A New Family of Membrane Electron Transporters and Its Substrates, Including a New Cell Envelope Peroxiredoxin, Reveal a Broadened Reductive Capacity of the Oxidative Bacterial Cell Envelope

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ABSTRACT The *Escherichia coli* membrane protein DsbD functions as an electron hub that dispatches electrons received from the cytoplasmic thioredoxin system to periplasmic oxidoreductases involved in protein disulfide isomerization, cytochrome *c* biogenesis, and sulfenic acid reduction. Here, we describe a new class of DsbD proteins, named ScsB, whose members are found in proteobacteria and *Chlamydia*. ScsB has a domain organization similar to that of DsbD, but its amino-terminal domain differs significantly. In DsbD, this domain directly interacts with substrates to reduce them, which suggests that ScsB acts on a different array of substrates. Using *Caulobacter crescentus* as a model organism, we searched for the substrates of ScsB. We discovered that ScsB provides electrons to the first peroxide reduction pathway identified in the bacterial cell envelope. The reduction pathway comprises a thioredoxin-like protein, TlpA, and a peroxiredoxin, PprX. We show that PprX is a thiol-dependent peroxidase that efficiently reduces both hydrogen peroxide and organic peroxides. Moreover, we identified two additional proteins that depend on ScsB for reduction, a peroxiredoxin-like protein, PrxL, and a novel protein disulfide isomerase, ScsC. Altogether, our results reveal that the array of proteins involved in reductive pathways in the oxidative cell envelope is significantly broader than was previously thought. Moreover, the identification of a new periplasmic peroxiredoxin indicates that in some bacteria, it is important to directly scavenge peroxides in the cell envelope even before they reach the cytoplasm.

IMPORTANCE Peroxides are reactive oxygen species (ROS) that damage cellular components such as lipids, proteins, and nucleic acids. The presence of protection mechanisms against ROS is essential for cell survival. Bacteria express cytoplasmic catalases and thiol-dependent peroxidases to directly scavenge harmful peroxides. We report the identification of a peroxide reduction pathway active in the periplasm of *Caulobacter crescentus*, which reveals that, in some bacteria, it is important to directly scavenge peroxides in the cell envelope even before they reach the cytoplasm. The electrons required for peroxide reduction are delivered to this pathway by ScsB, a new type of membrane electron transporter. We also identified two additional likely ScsB substrates, including a novel protein disulfide isomerase. Our results reveal that the array of proteins involved in reductive pathways in the oxidative environment of the cell envelope is significantly broader than was previously thought.

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Until 20 years ago, the presence of redox reactions in the bacterial cell envelope was thought to be quite limited. However, this picture changed with the discovery of sets of enzymes that use the chemical properties of cysteines to form or reduce disulfide bonds in the bacterial periplasm (1). The oxidative enzymatic process that promotes protein disulfide bond formation derives its power from the transfer of electrons to membrane bound quinones. DsbA, a thioredoxin-like protein with a Cys-X-X-Cys active site, is the direct catalyst of the joining of two cysteines in a substrate protein in the periplasm (2). DsbA becomes reduced as a result of its oxidative reaction and is reoxidized via two pairs of redox-active cysteines of the membrane protein DsbB (3, 4). DsbB itself is reoxidized by transferring electrons to the quinones (5).

In addition, a number of enzymes were discovered in the periplasm that employ cysteines to provide electrons to various substrates. These enzymes include DsbC, a protein that is involved in disulfide bond isomerization (6–8), DsbG, which restores the reduced form of cysteines that have been oxidized to sulfenic acid (9), and CcmG, which reduces the oxidized cysteines of apo-cytochrome *c* so as to allow heme binding (10, 11). In *Escherichia coli*, these three proteins derive their reducing power from cytoplasmic thioredoxin via the cytoplasmic membrane protein DsbD. DsbD consists of three domains: an N-terminal periplas-
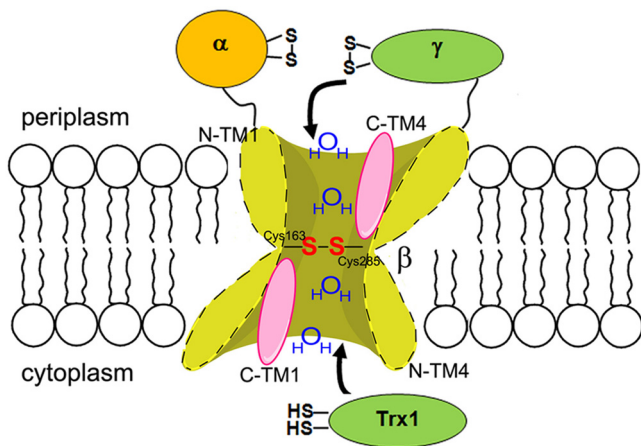
FIG 1 Structural model of DsbD. The model shows that DsbDβ adopts an hourglass-like structure where Cys163 and Cys285 are in the middle of the two water-exposed cavities. Halves of C-terminal TM1 and TM4 (C-TM1 and C-TM4) are water exposed (pink), while those of N-terminal ones (N-TM1 and N-TM4) are not (17, 18). Two thioredoxin modules (thioredoxin-1 and DsbDγ) may interact with Cys163 and Cys285 through these two water-exposed cavities. S, sulfur of thiol in a cysteine residue.

mic domain (DsbDα), a C-terminal periplasmic domain (DsbDγ), and an intervening hydrophobic membrane-embedded domain (DsbDβ) (Fig. 1). Each of the three domains contains two redox-active cysteines, essential for the protein's function (12). DsbD homologues are found in many other bacteria (13). However, some bacteria express a protein homologue that comprises only the cytoplasmic membrane domain of DsbD. The prototype of this protein, CcdA of *Rhodobacter capsulatus*, may be more limited in its specificity than DsbD, although it does reduce proteins that are homologues of CcmG (13, 14).

The transfer of electrons across the membrane by the protein DsbD occurs by a cascade of disulfide exchange reactions. This cascade begins with the reduction by cytoplasmic thioredoxin-1 of an intramembraneous disulfide bond in the hydrophobic DsbDβ domain. Electrons are then transferred from the reduced cysteines of DsbDβ to the cysteine pair of the periplasmic thioredoxin-like domain, DsbDγ (15, 16). This electron transfer occurs by an unusual mechanism whereby the two cysteines in DsbDβ are accessible to both thioredoxin-1 in the cytoplasm and the thioredoxin-like DsbDγ in the periplasm (Fig. 1) (17, 18). From DsbDγ, electrons are transferred to the immunoglobulin-like DsbDα domain, which then reduces its substrates, DsbC, DsbG, and CcmG (15, 19–21).

We have been investigating the extent to which reductive proteins related to DsbD are present in various bacterial species. Here, using bioinformatic programs to seek homologues of DsbD, we detected a third distinct class of reductive proteins (in addition to DsbD and CcdA). The prototype of this new class is *Salmonella enterica* serovar Typhimurium ScsB (suppression of copper sensitivity), a protein which has been shown to confer copper tolerance to copper-sensitive *E. coli* mutants (22).

