Identification of Redox Partners of the Thiol-Disulfide Oxidoreductase SdbA in *Streptococcus gordonii*

Naif A. Jalal,^{a,b,e} Lauren Davey,^{a,b,e} Scott A. Halperin,^{a,b,c,e} Song F. Lee^{a,b,c,d,e}

^aDepartment of Microbiology and Immunology, Dalhousie University, Halifax, Nova Scotia, Canada

^bCanadian Center for Vaccinology, Dalhousie University, Halifax, Nova Scotia, Canada

^cDepartment of Pediatrics, Faculty of Medicine, Dalhousie University, Halifax, Nova Scotia, Canada

^dDepartment of Applied Oral Sciences, Faculty of Dentistry, Dalhousie University, Halifax, Nova Scotia, Canada

^eIzaak Walton Killam Health Centre, Halifax, Nova Scotia, Canada

ABSTRACT We previously identified a novel thiol-disulfide oxidoreductase, SdbA, in *Streptococcus gordonii* that formed disulfide bonds in substrate proteins and played a role in multiple phenotypes. In this study, we used mutational, phenotypic, and biochemical approaches to identify and characterize the redox partners of SdbA. Unexpectedly, the results showed that SdbA has multiple redox partners, forming a complex oxidative protein-folding pathway. The primary redox partners of SdbA that maintain its active site in an oxidized state are a surface-exposed thioredoxin family lipoprotein called SdbB (Sgo_1171) and an integral membrane protein annotated as CcdA2. Inactivation of *sdbB* and *ccdA2* simultaneously, but not individually, recapitulated the *sdbA* mutant phenotype. The *sdbB-ccdA2* mutant had defects in a range of cellular processes, including autolysis, bacteriocin production, genetic competence, and extracellular DNA (eDNA) release. AtIS, the natural substrate of SdbA produced by the *sdbB-ccdA2* mutant lacked activity and an intramolecular disulfide bond. The redox state of SdbA in the *sdbB-ccdA2* mutant was found to be in a reduced form and was restored when *sdbB* and *ccdA2* were knocked back into the mutant. In addition, we showed that SdbB formed a disulfide-linked complex with SdbA in the cell. Recombinant SdbB and CcdA2 exhibited oxidase activity and reoxidized reduced SdbA *in vitro*. Collectively, our results demonstrate that *S. gordonii* uses multiple redox partners for oxidative protein folding.

IMPORTANCE *Streptococcus gordonii* is a commensal bacterium of the human dental plaque. Previously, we identified an enzyme, SdbA, that forms disulfide bonds in substrate proteins and plays a role in a number of cellular processes in *S. gordonii*. Here, we identified the redox partners of SdbA. We showed that SdbA has multiple redox partners, SdbB and CcdA2, forming a complex oxidative protein-folding pathway. This pathway is essential for autolysis, bacteriocin production, genetic competence, and extracellular DNA (eDNA) release in *S. gordonii*. These cellular processes are considered to be important for the success of *S. gordonii* as a dental plaque organism. This is the first example of an oxidative protein-folding pathway in Gram-positive bacteria that consists of an enzyme that uses multiple redox partners to function.

KEYWORDS CcdA, *S. gordonii*, SdbA, thiol-disulfide oxidoreductases, disulfide bond, redox partners

The formation of proper disulfide bonds is important for the correct folding, stability, and activity of extracytoplasmic proteins in bacteria (1). These proteins include secreted toxins and proteins of secretion systems, flagella, and pili (2). In bacteria, the formation and breakage of disulfide bonds are catalyzed by a group of enzymes named

Citation Jalal NA, Davey L, Halperin SA, Lee SF. 2019. Identification of redox partners of the thiol-disulfide oxidoreductase SdbA in *Streptococcus gordonii*. J Bacteriol 201:e00030-19. <https://doi.org/10.1128/JB.00030-19>.

Editor William W. Metcalf, University of Illinois at Urbana Champaign

Copyright © 2019 American Society for Microbiology. All Rights Reserved.

Address correspondence to Song F. Lee, song.lee@dal.ca.

Received 10 January 2019

Accepted 18 February 2019

Accepted manuscript posted online 25 February 2019

Published 24 April 2019

thiol-disulfide oxidoreductases (TDORs) (3). TDORs are well characterized in the Gram-negative bacterium *Escherichia coli*. DsbA-DsbB forms the major oxidative pathway that catalyzes disulfide bond formation in *E. coli* (4, 5). In this pathway, the soluble periplasmic oxidase DsbA introduces disulfide bonds in newly translocated substrate proteins via its active site CXXC motif (2). The reaction results in a disulfide-bonded substrate protein and a reduced DsbA. To perform another round of the reaction, the reduced DsbA needs to be reoxidized, and this is achieved by donating electrons to its redox partner, DsbB, which then transfers the electrons to the electron transport chain (2, 3). Approximately 300 extracytoplasmic proteins are predicted to be substrates of the DsbA-DsbB pathway (6). DsbA is a powerful oxidant, and it introduces disulfide bonds indiscriminately, resulting in nonnative disulfide bonds in substrates that have more than two cysteines (2). To correct these nonnative disulfide bonds, *E. coli* also has a disulfide bond isomerization system that consists of DsbC and DsbD, where DsbC functions as an isomerase and DsbD as a redox partner (7). In addition, the *E. coli* reductases DsbG and CcmG are important in protecting extracellular proteins from oxidation and in cytochrome *c* maturation, respectively (8).

In contrast to that in *E. coli*, disulfide bond formation has not been thoroughly investigated in Gram-positive bacteria (9, 10). Gram-positive bacteria are predicted to have a lower prevalence of disulfide-bonded proteins and a tendency to exclude cysteines from their extracytoplasmic proteins (11). Nevertheless, homologs of DsbA and DsbB exist in some Gram-positive bacteria such as *Bacillus subtilis* (BdbD and BdbC) (12). In contrast to DsbA and DsbB, BdbD and BdbC in *B. subtilis* have a limited number of substrates. To date, the only substrates of BdbD-BdbC that have been identified are the pseudopilin ComGC, the DNA translocation channel ComEC, and the osmoprotection membrane protein ProA (13). BdbC, together with a third enzyme called BdbB, is required for the production of the lantibiotic sublancin 168 (14). In addition to the Bdb proteins, *B. subtilis* possesses a reducing pathway that consists of an integral membrane protein, CcdA, which shows high homology to the membrane portion of DsbD (15). In *B. subtilis*, CcdA cooperates with ResA in cytochrome *c* maturation and with StoA in endospore production (16–18). Interestingly, *Bacillus anthracis* possesses two CcdA proteins, CcdA1 and CcdA2. While both are involved in cytochrome *c* maturation and virulence regulation, only CcdA2 is required for sporulation (19).

Similar to that in *B. subtilis*, *Staphylococcus aureus* DsbA is also essential for the stability of the pseudopilin ComGC; however, *S. aureus* appears to lack a DsbB homolog, and it is proposed that DsbA is reoxidized by extracellular oxidants (20–22).

Recently a TDOR named MdbA was characterized in *Corynebacterium diphtheriae*. MdbA has been shown to play an essential role in pilus assembly and diphtheria toxin production (23). Unlike DsbA, however, *C. diphtheriae* MdbA also has disulfide isomerase/reductase activity and is thought to maintain CueP (copper-binding protein) in the reduced active form (24). Similar to that in *C. diphtheriae*, *Actinomyces oris* MdbA is found to cooperate with its redox partner, the membrane protein vitamin K epoxide reductase (VKOR), to catalyze disulfide bond formation in the major pilin subunit FimA (25).

