Disulfide bonds are important for the stability of many extracellular proteins, including bacterial virulence factors. Formation of these bonds is catalyzed by thiol-disulfide oxidoreductases (TDORs). Little is known about their formation in Gram-positive bacteria, particularly among facultative anaerobic Firmicutes, such as streptococci. To investigate disulfide bond formation in *Streptococcus gordonii*, we identified five putative TDORs from the sequenced genome. Each of the putative TDOR genes was insertionally inactivated with an erythromycin resistance cassette, and the mutants were analyzed for autolysis, extracellular DNA release, biofilm formation, bacteriocin production, and genetic competence. This analysis revealed a single TDOR, SdbA, which exhibited a pleiotropic mutant phenotype. Using an *in silico* analysis approach, we identified the major autolysin AtlS as a natural substrate of SdbA and showed that SdbA is critical to the formation of a disulfide bond that is required for autolytic activity. Analysis by BLAST search revealed homologs to SdbA in other Gram-positive species. This study provides the first indication that thiol-disulfide oxidoreductases are important to the physiology of Firmicute bacteria.

Disulfide bonds are important for the folding and activity of many extracellular proteins, including bacterial virulence factors, such as flagella, secretion systems, pili, and toxins (1). Although spontaneous disulfide bond formation can occur, the process is extremely slow, and most disulfide bonds formed in vivo are catalyzed by thiol-disulfide oxidoreductases (TDORs) (2).

The *Escherichia coli* Dsb pathway is the paradigm for disulfide bond formation. In this system, the periplasmic oxidase DsbA forms disulfide bonds in substrate proteins as they are translocated from the reducing environment of the cytoplasm. DsbA contains a catalytic disulfide bond with a Cys-X-X-Cys motif that forms a mixed disulfide intermediate with its substrates, which, once resolved, leaves DsbA in a reduced state. Its reductox partner DsbB then reoxidizes DsbA back to its active state, allowing the cycle to continue (2, 3). DsbA has a broad substrate specificity, with an estimated 300 substrates in *E. coli* (2), and homologs of DsbA appear to be widely distributed among Gram-negative bacteria (1, 4). Additional components of the pathway include a disulfide isomerase, DsbC, and its reductox partner DsbD (5, 6) as well as a reductase, DsbE (CcmG), that also partners with DsbD and functions to reduce the cysteines in apocytochrome *c* to enable heme to bind (5, 7).

In contrast, little is known about disulfide bond formation in Gram-positive bacteria, which are predicted to have a lower prevalence of disulfide-bonded proteins (4, 8). Although Gram-positive DsbA homologs have been identified, few have demonstrated functions, and those that do appear to perform specialized functions in the cell. For example, substrates of the DsbAB homologs in *Bacillus subtilis*, BdbDC, are limited to proteins required for genetic competence, such as the ComGC pseudopilus and ComEC channel protein (9, 10). In *Staphylococcus aureus*, SaDsbA has also been shown to be required for the formation of disulfide bonds.
stable production of ComGC (11); however, no other substrates or phenotypes have been identified despite detailed functional and structural characterization (12, 13). Similarly, the biological function of the Bacillus brevis DsbA homolog, Bdb, has not been determined (14).

Emerging evidence suggests that Gram-positive Actinobacteria may use different types of TDORs, with low homology to E. coli DsbA, to form disulfide bonds (8, 15, 16). Novel oxidases identified in these species include Mycobacterium tuberculosis DsbE and DsbF (15, 16), which show greater homology to E. coli reductases than to DsbA, and a TDOR from Corynebacterium glutamicum, CG.0026, which is predicted to be widespread among the Actinobacteria (8). In vitro analyses have confirmed that these enzymes function as oxidases, although no in vivo functions or phenotypes have been identified (8, 15, 16).

Bioinformatic screens for TDORs among diverse groups of bacteria have noted that many anaerobic Firmicutes, including streptococci and clostridia, lack homologs to E. coli DsbA and DsbB (1, 4, 8). It has been proposed that these bacteria do not engage in disulfide bond formation; however, there are important examples of disulfide-bonded proteins produced by these species, including secreted toxins that contribute to virulence (17, 18). Moreover, our laboratory has previously reported the presence of the thiol-disulfide oxidoreductase (TDOR) SdbA in S. gordonii DL-1 that secretes a single-chain variable fragment antibody (scFv) against complement receptor 1 (CR1) (19). Production of recombinant SpaP-S1 was tested using S. gordonii RIM4 as the parent strain (25). Unless otherwise noted, S. gordonii was grown in HTVG (per ml: 5 mg of glucose, 35 mg of tryptone, 100 mm HEPES, 0.04 μg of p-aminobenzoic acid, 0.2 μg of thiamine-HCl, 1 μg of nicotinamide, and 0.2 μg of riboflavin, pH 7.6) (26) at 37 °C, 5% CO2, without shaking. Streptococcus oralis 34 and Streptococcus mitis 118 were grown in brain-heart infusion medium (BHI; Difco). E. coli XL-1 Blue and E. coli BL21(DE3) were grown in Luria-Bertani medium (LB) at 37 °C with shaking. Antibiotics were used at the following concentrations: for S. gordonii, erythromycin (10 μg/ml), tetracycline (10 μg/ml), spectinomycin (250 μg/ml), kanamycin (250 μg/ml), and rifampin (100 μg/ml); for E. coli, ampicillin (100 μg/ml) and tetracycline (10 μg/ml).

**Genetic Manipulations**—The TDOR mutants were constructed by insertional inactivation with an erythromycin resistance cassette (ermAM) (27) amplified by polymerase chain reaction (PCR) using the primers listed in supplemental Table S1. PCR products were digested with restriction enzymes as indicated in supplemental Table S1 and ligated together with T4 DNA ligase (New England Biolabs). The ligation products were amplified using the outside primers, and the resulting constructs were used to transform S. gordonii SecCR1 and S. gordonii RIM4 as described previously (19). Transformants were selected on BHI containing the appropriate antibiotics, and insertion of the ermAM cassette was confirmed by PCR.