The ScsB class has domains comparable to those of DsbD. However, the N-terminal periplasmic domain of ScsB (ScsBα) is quite different from DsbDα, suggesting that it acts on an array of substrate proteins different from those already known for DsbD. Indeed, we found that ScsB transfers electrons to a thioredoxin-like protein (TlpA), which in turn reduces a peroxidase (peroxiredoxin-Q type), PprX. PprX is the first peroxiredoxin

identified in the bacterial cell envelope. ScsB also reduces a peroxiredoxin-like1 protein (PrxL) whose function remains unknown and a novel bacterial protein, disulfide isomerase (ScsC). We have established the *in vivo* functions of some of these proteins and their interactions with ScsB using a model organism, *Caulobacter crescentus*. Our results reveal that the range of such electron transfer reactions taking place in the periplasm is much broader than was previously thought. Moreover, the identification of a periplasmic peroxiredoxin indicates that in some bacteria, it is important to directly scavenge peroxides in the cell envelope before they even reach the cytoplasm.

RESULTS

A new class of DsbD family, ScsB. We showed previously that the DsbD family is divided into two classes, the DsbD-like and CcdA-like proteins (13). The results presented here indicate that there is a third distinct class which comprises DsbD homologues with quite different α domains [Cluster of Orthologous Groups (COG) 4233] (23). The most studied member of this third class has been identified in *Salmonella* Typhimurium (22). Because the gene for this protein is a member of an operon that confers suppression of copper sensitivity to *E. coli*, the operon was annotated as *scs*, and *scsB* encodes the homologue of DsbD. Although it seemed possible that ScsB is a new type of DsbD, there has been no information on the reductive reactions it may carry out and how it may differ functionally from the other DsbD families.

We have identified ScsBs in many other bacteria by doing a blast search for homologues of the *S.* Typhimurium ScsB (Table 1; also, see Table S1 in the supplemental material [Table 1 is a summary table of Table S1]) (24). To show the relationship between members of the DsbD, CcdA, and ScsB classes, we constructed a phylogenetic tree using members of each class (Fig. 2). The tree shows that ScsB proteins form a group distinct from the DsbD and CcdA classes of reductive enzymes. ScsB is positioned close to DsbD in the phylogenetic tree because both DsbDs and ScsBs have a similar domain organization (the α, β, and γ domains) and eight transmembrane segments in the β domain (see Fig. S1A and B in the supplemental material). The distribution of ScsB is quite diverse among bacteria. Most proteobacteria, excluding the epsilonproteobacteria, and all *Chlamydia* strains tested have *scsB* (Table 1; also, see Table S1). Many gammaproteobacteria, including *S.* Typhimurium and some *E. coli* strains (e.g., strain TA280), have both *scsB* and *dsbD* on their chromosomes.

Interestingly, we observed that many alphaproteobacteria do not have full-length ScsB but appear to express ScsBα as a separate protein (Table 1; also, see Table S1 and Fig. S1C in the supplemental material), suggesting that the full-length ScsB class of proteins may have evolved from fusion of independently expressed polypeptide chains corresponding to the three domains of the protein. We had previously postulated (13), based on subcloning of DsbD domains and bioinformatic analysis, that DsbD had evolved as a fusion protein between three different proteins (the α, β, and γ domains). Therefore, we searched for genes encoding the other two domains of ScsB, ScsBβ and ScsBγ, in organisms that expressed an independent ScsBα. We found that all but one of those organisms contain genes that encode, in addition to ScsBα, a membrane protein corresponding to either CcdA (the homologue of DsbDβ) or a ScsBβγ fusion protein (Table 1; also, see Table S1). In the former case, while we did not find any separately expressed ScsBγ homologues, we think it likely that a thioredoxin-like pro-

FIG 2 Phylogenetic analysis of the DsbD superfamily in eubacteria. The tree was constructed using the neighbor-joining method from the MEGA5 sequence analysis program (60) based on the amino acid sequences of the DsbD superfamily members listed in Table 1. A more detailed phylogenetic tree can be found in Fig. S1E in the supplemental material. The scale bar indicates the number of substitutions per site. All the family members are represented by the organism names. CcdA family members are circled in light yellow, DsbD family members in light red, and ScsB family members in light blue. We colored the DsbD homologues from *Bacteroides* and *Leptospira* and that from *Mycobacterium* as belonging to the DsbD and CcdA subgroup, respectively, because of their domain compositions and sequence similarities. However, these proteins appear to form distinct branches within the DsbD superfamily. Moreover, we observed that CcdA family members appear to be quite diverse compared to others. In the schematic drawings, the two essential cysteine residues in each domain of DsbD and ScsB or those in CcdA are depicted as "S" in red in yellow circles. "No_alpha" means that the corresponding DsbD or ScsB protein has no α domain. *Caulobacter crescentus* CB15N has ScsB and is the organism used for further study (red circle). *Escherichia coli* K-12 DsbD is indicated by a blue circle. Sequences are provided in Text S1 in the supplemental material.

tein predicted to be exported would replace the function of ScsBγ in those organisms. The finding of ScsBβγ, in addition to ScsBα, is consistent with evolution of these proteins by gene fusion and suggests a possible order of gene fusion events (Table 1; also, see Table S1). Thus, ScsBα as a separate protein might represent a component of a more primitive electron transpon which CcdA (β) transfers electrons to a thioredoxin-like protein(γ) and thence to ScsBα (α), by analogy to the order of electron transfer in the three-domain protein DsbD.

While the DsbD and ScsB classes of proteins show different conservation throughout much of their polypeptide sequences (see Fig. S1A and B in the supplemental material), they differ particularly in their α domains, which cannot be aligned at all. Furthermore, the number of amino acid residues of ScsBα is greater than that of DsbDα (compare Fig. S1A and B in the supplemental material; for details, see the Discussion). Nevertheless, ScsBα is predicted to have a largely beta-sheet structure like that of DsbDα (see Fig. S1D). The position of the two cysteines is conserved in the ScsBα family, and they are presumably redox active in that domain for electron transfer (see Fig. S1A and C).

Predicting substrates of ScsB. Since ScsBα appears to have a secondary structure similar to that of DsbDα, and ScsB has a domain organization similar to that of DsbD, we postulate that ScsBα likely plays the same role as DsbDα, which is to donate electrons to substrate proteins. However, given the difference in sequence conservation and length of these two domains, we suspected that ScsB may have a different array of substrate proteins than DsbD. Since *scsB* forms an *scs* operon with *scsC*, which encodes thioredoxin fold proteins in some bacteria (22), we considered ScsC a likely substrate of ScsB. No significant homologues of ScsC are found in *E. coli* K-12 by a BLAST search (see Fig. S2A and S2B in the supplemental material; DsbG and DsbC are the closest homologues, but the expectation values are 3.5 and 7.5, respectively). Therefore, we chose ScsC as a potential substrate of ScsB for further characterization below.

In a number of bacteria, we have also found a protein with a putative thioredoxin fold encoded by a gene near *scsB* on the chromosome (Table 1; also, see Table S1 in the supplemental material). The gene product is annotated as a peroxiredoxin-like1 (PrxL) by Conserved Domains (CDD) in National Center for Biotechnology

Information (NCBI) and it has a signal sequence for export. PrxL has not been characterized before. Therefore, we chose PrxL as a second potential substrate of ScsB for further characterization.

As another means of identifying possible substrates of ScsB, we used STRING, an interacting-protein database, entering the amino acid sequence of *Caulobacter crescentus* ScsB (CC0217) (see Fig. S2C in the supplemental material; the choice of *C. crescentus* ScsB is explained below). The branches revealed by this analysis could represent directly interacting partners, regulators, or false positives. Among potential partners found were ScsC and PrxL, showing the likely utility of a STRING analysis for this purpose. In addition, the STRING analysis pointed to a second potential protein interacting with ScsB, a *C. crescentus* peroxiredoxin homologue (CC1673) which has a signal sequence for export. Peroxiredoxins (Prx) are peroxidases that utilize redox-active cysteines to detoxify peroxides (25, 26). While Prxs are widespread amongst bacteria throughout nature and protect against oxidative stress, no Prx has been identified in the bacterial cell envelope. [*E. coli* Tpx (27) was originally reported as a periplasmic Prx. However, Tpx does not possess a signal sequence and was subsequently demonstrated to be located in the cytoplasm (28).] For this reason, we also chose this interesting periplasmic Prx as a substrate of ScsB for further characterization. For clarity and simplicity, we designate *C. crescentus* periplasmic Prx as PprX.

