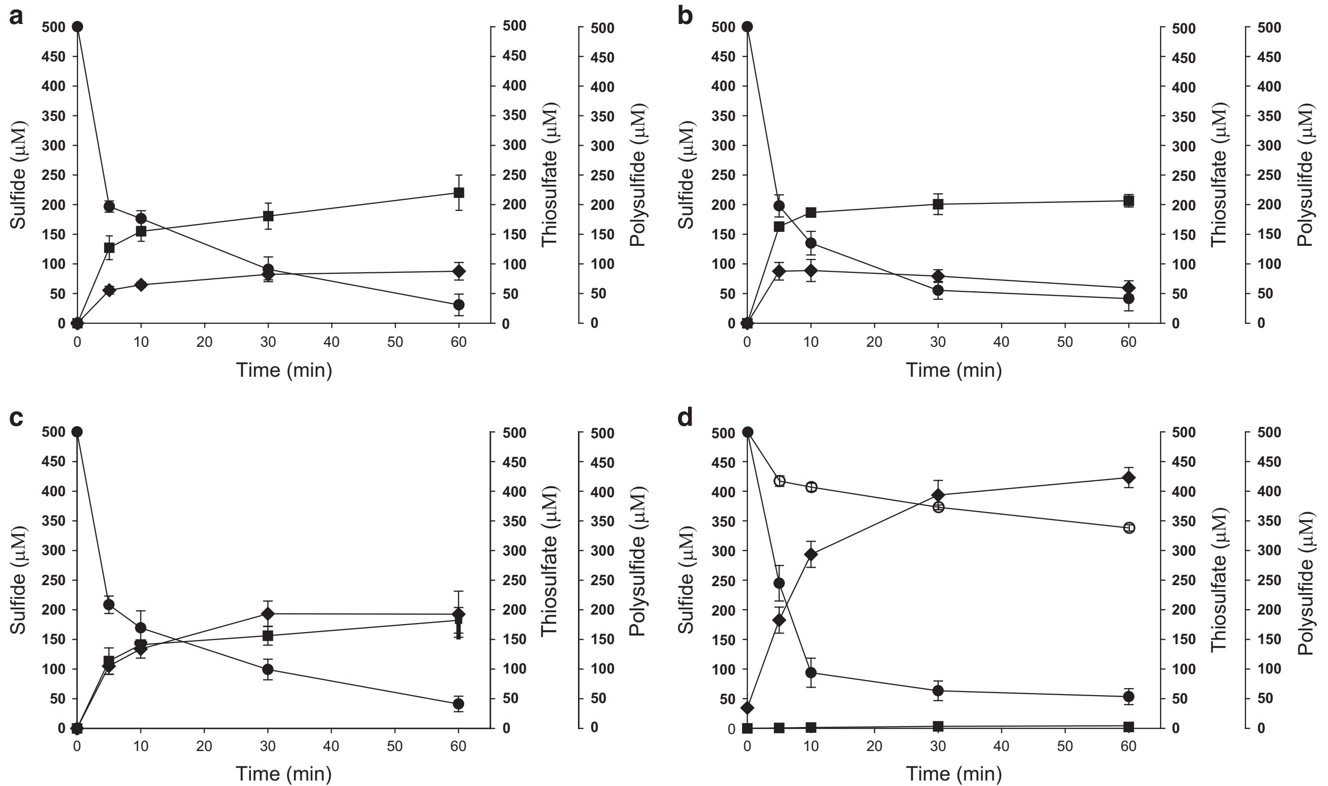

Figure 2 Sulfide oxidation by recombinant *E. coli* BL21(DE3) cells. Cells were suspended in 100 mM Tris buffer (pH 8) with 50 μM DTPA at OD_{600nm} of 2. Sulfide (500 μM) was added to initiate the reaction. (**a**) *E. coli* BL21(DE3) (Ec) with cloned Zp*pdo*-Pa*sqr2*, Ec(Zp*pdo*-Pa*sqr2*); (**b**) Ec(Bc*pdo*-Pa*sqr2*); (**c**) Ec(Sa*pdo*-Pa*sqr2*); (**d**) Ec(Pa*sqr2*) and Ec(pMCS5). Sulfide, ●; thiosulfate, ■; and sulfane sulfur, ♦. In Figure 2d, control Ec(pMCS5): sulfide, ○; thiosulfate, □; and sulfane sulfur, ◇. Sulfide oxidation, polysulfide production and thiosulfate production by *E. coli* with cloned Zp*pdo*, Bc*pdo* or Sa*pdo* were essentially the same as Ec(pMCS5) (Data not shown).