Recently, our laboratory identified a TDOR in *Streptococcus gordonii* named SdbA (*Streptococcus* *disulfide* *bond* protein *A*) that forms disulfide bonds in substrate proteins. *S. gordonii* is an inhabitant of the human oral cavity; it is considered a pioneer colonizer of the tooth surface, playing an important role in dental plaque formation (26). *sdbA* mutants are defective in autolysis, extracellular DNA (eDNA) release, bacteriocin production, and genetic competence but form more biofilm (27). SdbA shares little sequence homology to DsbA and BdbD. Homologs of SdbA appear to be present in a range of Gram-positive bacteria that lack DsbA (27). SdbA is able to introduce a disulfide bond into its natural substrate, the major autolysin AtlS. This can be achieved with a single C-terminal cysteine in its CPDC active site, further suggesting SdbA is quite different from DsbA (28). Interestingly, inactivation of *sdbA* upregulated the CiaRH two-component regulatory system in *S. gordonii*, leading to the repression of the

FIG 1 Organization of genes coding for redox partners of SdbA in *S. gordonii*. (A) Sequence alignment of CcdA1 and CcdA2. The two conserved cysteines are boxed. Predicted transmembrane regions are underlined (<http://www.cbs.dtu.dk/services/TMHMM-2.0>). (B) Sequence alignment of SdbB and Sgo_1177 using Clustal Omega (49). The predicted CXXC active site and the conserved *cis*-proline residue are boxed. (C) A schematic showing the genetic loci of *ccdA1* and *ccdA2* with their adjacent genes. HK and RR, predicted histidine kinase and response regulator of a two-component regulatory system. Downstream of *ccdA1* and *ccdA2* are *sgo_1177* and *sdbB* (*sgo_1171*), respectively. Bent arrows, predicted promoters (http://www.fruitfly.org/seq_tools/promoter.html); lollipops, predicted rho-independent terminators (http://transterm.cbcb.umd.edu/tt/Streptococcus_gordonii_Challis_substr_CH1.tt); small arrows, primers used for RT-PCR analysis. The sizes of the DNA fragments generated by the primer pairs are indicated. (D) Transcript analysis of the *sgo_1177-ccdA1* and *sdbB-ccdA2* operons by RT-PCR. PCR fragments generated from cDNA using the indicated primer combinations (bottom) are shown. The predicted sizes of these fragments are indicated in panel B. (E) Transcript analysis of *sgo_1170* and *sgo_1174* by qRT-PCR in the parent (SecCR1), *sdbB-ccdA2* mutant, and *sdbB-ccdA2* knock-in mutant. Results are means $\pm$ standard deviations (SDs) from three experiments. NS, no significant difference.

ComDE quorum sensing system, which resulted in the enhanced biofilm formation and the lack of bacteriocin production (29, 30).

Here, we report the identification and characterization of the SdbA redox partners SdbB and CcdA2 in *S. gordonii*. The results indicate that SdbB together with CcdA2 constitutes the main pathway for SdbA reoxidation.

RESULTS

Identification and genetic organization of SdbA redox partners. Our initial approach to find the redox partner of SdbA was to perform a BLAST search of the sequenced genome of *S. gordonii* using *E. coli* DsbB and *B. subtilis* BdbC as queries. The search yielded no potential candidates, even under low stringency conditions. Because SdbA has homology to ResA from *B. subtilis* and ResA interacts with the integral membrane protein CcdA (16), we performed an additional search using *B. subtilis* CcdA as the query. The search found two CcdA proteins, CcdA1 and CcdA2, in *S. gordonii*. CcdA1 and CcdA2 show 23.7% and 27.1% sequence identity, respectively, to the transmembrane domain of *E. coli* DsbD (31). CcdA1 and CcdA2 are predicted to be integral membrane proteins with six transmembrane regions. CcdA1 and CcdA2 both consist of 236 amino acids and are highly homologous, with 69.8% sequence identity (Fig. 1A; see also Fig. S1 in the supplemental material).

Examination of the genetic locus of *ccdA1* revealed that it has two genes located immediately downstream, namely, *sgo_1177* and *sgo_1176*, which are annotated as a TDOR gene and a methionine sulfoxide reductase (MsrAB) gene, respectively (Fig. 1C). The *ccdA2* locus contains one gene, *sgo_1171*, immediately downstream that is anno-

TABLE 1 Bacterial strains and plasmids used in this study

Strain, genotype, or plasmid	Relevant characteristics	Source or reference
Strains		
<i>S. gordonii</i> Challis DL-1		
SecCR1	<i>hpgG::tet</i> , secretes anti-CR1 scFv, Tet ^r Spec ^r	44
<i>sdbA</i>	SdbA-negative mutant in SecCR1, <i>sdbA::ermAM</i>	27
<i>ccdA1</i>	CcdA1-negative mutant in SecCR1, <i>ccdA1::aphA3</i>	This study
<i>ccdA2</i>	CcdA2-negative mutant in SecCR1, <i>ccdA2::ermAM</i>	This study
<i>sdbB</i>	SdbB-negative mutant in SecCR1, <i>sgo_1171::ermAM</i>	27
<i>sgo_1177</i>	SGO_1177-negative mutant in SecCR1, <i>sgo_1177::ermAM</i>	27
<i>sgo_1177 ccdA1</i>	SGO_1177 and CcdA1-negative mutant in SecCR1, <i>sgo_1177::ermAM ccdA1::aphA3</i>	This study
<i>sdbB ccdA2</i>	SdbB and CcdA2-negative mutant in SecCR1, <i>sgo_1171::aphA3 ccdA2::ermAM</i>	This study
<i>sdbB ccdA2</i> knock-in	<i>sdbB</i> and <i>ccdA2</i> gene knock-in the <i>sdbB-ccdA2</i> mutant, Cm ^r	This study
<i>sdbB ccdA1</i>	SdbB and CcdA1-negative mutant in SecCR1, <i>sgo_1171::ermAM ccdA1::aphA3</i>	This study
<i>sgo_1177 ccdA2</i>	SGO_1177 and CcdA2-negative mutant in SecCR1, <i>sgo_1177::aphA3 ccdA2::ermAM</i>	This study
<i>ccdA1 ccdA2</i>	CcdA1 and CcdA2-negative mutant in SecCR1, <i>ccdA1::aphA3 ccdA2::ermAM</i>	This study
<i>sdbB sgo_1177</i>	SdbB and SGO_1177-negative mutant in SecCR1, <i>sgo_1171::ermAM sgo_1177::aphA3</i>	This study
<i>E. coli</i>		
M15	Protein expression host, pRE4 Kan ^r	Qiagen
M15-SdbB	M15 carrying pQE-30-SdbB, Kan ^r Amp ^r	This study
M15-Sgo_1177	M15 carrying pQE-30-Sgo_1177, Kan ^r Amp ^r	This study
XL1-Blue	Cloning host, Tet ^r	Stratagene
XL1-Blue-SdbA	XL1-Blue carrying pQE-30-SdbA, Tet ^r Amp ^r	27
XL1-Blue-CcdA2	XL1-Blue carrying pQE-30-CcdA2, Tet ^r Amp ^r	This study
Plasmids		
pQE-30-SdbA	<i>sdbA</i> cloned into BamHI and HindIII sites on pQE-30, His ₆ tag	27
pQE-30-SdbB	<i>sdbB</i> cloned into BamHI and HindIII sites on pQE-30, His ₆ tag	This study
pQE-30-Sgo_1177	<i>sgo_1177</i> cloned into BamHI and HindIII sites on pQE-30, His ₆ tag	This study
pQE-30-CcdA2	<i>ccdA2</i> cloned into BamHI and EcoRV sites on pQEDegP, N- and C-terminal His ₆ , C-terminal HA tag	This study

tated as another TDOR gene. We previously identified *Sgo_1171* and *Sgo_1177* as homologs of SdbA that contained the characteristic Cys-X-X-Cys active site of TDORs and the conserved *cis*-proline residue (27) (Fig. 1B).

In silico analysis identified predicted promoters and rho-independent transcription terminators on the *ccdA1* and *ccdA2* loci. For the *ccdA1* locus, the promoter and terminator were located upstream of *sgo_1181* and downstream of *sgo_1176* (*msrAB*), respectively (Fig. 1C). The promoter and terminator of the *ccdA2* locus were located upstream and downstream of *sgo_1175* and *sgo_1171*, respectively. To confirm that these genes form operons, reverse transcription-PCR (RT-PCR) was carried out using primers between two adjacent genes. The results showed that *sgo_1181*, *sgo_1180*, *ccdA1*, *sgo_1177*, and *sgo_1176* form a five-gene operon, while *sgo_1175*, *sgo_1174*, *ccdA2*, and *sgo_1171* form a four-gene operon (Fig. 1D). The results are in agreement with a recent study showing that *sgo_1180*, *ccdA1*, *sgo_1177*, and *sgo_1176* were cotranscribed and that *sgo_1175*, *sgo_1174*, and *ccdA2* were cotranscribed (32).

Based on the findings described below, we named *Sgo_1171* SdbB (*Streptococcus* disulfide bond protein B).

The *sdbB-ccdA2* double-gene mutant exhibits a pleiotropic mutant phenotype identical to that of the *sdbA* mutant. To help determine if any of the genes identified above might be a redox partner of SdbA, we systematically inactivated these genes, creating single-gene and double-gene mutants (Table 1). We reasoned that inactivation of the SdbA redox partner would produce the same phenotype as the *sdbA* mutant. Thus, the mutants were tested for *sdbA*-associated phenotypes, namely, autolysis, eDNA release, bacteriocin production, and genetic competence.