CcmG homologues are also found in ScsB-containing organisms. However, we decided not to investigate them further because they have already been well studied in diverse bacteria as substrates of CcdA or DsbD.

ScsB is essential in *C. crescentus* and maintains ScsC, PprX, and PrxL reduced in the periplasm. We proceeded to seek direct evidence that the putative substrate proteins identified in the search described above depend on ScsB for reduction. Since ScsB is likely to be involved in reductive pathways in the bacterial cell envelope, the substrate proteins would be ones that are maintained by ScsB in the reduced state *in vivo*. Thus, deletion of *scsB* should lead to oxidation of the substrate proteins. This has previously been shown for *E. coli* DsbD and its substrate proteins (8, 29). Among the organisms with a *scsB* gene, *C. crescentus* appeared the most amenable to genetic analysis. Furthermore, it has no *ccdA* and no *dsbD*, having only one *scsB* gene along with each of the candidate substrate genes mentioned above (Table 1).

Initial attempts to knock out *scsB* by inserting an antibiotic cassette failed, suggesting that ScsB may be essential. However, we were able to obtain the knockout mutations of *scsB* in the presence of a complementing plasmid (Fig. S3 in the supplemental material shows the essentiality of ScsB; also, see the Discussion). When we then sought to assess the redox state of potential substrate proteins after depletion of ScsB by removing an inducer, we found that the basal level of expression was too leaky. Therefore, we chose an alternative approach in which we grew the *scsB* strains carrying the complementing plasmid in the absence of antibiotic, under which conditions the plasmid is reduced in copy number, as it does not have a partitioning system (30). This approach allows the cells to reduce by attrition the copy number of the plasmid to the point where they maintain the smallest number of plasmids needed to survive with the *scsB* null mutation.

Thus, we depleted cells of ScsB in this way and analyzed the redox state of the potential substrates ScsC, PrxL, and PprX. Each of these proteins has only two putative redox-active cysteines. To detect the proteins and analyze the oxidation state of their cys-
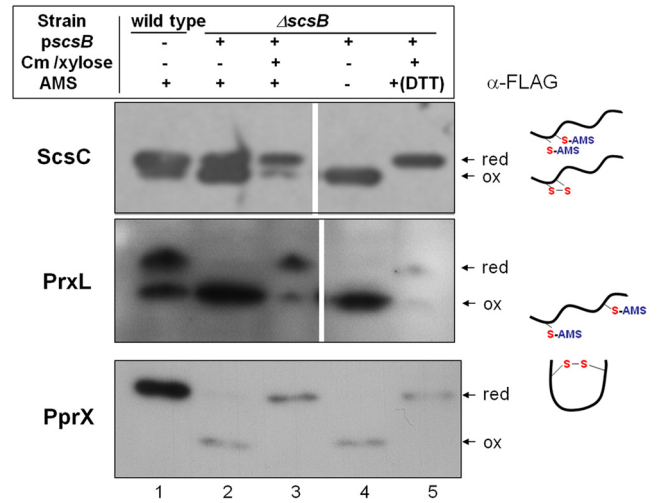

FIG 3 *In vivo* redox states of CcScsC, CcPrxL, and PprX in ScsB-depleted *C. crescentus* strains. Structural genes for CcScsC, CcPrxL, and PprX were fused with a Flag tag and expressed from pSC160, pSC171, and pSC159 plasmids, respectively. Each cell was cotransformed with a ScsB-expressing plasmid (p*scsB*; pSC161) (lanes 2 to 5) or left untransformed (lane 1). Cells were induced by 0.2% D-xylose for p*scsB* or 0.5 mM vanillate for the other plasmids and harvested at mid-log phase, and then proteins were precipitated with TCA and subjected to AMS alkylation. Proteins were then separated by SDS-PAGE under nonreducing conditions and visualized by Western blotting using an anti-Flag antibody. AMS was used to treat samples in lanes 1 to 3 and lane 5. In lane 5, treatment of samples with 50 mM DTT was followed by AMS alkylation. For depletion of ScsB, chloramphenicol (Cm) and d-xylose were not added (lanes 2 and 4). The strain backgrounds used were *C. crescentus* CB15N (wild type; lane 1) and SEN 233 (the *scsB* mutant with p*scsB*). Cartoons explain the shifts in the mobility of proteins which reflect either cysteine modification by AMS (higher bands) or a modified and more compact structure due to disulfide bond formation. CcPrxL and PprX have distantly located cysteines such that disulfide bond formation causes more compact structures and therefore a fast mobility in gels compared to the structure of AMS-modified reduced proteins, whereas CcScsC has CXXC, leading to a smaller mobility shift caused solely or primarily by AMS modification. The depletion experiment was repeated several times. Although the quantities of protein recovered after the TCA precipitation varied from sample to sample, the results were always consistent with those shown in this figure.

teins by Western blotting, we tagged the three candidate substrates with Flag epitopes and alkylated cysteine residues with 4-acetamido-4'-maleimidylstilbene-2,2'-disulfonic acid (AMS) (12). AMS adds 0.5 kDa of molecular mass and causes a shift in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (31). When reduction of the three putative substrate proteins by dithiothreitol (DTT) is followed by AMS treatment and cell extracts are run on SDS-PAGE, all three proteins run with lower mobility (Fig. 3, lane 5) than they do in samples without DTT and AMS treatment (lane 4). In the case of PrxL and PprX, the shifts are bigger than that of ScsC because disulfide bonding of the two distantly located cysteines in the primary sequences probably leads to more significant conformational changes and a more compact protein structure. Our results show that in both the wild-type strain and the *scsB*-null strain expressing high levels of ScsB from the plasmid, most or significant portions of ScsC, PrxL, and PprX are in the reduced state (Fig. 3, lanes 1 and 3, respectively), suggesting that they are involved in reductive pathways. In contrast, significant portions of the three proteins are oxidized when cells are depleted of ScsB by removal of the antibiotic and inducer

TABLE 1 Distribution of DsbD, CcdA, ScsB, ScsC, PprX, and PrxL among diverse bacteria