Table 1 The classification and distribution of SQRs and PDOs from sequenced bacterial genomes at the phylum level

Phylum	*SQR types*						*PDO types*			*Total genomes at phylum levels*
	Type I	*Type II*	*Type III*	*Type IV*	*Type V*	*Type VI*	*Type I*	*Type II*	*Type III*	
Acidobacteria	0	0	0	0	0	0	0	0	1	8
Actinobacteria	1	61	11	13	0	0	0	0	210	531
Aquificae	10	0	5	3	0	10	0	0	0	16
Armatimonadetes	0	0	0	0	0	0	0	0	1	1
Bacteroidetes	1	22	2	5	0	1	0	0	194	185
Candidate division SR1	0	0	0	0	0	0	0	0	1	2
Chlorobi	0	0	9	13	2	4	0	0	0	11
Chloroflexi	0	5	5	0	0	0	0	0	8	25
Cyanobacteria	15	24	0	0	0	0	58	0	0	91
Deinococcus-Thermus	1	4	1	0	0	0	0	0	19	24
Firmicutes	2	158	10	2	6	0	1	0	246	1086
Gemmatimonadetes	0	0	0	0	0	0	0	0	2	2
Ignavibacteriae	0	0	1	1	1	0	0	0	0	2
Nitrospirae	7	0	4	0	0	3	0	0	2	8
Planctomycetes	0	2	0	0	0	0	0	0	4	6
Proteobacteria	130	532	143	15	8	53	272	852	34	2504
Spirochaetes	2	0	1	0	0	0	2	0	0	65
Thermobaculum	0	0	0	0	0	0	0	0	1	1
Verrucomicrobia	0	0	1	0	0	0	0	0	0	9

CpΔ*pdo*2Δ*sqr* (Cp2K) released about 10 μM H_2S. However, the complementation strains Pa3K (Pa*pdo*-P*sqr*2) or Cp2K (Cp*pdo*2-Cp*sqr*) did not release H_2S (Figures 3b and c). Further, the resting cells of *P. aeruginosa* PAO1 and *C. pinatubonensis* JMP134 oxidized spiked sulfide, but Pa3K and Cp2K did not (Figures 4a and b). None of the tested H_2S-releasing bacteria was able to oxidize spiked sulfide

Table 2 H_2S production by bacteria in rich media

Strains	*sqr*	*pdo*	*Adjacently linked*[a]	*H_2S production*
Agrobacterium tumefaciens C58	0	1	NA	+
Bacillus subtilis 168	0	0	NA	+
Corynebacterium glutamicum RES167	0	1	NA	+
Corynebacterium vitaeruminis DSM20294	2	0	NA	+
Enterobacter cloacae ATCC 13047	0	0	NA	+
Enterococcus faecalis ATCC29200	0	0	NA	+
Escherichia coli BL21(DE3)	0	0	NA	+
Klebsiella pneumonia DSM30104	0	0	NA	+
Serratia fonticola DSM4576	1	0	NA	+
Sinorhizobium meliloti 1021	0	2	NA	+
Sphingobium chlorophenolicum L-1	0	0	NA	+
Staphylococcus sciuri Z8	0	0	NA	+
Streptomyces coelicolor M145	0	0	NA	+
Streptomyces griseus NBRC13350	0	1	NA	+
Zymomonas mobilis ATCC 31821	0	0	NA	+
Burkholderia cepacia ATCC 25416	1	2	+	−
Cupriavidus pinatubonensis JMP134	1	2	+	−
Gluconobacter oxydans 621H	2	1	+	−
Pseudomonas aeruginosa PAO1	2	1	−	−
Pseudomonas putida ML2	1	1	+	−
Serratia marcescens ATCC13880	2	1	−	−
Bacillus cereus ATCC10876	1	1	+	−
Staphylococcus aureus ATCC6538P	1	1	+	−
Zunongwangia profunda SM-A87	1	4	+	−

Abbreviation: NA, not applicable.
The accession numbers of PDOs and SQRs are given in Supplementary Table 5.
[a]The *sqr* and *pdo* genes are adjacently located on the chromosome.

(Figure 4c), and the slow decrease of sulfide is likely due to volatilization (Xin *et al.*, 2016) and non-specific enzymatic reactions (Luther *et al.*, 2011a). *C. vitaeruminis* DSM20294 with only *sqr* oxidized sulfide after induction with sulfide (Supplementary Figure 6A), but *S. fonticola* DSM4576 carrying a *sqr* gene did not metabolize spiked sulfide even after induction with sulfide (Supplementary Figure 6B).

Rapid sulfide oxidation was observed in samples containing sulfide-induced *C. vitaeruminis* DSM20294 either alone or mixed with *S. meliloti* 1021 (Figure 5a). *C. vitaeruminis* DSM20294 with only *sqr* oxidized sulfide mainly to polysulfide. *S. meliloti* 1021 with only *pdo* did not oxidize sulfide to polysulfide, sulfite or thiosulfate. When combined, the two bacteria oxidized sulfide to polysulfide and then to thiosulfate (Figure 5a). The results suggested that in mixed cultures, cells with *sqr* and cells with *pdo* can collectively oxidize sulfide to polysulfide and then to sulfite, which reacts with polysulfide to produce thiosulfate (Xin *et al.*, 2016). The collaboration of *E. coli* BL21(DE3) expressing either *sqr* or *pdo* for sulfide oxidation to thiosulfate was also confirmed (Figure 5b). The results indicate that polysulfide or persulfide can be transferred from one cell to another during sulfide oxidation by the combined cells.