None of the single-gene mutants displayed any defect in eDNA release, bacteriocin production, or genetic competence. The *ccdA1* and *ccdA2* single-gene mutants were partially defective in autolysis, and the *sdbB* mutant was slightly defective in autolysis (Fig. 2).

Interestingly, five of the double-gene mutants displayed some phenotypes similar to that of the *sdbA* mutant. In particular, the *sdbB-ccdA2* mutant produced all of the

FIG 2 Phenotypic analysis of single- and double-gene mutants of *S. gordonii*. (A) Autolysis of single-gene mutants. The parent strain and *sdbA* mutant served as controls. (B) Autolysis of double-gene mutants compared to that of the parent and *sdbA* mutant. (C) Extracellular DNA (eDNA) release by the mutants analyzed on a 0.8% agarose gel. (D) Bacteriocin production as assessed by growth inhibition of the target strain *S. mitis*. (E) Transformation frequency. In panels A, B, D, and E, results are means $\pm$ SDs from three independent experiments. Results in panel C are representative of at least three separate experiments. *, $P < 0.05$; SecCR1, parent strain.

phenotypes displayed by the *sdbA* mutant (Fig. 2). The *sdbB-sgo_1177* mutant was defective in eDNA release and bacteriocin production but not autolysis or genetic competence. The *sdbB-ccdA1* mutant was defective in autolysis but not eDNA release, bacteriocin production, or genetic competence. The *sgo_1177-ccdA2* mutant was partially defective in autolysis but not in other phenotypes. The *ccdA1-ccdA2* mutant was defective only in bacteriocin production.

Thus, it is clear from the above results that the only mutant that duplicated the phenotypes observed in the *sdbA* mutant is the *sdbB-ccdA2* mutant, suggesting that SdbB and CcdA2 may be redox partners of SdbA. The phenotypes exhibited by the *sdbB-ccdA2* mutant were reversed when functional copies of *sdbB* and *ccdA2* were knocked back into the same location on the chromosome (Fig. 2). Quantitative real-time PCR (qRT-PCR) analysis of the *sdbB-ccdA2* mutant showed that the genes immediately upstream (*sgo_1174*) and downstream (*sgo_1170*) of the mutated genes were transcribed, indicating that the mutation did not affect the expression of adjacent genes (Fig. 1E). The levels of *sgo_1174* and *sgo_1170* expression were similar between the parent, *sdbBccdA2* mutant, and *sdbB-ccdA2* knock-in mutant. The above results established that SdbB and CcdA2 are responsible for the *sdbA*-associated phenotypes.

AtIS, the natural substrate of SdbA, in the *sdbB-ccdA2* mutant lacks activity and a disulfide bond. Previously, we showed that the major autolysin AtIS is a natural substrate of SdbA (27). SdbA forms an intramolecular disulfide bond between the two cysteine residues in AtIS that are required for proper folding and autolytic activity (27). Thus, examination of the activity and redox state of AtIS would provide evidence of the role of redox partners in protein oxidation by SdbA.

FIG 3 The major autolysin AtIS is inactive and lacks a disulfide bond in the *sdbB-ccdA2* mutant. (A, left) Zymogram analysis shows autolytic activity of the autolysin AtIS. The zymogram is shown as an inverted image of a digital scan. (Right) Western blotting showing total AtIS in samples used in zymogram analysis. (B) Alkylation of AtIS. AtIS in samples were alkylated with maleimide-PEG₂-biotin and detected by either avidin-alkaline phosphatase (AP) or anti-AtIS for the total amount of AtIS. From each strain, a positive control was prepared by reducing the sample with DTT prior to alkylation. (C) Densitometry analysis of AtIS alkylation using ImageJ. The intensity of the 130-kDa AtIS band detected by avidin-AP is divided by that detected by anti-AtIS. Results are means $\pm$ SDs from three independent experiments. NS, no significant difference.

The activity of AtIS was examined by the zymogram assay. The parent strain showed AtIS activity bands at 130 kDa (intact) and 90 kDa (processed), and the *sdbA* mutant showed a much-diminished activity band consistent with previous findings (27, 28). The *sdbB-ccdA2* and *sdbB-ccdA2-degP* mutants also showed reduced activity (Fig. 3A). The

reason for including the triple mutant *sdbB-ccdA2-degP* in the analysis was to ensure that the results were not due to the different levels of SdbA in the mutants. Our results below (see Fig. 4) showed that the level of SdbA in the *sdbB-ccdA2* mutant was reduced, and in the *sdbB-ccdA2-degP* mutant, it was comparable to that in the parent strain. Western blotting results confirmed that AtIS was produced by the *sdbB-ccdA2*, *sdbB-ccdA2-degP*, and *sdbA* mutants. Western blotting results also revealed that the AtIS protein in the *sdbB-ccdA2* and *sdbB-ccdA2-degP* mutants was not processed similar to that in the *sdbA* mutant. When *sdbB* and *ccdA2* were returned to the *sdbB-ccdA2* mutant, AtIS activity and processing were restored (Fig. 3A). These results confirmed that the lack of autolysis in the *sdbB-ccdA2* mutant was due to a defect in the activity of AtIS.

Next, we investigated the redox state of AtIS. AtIS from the parent strain was alkylated by maleimide-polyethylene glycol (PEG₂)-biotin and detected as a 130-kDa protein. The intensity of the band was markedly increased in samples reduced with dithiothreitol (DTT) prior to alkylation, indicating that AtIS from the parent strain contains a disulfide bond (Fig. 3B and C). In contrast, the intensity of the 130-kDa AtIS band from the *sdbB-ccdA2* and *sdbB-ccdA2-degP* mutants remained the same with or without DTT treatment, suggesting that AtIS from these mutants lacked a disulfide bond. The same was observed in the *sdbA* mutant.

The above results showed that SdbB or CcdA2 is required for disulfide bond formation in AtIS, the natural substrate of SdbA.

SdbA is in a reduced state in the *sdbB-ccdA2* mutant. We next sought to determine the redox state of SdbA in *S. gordonii*. We reasoned that the inactivation of the redox partners of SdbA would have a direct impact on the redox state of SdbA in the cells. Proteins from the parent and mutant strains were prepared, alkylated, and analyzed in immunoblots. The results showed that SdbA from the parent strain was present as an oxidized protein and that it became reduced after DTT treatment (Fig. 4A). In contrast, SdbA from the *sdbB-ccdA2* mutant was present in a reduced form. *sdbB-ccdA2* knock-in restored SdbA to the oxidized form.

We noted that the amount of SdbA in the *sdbB-ccdA2* mutant was low when compared to that in the parent and knock-in strains. We previously showed that the serine protease DegP degraded misfolded anti-CR1 single-chain antibody, a protein that requires two disulfide bonds for stability in *S. gordonii* (27, 28). Thus, we mutated *degP* in the *sdbB-ccdA2* mutant and also in the parent strain as a control. Inactivation of *degP* in the *sdbB-ccdA2* mutant returned SdbA to a level similar to that in the parent and knock-in strains (Fig. 4B). Importantly, SdbA in the *sdbB-ccdA2-degP* mutant remained reduced. The *degP* mutation in the parent strain did not change the redox state of SdbA, indicating that the alteration of SdbA redox state in the *sdbB-ccdA2-degP* mutant was not due to *degP* mutation. SdbA in the single-gene mutants (*sdbB* and *ccdA2* mutants) was present as an oxidized protein (Fig. 4C).

SdbA_{C89A} variant forms mixed disulfide with SdbB *in vivo*. To provide direct evidence that SdbA and SdbB interacted in *S. gordonii*, we employed an *sdbA*_{C89A} Δ *degP* mutant for the detection of the SdbA-SdbB complex. We reported previously that the single-cysteine active site variant, SdbA_{C89A}, formed a number of mixed disulfide complexes in this mutant (28). Proteins were extracted from the cells and analyzed by Western blotting using anti-SdbA and anti-SdbB antibodies as probes. As shown in Fig. 5A, a number of complexes were detected by the antibodies. A band of ca. 40 kDa, the expected size of the SdbA (21.9 kDa)-SdbB (17.8 kDa) complex, was detected by both antibodies. This 40-kDa band as well as other complexes dissociated under reducing conditions indicated that they were disulfide-linked proteins (Fig. 5B).