Phylum	Class or order	Organism name[a]	DsbD	CcdA	ScsB α[b]	ScsB Whole[c]	ScsB βγ[c]	ScsC[b,d]	PprX[b]	PrxL[b]
Proteobacteria	Alphaproteobacteria	*Rhodospirillum rubrum* ATCC 11170	-	-	-	+	-	+	-	-
		Bartonella quintana Toulouse	-	+	-	-	-	-	-	-
		Brucella suis 1330	-	+[e]	+	-	-	+	-	-
		Xanthobacter autotrophicus Py2	+	+[e]	+	-	-	+	-	-
		Ehrlichia ruminantium Gardel	-	-	+[f]	-	+[e]	+	-	-
		Wolbachia sp. Dm	-	-	-	-	-	+	-	-
		***Caulobacter crescentus* CB15**	-	-	-	+	-	+	+	+
		Rhodobacter sphaeroides 2.4.1	-	+[e]	+	-	-	+	-	-
	Betaproteobacteria	*Acidovorax* sp. JS42	+	-	-	+	-	-	-	-
		Burkholderia cenocepacia AU 1054	+	-	-	-	-	-	+	-
		Methylibium petroleiphilum PM1	+	-	-	+	-	-	-	+
		Neisseria gonorrhoeae FA 1090	+	-	-	-	-	-	-	-
		Nitrosospira multiformis ATCC 25196	+	-	-	-	-	-	+	-
	Gammaproteobacteria	*Pseudomonas aeruginosa* PAO1	+	-	-	-	-	-	-	-
		Hahella chejuensis KCTC 2396	+	-	-	+	-	-	-	+
		Buchnera aphidicola APS	-	-	-	-	-	-	-	-
		Enterobacter sp. 638	+	-	-	-	+	+	+	-
		Escherichia coli K-12	+	-	-	-	-	-	-	-
		Photorhabdus luminescens	+	-	-	-	-	-	-	-
		Salmonella enterica LT2	+	-	-	+	-	+[g]	-	-
		Shigella dysenteriae	+	-	-	-	-	-	-	-
		Yersinia pestis Antiqua	+	-	-	-	-	-	-	-
		Saccharophagus degradans 2-40	+	-	-	+	-	+	-	+
		Shewanella amazonensis SB2B	+	-	-	+	-	-	-	-
		Coxiella burnetii 493	+	-	-	-	-	+	-	-
		Francisella tularensis FSC 198	-	-	-	-	-	-	-	-
		Methylococcus capsulatus Bath	+	-	-	-	-	-	-	-
		Xanthomonas campestris 33913	-	-	-	+	-	-	-	-
	Deltaproteobacteria	*Myxococcus xanthus* DK 1622	+	+	-	+	-	-	-	+
		Desulfovibrio desulfuricans G20	-	-	-	+	-	-	-	-
		Geobacter metallireducens GS-15	-	+	-	-	-	-	-	-
	Epsilonproteobacteria	*Campylobacter jejuni* 11168	+	-	-	-	-	-	-	-
		Helicobacter pylori J99	-	+	-	-	-	-	-	-
	Others	"*Magnetococcus*" sp. MC-1	-	+	-	-	-	-	-	-
Acidobacteria	Acidobacteriales	Candidatus "Koribacter versatilis" Ellin345	-	+	-	-	-	-	+	-
Actinobacteria	Actinobacteridae	*Arthrobacter aurescens* TC1	-	+	-	-	-	-	-	-
		Mycobacterium tuberculosis H37Rv	-	+	-	-	-	-	-	-
		Streptomyces coelicolor	-	+	-	-	-	-	-	-
		Bifidobacterium adolescentis	-	-	-	-	-	-	-	-
	Rubrobacteridae	*Rubrobacter xylanophilus* DSM 9941	-	+	-	-	-	-	-	-
Firmicutes	Clostridia	"*Alkaliphilus metalliredigens*" QYMF	-	-	-	-	-	-	-	-
		Clostridium botulinum 19397	-	+	-	-	-	-	-	-
		Symbiobacterium thermophilum IAM 14863	-	+	-	-	-	-	-	-
	Bacillales	*Bacillus subtilis*	-	+	-	-	-	-	-	-
		Geobacillus kaustophilus HTA426	-	+	-	-	-	-	-	-
		Staphylococcus aureus RF122	-	-	-	-	-	-	-	-
	Lactobacillales	*Lactobacillus salivarius* UCC118	-	-	-	-	-	-	-	-
		Streptococcus pneumoniae D39	-	+	-	-	-	-	-	-
Tenericutes	Mollicutes	*Mycoplasma agalactiae*	-	-	-	-	-	-	-	-
		Candidatus "Phytoplasma asteris"	-	-	-	-	-	-	-	-
Cyanobacteria	Nostocales	*Anabaena variabilis* ATCC 29413	-	+	-	-	-	+	+	-
	Gloeobacteria	*Gloeobacter violaceus*	-	+	-	+	-	+	+	+
	Prochlorales	*Prochlorococcus marinus* 9215	-	+	-	-	-	-	+	-
	Chroococcales	*Synechococcus elongatus* PCC 6301	-	+	-	-	-	-	+	-
	Oscillatoriales	*Trichodesmium erythraeum* IMS101	-	+	-	-	-	+	+	-
Aquificae	Aquificales	"*Aquifex aeolicus*"	-	+	-	-	-	-	+	-
Bacteroidetes	Bacteroidia	*Bacteroides fragilis* NCTC 9343	+	-	-	-	-	-	-	-
	Sphingobacteria	*Cytophaga hutchinsonii* ATCC 33406	+	-	-	-	-	-	-	+
	Flavobacteriales	*Flavobacterium johnsoniae* UW101	+	-	-	-	-	-	-	+
Chlorobi	Chlorobia	"*Chlorobium chlorochromatii*" CaD3	+	-	-	-	-	-	-	-
Spirochaetes	Spirochaetales	*Leptospira borgpetersenii* serovar Hardjobovis JB197	+	-	-	-	-	-	-	-
Chlamydiae	Chlamydiales	*Chlamydophila pneumoniae* TW 183	-	-	-	+	-	-	-	-
		Candidatus "Protochlamydia amoebophila" UWE25	-	-	-	+	-	-	-	-
Chloroflexi	Dehalococcoidetes	"*Dehalococcoides ethenogenes*" 195	-	+	-	-	-	-	-	-
Deinococcus	Deinococci	*Deinococcus geothermalis* DSM 11300	-	+	-	-	-	-	-	-
Thermotogae	Thermotogales	*Thermotoga petrophila* RKU-1	-	+	-	-	-	-	-	-
Fusobacteria	Fusobacteriales	*Fusobacterium nucleatum*	-	+	-	-	-	-	-	-
Planctomycetes	Planctomycetales	*Rhodopirellula baltica* SH 1	-	-	-	+	-	-	-	+

[a] The organisms were chosen so that they become representatives of the branches in the bacterial phylogenetic tree in Table S1 in the supplemental material.

[b] The presence of signal sequence or N-terminal transmembrane segment was analyzed using SignalP 3.0 (http://www.cbs.dtu.dk/services/SignalP/) or TMHMM 2.0 (http://www.cbs.dtu.dk/services/TMHMM-2.0/), respectively.

[c] In ScsBβ, the transmembrane segment 1, which contains the first putative redox-active cysteine, appears to be highly conserved, as shown in Fig. S1A in the supplemental material. A Lys residue in the 8th position at the C terminus of the first cysteine in ScsBβ is especially conserved and typical in ScsB (not found in DsbD and CcdA). All the tested ScsBβ proteins have this Lys residue. Therefore, the presence of this residue was used as one method to distinguish ScsBβ from DsbDβ and CcdA.

[d] Selected are ScsC homologues which have the N-terminal helical domain (see the text). psi-blast and STRING analysis were used for the primary selection, and then the homologous sequence motif with EHPE was used for the secondary selection since this sequence motif is well conserved in the putative hinge region of the N-terminal helical domain as shown in Fig. S6A. Many ScsC variants, including prototypic ScsCs, are found in diverse bacteria. It appears that ScsC is widely used for many kinds of redox reactions in many ways in bacteria. Because of this diversity of ScsC and the established relationships between CcScsB and CcScsC, we included only the CcScsC type in Table 1 and Table S1, and its occurrence correlation with ScsB appears to be very good.

[e] These organisms containing CcdA or ScsBβγ contain separate ScsBα but not full-length ScsB in most cases (see the text).