Both sulfide oxidizers and non-oxidizers were isolated from environmental samples

Various soil and water samples were collected and inoculated in LB; mixed cultures grew rapidly, but none released any detectable H_2S (Supplementary Figure 7). The samples were also diluted and spread on LB agar plates. Without exception, both H_2S-releasing and H_2S non-releasing bacteria were isolated from these samples (Supplementary Table 6). When the H_2S-releasing bacteria and H_2S-non-releasing bacteria were mixed at different ratios and incubated in LB, H_2S was not accumulated in the cultures of the farm, forest and lake isolates (Supplementary Figure 7); the mixed marine isolates released some H_2S, but much lower than the control with only the marine H_2S-releasing bacterium (Supplementary Figure 7). Thus, the sulfide oxidizers did not release H_2S, while the non-oxidizers released H_2S in rich medium. The selected bacteria were identified to their closest relatives according to 16 S rRNA gene sequences (GenBank: KT443871-KT443878). Sequence search of the genomes of these close relatives revealed that the sulfide oxidizers were closely related to bacteria containing both *sqr* and *pdo* genes and the non-oxidizers were related to bacteria without the genes (Supplementary Figure 7 legend).

The abundance and diversity of PDOs and SQRs in the metagenomes from the GOS Expedition

175 PDOs and 82 SQRs were identified from 124 genomes of the 177 marine bacterial genomes of the Gordon and Betty Moore Foundation Marine Microbial Genome Sequencing project (http://www.jcvi.org/cms/research/past-projects/microgenome/overview/) (Supplementary Figure 8). SQRs and PDOs

are common in the Roseobacter clade, but rare in the SAR11 clade (Supplementary Table 7). The marine bacterial PDOs and SQRs were used to search the metagenomic database of the GOS Expedition (Rusch *et al.*, 2007). 1895 PDOs and 439 SQRs were found in the GOS data set. The type I PDOs and type II SQRs were dominant (Supplementary Table 8). The average percentages of bacterial genomes with *sqr* and *pdo* were 4 and 34.9%, respectively, in the GOS data set (Supplementary Table 9). The percentages of genomes with SQR and PDO from each GOS sampling site ranged from 0% to 53.9% and 10.7% to 74.9%, respectively (Table 3). The numbers of *sqr* in hypersaline waters were significantly higher than that in other sites (Table 3). The *pdo* genes were detected at all sampling sites, and the *sqr* genes were detectable from 42 of the 58 sampling sites. Thus, the *sqr* genes were also commonly occurring in marine surface waters, but its abundance was lower than that of *pdo* (Table 3).