To provide further evidence that the 40-kDa band was an SdbA-SdbB complex, immunoprecipitation using anti-SdbB antibody-coupled beads was performed. The eluate was analyzed by Western blotting. The results showed that eluate contained the 40-kDa band that was recognized by both the anti-SdbA and anti-SdbB antibodies (Fig. 5C). The eluate also contained the SdbB monomer and a weak 37-kDa band, which

FIG 4 The redox state of SdbA in *S. gordonii* parent and mutant strains. SdbA in *S. gordonii* strains was alkylated with maleimide (Mal) and analyzed by immunoblotting using anti-SdbA antibody as the probe. Alkylation adds 0.5 kDa per thiol to reduced SdbA, causing an upshift in migration, while oxidized SdbA will not be alkylated and migrated faster during electrophoresis. SecCR1, parent strain; SdbA_{oxi}, oxidized form of SdbA; SdbA_{red}, reduced form of SdbA; DTT, dithiothreitol. Arrowheads indicate the position of reduced or oxidized SdbA. Results are representative of three independent experiments.

might be the SdbB homodimer. The blot probed with anti-SdbA showed the 40-kDa band as well as an unknown 28-kDa band. Nonetheless, the recovery of the 40-kDa band by immunoprecipitation strongly indicates that it was an SdbA-SdbB complex.

SdbB and CcdA2 possess oxidase activities. The above results collectively suggest that SdbB and CcdA2 are redox partners of SdbA. For these proteins to function as a redox partner of SdbA, they must possess oxidase activities. To provide support of this notion, recombinant SdbB and CcdA2 were produced and purified from *E. coli* (Fig. 6A) and assayed in the RNase A refolding assay (Fig. 6B). Recombinant SdbA was also purified and assayed as a positive control. The results clearly showed that SdbB and CcdA2 were able to refold denatured and reduced RNase A, indicating oxidase activities. We further examined the ability of SdbB and CcdA2 to reoxidize SdbA in a disulfide exchange reaction. The results showed that SdbB could reoxidize reduced SdbA (Fig. 6C). Similarly, CcdA2 was able to reoxidize SdbA (Fig. 6D). This reoxidation of SdbA required oxidized SdbB or CcdA2 and was not a result of spontaneous oxidation, since reduced SdbA alone (see Fig. S2A) or incubation with reduced SdbB (Fig. S2B) or reduced CcdA2 (Fig. S2C) did not result in reoxidation of SdbA. Collectively, these results indicate that SdbB and CcdA2 possess the enzymatic capability to reoxidize SdbA.

FIG 5 SdbB forms a disulfide-linked complex with SdbA_{C89A} in *S. gordonii*. (A) Nonreducing blots of proteins prepared from *S. gordonii* SdbA_{C89A} probed with anti-SdbA and anti-SdbB antibodies. (B) Reducing blots of proteins prepared from *S. gordonii* SdbA_{C89A} probed with anti-SdbA and anti-SdbB antibodies. (C) Proteins recovered from immunoprecipitation (IP) were analyzed by Western blotting. Results are representative of at least three independent experiments. M, prestained protein markers; *, SdbA-SdbB complex; >, SdbA monomer; o, SdbB monomer; ?, unknown protein.

DISCUSSION

Despite several TDORs having now been identified in a number of Gram-positive species, the oxidative protein-folding pathway in Gram-positive bacteria remains poorly understood. We previously identified a major TDOR, SdbA, in *S. gordonii* that introduces

FIG 6 SdbB and CcdA2 exhibit oxidase activity. (A) SDS-PAGE of recombinant SdbA, SdbB, and CcdA2 purified from *E. coli*. M, prestained protein markers. (B) Oxidative folding of reduced denatured RNase A by SdbB and CcdA2. SdbA was included as a positive control, and reduced denatured RNase A alone was used as a negative control. The data are means $\pm$ SDs from three separate experiments. (C) Oxidation of reduced SdbA by oxidized SdbB. The reoxidation of SdbA was not a result of spontaneous oxidation, since reduced SdbA alone (see also Fig. S2A in the supplemental material) or incubation with reduced SdbB did not result in the reoxidation of SdbA (Fig. S2B). (D) Oxidation of reduced SdbA by oxidized CcdA2. Reoxidation of SdbA did not occur when it was incubated with reduced CcdA2 (Fig. S3C). Results in panels C and D are representative of three independent experiments.

disulfide bonds in substrate proteins and plays a role in multiple phenotypes in this bacterium (9, 27).

In the present study, we have identified the redox partners of SdbA. The results show that SdbB and CcdA2 are the primary redox partners of SdbA. Several lines of evidence support this statement. First, mutational results showed that the *sdbB-ccdA2* mutant duplicated phenotypes exhibited by the *sdbA* mutant, and *sdbB-ccdA2* knock-in restored the parental phenotypes. Second, SdbA was locked in a reduced state when *sdbB* and *ccdA2* were inactivated, and knock-in returned SdbA to the oxidized state as in the parent strain. Third, SdbB formed a mixed disulfide with SdbA_{C89A} in the cells. Fourth, AtfS, the natural substrate of SdbA, was in a reduced state in the *sdbB-ccdA2* mutant. And fifth, SdbB and CcdA2 possess oxidase activities capable of refolding RNase A and reoxidizing SdbA *in vitro*. Collectively, these results strongly support that SdbB and CcdA2 are the redox partners of SdbA.

Our results showed that mutation of *sdbB* or *ccdA2* alone did not result in *sdbA* mutant phenotypes; mutation of both genes was needed to reproduce the *sdbA* mutant phenotypes. This suggests that SdbB and CcdA2 can both serve as independent redox partners of SdbA. Accordingly, SdbA remains in an oxidized state in the *sdbB* and *ccdA2* single-gene mutants. *In vitro*, both SdbB and CcdA2 reoxidized SdbA independently, supporting the notion that SdbB and CcdA2 are independent redox partners of SdbA. These results indicate that the protein-folding pathway in *S. gordonii* is quite complex and very different from the two-protein pathway (DsbA-DsbB) of *E. coli* (33) or the MdbA-VKOR pathway of *A. oris* (25, 33).

It is interesting that a second set of proteins, SGO_1177 and CcdA1, which are homologous to SdbB and CcdA2, respectively, appear to have limited ability to serve as redox partners of SdbA. The *sgo_1177-ccdA1* mutant failed to reproduce the *sdbA* mutant phenotypes except bacteriocin production. However, it remains possible that SGO_1177 and CcdA1 can serve as minor partners of SdbA in the absence of SdbB or CcdA2. Because of this cross-interaction, the phenotypes of some of the cross-operon mutants are difficult to explain.

SdbB and SGO_1177 are predicted to belong to the thioredoxin family of redox proteins with a typical active site CXXC motif. As demonstrated in this study, SdbB exhibited oxidase activity and the ability to reoxidize SdbA. SGO_1177 also has the ability to refold denatured and reduced RNase A (data not shown); however, it may play a role in oxidative stress resistance by maintaining methionine sulfoxide reductase (Msr) in its reduced form. The genetic organization of the *sgo_1177* operon closely resembles the recently characterized *ccdA1-etrx1* (*sgo_1177* homolog)-*msrAB* operon in *Streptococcus pneumoniae* (34). In *S. pneumoniae*, the *etrx1* operon plays an important role in oxidative stress resistance.

CcdA1 and CcdA2 are annotated as cytochrome *c* biogenesis protein A. In *B. subtilis*, CcdA was part of the reducing pathway required for cytochrome *c* maturation and sporulation (15, 18). Given that streptococci lack cytochrome *c* and do not sporulate, it is unlikely that they play a role in these two processes. Both CcdA1 and CcdA2 contain two conserved cysteines separated by two transmembrane domains, a feature that is similar to the β domain of the *E. coli* DsbD. In *E. coli*, electrons from thioredoxin flow to the β domain of DsbD initially during the reduction of DsbC in the isomerization pathway (33). A recent study showed that the archaeal CcdA is capable of relaying electrons from thioredoxins across the cytoplasmic membrane in a substrate-specific pathway (35). Hence, it is conceivable that CcdA2 is capable of receiving electrons from SdbA or SdbB, though these are extracytoplasmic proteins. In agreement with this notion, our results showed that CcdA2 is able to reoxidize reduced SdbA. The phenotypic analysis results of the *sdbB-ccdA2* mutant suggested a more active role of CcdA2 in SdbA reoxidation *in vivo*. The novelty of our results is the oxidase activity possessed by CcdA2. To the best of our knowledge, this is the first report of such an activity displayed by CcdA-like proteins. This suggests a potential role of CcdA proteins as interchangeable modules in different disulfide exchange pathways connecting the oxidation and the reduction pathways.

The roles of SdbB and CcdA2 in oxidative protein folding are further demonstrated in the examination of the redox state and activity of AtIS, the natural substrate of SdbA. The results showed that AtIS from the *sdbB-ccdA2* mutant lacked a disulfide bond and was inactive. These results suggest that SdbB and CcdA2 play critical roles in working with SdbA in the oxidative folding of AtIS.