[f] The ScsBα domain appears to have two subdomains (see the text). The N-terminal subdomain is relatively well conserved, while the C-terminal subdomain is not. Related to this observation, we could find ScsBα in some bacteria which do not have the C-terminal subdomain.

[g] In *Salmonella*, in addition to StScsC, which is not included in this table, another ScsC homologue is found which has the N-terminal helical domain like CcScsC. The gene is annotated as *bcfH*, a gene in a *bcf* operon to encode proteins for fimbriae.

(Fig. 3, lane 2), indicating that they are normally reduced by ScsB. The dependence of PprX, PrxL, and ScsC on ScsB for reduction has also been confirmed by heterologous expression of these proteins in *E. coli* (see Fig. 5 for PprX; see Fig. S7 in the supplemental material for ScsC; data not shown for PrxL). Altogether, these results indicate that ScsB provides electrons for reduction of these periplasmic proteins.

Discovery of a new periplasmic peroxiredoxin (PprX). We describe above the detection of a gene encoding a protein that appears to be a periplasmic Prx, PprX. We have pursued characterization of this protein since, while Prxs have been found in the cytoplasm of bacteria, none have been confirmed to be expressed in the bacterial cell envelope. These enzymes utilize their peroxidatic cysteine residue to attack the O—O bond of the hydroperoxide substrate, thus becoming oxidized to a sulfenic acid form. A second, resolving cysteine, attacks the cysteine-sulfenic acid, generating an inter- or intrachain disulfide bond. The activity of the Prx enzyme is then regenerated by reduction of the disulfide bond, usually by thioredoxin-like proteins. In *E. coli*, three Prxs, alkyl hydroperoxide reductase (AhpC), bacterioferritin-comigratory protein (BCP), and thiol peroxidase (Tpx), are present in the cytoplasm and are considered major scavengers of hydrogen peroxide (28, 32–34). The closest homologue of *C. crescentus* PprX in *E. coli* is the Prx BCP, and a bioinformatic analysis based on active site profiling places this Prx clearly in the BCP/PrxQ subfamily of Prx (http://csb.wfu.edu/prex) (35, 36). Sequence alignment of some PrxQ members, including these two proteins, shows strong sequence conservation, including the absolutely conserved PXXXTXXC active site, which contains the cysteine that attacks H_2O_2, and a critical arginine residue which is conserved across the broad Prx family (Fig. 4A). However, the position of the resolving cysteine is different, as is the case for a subset of members of this subfamily (35, 37). The 20-amino-acid extension present at the N terminus of PprX corresponds to a signal sequence, indicating that this possible Prx is an exported protein. We have also found PprX homologues in bacteria other than *C. crescentus*, some among members of the alpha- and betaproteobacteria, and in most cyanobacteria (Table 1 and Table S1; see details in the Discussion).

***C. crescentus* PprX is a thiol-dependent peroxidase.** We asked whether PprX actually had thiol peroxidase activity and what its activity was toward various peroxides. To do this, we purified a signal sequence-less engineered version of PprX and performed a coupled assay, using H_2O_2 or cumene hydroperoxide as the substrate in the presence of thioredoxin-1 and thioredoxin reductase from *E. coli* to maintain thiol peroxidase activity (Fig. 4B). PprX shows almost identical apparent V_{max} values for both H_2O_2 and cumene hydroperoxide reactions. There is a difference in the apparent K_m values for the two peroxide substrates, with the enzyme displaying a lower K_m for the bulkier substrate, cumene hydroperoxide ($K_{m,app}$ of 23 μM versus 104 μM for H_2O_2). PprX shows peroxidase activity that is similar (within a factor of 2) to that measured for the *E. coli* BCP at the 10 μM concentration of thioredoxin-1 used in the assays (38). These results indicate that PprX is a thiol-dependent peroxidase.

Electron donor of PprX. The *in vitro* results reported above suggest that the direct *in vivo* electron donor for PprX in the periplasm is a thioredoxin-like protein, as is generally the case for peroxiredoxins located in the cytoplasm. If the pathway for electron transfer by ScsB is the same as in DsbD, one might expect that the domain that donates electrons to substrates would be ScsBα; however, ScsBα does not have a thioredoxin fold and is predicted to be largely a beta-sheet structure (see Fig. S1D in the supplemental material) (20, 39). To determine whether ScsBα can directly reduce PprX, we coexpressed *C. crescentus* ScsB (CcScsB) along with PprX in an *E. coli dsbD* mutant. However, we observed that PprX cannot be reduced by CcScsB (Fig. 5, lane 3), suggesting that there is a missing component, presumably another thioredoxin-like protein, that mediates electron transfer from ScsB to PprX in *C. crescentus*.

We then set out to identify the missing link between ScsB and PprX by searching the *C. crescentus* genome for thioredoxin-like proteins with export signals. Several proteins that meet the criteria were identified (see Fig. S4A in the supplemental material). Among those, TlpA (thioredoxin-like protein A) drew our attention because, in certain microorganisms, this protein is fused to a periplasmic methionine sulfoxide reductase based on STRING analysis (see Fig. S4B in the supplemental material). Methionine sulfoxide reductase is an antioxidant enzyme that reduces oxidized methionine residues and, like Prx, also requires a thioredoxin-like protein as an electron donor (40). Therefore, we decided to test whether CcTlpA can provide electrons to PprX by introducing a CcTlpA expression plasmid in the *E. coli dsbD* strain expressing CcScsB and PprX. Interestingly, we observed that the expression of CcTlpA leads to the reduction of PprX (Fig. 5, compare lane 4 with lane 3). The reduction is dependent on the presence of CcScsB (Fig. 5, compare lane 4 with lane 2). Consistently, a larger fraction of CcTlpA is in the reduced state in the presence of CcScsB (Fig. 5, compare lane 4 with lanes 1, 2, 5, and 6). Thus, we can conclude from these experiments that ScsB transfers electrons to PprX via TlpA in *C. crescentus*.

CcPrxL does not have a detectable peroxidase activity. While the second potential peroxiredoxin-like (PrxL) protein (cd02969) that is a likely substrate of ScsB does not belong to the Prx family (cd02971) according to the classification of CDD and the PREX database (this protein has two Cys residues but lacks any of the other stringently conserved residues mentioned above), the name originated from its sequence similarity to Prx family members according to the description in CDD. Therefore, we also attempted to measure thiol-dependent peroxidase activity for PrxL *in vitro*. Using the FOX assay to detect loss of peroxide and DTT as a nonspecific reductant of the potential peroxiredoxin, we could not detect significant thiol-dependent peroxidase activity. Thus, it appears that PrxL, which is probably not a peroxidase, has a yet-to-be defined redox function.

ScsC is a bacterial protein disulfide isomerase in *C. crescentus*. The third potential ScsB substrate we studied is ScsC. Results presented above suggested that ScsC, which has a thioredoxin fold, is involved in a reductive pathway in the envelope of *C. crescentus*. *C. crescentus* has two putative *dsbAs* (CC0374 and CC0375) and one putative *dsbB* (CC2196) (41, 42) but no *dsbC* or *dsbG* in its genome (see the next section for details; also, see Fig. S5A in the supplemental material) (13). However, the closest homologue of CcScsC in *E. coli* K-12 is DsbC (see Fig. S5A). Therefore, we hypothesized that the *in vivo* role of ScsC in *C. crescentus* is protein disulfide isomerization.