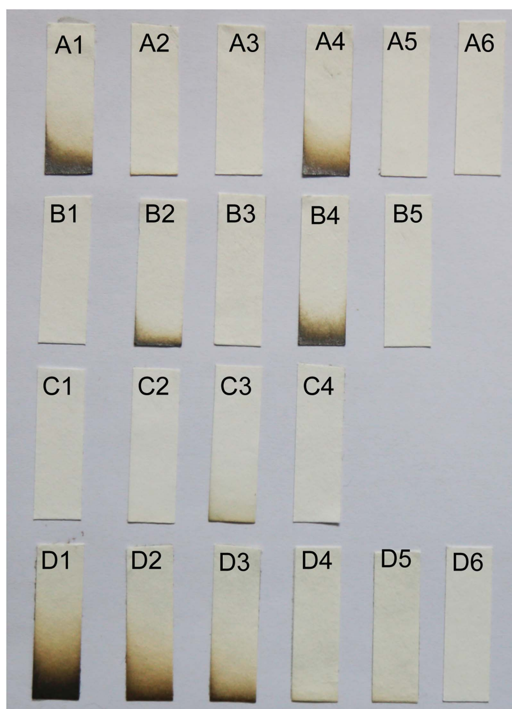

Figure 3 Testing H_2S Production by bacteria in LB. Bacteria were inoculated in 2 ml of LB in 15-ml tubes with the lead-acetate filter paper fixed at the top of the headspace. (Row A) A1, *E. coli* BL21 (DE3) (Ec); A2, Ec(Pa*sqr*1); A3, Ec(Pa*sqr*2); A4, Ec(Pa*pdo*); A5, Ec(Pa*pdo*-Pa*sqr*2); A6, Ec(Cp*pdo*2-Cp*sqr*). (Row B) B1, *P. aeruginosa* PAO1 (Pa); B2, PaΔ*sqr*1Δ*sqr*2; B3, PaΔ*pdo*; B4, PaΔ*sqr*1Δ*sqr*2Δ*pdo* (Pa3K), B5: Pa3K(Pa*pdo*-Pa*sqr*2). (Row C) C1, *C. pinatubonensis* JMP134 (Cp); C2, CpΔ*sqr*; C3, CpΔ*pdo*2Δ*sqr* (Cp2K); C4, Cp2K(Cp*pdo*2-Cp*sqr*). (Row D) NaHS standards in LB: D1, 100 μM; D2, 50 μM; D3, 25 μM; D4, 10 μM; D5, 5 μM; D6, 0 μM. *E. coli* and *P. aeruginosa* were incubated at 37 °C, and *C. pinatubonensis* was incubated at 30 °C for 18 h.

Identification of pdo and sqr transcripts in marine metatranscriptomic data
The transcripts of *sqr* and *pdo* were further detected in three metatranscriptomic data sets of marine samples, and the ratios of *pdo* and *sqr* transcripts over total transcripts were 7.6×10^{-6} and 5.5×10^{-7} from station ALOHA (NCBI accession: PRJNA244754), 2.2×10^{-5} and 4.5×10^{-6} from Monterey Bay (PRJNA183166), 5.1×10^{-6} and 6.8×10^{-7} from a coastal California water (PRJNA268385), respectively. Waters from different locations vary in chemical and physical characteristics, being nutrient-rich from the Monterey Bay and coastal waters and oligotrophic at station ALOHA in the North Pacific Gyre (Amin *et al.*, 2015). The *sqr* transcripts doubled in the samples taken at night, but the *pdo* transcripts were essentially the same (Supplementary Figure 9). The data suggest that bacterial *pdo* and *sqr* are present and expressed in these natural habitats.

Discussion

Sulfide production and benefits
During heterotrophic growth, microorganisms are likely released H_2S from sulfur-containing amino acids by using a variety of enzymes, including

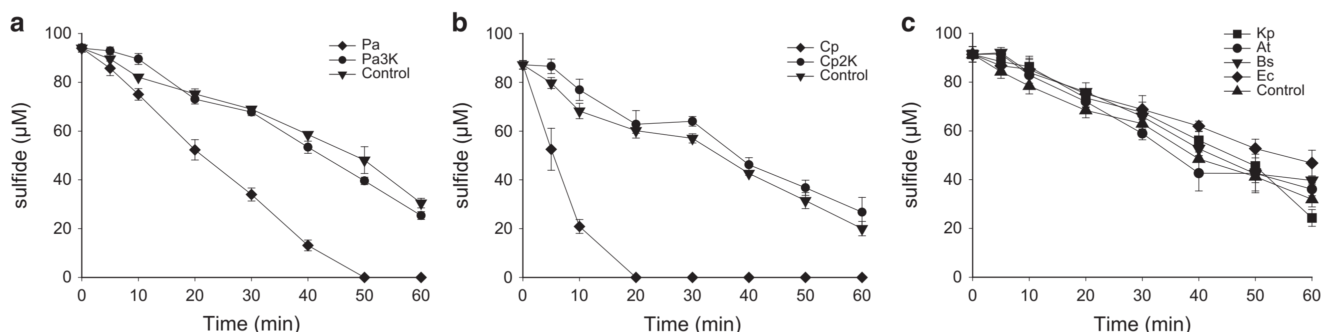

Figure 4 Resting bacterial cells oxidize spiked sulfide. Cells were suspended in 50 mM Tris buffer, pH 8.0, with 50 μM DTPA at OD_{600nm} of 1, and NaHS was added to initiate the reaction. Controls were done in the same buffer without bacterial cells. All data are average of three samples with standard deviation (error bar). (**a**) *C. pinatubonensis* JMP134 (Cp) and *C. pinatubonensis* 2 K (Cp2K); (**b**) *P. aeruginosa* PAO1 (Pa) and *P. aeruginosa* 3 K (Pa3K); (**c**) *B. subtilis* 168 (Bs), *A. tumefaciens* C58 (At), *K. pneumonia* DSM30104 (Kp) and *E. coli* BL21 (DE3) (Ec).