Construction of a sdbA-complemented mutant was achieved by introducing a functional sdbA gene back onto the chromosome. To this end, the entire sdbA reading frame and a portion of the upstream gene, nusG, was amplified with the primer pair SL756/SL803. This fragment was digested with BamHI and ligated to a kanamycin resistance cassette (aphA3) amplified from plasmid pDL276 (28). The ligation product was amplified by PCR and digested with EcoRI. This construct was then ligated to a 557-bp segment of sdbA and the downstream gene, padA, amplified with the primers SL758/SL759. The resulting construct was used to transform the sdbA mutant, replacing the ermAM cassette with a functional sdbA gene and a kanamycin resistance marker by double crossover homologous recombination. Transformants were selected on BHI with kanamycin, and replica plating was used to identify erythromycin-sensitive, kanamycin-resistant colonies. Complementation of the sdbA gene was confirmed by PCR analysis and with Western blots using anti-SdbA antisera.

**RT-PCR**—RNA was extracted from cultures of the parent, sdbA mutant, and sdbA-complemented mutant grown in HTVG to a density of A600 = 0.6 using the hot acid phenol method, as described previously (29).

The RNA (1 μg) was treated with 1 unit of amplification grade DNase I (Invitrogen) for 15 min at room temperature, and removal of DNA was confirmed by PCR with 16 S rRNA

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**Novel Thiol-disulfide Oxidoreductase in S. gordonii**

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Culture Conditions**—Experiments were carried out using S. gordonii SecCR1 as the parent strain. S. gordonii SecCR1 is a recombinant strain of S. gordonii Challis DL-1 that secretes a single-chain variable fragment antibody (scFv) against complement receptor 1 (CR1) (19). Production of recombinant SpaP-S1 was tested using S. gordonii RIM4 as the parent strain (25). Unless otherwise noted, S. gordonii was grown in HTVG (per ml: 5 mg of glucose, 35 mg of tryptone, 100 mm HEPES, 0.04 μg of p-aminobenzoic acid, 0.2 μg of thiamine-HCl, 1 μg of nicotinamide, and 0.2 μg of riboflavin, pH 7.6) (26) at 37 °C, 5% CO2, without shaking. Streptococcus oralis 34 and Streptococcus mitis 118 were grown in brain-heart infusion medium (BHI; Difco). E. coli XL-1 Blue and E. coli BL21(DE3) were grown in Luria-Bertani medium (LB) at 37 °C with shaking. Antibiotics were used at the following concentrations: for S. gordonii, erythromycin (10 μg/ml), tetracycline (10 μg/ml), spectinomycin (250 μg/ml), kanamycin (250 μg/ml), and rifampin (100 μg/ml); for E. coli, ampicillin (100 μg/ml) and tetracycline (10 μg/ml).

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Novel Thiol-disulfide Oxidoreductase in S. gordonii

primers (SL525/697). cDNA synthesis was carried out using random primers (Invitrogen) and SuperScript II reverse transcriptase (Invitrogen) according to the manufacturer’s directions. The cDNA (3 µl) was used as the template to amplify nusG (SL783/784) and padA (SL785/SL786). Total cDNA was assessed by amplification of 16 S rRNA.

Biofilm Formation—Biofilms were grown as described by Loo et al. (22), with the following modifications. Overnight cultures of S. gordonii grown in HTVG were harvested by centrifugation (3000 × g for 10 min), washed once with phosphate-buffered saline (PBS), and suspended in biofilm medium to an A600 of 0.250. Flat bottom 24-well plates (Costar) were inoculated with 1 ml/well of the cell suspension, and the plates were incubated for 24 h at 37 °C, 5% CO2. Following incubation, the medium was removed, and the wells were washed once with 1 ml of distilled water to remove loosely attached cells. The plates were air-dried for 15 min and fixed with 10% (v/v) formaldehyde and 5% (v/v) acetic acid in PBS for an additional 15 min. The biofilms were then stained with 0.5 ml of 0.1% crystal violet. After 15 min, the wells were rinsed three times with distilled water, and the bound stain was solubilized in 1 ml of acetone/ethanol solution (1:1). The liquid was transferred to a new microtiter plate, and the optical density was measured at 600 nm in a BioTek microplate reader. The biofilm assays were carried out in triplicate with three or more separate experiments.

Biofilms for SEM analysis were grown in biofilm medium on glass coverslips. Following growth for 24 h at 37 °C, 5% CO2, the medium was removed, and planktonic cells were removed by washing once with PBS. The biofilms were fixed with 2.5% glutaraldehyde in 90 mM cacodylate buffer (pH 7.3) for 24 h at room temperature. The samples were then osmicated and dehydrated to 100% ethanol by standard methods. The dehydrated samples were processed by critical point drying and gold sputter-coated. Biofilms were examined using a Hitachi S-4700 field emission scanning electron microscope (Hitachi High-Technologies Canada, Inc.) at the Institute for Research in Materials at Dalhousie University.

Autolysis and eDNA Release—Autolysis was tested as described by Ahn and Burne (30) with the following modifications. Overnight cultures of S. gordonii were diluted 1:20 into HTVG and grown at 37 °C, 5% CO2, to A600 = 1.0. The cells were pelleted by centrifugation (3000 × g, 10 min) and washed twice with PBS. Pellets were then suspended in prewarmed (44 °C) 20 mM potassium phosphate buffer (pH 6.5) containing 1 M KCl, 1 mM CaCl2, 1 mM MgCl2, 0.4% sodium azide, 0.2% Triton X-100. The cell suspensions were incubated in a 44 °C water bath, and autolysis was monitored by measuring the A600 at regular intervals.