Our results showed that SdbB and CcdA2 are required to maintain SdbA in an oxidized state in the cell. This statement is supported by the findings that inactivation of *sdbB* and *ccdA2* resulted in a reduced SdbA, and SdbA was restored to the oxidized form upon the return of *sdbB* and *ccdA2* genes back into the mutant. The conversion of SdbA from an oxidized to a reduced state is achieved only by the inactivation of *sdbB* and *ccdA2* simultaneously but not individually, which lends support to the notion that SdbB and CcdA2 are independent redox partners of SdbA. It is interesting to note that the level of SdbA was lower in the *sdbB-ccdA2* mutant than in the parent and knock-in strains. Inactivation of the serine protease DegP restored the SdbA level in the *sdbB-ccdA2* mutant to a level similar to those of the parent and knock-in strains. These results suggest that the reduced form of SdbA is unstable and prone to degradation in the cell and may explain why only oxidized SdbA was detected in the parent strain. This stability issue helps to explain the need of multiple redox partners to ensure SdbA is maintained in the most stable (i.e., oxidized) form in *S. gordonii*.

To provide further evidence that SdbB is a redox partner, we investigated the formation of disulfide-linked complexes between SdbB and the active site variant SdbA_{C89A} in the cell. We previously showed that SdbA_{C89A} formed mixed disulfide complexes and that one of the complexes was SdbA_{C89A} and its natural substrate AtIS (28). Here, we show that SdbA_{C89A} formed a disulfide-linked complex with SdbB. Oxidoreductases with mutations to the C-terminal cysteine of the CXXC motif, including DsbA (36), protein disulfide isomerase (PDI) (37), and thioredoxin (38), can form disulfide-linked complexes with their redox partners and substrates. Thus, our results are consistent with the literature. We were not able to investigate complex formation between CcdA2 and SdbA because of the lack of a specific anti-CcdA2 antibody. CcdA2 and CcdA1 share a high sequence homology, and distinguishing reactions to CcdA2 but not CcdA1 would require a highly specific antibody such as a monoclonal antibody.

Homologs of SdbB CcdA2 and Sgo_1177 CcdA1 are found in other Gram-positive bacteria (*Streptococcus pneumoniae*, *Streptococcus sanguinis*, and *Clostridium botulinum*) that lack a DsbA homolog (see Fig. S3 in the supplemental material). The roles of these proteins in these bacteria are largely unknown, except in *S. pneumoniae*, where they are involved in a reducing pathway coping with oxidative stress (34).

In conclusion, we have shown that SdbA has multiple redox partners, forming a complex oxidative protein-folding pathway in *S. gordonii* (Fig. 7). We propose that SdbA is the oxidase that introduces disulfide bonds in protein substrates. This results in a disulfide-bonded substrate protein and a reduced SdbA. SdbB pairs up with CcdA2 to reoxidize SdbA, allowing SdbA to continue its function. The SdbA-SdbB and SdbA-CcdA2 pathways represent the main oxidative pathways affecting autolysis, bacteriocin production, genetic competence, and eDNA release.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Bacterial strains and plasmids used in this study are listed in Table 1. *S. gordonii* strains were grown at 37°C, 5% CO₂ in HTVG (per ml: 5 mg of glucose, 35 mg of tryptone, 0.04 μg of *p*-aminobenzoic acid, 0.2 μg of thiamine-HCl, 1 μg of nicotinamide, 0.2 μg of riboflavin, and 100 mM HEPES, pH 7.6) (39). For bacteriocin production and genetic competence assays, *S. gordonii* and *Streptococcus mitis* I18 strains were grown in brain heart infusion (BHI) broth. *E. coli* was grown at 37°C in Luria-Bertani broth (LB) with shaking. When needed, antibiotics were used at the following concentrations: for *S. gordonii*, spectinomycin (250 μg/ml), kanamycin (250 μg/ml), tetracycline (10 μg/ml), erythromycin (10 μg/ml), chloramphenicol (5 μg/ml), and rifampin (100 μg/ml); and for *E. coli*, ampicillin (100 μg/ml), kanamycin (50 μg/ml), and tetracycline (10 μg/ml).

Mutant construction. The TDOR single- and double-gene mutants were constructed by insertional inactivation. To achieve this, the erythromycin resistance gene (*ermAM*) (40) or kanamycin resistance gene (*aphA3*) (41) with its promoter was amplified by PCR using primers listed in Table S1 in the supplemental material. The upstream and downstream regions of the target gene were individually amplified by PCR and ligated to *ermAM* or *aphA3* in such a way that the antibiotic resistance gene was

FIG 7 A proposed model for oxidative protein-folding pathway in *S. gordonii*. SdbA is the oxidase that introduces a disulfide bond in substrate proteins. The reduced SdbA can be oxidized by CcdA2 or SdbB. This SdbA-CcdA2 or SdbA-SdbB pathway is the main oxidative pathway affecting autolysis, bacteriocin production, genetic competence, and eDNA release. The downstream partner of CcdA2 and SdbB is unknown and is depicted as question marks.

in the opposite orientation of the target gene. The ligation products were amplified using the outside primers. The resulting PCR products were transformed into the *S. gordonii* parent strain (SecCR1), and transformants were selected on BHI agar containing appropriate antibiotics. The insertion of the *aphA3* or *ermAM* cassette was confirmed by PCR.

Construction of knock-in mutant strain. To construct the *sdbB-ccdA2*-knock-in mutant, functional copies of *sdbB* and *ccdA2* genes were introduced back into the chromosome of the *sdbB-ccdA2* double mutant. To achieve this, the *sdbB-ccdA2* operon was amplified together with a portion of the upstream gene (*sgo_1174*) using primers SL1061/SL1235, and the PCR product was digested with SphI. A downstream fragment carrying portions of *sdbB* and *sgo_1170* was also amplified using primers SL869/SL1226 and digested with BamHI. Next, the chloramphenicol acetyltransferase (*cat*) resistance cassette was cut from pCopCAT/pUC18 using SphI and BamHI (42). The three fragments were then ligated using T4 DNA ligase, and the ligation product was amplified using the outside primers SL1061/SL1226. The resulting PCR product was used to transform into the *sdbB-ccdA2* double mutant. Transformation resulted in replacing the *ermAM* and *aphA3* cassettes with the functional *sdbB-ccdA2* genes and the *cat* cassette. Transformants were selected on BHI agar containing chloramphenicol and replica plated to identify kanamycin- and erythromycin-sensitive colonies. The reintroduction of the *sdbB* and *ccdA2* genes was verified by PCR.

Reverse transcription-PCR and quantitative real-time PCR. Total RNA was extracted from mid-exponential-phase *S. gordonii* cells using the hot acid phenol method (43). DNA was removed from RNA (1 μ g) samples by digestion with amplification grade DNase I (Life Technologies) for 15 min at room temperature. The resulting RNA samples were confirmed to be DNA-free by performing PCR for the 16S rRNA gene using primers SL525/SL697. cDNA was synthesized from the RNA using random primers and SuperScript II reverse transcriptase (Life Technologies) according to the manufacturer's instructions. The resulting cDNA was used as the template in RT-PCR for the analysis of the *sgo_1177-ccdA1* and *sdbB-ccdA2* operons. The cDNA was also used in qRT-PCR for the expression of *sgo_1170* and *sgo_1174* in the parent, *sdbB-ccdA2* mutant, and *sdbB-ccdA2*-knock-in mutant. qRT-PCR was performed using the PowerUp Sybr green master mix (Invitrogen) on an Applied Biosystems StepOne machine with a relative standard curve analysis. Each reaction was performed in duplicates using cDNA from at least three biological replicates. Results are presented as relative quantification ($2^{-\Delta\Delta CT}$).

Recombinant proteins. pQE-30 (Qiagen) was used as the vector to express recombinant SdbB with an N-terminal His₆ tag. Briefly, DNA coding for the mature SdbB was PCR amplified using primers

SL1157/SL1158, and the PCR product was cloned into the BamHI and HindIII sites of pQE-30. The cloning placed SdbB without the predicted N-terminal signal sequence behind the His₆ tag. The recombinant protein was produced in *E. coli* M15 and purified from cell lysates using affinity chromatography on His60 Ni Superflow columns (Clontech Laboratories Inc.) using a protocol similar to that previously described (27). Recombinant SdbA protein was purified from *E. coli* XL1-Blue as described previously (27). Anti-SdbB antibodies were raised in BALB/c mice using methods similar to those described previously for anti-SdbA antibodies (27). The usage of mice was approved by the Dalhousie University's Animal Care Committee.