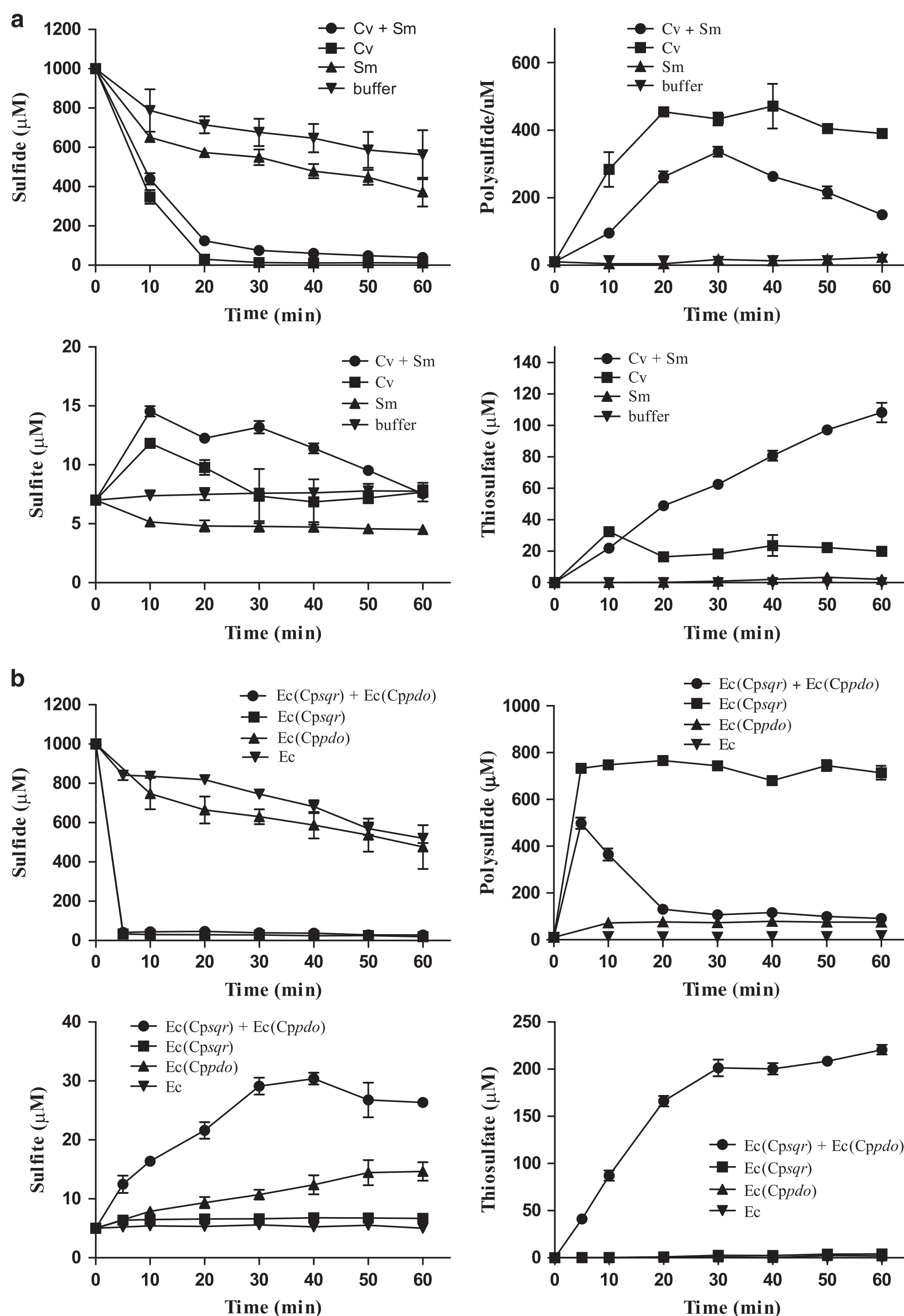

Figure 5 Sulfide oxidation by mixed bacterial cells with SQR or PDO. (**a**) Combination of *C. vitaeruminis* DSM20294 (Cv) (with *sqr*) and *S. meliloti* 1021 (Sm) (with *pdo*). (**b**) Combination of *E. coli* BL21(DE3) (Ec) with Cp*sqr* [Ec(Cpsqr)] or Cp*pdo*2 [(Ec(Cp*pdo*2)]. Cv and Sm were induced with sulfide before harvesting, and the recombinant *E. coli* cells were induced with IPTG before harvesting. Cells were suspended in 50 mM Tris buffer, pH 8.0, with 50 μM DTPA at OD_{600nm} of 2 for individual strains or 4 for mixed strains and 1 mM NaHS was added to initiate the reaction. Controls were done with individual bacterial strains. Sulfide oxidation and the production of polysulfide, sulfite and thiosulfate were detected. All data are average of three samples with standard deviation (error bar).

cysteine desulfhydrase, 3-mercaptopyruvate sulfurtransferase, cystathionine β-synthase and cystathionine γ-lyase (Morra and Dick, 1991; Shatalin *et al.*, 2011; Oguri *et al.*, 2012). In agreement with these well documented mechanisms, we have observed H_2S releasing from all tested bacteria without SQR and PDO as well as the mutants without SQR and PDO activities in rich media (Table 2). The sulfur in LB is from yeast extract and tryptone, and the determined sulfur contents are similar to related

Table 3 The distribution of *pdo* and *sqr* homologs in the GOS data set per sample sites