In *E. coli*, several proteins have nonconsecutive disulfide bonds whose formation depends on DsbC (43–46). To detect *in vivo* protein disulfide isomerase activity of CcScsC, we sought to identify *C. crescentus* periplasmic proteins with nonconsecutive disulfide bonds. We found a periplasmic ribonuclease (RNase) (CC0030; RNase I) which is homologous to a tobacco RNase that has nonconsecutive disulfide bonds (47). Two of the cysteines that
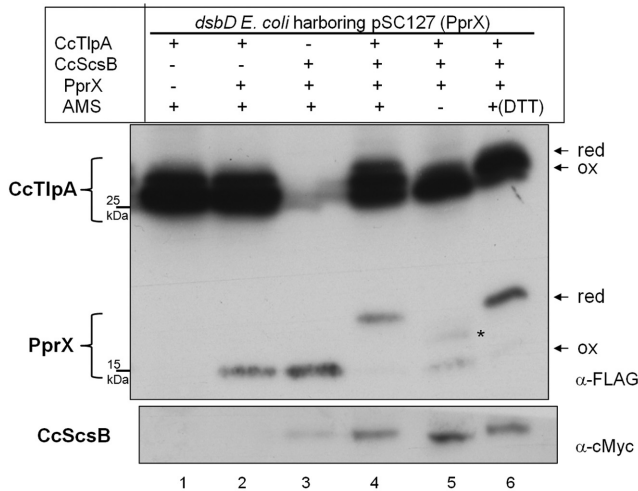
FIG 4 PprX is a true, thiol-dependent peroxidase. (A) Sequence alignment of PprX (CC1673) and five other Prxs from the BCP/PrxQ subfamily. Absolutely conserved Prx residues (Pro, Thr, Cys, and Arg) are shown in red. The putative signal sequence of PprX from *C. crescentus* is underlined. Known resolving cysteines of other BCP/PrxQ members are double-underlined. Sequences shown are from *Saccharomyces cerevisiae* (nuclear Prx, UniProt accession P40553), *Sulfolobus solfataricus* (Q97WP9), *Xylella fastidiosa* (Q9PER7), *Xanthomonas campestris* pv. campestris (Q8P9V9), and *E. coli* (POAE52). PDB identifiers are shown in parentheses for the first four for which structures have been determined. (B) Thiol-dependent peroxidase activity of the purified PprX. A coupled assay was used to show peroxidase activity. The assay uses thioredoxin-1 and thioredoxin reductase from *E. coli* as the electron donating system and H_2O_2 or cumene hydroperoxide (OOH) as the oxidizing substrate. The NADPH consumption rate was monitored to measure the activity. Kinetic parameters determined under these conditions for H_2O_2 and cumene OOH, respectively, were 17.8 ± 0.8 and 17.0 ± 0.8 μmol/min for $V_{max,app}$ and 104 ± 18 and 23 ± 6 for $K_{m,app}$.

are involved in the formation of a nonconsecutive disulfide bond are conserved in *C. crescentus* RNase I (Fig. 6A), strongly suggesting that *C. crescentus* RNase I also contains a nonconsecutive disulfide in its native conformation (Fig. 6A).

To ask whether CcScsC is involved in RNase I folding, we generated an *scsC* mutant of *C. crescentus*, cloned *rnaI* for expression in *C. crescentus*, and tested for RNase activity in wild-type and *scsC* strains. Using a zymogram to assess RNase activity, we found that the activity was significantly reduced in the *scsC* mutant compared to the wild type (Fig. 6B, compare lane 4 and lane 2). This result suggests that CcScsC is involved in RNase I folding, likely via disulfide bond rearrangement. The protein disulfide isomerization activity of CcScsC was further confirmed by expressing it in a *dsbC*

E. coli mutant and finding that it restores the folding of RcsF, an outer membrane lipoprotein with two nonconsecutive disulfide bonds whose assembly normally requires DsbC (for details, see Fig. S5B in the supplemental material) (44).

C. crescentus ScsC forms a homodimer in a different way from DsbC. The dimeric nature of DsbC is important for its protein disulfide isomerization activity (48–50). To determine whether CcScsC also forms dimers, we performed gel filtration chromatography to estimate the molecular weight of CcScsC. CcScsC with a His tag (25 kDa in monomer size) runs similarly to DsbC with a His tag (25 kDa in monomer size) and elutes with an apparent molecular mass of 47 ± 10 kDa, indicating that it forms a dimer in solution (see Fig. S6B in the supplemental material). We

FIG 5 *In vivo* redox states of PprX expressed in *dsbD E. coli* strain assessed in the presence of CcTlpA or CcScsB. Cells were induced by 0.2% L-arabinose for p*tlpA* encoding CcTlpA with triple Flag tags and p*scsB* encoding CcScsB with a c-Myc tag, and 250 μM IPTG for pSC127 (PprX with a Flag tag). All strains were transformed with pSC127 and cotransformed with a second plasmid, pBAD18 (a vector control instead of p*scsB*), and a third plasmid, p*tlpA* (lanes 1 and 2), pBAD43 (a vector control instead of p*tlpA*) and p*scsB* (lane 3), or p*tlpA* and p*scsB* (lanes 4–6). To distinguish the PprX band from that of TlpA, no IPTG was added in lane 1. Cells were treated to determine redox states of proteins as described for Fig. 3. Proteins were visualized by Western blots using an anti-Flag and an anti-c-Myc antibody. The strain backgrounds used were FED126 (the *dsbD* mutant). The asterisk indicates portions of reduced proteins which are not alkylated by AMS.

then predicted the secondary structure of CcScsC to see whether this protein contains a dimerization domain similar to that of DsbC (see Fig. S6A). The C-terminal domain of CcScsC is predicted to have a thioredoxin-like fold like that of DsbC. The dimerization domain of DsbC is located at the N terminus of the protein and consists of beta-strands. However, the N terminus of CcScsC is predicted to form a long alpha helix (see Fig. S6A). We hypothesize that this region mediates dimerization of CcScsC. *S.* Typhimurium ScsC (StScsC) does not have the long N-terminal alpha helices found in CcScsC (see Fig. S6A). Interestingly, it behaves in gel filtration chromatography like a monomer (see Fig. S6C). This result suggests that the N-terminal helical domain of CcScsC mediates homodimerization of the molecule.

DISCUSSION

We present evidence that a family of prevalent electron transporters in bacterial membranes can be divided into the DsbD, CcdA, and ScsB classes. Each of these protein families uses two similarly positioned redox-active cysteines in its membrane-embedded domain to transfer electrons from the cytoplasmic thioredoxin system to a periplasmic thioredoxin-like protein or domain. The three families differ first in that the CcdA protein comprises only the membrane-embedded domain, while, usually, DsbD and ScsB both contain an amino-terminal periplasmic domain (the α domain) and a carboxy-terminal periplasmic domain (the γ domain). DsbD and ScsB themselves differ in that their α domains show no evidence of homology to each other.