Recombinant CcdA2 was produced in *E. coli* XL1-Blue. Briefly, the DNA coding for the entire CcdA2 was amplified by PCR using primers SL1096/SL1295, and the PCR product was digested with SpeI and ligated to similarly digested pSecCR1 (44). The ligation product was amplified using Phusion DNA polymerase (New England BioLabs) with the primers SL1096/SL334. The PCR product was digested with BamHI and cloned into the BamHI-EcoRV site of pQEDegP (pQE-30 with a *degP* insert; unpublished) generating pQE-30-CcdA2. The cloning placed *ccdA2* behind a His₆ tag and before a second His₆ and hemagglutinin (HA) tag sequences. The expression of recombinant CcdA2 was induced with 1 mM IPTG (isopropyl- β -D-thiogalactopyranoside), and the protein was found to localize to the cytoplasmic membrane. The isolation of CcdA2 was performed as described previously (35) with modifications. Briefly, cells in buffer 1 (50 mM sodium phosphate buffer, 300 mM NaCl, pH 8) were broken by sonication, and intact cells were removed by centrifugation ($5,000 \times g$ for 10 min). The broken cell fragments were subsequently pelleted by centrifugation ($27,000 \times g$ for 1 h at 4°C) and washed once with buffer 1. The resulting pellet was resuspended in buffer 1 containing 1% (wt/vol) *n*-dodecylphosphocholine (DPC; Anatrace) and incubated at 4°C for 2 h with gentle rotation. Insoluble material was removed by centrifugation ($27,000 \times g$ for 1 h). The solubilized proteins were applied to a His60 Ni Superflow column, which was equilibrated with buffer 1 containing 15 mM imidazole and 4.5 mM DPC. Following washing, the recombinant CcdA2 was eluted in 1-ml fractions using buffer 2 (20 mM sodium phosphate buffer, 40 mM NaCl, 200 mM imidazole, and 4.5 mM DPC, pH 7.5).

Phenotypic assays and zymographic analysis of autolysin. Analyses of eDNA release, bacteriocin production, genetic competence, and autolysis and zymographic analysis of AtlS were performed as described previously (27).

Determination of the redox state of AtlS. The redox state of AtlS was determined as described previously (27). Briefly, surface proteins were extracted from the parent, *sdbA* mutant, and *sdbB-ccdA2* mutant cells with 4% SDS. The proteins were then precipitated with 9% trichloroacetic acid (TCA) on ice for 30 min and then pelleted by centrifugation at $15,000 \times g$ for 10 min. The pelleted proteins were washed twice with cold acetone and resuspended in 100 mM Tris-HCl (pH 7.0) containing 1% (wt/vol) SDS and 5 mM maleimide-PEG₂-biotin (525 Da; American Peptide Co.). Proteins in the samples were alkylated by incubating at room temperature for 30 min, followed by 10 min at 37°C. Excess maleimide-PEG₂-biotin was then removed by TCA precipitation, and the protein pellets were resuspended in 100 mM Tris-HCl (pH 7.0), 1% (wt/vol) SDS, and 8 M urea. A positive control for each sample was prepared by incubating the sample with 100 mM DTT in 10 mM Tris-HCl (pH 8.1) for 30 min at room temperature before alkylation with maleimide-PEG₂-biotin. The samples were analyzed by Western blotting and reacted with avidin-alkaline phosphatase (Sigma-Aldrich) to detect biotinylated proteins; duplicate samples were reacted with anti-AtlS antisera (1:1,000) (27) as loading controls. The level of alkylated AtlS was analyzed by densitometry using ImageJ (45).

Preparation of oxidized and reduced recombinant proteins. To prepare fully oxidized SdbA and SdbB, proteins were incubated in the oxidizing buffer (50 mM oxidized glutathione, 100 mM Tris-HCl [pH 8.8], 200 mM KCl, 1 mM EDTA) for 1 h at room temperature. Samples were then dialyzed against 100 mM sodium phosphate buffer (pH 7) to remove the unreacted glutathione. CcdA2 was oxidized similarly but in the presence of 3 mM DPC. Excess glutathione was removed from CcdA2 using desalting columns (PD-10; Amersham) equilibrated with 50 mM sodium phosphate buffer (pH 7) containing 3 mM DPC.

To fully reduce the purified SdbA and SdbB, proteins were incubated with 50 mM DTT for 30 min on ice. After incubating, DTT was removed by passing the samples through PD-10 columns (46). Reduced CcdA2 was similarly prepared but in the presence of 3 mM DPC. The complete oxidation and reduction of the recombinant proteins was confirmed by reaction with Ellman's reagent (dithionitrobenzoic acid) and by alkylation with maleimide-PEG₂-biotin.

Oxidase activity assay. The oxidase activity of SdbA, SdbB, and CcdA2 was analyzed in the RNase A refolding assay as described previously (11, 27). In this assay, denatured and reduced RNase A was incubated with the test protein, and the activity of refolded RNase A to cleave cCMP was monitored at A_{296} .

The disulfide exchange reactions between SdbA and SdbB were performed as described previously with modifications (46). Briefly, equal molar amounts of the oxidized and reduced proteins (10 μ mol) were incubated in 1 ml of 50 mM sodium phosphate buffer (pH 8.0) with 100 mM NaCl and 0.5 mM EDTA at room temperature. Aliquots (200 μ l) of the reaction mixture were transferred to new tubes after 5, 15, 30, and 90 min, and the reaction was terminated by TCA precipitation. Samples were then alkylated with 5 mM maleimide-PEG₂-biotin as described above, and excess maleimide-PEG₂-biotin was removed by TCA precipitation and an acetone wash. Pellets were then resuspended in 100 mM Tris-HCl (pH 7.0) containing 1% (wt/vol) SDS and analyzed by Western blotting. Disulfide exchange reactions between CcdA2 and SdbA were performed as described above except 1 μ mol of each protein was used in the reactions. The rabbit anti-SdbA (27) antiserum was used for Western blot analysis.

Determination of the redox state of SdbA. Overnight cultures of *S. gordonii* grown in HTVG were diluted 1:10 and grown to mid-exponential phase of growth (optical density at 600 nm [OD₆₀₀] of 0.8). The cells were collected by centrifugation (4,000 × *g* for 10 min) and resuspended in 100 mM sodium phosphate buffer (pH 7.5) containing 2% SDS with or without 10 mM maleimide-PEG₂-biotin. The cells were incubated for 30 min at room temperature followed by 10 min at 37°C. The cell suspensions were then boiled for 5 min and centrifuged (12 000 × *g* for 10 min). The supernatant fluids, which contained the extracted SdbA, were analyzed by Western blotting using anti-SdbA (1:1,000) as the probe. To prepare a positive control, cells were incubated with 100 mM DTT in 10 mM sodium phosphate buffer (pH 7.5) for 1 h at room temperature, centrifuged to remove excess DTT, and then treated with maleimide-PEG₂-biotin and processed as stated above.

SdbA-SdbB complex preparation. To prepare sample containing the SdbA-SdbB complex, cells from 500 ml HTVG overnight cultures of the *S. gordonii* SdbA_{C89A} ΔDegP mutant (28) were pelleted by centrifugation (12,000 × *g* for 10 min), resuspended in 5 ml of 4% SDS, and incubated for 1 h at room temperature with slow rotation. The cell suspension was centrifuged (20,000 × *g* for 15 min), and the clear supernatant was saved. SDS was removed by incubating with 50 mM KCl on ice for 30 min followed by centrifugation (15,000 × *g* for 15 min at 4°C) (47). The sample was chromatographed onto a gel filtration column (Sephadex G-50, 2.5 cm by 100 cm) using 10 mM Tris-HCl (pH 7.5) plus 100 mM NaCl as the eluent. Fractions of 5-ml volumes were collected and analyzed by Western blotting using anti-SdbA antibodies as the probe. Fractions containing SdbA mixed disulfide complexes were pooled and used as the source for SdbA-SdbB complex analysis and immunoprecipitation (described below).