Site	*Site names*	*Types*	*% genomes with pdo*	*% genomes with sqr*
0	Sargasso Station	Open Ocean	35.2	7.7
1	Hydrostation S	Open Ocean	35.3	0.9
2	Gulf of Maine	Coastal	64.9	4.7
3	Browns Bank, Gulf of Maine	Coastal	74.9	2.4
4	Outside Halifax, Nova Scotia	Coastal	25.7	5.4
5	Bedford Basin, Nova Scotia	Embayment	32.7	20.5
6	Bay of Fundy, Nova Scotia	Estuary	52.2	4.2
7	Northern Gulf of Maine	Coastal	32.1	0.0
8	Newport Harbor, RI	Coastal	46.3	12.9
9	Block Island, NY	Coastal	53.8	0.0
10	Cape May, NJ	Coastal	59.4	6.0
11	Delaware Bay, NJ	Estuary	30.3	12.3
12	Chesapeake Bay, MD	Estuary	23.0	1.3
13	Off Nags Head, NC	Coastal	58.6	12.2
14	South of Charleston, SC	Coastal	42.0	6.5
15	Off Key West, FL	Coastal	31.9	0.0
16	Gulf of Mexico	Coastal Sea	38.3	1.1
17	Yucatan Channel	Open Ocean	34.8	2.6
18	Rosario Bank	Open Ocean	33.0	0.8
19	Northeast of Colon	Coastal	21.3	0.0
20	Lake Gatun	Fresh Water	18.9	5.3
21	Gulf of Panama	Coastal	51.7	0.0
22	250 miles from Panama City	Open Ocean	40.2	0.0
23	30 miles from Cocos Island	Open Ocean	25.9	0.9
25	Dirty Rock, Cocos Island	Fringing Reef	11.5	0.0
26	134 miles NE of Galapagos	Open Ocean	42.6	1.4
27	Devil's Crown, Floreana Island	Coastal	37.6	1.1
28	Coastal Floreana	Coastal	40.3	1.3
29	North James Bay, Santigo Island	Coastal	31.1	1.3
30	Warm seep, Roca Redonda	Warm Seep	26.4	1.6
31	Upwelling, Fernandina Island	Coastal upwelling	43.4	0.9
32	Mangrove on Isabella Island	Mangrove	36.7	3.8
33	Punta Cormorant, Hypersaline Lagoon, Floreana Island	Hypersaline	56.9	53.9
34	North Seamore Island	Coastal	26.7	1.1
35	Wolf Island	Coastal	49.5	1.1
36	Cabo Marshall, Isabella Island	Coastal	34.6	0.0
37	Equatorial Pacific TAO Buoy	Open Ocean	17.6	2.6
47	201 miles from F. Polynesia	Open Ocean	46.4	0.0
48	Moorea, Cooks Bay	Coral Reef	39.8	6.3
49	Moorea, Outside Cooks Bay	Coastal	29.2	0.0
51	Rangirora Atoll	Coral Reef Atoll	62.0	1.2
108	Coccos Keeling, Inside Lagoon	Lagoon Reef	39.8	14.5
109	Indian Ocean	Open Ocean	21.6	0.0
110	Indian Ocean	Open Ocean	23.4	0.6
111	Indian Ocean	Open Ocean	37.8	1.8
112	Indian Ocean	Open Ocean	19.9	0.6
113	Indian Ocean	Open Ocean	25.4	1.9
114	500 Miles west of the Seychelles in the Indian Ocean	Open Ocean	25.9	0.0
115	Indian Ocean	Open Ocean	20.1	0.0
116	Outside Seychelles, Indian Ocean	Open Ocean	37.7	0.0
117	St. Anne Island, Seychelles	Coastal	20.6	0.5
119	International Water Outside of Reunion Island	Open Ocean	24.8	0.0
120	Madagascar Waters	Open Ocean	30.3	4.4
121	International water between Madagascar and South Africa	Open Ocean	26.8	5.3
122	International waters between Madagascar and South Africa	Open Ocean	21.7	2.8
123	International water between Madagascar and South Africa	Open Ocean	33.2	3.0
148	East coast Zanzibar (Tanzania), offshore Paje lagoon	Fringing Reef	29.7	1.0
149	West coast Zanzibar (Tanzania), harbor region	Harbor	33.2	0.0
		Average	34.9	4.0

products, in which most sulfur is in cysteine and methionine (Sugata and Koch, 1926; Kassell and Brand, 1938; McManus *et al.*, 1950). The observation highlights the common production of sulfide from organic sulfur by heterotrophic bacteria. We suggest that aerobic sulfide production by heterotrophic bacteria can be significant in soils with high organic contents and in water bodies with eutrophication, during algal bloom, or on organic particulate matter.