Extracellular DNA was recovered from stationary phase cultures grown in HTVG. Cultures were standardized to A600 = 1.0 and centrifuged (14,000 × g, 5 min). The supernatants (750 µl) were mixed 1:1 with cold acetone and incubated overnight at 4 °C. The precipitated DNA was centrifuged and dissolved in 50 µl of TE buffer. The samples were analyzed on 0.8% agarose gels.

Genetic Competence and Bacteriocin Production—Genetic competence in the parent and mutant strains was tested by transformation with genomic DNA isolated from S. gordonii Wicky WK1, a strain that contains a spontaneous mutation in the β-subunit of RNA polymerase conferring resistance to rifampin (31). Cultures were grown to A600 = 0.2 in BHI with 5% heat-inactivated calf serum (Invitrogen) prior to the addition of 1 µg of S. gordonii Wicky WK1 genomic DNA. Cultures were incubated for an additional 2 h to allow for DNA uptake, and serial dilutions were drop-plated on either BHI or BHI with 100 µg/ml rifampin. The plates were incubated for 48 h, and the transformation efficiency was calculated as the percentage of rifampin-resistant transformants to the total cfu/ml.

Activity of Sth1 and Sth2 was tested exactly as described by Heng et al. (24), using S. oralis 34 and S. mitis 118 as the indicator strain, respectively.

Foreign Protein Production—Production of anti-CRI scFv and SpaP/S1 was tested by Western blotting. Cells from 1.5 ml of overnight culture grown in HTVG were pelleted by centrifugation (14,000 × g, 3 min, 4 °C) and suspended in 50 µl of SDS-PAGE sample buffer. Samples were boiled for 5 min and separated by SDS-PAGE, followed by Coomassie Blue staining to ensure equal protein loading. The same volume of protein was loaded for Western blotting. Anti-CRI scFv was detected with the anti-HA antibody (1:30,000 dilution; Sigma) and SpaP/S1 was detected with the anti-S1 monoclonal antibody (1:7000).

Cloning and Expression of Recombinant Proteins—Recombinant plasmids to produce AtlS and SdbA were constructed by cloning in frame fragments of the genes into the expression vector pQE-30 (Qiagen) behind an N-terminal His6 tag. The primers and restriction enzymes used for each gene are listed in supplemental Table S1. His6-SdbA consisted of a 17.13-kDa portion of SdbA after the signal sequence, and His6-AtlS contained a 30-kDa portion of the C-terminal region of the protein. His6-tagged proteins were expressed in E. coli XL-1 Blue and then purified from lysates using a NiCAM affinity column (Sigma) using standard techniques. E. coli DsbA was expressed from pDsbaCyt in E. coli BL21(DE3) and purified as described by Hennecke et al. (32). Antiserum was raised against SdbA and AtlS in BALB/c mice using methods similar to those described previously (33).

Analysis of Oxidase Activity—Recombinant SdbA expressed in E. coli XL-1 Blue was obtained in a mix of oxidized and reduced forms as shown by alkylation with maleimide-PEG2-biotin (Thermo). To produce fully oxidized SdbA, 0.5 mg/ml protein in 100 mM Tris (pH 8.8), 200 mM KCl, 1 mM EDTA was incubated with 100 mM oxidized glutathione (Sigma) (32). Glutathione was then removed by dialysis against 100 mM sodium phosphate buffer (pH 7), and aliquots were stored at −80 °C. Production of reduced, denatured RNase A (Sigma) and analysis of oxidase activity by RNase A-catalyzed cCMP hydrolysis were carried out as described by Daniels et al. (8).

Zymogram Analysis—Zymogram analysis of SDS-extracted surface proteins was carried out as described by Liu and Burne (21). Surface protein extracts were prepared from 100-ml cultures of S. gordonii grown in HTVG to A600 = 0.9–1.0. Cells were pelleted by centrifugation (5000 × g, 10 min) and suspended in 1 ml of 4% SDS. After incubation for 60 min at room temperature, the cells were removed by centrifugation.
were washed twice with distilled water and incubated in 0.2M Tris-HCl (pH 6.5), 10% glycerol. Following electrophoresis of the SDS-extracted proteins, the gels of the cells in the polyacrylamide gel was 1% wet weight. Following electrophoresis of the SDS-extracted proteins, the gels were washed twice with distilled water and incubated in 0.2M sodium phosphate buffer (pH 7.0), 1% SDS, 8M urea. To prepare positive controls, the extracts were reduced with 100 mM dithiothreitol (DTT) and alkylated with maleimide-PEG2-biotin. Surface protein extracts containing the autolysin were obtained from the sdbA mutant by extraction with 4% SDS as described above. Attempts to analyze SDS-extracted proteins from the parent strain were unsuccessful due to the high concentration of competing proteins in the sample. Thus, AtlS from the parent strain was obtained by extraction with 5 mM LiCl for 60 min at 4 °C, which extracted surface proteins with a lower efficiency than SDS, resulting in a less complex sample. Alkylation of the protein extracts was carried out as described previously, with the following modifications (34, 35). Surface protein extracts were precipitated with 9% trichloroacetic acid (TCA) on ice for 30 min, followed by centrifugation at 15,000 × g for 10 min at 4 °C and washed twice with acetone. Pellets were then suspended in 5 mM maleimide-PEG2-biotin in 100 mM Tris (pH 7.0) and 1% SDS and incubated for 30 min at room temperature, followed by 10 min at 37 °C. Excess maleimide-PEG2-biotin was removed by TCA precipitation. The resulting pellets were solubilized in 100 mM Tris (pH 7.0), 1% SDS, 8 mM urea. To prepare positive controls, the extracts were reduced with 100 mM dithiothreitol (DTT) in 10 mM Tris (pH 8.1) for 30 min at room temperature and TCA-precipitated prior to the addition of maleimide-PEG2-biotin.