Our studies of ScsB expand the range of protein classes that are substrates of these reductive enzymes. Specifically, we have shown (i) that periplasmic peroxiredoxin homologues exist in bacteria,
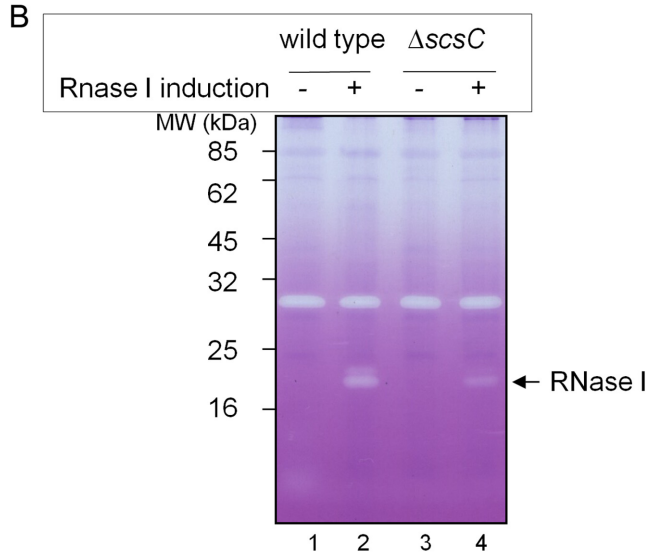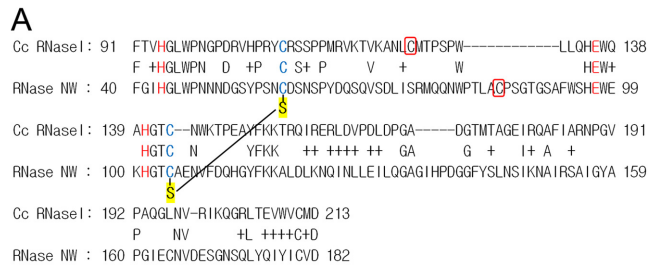
FIG 6 *In vivo* RNase I activity depends on *scsC* in *C. crescentus*. (A) Sequence alignment of *C. crescentus* RNase I (CC0030) and a tobacco RNase (RNase NW) after Jpred3 analysis. The high-resolution structure of the latter has been solved, and disulfide bonds have been assigned from the structure (PDB: 1iyb). The letters in red in the alignment are predicted to be catalytic residues in the active sites of both RNases. Two cysteine residues in blue are conserved in both RNases, and those from RNase NW form a disulfide bond which is represented with an S (sulfur in thiol) in a yellow background. One additional cysteine residue between these conserved cysteines in each of the *C. crescentus* RNase I and RNase NW proteins is indicated in a red box. (B) Zymogram to show RNase activity in the wild-type and the *scsC C. crescentus* strains. Cells were induced by 0.5 mM vanillate for expression of *C. crescentus* RNase I from pSC164. Cells were harvested at mid-log phase, broken by sonication, and subjected to SDS-PAGE. Poly(C) as an RNA substrate and toluidine blue as an intercalating dye were added to visualize RNase activity on a gel. The strain backgrounds used were *C. crescentus* CB15N (wild type; lanes 1 and 2) and SEN 224 (the *scsC* mutant; lanes 3 and 4).

(ii) that they are maintained in the active state by a reducing system that involves ScsB and a thioredoxin-like protein (TlpA), and (iii) that a novel type of protein disulfide isomerase depends on ScsB for reduction. In an example similar to the periplasmic peroxiredoxins, it has been shown that *Neisseria gonorrhoeae* expresses a periplasmic methionine sulfoxide reductase, an enzymatic activity previously considered to be restricted to the cytoplasm (51, 52). Like cytoplasmic peroxiredoxins, cytoplasmic methionine sulfoxide reductases are reduced by thioredoxins. And by analogy to periplasmic methionine sulfoxide reductase, periplasmic peroxiredoxins can be reduced via electrons transferred from DsbD or ScsB. In many ways, the functioning of the membrane domain of members of the DsbD family fills the same role in the periplasm as thioredoxin reductase does in the cytoplasm.

Subdomain structure of ScsBα. The number of amino acid residues of ScsBα is greater than that of DsbDα (compare Fig. S1A and B in the supplemental material). Among ScsBα domains, especially within the N-terminal portion, which has the two conserved cysteines, the sequences are relatively well conserved compared to those in the C-terminal part (see Fig. S1A and C). This finding, along with the observation that *Ehrlichia ruminantium* has ScsBα lacking the less-conserved C terminus, suggests that ScsBα may have two subdomains (the N subdomain and the C subdomain) (Table 1; see Table S1 in the supplemental material for more examples; see also Fig. S1C). One possibility is that the C subdomain of CcScsBα modulates the substrate specificity of the N subdomain, which on its own would function as a general electron donor. Some of our data are consistent with this idea. *E. coli* DSbDα (EcDsbDα) has only one domain and is missing the hypothetical modulator domain. EcDsbDα appears to have broader substrate specificity than CcScsB, since EcDsbDα can reduce either EcDsbC or CcScsC, while CcScsBα can reduce only CcScsC (see Fig. S7A, lanes 2 and 3). It would be interesting to see whether ScsBα without the C subdomain has broader substrate specificity than the ScsBα with the two subdomains.

Periplasmic peroxiredoxin in bacteria. Detoxification of peroxides in the cytoplasm has been extensively studied in bacteria. Prxs are very efficient scavengers for peroxides generated at physiological concentration (micromolar range), while catalase can deal with peroxides only at much higher concentrations (millimolar range) (25). In *E. coli*, two of the three Prxs, all cytoplasmic, are particularly specialized in efficiently reducing either H_2O_2 (AhpC) (53) or bulkier hydroperoxide substrates (Tpx) (32, 33). The third, BCP, exhibits broader specificity but also lowered catalytic efficiencies (from about $10^7 \, M^{-1} \, s^{-1}$ for AhpC and Tpx with their best substrates to around $10^5 \, M^{-1} \, s^{-1}$ for BCP with the same substrates, hydrogen peroxide and cumene hydroperoxide) (38). Another member of this BCP/PrxQ subfamily, PrxQ from *Xylella fastidiosa*, also exhibited a catalytic efficiency (k_{cat}/K_m for peroxides) of $\sim10^5 \, M^{-1} \, s^{-1}$ but was shown in rapid-reaction studies to degrade hydroperoxides and peroxynitrite with second-order rate constants of $\sim10^6$ to $10^7 \, M^{-1} \, s^{-1}$ (54).

Several reports highlight the importance of oxidative stress defense mechanisms to protect envelope proteins from oxidative damage, including the *Neisseria gonorrhoeae* methionine sulfoxide reductase described above (52). Moreover, DsbG, a protein homologous to DsbC, regenerates cysteines in oxidatively damaged (sulfenylated) proteins in the *E. coli* periplasm (9). Tpx, a peroxiredoxin family member in *E. coli* was originally reported to be a periplasmic protein (27) but has since been shown conclusively to be cytoplasmically localized (28). Here, we report the identification of a new periplasmic peroxiredoxin, which acts as a direct scavenger of peroxides in the bacterial cell envelope. Deletion of *pprX* does not seem to increase the sensitivity of *C. crescentus* to peroxides (data not shown), and the *in vivo* significance of PrxP needs further investigation.

Electron donor of PprX. We have presented evidence that CC2210 directly donates electrons to PprX. CC2210 is a TlpA (thioredoxin-like protein) homologue which is known to be involved in the maturation of aa_3-type cytochrome oxidase in *Bradyrhizobium japonicum* (55) (Fig. S4C), but the molecular mechanism involved has not been explored. *B. japonicum* is an alphaproteobacterium, like *C. crescentus*, so it would be interest-

ing to see whether deletion of CcTlpA affects the maturation of aa_3-type cytochrome oxidase in *C. crescentus*.