Anti-SdbB antibody purification and immunoprecipitation. To prepare the antibody for immunoprecipitation, anti-SdbB antiserum (3 ml) was precipitated with 40% saturation of (NH₄)₂SO₄, and the precipitated antibodies were dialyzed against phosphate-buffered saline (PBS; pH 7.4). The antibodies were then affinity purified using SdbB-coupled cyanogen bromide (CNBr)-activated Sepharose 4B Fast Flow beads (GE Healthcare), which were prepared as follows. Purified recombinant SdbB (5 mg) was coupled to CNBr-activated Sepharose (0.3 g) at 4°C with slow rotation for 18 h. The beads were then washed once with 0.1 M NaHCO₃-0.5 M NaCl (pH 8.3), and excess reactive sites were blocked with 0.1 M Tris-HCl (pH 8.0). The beads were then washed 5 times with 0.1 M acetic acid-0.5 M NaCl (pH 3.5) followed by 0.1 M Tris-HCl-0.5 M NaCl (pH 8.8). The SdbB-Sepharose beads were packed into a 3-ml syringe and used to affinity purify the anti-SdbB antibodies prepared above according to standard procedures (48). Briefly, the anti-SdbB antibodies were applied to the column followed by two washes with 10 mM Tris-HCl (pH 7.5) and 10 mM Tris-HCl (pH 7.5) plus 0.5 M NaCl. The anti-SdbB antibodies were eluted from the column with 100 mM glycine (pH 2.5) and neutralized immediately with 1 M Tris-HCl (pH 8). The eluted anti-SdbB antibodies were confirmed to contain activity against SdbB by enzyme-linked immunosorbent assay (ELISA). SDS-PAGE analysis of the eluted antibody showed 95% purity and a protein concentration of 276 μg/ml.

The affinity-purified anti-SdbB antibodies were used to prepare antibody-protein A magnetic beads (Sure Beads; Bio-Rad Laboratories) according to the manufacturer's instructions with modifications. Briefly, 36 μl of anti-SdbB (10 μg) was rotated with 1 mg of protein A magnetic beads in 200 μl PBS containing 0.1% Tween 20 (PBST) for 1 h at room temperature. The beads were magnetized, and unbound antibodies were removed followed by 3 washes with PBST. Triton X-100 (1% [vol/vol]) and EDTA (1 mM) were added to 500 μl of sample containing the SdbA-SdbB complex, and the mixture was rotated with the beads at 4°C for 16 h. The beads were magnetized, and unbound antigen was removed followed by 3 washes with PBST. SdbA-SdbB complex was eluted with 20 mM glycine (pH 2.0) at room temperature and neutralized immediately with 1 M sodium phosphate buffer (pH 7.4). The eluted SdbA-SdbB complex was analyzed by Western blotting as indicated in Results.

Statistical analysis. The results were analyzed using one-way analyses of variance (ANOVAs). A *P* value of <0.05 was considered statistically significant.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/JB.00030-19>.

SUPPLEMENTAL FILE 1, PDF file, 0.6 MB.

ACKNOWLEDGMENTS

Funding for this study was provided by the Natural Sciences and Engineering Research Council of Canada (NSERC). N.A.J. is a recipient of a postgraduate scholarship from the Saudi Cultural Bureau and Umm Al-Qura University.

REFERENCES

- Kadokura H, Beckwith J. 2010. Mechanisms of oxidative protein folding in the bacterial cell envelope. *Antioxid Redox Signal* 13:1231–1246. <https://doi.org/10.1089/ars.2010.3187>.
- Heras B, Shouldice SR, Totsika M, Scanlon MJ, Schembri MA, Martin JL. 2009. DSB proteins and bacterial pathogenicity. *Nat Rev Microbiol* 7:215–225. <https://doi.org/10.1038/nrmicro2087>.
- Kadokura H, Katzen F, Beckwith J. 2003. Protein disulfide bond formation in prokaryotes. *Annu Rev Biochem* 72:111–135. <https://doi.org/10.1146/annurev.biochem.72.121801.161459>.
- Bardwell JC, McGovern K, Beckwith J. 1991. Identification of a protein required for disulfide bond formation *in vivo*. *Cell* 67:581–589. [https://doi.org/10.1016/0092-8674\(91\)90532-4](https://doi.org/10.1016/0092-8674(91)90532-4).