To specifically detect disulfide-bonded cysteines, iodoacetamide was used to block the free thiol groups. Surface protein extracts were prepared as described above and subsequently TCA-precipitated and washed twice with ice-cold acetone. The resulting pellets were suspended in 200 mM Tris, pH 8.1, 100 mM iodoacetamide and incubated for 20 min on ice in the dark, followed by TCA precipitation to remove excess iodoacetamide. Disulfide bonds were then reduced with DTT and alkylated with maleimide-PEG2-biotin. To detect biotinylated proteins, the samples were boiled in sample buffer and run in duplicate on a 10% SDS-PAGE. Proteins were transferred to a nitrocellulose membrane and reacted with avidin-alkaline phosphatase (Sigma). Total AtlS concentration in the samples was used as a loading control and was detected with anti-AtlS antiserum (1:1000). Experiments were repeated at least three times to ensure reproducibility.

**Determination of the Redox State of AtlS**—Surface protein extracts containing the autolysin were obtained from the sdbA mutant by extraction with 4% SDS as described above. Attempts to analyze SDS-extracted proteins from the parent strain were unsuccessful due to the high concentration of competing proteins in the sample. Thus, AtlS from the parent strain was obtained by extraction with 5 mM LiCl for 60 min at 4 °C, which extracted surface proteins with a lower efficiency than SDS, resulting in a less complex sample. Alkylation of the protein extracts was carried out as described previously, with the following modifications (34, 35). Surface protein extracts were precipitated with 9% trichloroacetic acid (TCA) on ice for 30 min, followed by centrifugation at 15,000 × g for 10 min at 4 °C and washed twice with acetone. Pellets were then suspended in 5 mM maleimide-PEG2-biotin in 100 mM Tris (pH 7.0) and 1% SDS and incubated for 30 min at room temperature, followed by 10 min at 37 °C. Excess maleimide-PEG2-biotin was removed by TCA precipitation. The resulting pellets were solubilized in 100 mM Tris (pH 7.0), 1% SDS, 8 mM urea. To prepare positive controls, the extracts were reduced with 100 mM dithiothreitol (DTT) in 10 mM Tris (pH 8.1) for 30 min at room temperature and TCA-precipitated prior to the addition of maleimide-PEG2-biotin.

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**Site-directed Mutagenesis**—A cysteine to serine point mutation in atlS was generated in the parent SecCR1 background by introducing a mutation to replace the codon for cysteine at position 1069 (TGT) to serine (TCC). To achieve this, the upstream region of atlS through to the cysteine codon was amplified with Platinum Pfx DNA polymerase (Invitrogen) using the primer pair SL893/SL896 and digested with BspEI. The fragment was then ligated to a second amplicon that contained the downstream portion of atlS, from the cysteine codon to the end of the reading frame, amplified with the primer pair SL897/SL898. The resulting construct consisted of the atlS gene with a Cys → Ser mutation, which also created a novel BspEI restriction site (TCCGGA) from the parent sequence (TGTTGA). To enable selection, an ermAM cassette was digested with BamHI and ligated to a 628-bp fragment amplified from the region located immediately downstream of atlS with the primers SL899/SL900. Finally, the two constructs were ligated via an EcoRV restriction site and amplified by PCR using the outside primers SL893/SL900. The PCR product was used to transform S. gordonii SecCR1, and transformants were selected on BHI with erythromycin. The mutation was confirmed by PCR and restriction analysis of the BspEI site.

**Sequence Analysis**—To identify TDORS in S. gordonii, the sequences for B. subtilis 168 BdbD and BdbC were used as the query for a BLASTP (36) search in S. gordonii Challis DL-1. A second search for proteins similar to the query S. gordonii SecCR1, and transformants were selected on BHI with erythromycin. The mutation was confirmed by PCR and restriction analysis of the BspEI site.

**Screening for potential SdbA substrates** was carried out using a protocol modified from Daniels et al. (8). The S. gordonii proteome was downloaded from the UniProtKB database. Extracytoplasmic proteins were identified using the prediction servers SignalP 3.0 (38) and LipoP 1.0 (39) to identify predicted secreted proteins and lipoproteins, respectively. Cytoplasmic proteins were discarded, and proteins that were predicted to encode a signal sequence by either the neural network or hidden Markov model were considered as extracellular and analyzed for cysteine content. Extracellular portions of the proteins were identified using the transmembrane prediction server SCAMPI (40), and proteins with two or more cysteine residues predicted to localize on the outside of the membrane were collected in a list of potential substrates. The annotated functions of the genes were obtained from the Oralgen database. To identify homologs of S. gordonii SdbA in other Gram-positive species, a DELTA BLAST search was carried out using SdbA (YP_001451255) as the query sequence with a homology cut-off of e < 10^-20.

**Statistical Analysis**—Results were analyzed by one-way analysis of variance with Bonferroni post-tests using GraphPad Prism version 5 (GraphPad Software Inc.).

**RESULTS**

**Identification of Putative TDORs in S. gordonii**—We hypothesized that previous screens failed to identify TDORs in some Firmicutes because these species use different enzymes, with low homology to DsbA, which could be missed due to overly stringent search criteria. Therefore, we conducted a BLASTP...
search in *S. gordonii* using *B. subtilis* 168 BdbC and BdbD as a query, looking for proteins with even very low homology. Although there were no hits to BdbC, a homolog of *E. coli* DsbB (10), the search identified a thioredoxin signature protein with limited homology to BdbD called Sgo.2006 (e value of 0.14). A search for additional TDORs, similar to Sgo.2006, identified three uncharacterized thioredoxin family proteins: Sgo.1267, Sgo.1171, and Sgo.1177. The proteins ranged in size from 160 to 187 amino acids, and all were predicted to belong to the TlpA/DsbE/ResA family. We also chose to investigate a fifth protein, Sgo.1216, annotated as a bacteriocin transport accessory protein (Bta), based on its similarity to *S. thermophilus* LMD-9 BlpG(ST) (BLASTP e value of 10⁻¹⁸, 13.2% identity and 23% similarity), a thiol-disulfide oxidase dedicated to disulfide bond formation in the bacteriocin thermophilin-9 (37).

