⌘ *Author's Choice*

Disulfide Bond Formation and Cysteine Exclusion in Gram-positive Bacteria*[s]◆

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Most secretion pathways in bacteria and eukaryotic cells are challenged by the requirement for their substrate proteins to mature after they traverse a membrane barrier and enter a reactive oxidizing environment. For Gram-positive bacteria, the mechanisms that protect their exported proteins from misoxidation during their post-translation maturation are poorly understood. To address this, we separated numerous bacterial species according to their tolerance for oxygen and divided their proteomes based on the predicted subcellular localization of their proteins. We then applied a previously established computational approach that utilizes cysteine incorporation patterns in proteins as an indicator of enzymatic systems that may exist in each species. The Sec-dependent exported proteins from aerobic Gram-positive Actinobacteria were found to encode cysteines in an even-biased pattern indicative of a functional disulfide bond formation system. In contrast, aerobic Gram-positive Firmicutes favor the exclusion of cysteines from both their cytoplasmic proteins and their substantially longer exported proteins. Supporting these findings, we show that Firmicutes, but not Actinobacteria, tolerate growth in reductant. We further demonstrate that the actinobacterium *Corynebacterium glutamicum* possesses disulfide-bonded proteins and two dimeric Dsb-like enzymes that can efficiently catalyze the formation of disulfide bonds. Our results suggest that cysteine exclusion is an important adaptive strategy against the challenges presented by oxidative environments.

Proper protein targeting and maturation are fundamental to the homeostasis of all organisms. Whereas many of the features that direct the membrane topology and localization of a protein are known, the strategies employed by cells to promote the proper maturation of their proteins under different environmental conditions are not entirely understood.

Protein secretion is a dynamic and complex process due to the membrane separation of synthesis and folding (1–3). The majority of secreted proteins are translocated across a membrane into an oxidizing environment, where their folding and maturation occur. Bacteria have evolved several secretory pathways for translocating their proteins across the plasma membrane that can be categorized as either Sec-dependent or Sec-independent (4, 5). The Sec-dependent pathway, which is essential in all organisms, is the major route of protein secretion.

In bacteria, the majority of proteins that are targeted for Sec-dependent secretion contain a canonical N-terminal signal peptide that is either directly recognized by SecA or delivered to SecA with the aid of the secretion-dedicated chaperone SecB (6). SecA then drives the translocation of the protein through the SecYEG channel. Alternatively, most plasma membrane proteins and a small number of secretory proteins are recognized as ribosome-bound nascent chains by the signal recognition particle, which directs them to SecYEG, where they are co-translationally inserted into or translocated across the plasma membrane. Upon emergence from the translocon, nascent secretory proteins mature after removal of their signal peptide by either the type I signal peptidase or, in the case of lipoproteins, the type II signal peptidase (7).

Although the obstacles associated with protein folding are fundamentally similar across all organisms, the machinery involved in this process and the environment where it occurs can differ dramatically (8–10). In Gram-negative bacteria, secreted proteins mature in the oxidative periplasm with the aid of molecular chaperones and disulfide bond-forming machinery (11–13). Gram-positive bacteria lack this protected compartment due to the absence of an outer membrane. Thus, their secreted proteins have to mature in the unregulated environment of the thick, negatively charged cell wall composed of multilayered networks of peptidoglycan and teichoic acids (supplemental Fig. S1).

Cysteine residues perform a variety of essential functions in proteins, from metal binding and enzyme catalysis to structural stability through the formation of disulfide bonds (14). However, the reactive thiol side chain that is amenable to these roles can also negatively impact the function of a protein by the formation of incorrect disulfide bonds or other deleterious modi-

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[s] The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S13 and Table 1.

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fications. The high reactivity of cysteine thiols and their susceptibility to attack by reactive oxygen species likely create an oxidative selection pressure on cysteine-containing proteins that relates directly to their environment and localization (15). Consequently, bacteria have evolved systems that keep their cytoplasm in a reducing state and maintain the proper oxidation state of the cysteines in their extracellular proteins (16–19).