5. Bardwell JC, Lee JO, Jander G, Martin N, Belin D, Beckwith J. 1993. A pathway for disulfide bond formation *in vivo*. *Proc Natl Acad Sci U S A* 90:1038–1042. <https://doi.org/10.1073/pnas.90.3.1038>.
6. Depuydt M, Messens J, Collet JF. 2011. How proteins form disulfide bonds. *Antioxid Redox Signal* 15:49–66. <https://doi.org/10.1089/ars.2010.3575>.
7. Rietsch A, Belin D, Martin N, Beckwith J. 1996. An *in vivo* pathway for disulfide bond isomerization in *Escherichia coli*. *Proc Natl Acad Sci U S A* 93:13048–13053. <https://doi.org/10.1073/pnas.93.23.13048>.
8. Denoncin K, Collet JF. 2013. Disulfide bond formation in the bacterial periplasm: major achievements and challenges ahead. *Antioxid Redox Signal* 19:63–71. <https://doi.org/10.1089/ars.2012.4864>.
9. Davey L, Halperin SA, Lee SF. 2016. Thiol-disulfide exchange in Gram-positive *Firmicutes*. *Trends Microbiol* 24:902–915. <https://doi.org/10.1016/j.tim.2016.06.010>.
10. Reardon-Robinson ME, Ton-That H. 2016. Disulfide-bond-forming pathways in Gram-positive bacteria. *J Bacteriol* 198:746–754. <https://doi.org/10.1128/JB.00769-15>.
11. Daniels R, Mellroth P, Bernsel A, Neiers F, Normark S, von Heijne G, Henriques-Normark B. 2010. Disulfide bond formation and cysteine exclusion in Gram-positive bacteria. *J Biol Chem* 285:3300–3309. <https://doi.org/10.1074/jbc.M109.081398>.
12. Bolhuis A, Venema G, Quax WJ, Bron S, van Dijk JM. 1999. Functional analysis of paralogous thiol-disulfide oxidoreductases in *Bacillus subtilis*. *J Biol Chem* 274:24531–24538. <https://doi.org/10.1074/jbc.274.35.24531>.
13. Goossens VJ, Mars RA, Akeroyd M, Vente A, Dreisbach A, Denham EL, Kouwen TR, van Rijk T, Olsthoorn M, van Dijk JM. 2013. Is proteomics a reliable tool to probe the oxidative folding of bacterial membrane proteins? *Antioxid Redox Signal* 18:1159–1164. <https://doi.org/10.1089/ars.2012.4664>.
14. Dorenbos R, Stein T, Kabel J, Bruand C, Bolhuis A, Bron S, Quax WJ, Van Dijk JM. 2002. Thiol-disulfide oxidoreductases are essential for the production of the lantibiotic sublancin 168. *J Biol Chem* 277:16682–16688. <https://doi.org/10.1074/jbc.M201158200>.
15. Schiott T, von Wachenfeldt C, Hederstedt L. 1997. Identification and characterization of the *ccdA* gene, required for cytochrome *c* synthesis in *Bacillus subtilis*. *J Bacteriol* 179:1962–1973. <https://doi.org/10.1128/jb.179.6.1962-1973.1997>.
16. Erlendsson LS, Acheson RM, Hederstedt L, Le Brun NE. 2003. *Bacillus subtilis* ResA is a thiol-disulfide oxidoreductase involved in cytochrome *c* synthesis. *J Biol Chem* 278:17852–17858. <https://doi.org/10.1074/jbc.M300103200>.
17. Erlendsson LS, Moller M, Hederstedt L. 2004. *Bacillus subtilis* StoA is a thiol-disulfide oxidoreductase important for spore cortex synthesis. *J Bacteriol* 186:6230–6238. <https://doi.org/10.1128/JB.186.18.6230-6238.2004>.
18. Schiott T, Hederstedt L. 2000. Efficient spore synthesis in *Bacillus subtilis* depends on the CcdA protein. *J Bacteriol* 182:2845–2854. <https://doi.org/10.1128/JB.182.10.2845-2854.2000>.
19. Han H, Wilson AC. 2013. The two CcdA proteins of *Bacillus anthracis* differentially affect virulence gene expression and sporulation. *J Bacteriol* 195:5242–5249. <https://doi.org/10.1128/JB.00917-13>.
20. Dumoulin A, Grauschopf U, Bischoff M, Thony-Meyer L, Berger BB. 2005. *Staphylococcus aureus* DsbA is a membrane-bound lipoprotein with thiol-disulfide oxidoreductase activity. *Arch Microbiol* 184:117–128. <https://doi.org/10.1007/s00203-005-0024-1>.
21. Heras B, Kurz M, Jarrott R, Shouldice SR, Frei P, Robin G, Čemažar M, Thöny-Meyer L, Glockshuber R, Martin JL. 2008. *Staphylococcus aureus* DsbA does not have a destabilizing disulfide. A new paradigm for bacterial oxidative folding. *J Biol Chem* 283:4261–4271. <https://doi.org/10.1074/jbc.M707838200>.
22. van der Kooi-Pol MM, Reilman E, Sibbald MJ, Veenstra-Kyuchukova YK, Kouwen TR, Buist G, van Dijk JM. 2012. Requirement of signal peptidase ComC and thiol-disulfide oxidoreductase DsbA for optimal cell surface display of pseudopilin ComGC in *Staphylococcus aureus*. *Appl Environ Microbiol* 78:7124–7127. <https://doi.org/10.1128/AEM.01565-12>.
23. Reardon-Robinson ME, Osipiuk J, Jooya N, Chang C, Joachimiak A, Das A, Ton-That H. 2015. A thiol-disulfide oxidoreductase of the Gram-positive pathogen *Corynebacterium diphtheriae* is essential for viability, pilus assembly, toxin production and virulence. *Mol Microbiol* 98:1037–1050. <https://doi.org/10.1111/mmi.13172>.
24. Um SH, Kim JS, Song S, Kim NA, Jeong SH, Ha NC. 2015. Crystal structure of DsbA from *Corynebacterium diphtheriae* and its functional implications for CueP in Gram-positive bacteria. *Mol Cells* 38:715–722. <https://doi.org/10.14348/molcells.2015.0099>.
25. Reardon-Robinson ME, Osipiuk J, Chang C, Wu C, Jooya N, Joachimiak A, Das A, Ton-That H. 2015. A disulfide bond-forming machine is linked to the sortase-mediated pilus assembly pathway in the Gram-positive bacterium *actinomyces oris*. *J Biol Chem* 290:21393–21405. <https://doi.org/10.1074/jbc.M115.672253>.
26. Gross EL, Leys EJ, Gasparovich SR, Firestone ND, Schwartzbaum JA, Janies DA, Asnani K, Griffen AL. 2010. Bacterial 16S sequence analysis of severe caries in young permanent teeth. *J Clin Microbiol* 48:4121–4128. <https://doi.org/10.1128/JCM.01232-10>.
27. Davey L, Ng CK, Halperin SA, Lee SF. 2013. Functional analysis of paralogous thiol-disulfide oxidoreductases in *Streptococcus gordonii*. *J Biol Chem* 288:16416–16429. <https://doi.org/10.1074/jbc.M113.464578>.
28. Davey L, Cohen A, LeBlanc J, Halperin SA, Lee SF. 2016. The disulfide oxidoreductase SdbA is active in *Streptococcus gordonii* using a single C-terminal cysteine of the CXXC motif. *Mol Microbiol* 99:236–253. <https://doi.org/10.1111/mmi.13227>.
29. Davey L, Halperin SA, Lee SF. 2016. Mutation of the *Streptococcus gordonii* thiol-disulfide oxidoreductase SdbA leads to enhanced biofilm formation mediated by the CiaRH two-component signaling system. *PLoS One* 11:e0166656. <https://doi.org/10.1371/journal.pone.0166656>.
30. Davey L, Halperin SA, Lee SF. 2016. Mutation of the thiol-disulfide oxidoreductase SdbA activates the CiaRH two-component system, leading to bacteriocin expression shutdown in *Streptococcus gordonii*. *J Bacteriol* 198:321–331. <https://doi.org/10.1128/JB.00800-15>.
31. Missiakas D, Schwager F, Raina S. 1995. Identification and characterization of a new disulfide isomerase-like protein (DsbD) in *Escherichia coli*. *EMBO J* 14:3415–3424. <https://doi.org/10.1002/j.1460-2075.1995.tb07347.x>.
32. Haase EM, Feng X, Pan J, Miecznikowski JC, Scannapieco FA. 2015. Dynamics of the *Streptococcus gordonii* transcriptome in response to medium, salivary alpha-amylase, and starch. *Appl Environ Microbiol* 81:5363–5374. <https://doi.org/10.1128/AEM.01221-15>.
33. Hatahet F, Boyd D, Beckwith J. 2014. Disulfide bond formation in prokaryotes: history, diversity and design. *Biochim Biophys Acta* 1844:1402–1414. <https://doi.org/10.1016/j.bbapap.2014.02.014>.
34. Saleh M, Bartual SG, Abdullah MR, Jensch I, Asmat TM, Petruschka L, Pribyl T, Gellert M, Lillig CH, Antelmann H, Hermoso JA, Hammerschmidt S. 2013. Molecular architecture of *Streptococcus pneumoniae* surface thioredoxin-fold lipoproteins crucial for extracellular oxidative stress resistance and maintenance of virulence. *EMBO Mol Med* 5:1852–1870. <https://doi.org/10.1002/emmm.201202435>.
35. Williamson JA, Cho SH, Ye J, Collet JF, Beckwith JR, Chou JJ. 2015. Structure and multistate function of the transmembrane electron transporter CcdA. *Nat Struct Mol Biol* 22:809–814. <https://doi.org/10.1038/nsmb.3099>.
36. Kishigami S, Kanaya E, Kikuchi M, Ito K. 1995. DsbA-DsbB interaction through their active site cysteines. Evidence from an odd cysteine mutant of DsbA. *J Biol Chem* 270:17072–17074. <https://doi.org/10.1074/jbc.270.29.17072>.
37. Walker KW, Lyles MM, Gilbert HF. 1996. Catalysis of oxidative protein folding by mutants of protein disulfide isomerase with a single active-site cysteine. *Biochemistry* 35:1972–1980. <https://doi.org/10.1021/bi952157n>.
38. Balmer Y, Koller A, del Val G, Manieri W, Schurmann P, Buchanan BB. 2003. Proteomics gives insight into the regulatory function of chloroplast thioredoxins. *Proc Natl Acad Sci U S A* 100:370–375. <https://doi.org/10.1073/pnas.232703799>.
39. Burne RA, Wen ZT, Chen YY, Penders JE. 1999. Regulation of expression of the fructan hydrolase gene of *Streptococcus mutans* GS-5 by induction and carbon catabolite repression. *J Bacteriol* 181:2863–2871.
40. Claverys JP, Dintilhac A, Pestova EV, Martin B, Morrison DA. 1995. Construction and evaluation of new drug-resistance cassettes for gene disruption mutagenesis in *Streptococcus pneumoniae*, using an ami test platform. *Gene* 164:123–128. [https://doi.org/10.1016/0378-1119\(95\)00485-0](https://doi.org/10.1016/0378-1119(95)00485-0).
41. Dunne GM, Lee LN, LeBlanc DJ. 1991. Improved electroporation and cloning vector system for Gram-positive bacteria. *Appl Environ Microbiol* 57:1194–1201.
42. Vats N, Lee SF. 2001. Characterization of a copper-transport operon, *copYAZ*, from *Streptococcus mutans*. *Microbiology* 147:653–662. <https://doi.org/10.1099/00221287-147-3-653>.
43. Tremblay YD, Lo H, Li YH, Halperin SA, Lee SF. 2009. Expression of the *Streptococcus mutans* essential two-component regulatory system VicRK is pH and growth-phase dependent and controlled by the LiaFSR three-component regulatory system. *Microbiology* 155:2856–2865. <https://doi.org/10.1099/mic.0.028456-0>.

44. Knight JB, Halperin SA, West KA, Lee SF. 2008. Expression of a functional single-chain variable-fragment antibody against complement receptor 1 in *Streptococcus gordonii*. *Clin Vaccine Immunol* 15:925–931. <https://doi.org/10.1128/CVI.00500-07>.
45. Schneider CA, Rasband WS, Eliceiri KW. 2012. NIH Image to ImageJ: 25 years of image analysis. *Nat Methods* 9:671–675. <https://doi.org/10.1038/nmeth.2089>.
46. Inaba K, Ito K. 2002. Paradoxical redox properties of DsbB and DsbA in the protein disulfide-introducing reaction cascade. *EMBO J* 21: 2646–2654. <https://doi.org/10.1093/emboj/21.11.2646>.
47. Zhou JY, Dann GP, Shi T, Wang L, Gao X, Su D, Nicora CD, Shukla AK, Moore RJ, Liu T, Camp DG, II, Smith RD, Qian WJ. 2012. Simple sodium dodecyl sulfate-assisted sample preparation method for LC-MS-based proteomics applications. *Anal Chem* 84:2862–2867. <https://doi.org/10.1021/ac203394r>.
48. Ed Harlow DL. 1988. *Antibodies: a laboratory manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
49. Sievers F, Wilm A, Dineen D, Gibson TJ, Karplus K, Li W, Lopez R, McWilliam H, Remmert M, Soding J, Thompson JD, Higgins DG. 2014. Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. *Mol Syst Biol* 7:539. <https://doi.org/10.1038/msb.2011.75>.