A multiple sequence alignment showed that each of the sequences contained a Cys-X-X-Cys motif, characteristic of the active site of TDOR enzymes. The proteins also contained a conserved proline residue located away from the active site (Fig. 1). This proline is a common feature of thioredoxin family proteins, which has several reported functions, including prevention of metal binding to the active site cysteines (41) and substrate release (42). Although each of the *S. gordonii* TDORs had similar sequences, only Sgo.2006 and Sgo.1177 were predicted to encode signal peptides (SignalP), and Sgo.1177 was predicted to be a lipoprotein (LipoP). Based on our findings described below, we named Sgo.2006 SdbA (*Streptococcus* disulfide bond protein A).

### SdbA Affects Multiple Biological Processes

To analyze the functions of the predicted TDORs in *S. gordonii*, we constructed mutants in each of the genes by insertion inactivation. A common first line approach to screen TDOR mutants is to test for sensitivity to reducing agents; however, this technique has had limited success in *Bacillus*, possibly due to its intrinsic resistance to oxidative stress (8). Similarly, our mutants did not show increased sensitivity to growth with DTT compared with the parent strain, which grew in the presence of 200 mM DTT (data not shown). In contrast, growth of *E. coli* DsbA mutants and wild-type strains is inhibited at 7 and 20 mM concentrations, respectively (43). As an alternative approach to detect disulfide bond formation, our phenotypic analysis of the mutants focused on traits required for the survival and persistence of *S. gordonii* in oral biofilms.

Biofilm formation was assessed in microtiter plates under aerobic conditions. Although four of the mutants formed biofilms similar to those formed by the parent, one mutant, sdbA, showed significantly enhanced biofilm formation (Fig. 2A). Analysis by scanning electron microscopy revealed that the sdbA mutant formed thick, multilayered biofilms, in contrast to the monolayer biofilms produced by the parent strain (Fig. 2B). Because *S. gordonii* forms thicker biofilms under anaerobic conditions (22), we also tested anaerobic biofilm formation to determine if the enhanced biofilm phenotype of the sdbA mutant was related to an inability to sense oxygen in the environment. When biofilms were grown under anaerobic conditions, the parent showed higher levels of biofilm formation as expected, and the sdbA mutant also showed a proportionate increase in adhesion, indicating that the enhanced biofilm phenotype is not a result of the atmospheric growth conditions (data not shown).
An important component of the biofilm matrix is eDNA, which contributes to cell-cell adhesion and exchange of genetic material (21, 44, 45). We tested eDNA release by the TDOR mutants to determine if this contributed to the enhanced biofilm phenotype of the \textit{sdbA} mutant and whether the other TDOR mutants showed changes in eDNA release. Surprisingly, high molecular weight eDNA could be extracted from the medium of stationary phase cultures for all of the mutants except \textit{sdbA} (Fig. 2C). The result suggests that SdbA plays an essential role in eDNA release, and the enhanced biofilm phenotype is a result of additional factors that have been altered in the absence of SdbA.

Next, the mutants were tested for autolysis, which has been reported to be required for both eDNA release and biofilm formation in streptococci (21, 30). Consistent with the absence of eDNA, the autolysis assay showed that the \textit{sdbA} mutant lacked autolytic activity, remaining at 88% of the initial optical density. Two additional mutants, \textit{sgo.1267} and \textit{sgo.1216}, also showed moderate defects in autolysis compared with the parent, which lyses rapidly under the test conditions (Fig. 2D).

Given the annotation of \textit{Sgo.1216} (Bta) as a bacteriocin transport accessory protein, we sought to test bacteriocin production by the mutants. \textit{S. gordonii} produces two bacteriocins, streptocins Sth\textsubscript{1} and Sth\textsubscript{2} (24); however, these proteins lack cysteines, and the role of \textit{Sgo.1216} in their production, if any, is unknown. Growth inhibition of the target strain \textit{S. oralis} 34 was used to test the activity of Sth\textsubscript{1}. Interestingly, only \textit{sdbA}, and not \textit{sgo.1216} or the other TDOR mutants, showed a defect in bacteriocin activity (Fig. 2E). The \textit{sdbA} mutant also lacked Sth\textsubscript{2} activity, as determined by testing inhibition of \textit{S. mitis} 118 as the target strain (data not shown). This indicates that SdbA is required for either processing or production of both Sth\textsubscript{1} and Sth\textsubscript{2} bacteriocins. Because bacteriocin activity coincides with development of genetic competence, and both are controlled under the ComDE regulon, we tested the transformation efficiency of the \textit{sdbA} mutant (24). The \textit{sdbA} mutant showed a dramatic defect in transformation frequency compared with the parent strain (Table 1). This suggests that a component of the ComDE regulon may be a natural substrate of SdbA.

In each of the assays, a single mutant, \textit{sdbA}, showed striking differences from the parent strain. The \textit{sdbA} mutant clumped when grown in liquid cultures (Fig. 3A), and SDS-PAGE analysis of SDS-extracted surface proteins showed that the protein profile of the mutant had diminished intensity compared with the parent (Fig. 3B). These results suggest that inactivation of \textit{sdbA} resulted in dramatic changes to the cell surface. A complemented mutant was constructed by introducing a functional \textit{sdbA} gene back onto the chromo-
some (Fig. 3C), which reversed the phenotypes observed in the mutant (Figs. 2 A–E, 3 A and B), and RT-PCR analysis of genes immediately upstream and downstream of sdbA confirmed that the mutation was non-polar (Fig. 3D). Based on these results, we selected the sdbA mutant for additional characterization.