The periplasm-localized disulfide oxidoreductases of the Dsb family have been best characterized in *Escherichia coli*. *E. coli* DsbA is a soluble monomeric enzyme with a thioredoxin-like fold and a Cys-*X*-*X*-Cys active site that aids in the maturation of secreted proteins by catalyzing their disulfide bond formation immediately after their translocation into the periplasm (supplemental Fig. S1) (20, 21). DsbA is maintained in its oxidized state by the membrane-bound protein DsbB (22, 23). DsbC and DsbG, homodimers that function as disulfide isomerases or reductases, and DsbE, a thioredoxin-like protein involved in cytochrome *c* maturation, are all kept in their reducing state by the membrane-bound protein DsbD (19, 24–26). Together, the Dsb proteins in Gram-negative bacteria aid in the maturation of exported proteins and protect them from improper oxidation.

Several studies have searched for homologs of the known disulfide bond-forming machinery to predict whether certain bacteria are capable of oxidizing their secreted proteins (16, 27, 28). Mallick *et al.* (29) were the first to demonstrate that hyperthermophilic Archaea, which possess disulfide bonds in their cytoplasmic proteins, have a bias for encoding proteins with an even number of cysteine residues. Recently, Beckwith and co-workers (27) applied a similar reasoning to examine whether the predicted exported proteins from a large number of bacterial species have an even-number cysteine bias. These results were then correlated to whether DsbA and DsbB homologs were present in the corresponding genomes.

We used a combined computational and experimental approach to investigate the different strategies Gram-positive bacteria have evolved to compensate for changes in the oxidative environment that accompanies the localization of their proteins and their tolerance for oxygen. In agreement with recent work, our predictions indicate that Gram-positive Actinobacteria likely form disulfide bonds in their exported proteins (as do Gram-negative Proteobacteria) and that aerobic Gram-positive Firmicutes tend to generally exclude cysteines from their exported proteins (27). Additionally, we also found that aerobic Firmicutes have substantially longer exported proteins and favor the exclusion of cysteines from their cytoplasmic proteins. Supporting these predictions, we show that Firmicutes, but not Actinobacteria, can tolerate high levels of reductant, indicating that Firmicutes do not rely on disulfide-bonded proteins for growth. We observed several disulfide-bonded proteins in Actinobacteria, but not in Firmicutes, and show that two different dimeric Dsb-like proteins from the actinobacterial species *Corynebacterium glutamicum* are capable of productive disulfide bond formation. These results provide further insight into how organisms combat oxidative challenges and how their proteins have coevolved in the absence or presence of various enzymatic redox systems to overcome these obstacles.

EXPERIMENTAL PROCEDURES

Acquisition, Classification, and Homology Reduction of Bacterial Genomes—Proteomes from the 340 analyzed bacterial species were downloaded from the European Bioinformatics Institute (EMBL-EBI Database). When several sequenced strains from a given species were available, one proteome from an ATCC strain was chosen to represent that species. The species were manually classified into their respective phyla (Proteobacteria, Firmicutes, and Actinobacteria) and for their ability to grow in the presence of oxygen (strict aerobes and facultative anaerobes *versus* strict anaerobes). Supplemental Table 1 shows the species distribution in each category. The individual bacterial proteomes were restricted to those proteins that were at least 80 amino acid residues in length. To prevent bias due to redundant protein families in the individual species, each bacterial proteome sequence set was internally homology-reduced using CD-HIT with a sequence identity threshold of 40% (30). These two initial screening steps retained 945,399 amino acid sequences for analysis and discarded 121,981 proteins based on homology reduction and 64,920 sequences as peptides.