The self-produced sulfide may benefit heterotrophic bacteria. It can protect bacteria from antibiotics through reacting and removing reactive oxygen species (Shatalin *et al.*, 2011). Sulfide may have special functions, as the human pathogen

Mycoplasma pneumoniae produces sulfide from cysteine for erythrocyte lysis (Großhennig *et al.*, 2016). Further, sulfide and its oxidation intermediate sulfane sulfur have been shown to function as signals by causing protein S-sulfhydration (Ida *et al.*, 2014; Miranda and Wink, 2014). The signaling role of H_2S in bacteria has been suggested (Lloyd, 2006), and it will likely join a growing number of volatile molecules produced by bacteria as signaling molecules for interactions among bacteria and with eukaryotic hosts or predators (Schmidt *et al.*, 2015; Schulz-Bohm *et al.*, 2016).

We have demonstrated that bacteria with SQR and PDO oxidized self-produced sulfide and did not release H_2S (Figure 3). In the assay of 2-ml bacterial cultures, as low as 5 μM H_2S, produced by bacteria without SQR and PDO during the course of incubation, was released into the headspace and clearly detected by the lead-acetate paper strip (Figure 3). Our incubation with shaking may also facilitate the volatilization of H_2S. Thus, the sulfide-oxidizing heterotrophic bacteria are able to oxidize sulfide at low levels to prevent the accumulation and volatilization of H_2S. We only tested 24 bacteria with sequenced genomes, and the result cannot predict that all bacteria with SQR and PDO will not release H_2S when growing in rich media, but can certainly expect most will follow the trend and oxidize sulfide as they produce it. This oxidation of sulfide at low concentrations may have ecological significance as abiotic oxidation of sulfide is slow with a half-life of 55 days in a trace metal-free solution at pH 12 and 25 °C (Luther *et al.*, 2011b) or a half-life of 26 h in seawater at 25 °C (Millero *et al.*, 1987). Without the oxidation, sulfide may accumulate (Figure 3).

Heterotrophic bacteria may get energy from sulfide oxidation. SQR oxidizes sulfide to polysulfide and passes the electrons to the electron transport chain of aerobic respiration, which will generate the proton motive force for ATP production (Marcia *et al.*, 2010). However, this system is not energy efficient, as PDO and rhodanese do not participate in energy conservation. Interestingly, the common marine Roseobacter members often carry the SQR-PDO system as well as the energy-generating sulfite oxidase and Sox systems that further oxidize sulfite and thiosulfate to sulfate (Lenk *et al.*, 2012). In the dark ocean bacteria are rich with sulfur oxidizing genes from metagenomic analysis, suggesting that these bacteria may use both organic and inorganic compounds to cope with the nutrient-poor environment (Swan *et al.*, 2011). However, the source of sulfide is unlikely from the anaerobic sediment, which is farther down below, and sulfide produced in the sediment hardly escapes the interphase of the water-sediment boundary. Our results suggest a possible source of the sulfide from the metabolism by heterotrophic bacteria, especially on sulfur rich compounds, for example, dimethylsulfoniopropionate (DMSP).

DMSP is mainly produced by marine macroalgae and single-cell phytoplankton, and it has major physiological roles in phytoplankton, heterotrophic bacteria and zooplankton (Yoch, 2002). Its annual production is estimated around 10^9 metric tons. The produced DMSP is rapidly converted to dimethylsulfide (DMS) by both phytoplankton and heterotrophic bacteria with DMPS lyases or demethylated to produce methanethiol by bacteria (Alcolombri *et al.*, 2015; Sun *et al.*, 2016). Only a small fraction of the produced DMS is released into the atmosphere, which is chemically oxidized to sulfite and sulfate to serve as nuclei for cloud formation (Vila-Costa *et al.*, 2006). DMS is either oxidized to dimethylsulfoxide by marine bacteria with trimethylamine monooxygenase (Lidbury *et al.*, 2016) or converted to MeSH by bacteria with dimethylsulfide monooxygenase (Boden *et al.*, 2011). MeSH can be metabolized to release sulfide or assimilated to produced methionine (Kiene *et al.*, 1999). Methionine is converted to homocysteine and cysteine, both of which can be further metabolized to release H_2S. Thus, it is speculated that the rapid turnover of the huge amount of DMSP may generate significant amounts of sulfide in marine waters, especially during algal bloom, and the heterotrophic bacteria with *sqr* and *pdo* present in the community are likely responsible for sulfide oxidation.