**SdbA Is Required for Production of the Disulfide-bonded Protein.** Anti-CR1 scFv—*S. gordonii* SecCR1 secretes scFv against CR1 fused to the signal peptide of *Streptococcus mutans* SpaP (19). Single-chain antibodies contain two intramolecular disulfide bonds, one in each of the VH and VL domains, and disruption of these bonds prevents stable folding, resulting in aggregation and proteolysis (46). Because correct disulfide bonding is well established as a critical factor in single-chain antibody production, anti-CR1 scFv levels were assessed as an indication of the cells’ capacity for production of disulfide-bonded proteins. Western blots revealed that SdbA is essential to anti-CR1 scFv production, because no protein was detected in the mutant, whereas complementation restored production to levels similar to the parent (Fig. 4 A). The other TDOR mutants produced anti-CR1 scFv at levels similar to those of the parent (data not shown).

The absence of anti-CR1 scFv production in the sdbA mutant indicates a possible role for SdbA in either the formation or isomerization of disulfide bonds necessary to stabilize the protein and prevent degradation. However, an alternative explanation could be that SdbA contributes to the function of the gen-

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**TABLE 1**

Transformation frequency of *S. gordonii* parent and mutant strains

| Strain          | Total Transformants | Frequency (%) |
|-----------------|---------------------|---------------|
| Parent SecCR1   | 2.20 × 10⁸ cfu/ml   | 0.0545        |
| sdbA            | 5.20 × 10⁷ cfu/ml   | 0.000001      |
| SdbA Compl.     | 1.94 × 10⁸ cfu/ml   | 0.0015        |

* Transformation frequency was calculated as the percentage of rifampin-resistant transformants divided by the total cfu/ml. Data are representative of at least three separate experiments.

* SdbA Compl, sdbA-complemented mutant.
eral secretory pathway. To distinguish between these two scenarios, we tested the production of a foreign protein without disulfide bonds. An sdbA mutant was generated in S. gordonii RJM4, a recombinant strain of S. gordonii DL-1 that secretes a fusion protein between SpaP and the N-terminal 179-amino acid fragment of the S1 subunit of pertussis toxin without disulfide bonds (25). Western blots showed that both the parent and the sdbA mutant produced similar levels of the SpaP-S1 protein (Fig. 4B). These findings indicate that the defect in anti-CR1 scFv production in the sdbA mutant may be the result of a general defect in secretion.

Next, the oxidase activity of recombinant SdbA was tested in vitro using the RNase A folding assay. Histagged SdbA lacking the N-terminal signal sequence and transmembrane domain was expressed in E. coli and purified by affinity chromatography (Fig. 5A). SdbA was incubated with reduced RNase A, and the oxidative folding of RNase A to its active conformation was monitored by cCMP hydrolysis. SdbA successfully catalyzed the correct folding of reduced, denatured RNase A, although the enzyme was slightly less active than E. coli DsbA, which served as a positive control (Fig. 5B). Taken together, these results indicate that SdbA is an oxidoreductase, which is directly involved in the production of disulfide-bonded proteins in S. gordonii.

AtlS Is a Natural Substrate of SdbA—The role of SdbA in the production of anti-CR1 scFv suggests that the multiple phenotypes observed in the sdbA mutant may be the result of impaired disulfide bond formation. To support this hypothesis, we sought to identify natural substrates of SdbA. Because there are no known disulfide-bonded proteins in S. gordonii, we used a computational approach to screen for candidate substrates. This identified 36 secreted proteins with two or more cysteines that may form disulfide bonds (supplemental Table S2), representing ~10% of the secreted proteins in S. gordonii.

Among the predicted substrates was the autolysin AtlS, a 1160-amino acid protein with an N-terminal GH25 (glycosyl hydrolase family 25) catalytic domain and two C-terminal cysteines at residues Cys-1048 and Cys-1069. Notably, AtlS was recently demonstrated to be crucial for autolysis in S. gordonii (21). Given the lack of autolytic activity in the sdbA mutant (Fig. 2D), we hypothesized that SdbA is required for the formation of a disulfide bond in AtlS and that this bond contributes to proper folding into a functional conformation.

Using a zymogram to test the autolytic activity of SDS-extracted surface proteins, we found that the activity of AtlS from the sdbA mutant was dramatically reduced compared with the parent and complemented mutant, which produced distinct bands of clearing at ~130 and 90 kDa (Fig. 6A). This banding pattern is consistent with previous reports showing that autolysins appear as doublets with full-length and processed forms (21, 47), and Western blots confirmed that these bands corresponded to AtlS (Fig. 6B). In comparison, only the 130 kDa band was detected in extracts from the sdbA mutant (Fig. 6B). This suggested that SdbA plays a part in the processing and activity of AtlS, possibly via the formation of a disulfide bond in the C-terminal cysteines.

To determine the in vivo disulfide status of AtlS in the parent and sdbA mutant, we carried out cysteine alkylation experiments with maleimide-PEG2-biotin. In this reaction, the maleimide moiety forms a thioether bond with free cysteine thiols, resulting in a biotinylated protein that can be detected with avidin-alkaline phosphatase (AP), whereas disulfidebonded cysteines are blocked from reaction. Bands detected with avidin-AP that corresponded to AtlS were identified based on molecular weight. To ensure that the bands aligned, control experiments were carried out by cutting individual lanes of Western blots in half and reacting with either avidin-AP or anti-AtlS.