Protein Topology Prediction and Cysteine Analysis—Protein sequences were classified according to predictions for the presence of transmembrane (TM)[4] regions using SCAMPI and signal peptidase (SPase) type I and II signal peptides using SignalP 3.0 and LipoP 1.0, respectively (supplemental Fig. S2) (31–33). The proteome sets for each of the four classifications were compiled and analyzed for their cysteine content: (i) lipoproteins, containing a SPase II signal peptide and no TM regions (21,826 or 2.3% of the sequences); (ii) secreted proteins, containing a SPase I signal peptide, no SPase II signal peptide, and no TM regions (77,419 or 8.2% of the sequences); (iii) TM proteins, containing at least one TM region, no SPase II signal peptide, but possibly a SPase I signal peptide (213,419 or 22.6% of the sequences); and (iv) cytoplasmic proteins, containing no signal peptide or TM regions (631,735 or 66.8% of the sequences). Sequences predicted to contain a SPase I signal peptide by only one of the neural network and hidden Markov model predictors and those with both a SPase II signal peptide and a TM region were discarded (1,000 or 0.1% of the sequences). In all sequence sets, the predicted signal peptides were removed and analyzed independently for cysteine occurrence. For lipoproteins, the N-terminal cysteine was excluded from the calculations.

Experimentally Investigated Bacterial Strains—*Arthrobacter luteus* (ATCC 21606), *Bacillus cereus* (ATCC 14579), *Bacillus subtilis* (ATCC 6051), *Corynebacterium glutamicum* (ATCC 13032), *E. coli* (UPEC (UTI89)) (34), *Enterococcus faecalis* (ATCC 29212), *Haemophilus influenzae* (SMI 120 strain 1 type B), *Micrococcus luteus* (ATCC 11880), *Pseudomonas aeruginosa* (ATCC 33357), *Rhodococcus equi* (ATCC 6939), *Salmonella typhimurium* (ATCC 14028), *Staphylococcus aureus* (ATCC 25923), and *Streptococcus pyogenes* (ATCC 11434) were used in this study.

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[4] The abbreviations used are: TM, transmembrane; SPase, signal peptidase; DTT, dithiothreitol; BisTris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol.

Two-dimensional SDS-PAGE—Bacterial cultures were grown in brain-heart infusion medium to mid-log phase and sedimented by centrifugation at $15,000 \times g$ for 10 min. Pellets were resuspended in 20 mM Tris-Cl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 20 mM *N*-ethylmaleimide, and 200 μg/ml mutanolysin (Sigma) and rotated for 30 min at 4 °C. Lysates were then adjusted to 1% SDS and 8 M urea and passed through a French press. Insoluble cell debris was removed by centrifugation at $15,000 \times g$ for 10 min. Samples were resolved in the first dimension via nonreducing SDS-PAGE. For the second dimension, the lanes were excised with a razor, placed in sample buffer containing 100 mM dithiothreitol (DTT), heated for 5 min at 95 °C, overlaid onto a 1.0-mm 2D-Well NuPAGE 10% BisTris gel, separated by electrophoresis, and resolved by Coomassie Blue staining (35).

Bacterial DTT Susceptibility Assay—Bacterial strains were grown at 37 °C to mid-log phase in brain-heart infusion medium supplemented with 10% glucose bouillon (10% horse serum, 1% meat extract, 1% peptone, 1% dextrose, and 0.5% NaCl, pH 7.5). Cultures were diluted to $OD_{600 \, nm} = 0.050$ in unconditioned growth medium and transferred to wells of a Honeycomb 2 plate (Isotron) containing DTT. The growth kinetics were monitored by changes in the $OD_{600 \, nm}$ at 37 °C using a Bioscreen C plate reader (Labsystems). For *H. influenzae,* the growth medium was supplemented with 0.2 mM NAD.