Another physiological role of sulfide oxidation by heterotrophic bacteria is likely detoxification when sulfide level is high. Baker's yeast without SQR and PDO accumulates sulfide, which then slows down respiration and growth due to sulfide inhibition of cytochrome C oxidase (Sohn *et al.*, 2000). Bacterial respiration with cytochrome bo_3 oxidase is also inhibited by sulfide with a half-maximal inhibitory concentration of 1.1 μM sulfide for the *E. coli* oxidase; however, *E. coli* cytochrome *bd* oxidase is less sensitive to sulfide (Forte *et al.*, 2016). The data we presented in Figure 3 and Supplementary Figure 7 show that bacteria with SQR and PDO oxidize sulfide and prevent its accumulation in pure and mixed cultures. Further, exogenous sulfide can also be oxidized and detoxified by bacteria with SQR and PDO (Figures 4 and 5). The detoxification role of sulfide oxidation has been reported, as *Staphylococcus aureus* with SQR and PDO is more resistant to added sulfide than the deletion mutant (Luebke *et al.*, 2014).

Bacteria may also use SQR or SQR and PDO to prevent the loss of sulfur through H_2S volatilization. Sulfide can be lost via H_2S volatilization (Figure 3; Supplementary Figure 7; Table 2), and bacteria with SQR or SQR and PDO can prevent the loss (Figure 3). The use of SQR for sulfur conservation is inferred from the genome of *Candidatus Evansia muelleri* Xc1, an endosymbiotic bacterium of the moss insect *Xenophyes cascus*. The endosymbiont has a very small genome but contains *sqr* that is proposed for sulfur conservation as the insect is on a sulfur-poor diet (Santos-Garcia *et al.*, 2014). Bacteria with *sqr*

and *pdo* genes, including some Pseudomonads, Bacilli and Roseobacter, are common in soil, freshwater and seawater (Table 1; Supplementary Table 7), and they oxidize biogenic sulfide to sulfite and thiosulfate (Xin *et al.*, 2016). Sulfite and thiosulfate can be directly used by plants and microorganisms as the sulfur source, or further oxidized by bacteria to sulfate (Anandham *et al.*, 2008; Marshall and Morris, 2013).

The *pdo* genes are more abundant than the *sqr* genes in marine waters (Table 3) and sequenced marine bacterial genomes (Supplementary Figure 8). Higher plants possess only PDO and its function is likely to prevent the accumulation of polysulfide and persulfide generated from sulfur-containing amino acids (Höfler *et al.*, 2016). Bacteria with PDO may have a similar role. However, bacteria with only SQR or PDO are able to collaborate in oxidizing sulfide to thiosulfate (Figure 5), suggesting the interspecies transfer of polysulfide or persulfide.

PDO classification. The PDO was initially classified into three groups, SdoA, ETHE1 and Blh (Liu *et al.*, 2014). Structure analysis grouped SdoA and Blh into the type II and ETHE1 related proteins as type I (Sattler *et al.*, 2015), and both used GSSH as the substrate (Liu *et al.*, 2014). The type I and II PDOs, present in Gram-negative bacteria, share about 20–30% sequence identities. The type III PDOs are further distanced from the type I and II PDOs with less than 20% sequence identity to them, and they are present in both Gram-positive and Gram-negative bacteria as well as in Archaea (Figure 1; Supplementary Figure 2; Table 1; Supplementary Table 3).

The distribution and transcription of sqr was affected by environmental conditions. The distribution and transcription of *sqr* in marine surface waters are possibly influenced by salt concentrations and light. The *sqr* genes were more abundant in hypersaline waters (Punta Cormorant, Hypersaline Lagoon, Floreana Island) (Table 3). This is possibly due to increased availability of sulfide in hypersaline waters because the dissolved oxygen content decreases with the increased salt contents (Wetzel, 2001), making sulfide more stable (Kolluru *et al.*, 2013). Further, in shallow hypersaline lagoons, sulfide may also come from dissimilatory sulfate reduction in the sediment (Cotner *et al.*, 2004). The transcription of *sqr* during the night was about 2 folds higher than that during the day (Supplementary Figure 9). We speculate that there is likely more sulfide during the night because phytoplankton respiration in the dark may contribute to additional sulfide and the lack of photosynthesis leads to reduced oxygen levels (Watt, 2000). The expression of *sqr* and *pdo* can be induced with increased sulfide as shown in this study (Supplementary Figure 6A) and as previously reported (Guimaraes *et al.*, 2011; Luebke *et al.*, 2014; Shimizu *et al.*, 2017).

In conclusion, bacterial production of sulfide from organic sulfur is common during aerobic growth. Due to the wide presence of sulfide-oxidizing heterotrophic bacteria and their ability to oxidize sulfide at low levels, the sulfide production by heterotrophic bacteria is not readily observed. Without the oxidation, sulfide accumulates and volatilizes as the H_2S gas. Since heterotrophic bacteria do not solely rely on sulfide to supply energy for growth, they can oxidize it at low levels and when the supply is intermittent. In the ocean, heterotrophic bacteria metabolize the large quantities of DMSP to release sulfide, and they are also likely to consume the released sulfide. Considering the scale of their presence on Earth, heterotrophic bacteria may significantly contribute to sulfide production and oxidation, a missed aspect of the sulfur cycle.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgements

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