Alkylated protein extracts from the same sample were run on Western blots and reacted with either avidin-AP to detect biotinylated proteins or anti-AtlS to determine the total amount of AtlS in the sample, thus serving as a loading control. The results for the parent strain showed a weak band detected by avidin-AP at the same molecular weight as the full-length AtlS. However, when samples were reduced with DTT prior to alkylation, there
was a marked increase in the intensity of this band, indicating the presence of a disulfide bond (Fig. 7A). No reaction was observed in the 90 kDa band, presumably because processing had removed the C-terminal cysteine residues. This is a logical explanation given that the active site of AtlS is located at the N terminus, and processing at the C terminus resulting in a reduction in molecular mass from 130 to 90 kDa would entail removal of ∼360 amino acids, including both cysteine residues. In contrast, cysteines in AtlS from the sdbA mutant were efficiently alkylated both with and without DTT treatment, showing that the protein lacked a disulfide bond (Fig. 7A).

Our analyses suggested that a portion of AtlS produced in the parent contained a disulfide bond, whereas the sdbA mutant lacked disulfide bonds entirely. To confirm these results and eliminate background from unbonded thiols, we used a differential thiol-trapping approach (35). Surface protein extracts containing AtlS were reacted with iodoacetamide to block free thiol groups, whereas cysteine residues already disulfide-bonded are protected from reaction. Following the removal of excess iodoacetamide, disulfide bonds were reduced with DTT, and the newly generated thiol groups were subsequently alkylated with maleimide-PEG₂-biotin. The results indicated that AtlS generated in the parent contains an intact disulfide bond, because biotinylated proteins were only detected in the sample reduced with DTT (Fig. 7B). In contrast, treatment with iodoacetamide efficiently blocked biotinylation of AtlS from the sdbA mutant, and reduction with DTT did not generate free thiol groups (Fig. 7B). Densitometry analysis indicated that ∼30% of the 130-kDa AtlS in the parent strain contained a disulfide bond, in contrast to none in the sdbA mutant (Fig. 7C).

To substantiate the importance of this disulfide bond for AtlS function, we constructed a C1069S point mutation in atlS in the parent strain. Importantly, AtlS is not predicted to contain NlpC/p60 or CHAP (cysteine- and histidine-dependent amidohydrolase/peptidase) domains, and it is unlikely that the point mutation eliminated a catalytic cysteine (48).

Similar to the sdbA mutant, the C1069S mutation resulted in a loss of autolytic activity (Fig. 6C), and zymogram analysis confirmed that AtlS was inactive (Fig. 6A). Western blots showed that the C1069S mutant produced three immunoreactive bands, two bands at the same molecular mass as the parent (130 and 90 kDa) in addition to a strong band at ∼85 kDa (Fig. 6B).
**Novel Thiol-disulfide Oxidoreductase in *S. gordonii***

Homologs of SdbA Are Present in Other Gram-positive Bacteria—Our results demonstrate that disulfide bond formation plays a more important role in *S. gordonii* physiology than previously recognized, and we wondered if this phenomenon extended to other Gram-positive species as well. Unlike the Actinobacteria and aerobic Firmicutes, the majority of facultatively anaerobic and anaerobic Firmicutes are not predicted to form disulfide bonds or to encode DsbA-like proteins (1, 4). Remarkably, a search for SdbA homologs identified similar proteins in a range of other species, including important pathogens, such as *Clostridium tetani* and *Streptococcus pneumoniae* (Fig. 8A). The top hit in *M. tuberculosis* H37Rv corresponded to *Mtb* DsbF (22.6% identity/37.9% similarity), which has been confirmed to function as an oxidase (16). Analysis with SignalP predicted that all of the SdbA homologs contain a signal peptide. In addition to the CXXC active site and conserved proline residue, the sequences also shared a conserved sequence immediately N-terminal to the CXXC active site with a consensus sequence of WAXW (Fig. 8B). Similar motifs have been observed in thioredoxin-like proteins (49), although their functional significance is unknown.

**DISCUSSION**

Disulfide bond formation in Gram-positive bacteria is a poorly understood process, and investigations have been hindered by a lack of mutant phenotypes and known substrates. Gram-positive bacteria produce fewer disulfide-bonded proteins than Gram-negative species (8) and in some cases use covalent amide bonds as an alternative to disulfide bond formation (50–52). Nevertheless, there are examples of disulfide bond-containing proteins produced by Gram-positive species, such as diphtheria and tetanus toxins, botulinum neurotoxin A, and streptococcal pyrogenic exotoxin (17, 18, 52, 53). In Gram-negative pathogens, DsbA forms disulfide bonds in secreted toxins, but the machinery that forms these bonds in Gram-positive species has not been identified (54, 55).

Among the few TDORs that have been identified in Gram-positive species, there are several examples of systems dedicated to bacteriocin production, including *B. subtilis* 168 BdbAB, *S. thermophilus* LMD BlpG STP, and *Streptococcus bovis* HJ50 Sdb1 (37, 56, 57). Despite the annotation as a bacteriocin transport accessory protein, Sgo.1216 did not affect bacteriocin production, and the function of this protein remains unknown. However, it is possible that Sgo.1216 affects production or activity of an unidentified bacteriocin, which inhibits strains of bacteria different from the ones used in our assays. Regardless, these types of TDORs probably perform specialized functions within the cell, although not necessarily related to bacteriocin production.

Two of the predicted TDORs, Sgo.1177 and Sgo.1171, did not exhibit any phenotypes in our assays but share a high level of homology with a recently reported TlpA protein from *S. pneumoniae* (BLASTP e values of 10^{-65} and 10^{-43}, respectively). *S. pneumoniae* TlpA is proposed to work in conjunction with MsrAB and CcdA to reduce oxidized methionines, and Sgo.1177 and Sgo.1171 may have the same function in *S. gordonii* (58). Notably, Sgo.1177 is located immediately upstream of a protein annotated as a methionine sulfoxide reductase.
Overall, it appears that most of the TDORs investigated here either perform specialized functions in the cell or exhibit functional redundancy that would require multiple mutations to produce observable phenotypes. In contrast, SdbA displayed a pleiotropic mutant phenotype, indicative of an important biological function and possibly broad substrate specificity. SdbA was essential for the production of anti-CR1 scFv, a protein that requires two disulfide bonds for stable folding. This suggests that SdbA directly contributes to disulfide bond formation, and in vitro analysis confirmed that SdbA exhibits oxidase activity.