In the experiments to show the interactions of PprX, CcScsB, and CcTlpA, we used the wild-type *E. coli* strain which expresses DsbD as a control (see Fig. S7B in the supplemental material). Surprisingly, portions of PprX are in the reduced state in the absence of CcTlpA. Overexpressed DsbD significantly increased the reduction of PprX. Since DsbD appears to reduce PprX, we asked whether it might do so through its DsbDγ, thioredoxin-like domain. This turned out to be the case: when we expressed a DsbD with a Cys mutation in DsbDα and another with Cys mutations in DsbDγ, we found that the former was still able to reduce PprX, while the latter was not. These results show that the periplasmic thioredoxin-like domain of DsbD can reduce PprX but the amino-terminal periplasmic domain cannot. Although this is an electron transfer by DsbD that is not known to take place in *E. coli*, we propose that such a mechanism of electron transfer by DsbD may exist in other bacteria, since certain bacteria containing PprX, for example *Bordetella* and *Burkholderia*, do not have ScsB but have DsbD as an electron donor (Table 1; also, see Table S1 in the supplemental material).

Diversity of ScsC. Our search for ScsC homologues using the StScsC sequence has revealed relatives of the protein in many bacteria, including the CcScsC (Table 1; also, see Table S1 in the supplemental material). These ScsC homologues showed significant diversity, the molecular difference in oligomeric state between CcScsC and StScsC described above being one example.

Essentiality of ScsB. While DsbD in *E. coli* is dispensable for growth under normal laboratory growth conditions, ScsB in *C. crescentus* is essential for growth, which was also recently reported in another study (56). We asked whether any of the three identified substrate proteins of ScsB are essential in *C. crescentus*. However, null mutants of each of the three genes still grow (data not shown). Interestingly, DsbD in *Neisseria meningitidis* has also been reported to be essential (57). *Neisseria* and *Caulobacter* share common membrane electron transport components which are not present in *E. coli*. These components involve the cytochrome bc_1 complex and the cytochrome *c* oxidase, which use cytochrome *c* as an electron acceptor and donor, respectively, for aerobic respiration. Interestingly, the synthesis of cytochrome *c* is known to require electrons donated by a periplasmic thioredoxin-like protein (CcmG). Therefore, it is likely that the nonviability of the *N. meningitidis dsbD* and Cc*scsB* mutants is caused by disruption of the cytochrome *c* synthesis pathway in these bacteria. Consistent with this hypothesis, *E. coli* uses the cytochrome *bd* or *bo* oxidase for aerobic respiration. These enzymes do not depend on cytochrome *c*, explaining why an *E. coli dsbD* null mutant is viable. To test this hypothesis, we asked whether *R. capsulatus* CcdA (RcCcdA) can complement the growth defect of a *scsB* mutant. The complementation was successful, which suggests that the essentiality of CcScsB is due to a defect in aerobic respiration (see Fig. S3 in the supplemental material). However, we cannot exclude the possibility of the existence of another, yet-unknown essential reductive pathway(s) which can receive electrons from RcCcdA.

MATERIALS AND METHODS

Strains and media. Strains and plasmids used in this work are listed in Table S2 in the supplemental material. *E. coli* cells were grown in NZ medium (7) at 37°C. When needed, 200 μg/ml of ampicillin, 30 μg/ml of chloramphenicol, and 100 μg/ml of spectinomycin were added. Expres-

sion of the genes was induced for 1 h by adding 0.2% L-arabinose or appropriate concentrations of isopropyl-β-d-thiogalactopyranoside (IPTG). *C. crescentus* cells were grown in PYE medium (58) at 30°C. When needed, media were supplemented with the following antibiotics (concentrations in liquid and solid media, in μg/ml); tetracycline (1 and 2), spectinomycin (25 and 100), chloramphenicol (2 and 1), and kanamycin (5 and 25). Expression of the genes was induced for 4 h by adding 0.2% D-xylose or 0.5 mM vanillate (Sigma)–NaOH (pH 7.5) (30).

Gene deletion in *C. crescentus*. To delete a gene in *C. crescentus*, we used a method described previously (59). A brief description can be found in the supplemental material.

Thiol-redox state analyses. To analyze the *in vivo* redox states of the proteins, free thiols were acid-trapped by trichloroacetic acid (TCA) and alkylated with AMS (Invitrogen) as previously described (12). When indicated, 50 mM DTT was added to reduce samples or AMS or DTT were not added to samples to obtain oxidized control proteins. Anti-Flag mouse monoclonal antibody (M2; Sigma) was used for Western blot analysis.

Thiol peroxidase assay using purified PprX and PrxL. To assess the *in vitro* thiol peroxidase activity of PprX and PrxL, we overexpressed the two proteins containing His tag but no signal sequences in *E. coli* and purified them using nickel affinity chromatography following the standard manufacturer's protocol (Novagen). Construction of the expression plasmids is described in the supplemental material.

To measure thiol peroxidase activity of PprX, a coupled assay using thioredoxin-1 (8 μM) and thioredoxin reductase (0.5 μM) from *E. coli* and 100 μM NADPH was used essentially as previously described (33). Briefly, various concentrations (5 to 500 μM) of H_2O_2 or cumene hydroperoxide were added to the 600-μl reaction mixtures. The reaction was initiated by the addition of 0.5 μM PprX. The NADPH consumption rate was measured at 340 nm. For each reaction, the rate of 340 nm absorbance change was corrected for the rate measured before addition of the PprX (typically 10% of the PprX-dependent rate). All the rates are the mean of at least two determinations. A more direct FOX assay to monitor the disappearance of peroxide (see Text S1 in the supplemental material) was used to detect any peroxidase activity of PrxL.

Bioinformatic analyses. To generate a phylogenetic tree of DsbD family proteins, DsbD, ScsB, and CcdA family members were randomly chosen from bacteria which are distantly related according to the phylogenetic classification of bacteria. The tree was constructed by the neighbor-joining method based on amino acid sequences using MEGA5 (60). For the sequence alignment, we used the internal MUSCLE alignment program from MEGA5.

STRING (http://string-db.org/) analysis was used to predict interacting proteins of a query protein. To determine the distribution of DsbD, CcdA, ScsB, PprX, ScsC, and PrxL in eubacteria, the "occurrence view" option in STRING analysis was used. To complement the STRING analysis for the distribution of homologues, protein-protein searches were performed using the algorithm PSI-BLAST (position-specific iterated blast) at the NCBI (61). The PSI-BLAST analysis was also used to obtain the amino acid sequence alignment of two specific proteins.

Secondary structures of proteins were predicted using PSIPRED (http://bioinf.cs.ucl.ac.uk/psipred/) (62). High-resolution structures of homologues of interest were found using Jpred3 analysis (http://www.compbio.dundee.ac.uk/www-jpred/) by an amino acid sequence query of a protein of interest. The presence of signal sequence or N-terminal transmembrane segment was analyzed using SignalP 3.0 (http://www.cbs.dtu.dk/services/SignalP/) or TMHMM 2.0 (http://www.cbs.dtu.dk/services/TMHMM-2.0/), respectively (63).

Zymogram for RNase activity. A zymogram to show RNase activity was performed according to a previous report (64). Briefly, cells expressing *C. crescentus* RNase I were harvested at mid-log phase, broken by sonication, and subjected to SDS-PAGE. Poly(C) (Sigma) as an RNA substrate was added to the solution for SDS-PAGE, and toluidine blue as an intercalating dye was added after electrophoresis to the gel-staining solution.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00291-11/-/DCSupplemental.

Text S1, DOC file, 0.1 MB.
Figure S1, PDF file, 2.3 MB.
Figure S2, PDF file, 0.5 MB.
Figure S3, PDF file, 1.1 MB.
Figure S4, PDF file, 0.4 MB.
Figure S5, PDF file, 0.7 MB.
Figure S6, PDF file, 0.8 MB.
Figure S7, PDF file, 0.1 MB.
Table S1, PDF file, 2.7 MB.
Table S2, DOC file, 0.1 MB.

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