Our results suggest that the autolysin AtlS is a natural substrate of SdbA and that SdbA contributes to the formation of an intramolecular disulfide bond in AtlS that is required for protein stability.

**FIGURE 8. SdbA homologs are present in other Gram-positive bacteria.** A. Clustal Omega alignment of homologs to *S. gordonii* SdbA (YP_001451255.1) identified using DELTA BLAST. The CXXC active site and conserved cis-proline residue are highlighted. B. Weblogo (63) showing the consensus sequence of the region surrounding the CXXC motif. **Spy**, *Streptococcus pyogenes* M1 (NP_269625.1); **Ssa**, *Streptococcus sanguinis* SK36 (YP_001035078.1, YP_001035083.1); **Sp**, *S. pneumoniae* TIG4 (NP_345164.1, NP_345477); **Smu**, *S. mutans* UA159 (NP_721554); **Smt**, *S. mitis* NCTC 1226 (ZP_07644877); **DsbF**, *M. tuberculosis* H37Rv (NP_216193.1); **TlpA**, *C. tetani* E88 (NP_780861.1); **H04402_00254**, *Clostridium botulinum* H04402_065 (YP_005676832.1).
cessing and activity. Consistent with this finding are previous reports of mutations that inhibit processing of the S. mutans autolysin, AtlA, also resulting in a loss of autolytic activity (29, 31). Inactivation of AtlS probably contributed directly to several of the phenotypes observed in the mutant, including impaired autolysis and eDNA release (21). However, not all of the sdbA mutant phenotypes can be attributed solely to inactivation of AtlS. For example, eDNA release in S. gordonii has also been reported to occur without cell lysis, via unknown mechanisms, which appear to be affected by SdbA as well (44, 59). In addition, atlS mutants are unable to form biofilms, whereas the sdbA mutant showed increased biofilm formation, a discrepancy that probably reflects the impact of SdbA on several substrates.

In addition to substrate proteins directly affected in the sdbA mutant, the observed phenotypes may also be the result of indirect effects. Because certain adhesins in S. gordonii can transcriptionally regulate the levels of others, it is plausible that inactivation of a single SdbA substrate could influence expression of multiple surface proteins (60). This situation is exemplified by a previous report that inactivation of the S. gordonii surface antigens SspA and SspB resulted in up-regulation of other surface proteins and a 20% increase biofilm formation (46). Moreover, the lack of competence and bacteriocin activity in the sdbA mutant strongly suggests an effect on the ComDE two-component system, which in turn could affect all of the genes in the regulon (24). We are currently investigating the relationship between SdbA and the ComDE system.

Along with AtlS, we identified 35 candidate SdbA substrates, including surface adhesins, cell wall–binding proteins, transporter proteins, and hypothetical proteins. It should be noted that some of these proteins contain catalytic cysteines unlikely to be involved in disulfide bond formation, such as Sgo.2107, a CHAP domain protein, and Sgo.1176, a methionine sulfoxide reductase (48, 61). However, given the ability of S. gordonii to produce hydrogen peroxide, it is plausible that the majority of these proteins would form disulfide bonds because single cysteines are vulnerable to irreversible oxidation (59).

SdbA has the highest homology to the TlpA/DsbE/ResA family of proteins, a diverse group of redox–active proteins that includes E. coli CcmG, a reductase that reduces apocytochrome c (7), as well as M. tuberculosis oxidases, DsbE and DsbF, capable of catalyzing disulfide bonds (15, 16). The results presented here show that SdbA is crucial for disulfide bond formation; however, additional biochemical analysis is required to determine the role that SdbA plays in this process. Although SdbA exhibits oxidase activity in vitro, suggesting that it may catalyze disulfide bond formation in vivo, it could also contribute to protein stability through disulfide isomerase activity to ensure correct disulfide connectivity. We are currently investigating the enzymatic activity of SdbA to address these questions.

SdbA homologs are distributed among a range of Gram-positive species, most of which are anaerobic Firmicutes that lack homologs to DsbA. This suggests that SdbA may belong to a novel class of TDOR that has gone undetected due to its lack of homology with DsbA. SdbA and its homologs are similar to DsbA and BdbD in having a CXXC catalytic domain and a conserved C-terminal proline residue but distinct from DsbA/BdbD in having a WAXW motif immediately next to the CXXC motif. SdbA is also distinct from the actinobacterial DsbA recently described by Daniels et al. (8), and we did not find homologs to this protein in S. gordonii or in other species with SdbA homologs. Thus, these enzymes may represent alternative strategies for disulfide bond formation, and SdbA may be favored by anaerobic species. Like S. gordonii, some species were found to encode more than one SdbA-like protein. Our analysis of TDORs in S. gordonii shows that these types of proteins may have similar sequences but very different effects on the cell, and experimental investigation is essential to determining the physiological roles of predicted TDORs.

In conclusion, our results show that SdbA plays an important role in disulfide bond formation and that inactivation of SdbA results in a pleiotropic phenotype. In contrast to previously described Gram-positive TDORs, many of which have no substrates or phenotypes associated with them, SdbA is unique in affecting multiple biological processes. This suggests that SdbA represents a novel class of thiol-disulfide oxidoreductase and a possible functional equivalent to E. coli DsbA, specific to certain Gram-positive bacteria.

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