DNA Constructs—The coding sequences for CG0026, CG0354, and CG2799 from *C. glutamicum* (ATCC 13032) and DsbA (C4447) from *E. coli* (UTI89) were PCR-amplified from chromosomal DNA with 5′-primers that excluded the N-terminal signal peptide and replaced it with a His$_6$ tag. The PCR products were subcloned into the pET21d expression vector (Novagen), and each construct was verified by DNA sequencing.

Recombinant Protein Expression, Purification, and Size Exclusion Chromatography—Bacterial cultures were grown to $A_{600 \, nm} = 0.6$; induced with 1 mM isopropyl β-D-thiogalactopyranoside for 3 h; sedimented; and resuspended in 20 mM Tris, pH 7.5, 500 mM NaCl, and 20 mM imidazole with 200 μg/ml lysozyme and 200 μg/ml DNase I. Following rotation for 30 min at 4 °C, the lysates were sonicated and clarified by centrifugation at $15,000 \times g$ for 10 min. Proteins were then isolated using nickel-Sepharose 6 Fast Flow (GE Healthcare); washed; eluted with 500 mM imidazole in 20 mM Tris, pH 7.5, and 500 mM NaCl; and dialyzed against 20 mM Tris, pH 7.5.

Protein concentrations were determined by the absorbance at 280 nm using their respective extinction coefficients and molecular masses. Size exclusion chromatography was performed on an ÄKTA FPLC system (GE Healthcare) equipped with a Superdex 75 column. The molecular mass standards were run according to the manufacturer's instructions; all protein samples were dialyzed into and separated with phosphate-buffered saline, pH 7.4; and the absorbance was monitored at 260 and 280 nm.

Determination of Oxidase and Reductase Activities—Oxidase activity was assayed by analyzing the cleavage of cCMP by reduced RNase A refolded in the presence of each enzyme (36). RNase A (Sigma) was reduced by overnight incubation at 27 °C in 100 mM Tris acetate, pH 8.0, containing 6 M guanidine HCl

and 140 mM DTT. DTT and guanidine were removed by size exclusion chromatography with Sepharose G-10 beads. RNase A reduction was verified by the mobility shift created by the large 525-Da free thiol-modifying agent maleimide-polyethylene oxide-biotin and with 5,5′-dithiobis(2-nitrobenzoic acid) (supplemental Fig. S11*A*). Each enzyme was assayed in a 1-ml solution containing 100 mM Tris acetate, pH 8.0, 2 mM EDTA, 0.2 mM GSSG, 1 mM GSH, and 10 μM reduced RNase A. Following a 2-min incubation, cCMP was added at a final concentration of 4.5 mM, and the absorbance at 296 nm was recorded. Reductase activity was determined at 27 °C by measuring the turbidity of insulin at $OD_{600 \, nm}$ that occurs upon precipitation of the reduced B chain (37). Insulin (Sigma) was resuspended at 5 mg/ml in 100 mM potassium acetate, pH 7.5, by dropping the pH to 3.0 with HCl and titrating back to pH 7.5 with KOH prior to adjusting to 2 mM EDTA and 10 μM DTT. The enzymes were assayed in a 200-μl solution containing 100 mM potassium acetate, pH 7.5, 2 mM EDTA, 320 μM DTT, and 200 μg of insulin.

RESULTS

Aerobic Gram-positive Actinobacteria Are Predicted to Stabilize Exported Proteins with Disulfide Bonds, Whereas Aerobic Firmicutes Exclude Cysteines—To gain an understanding of how Gram-positive bacteria have evolved to protect and stabilize their proteins in the different oxidative environments, we compared the cysteine incorporation patterns in different types of proteins from Gram-positive bacteria (Firmicutes and Actinobacteria) with those from Gram-negative bacteria (Proteobacteria). Initially, we downloaded the available proteomes from the 340 bacterial species of the major phyla of Gram-positive and Gram-negative bacteria (EMBL-EBI Database). The strict anaerobic species were then classified as "anaerobes," and all bacteria capable of aerobic respiration, including strict aerobes and facultative anaerobes, were grouped together as "aerobes" (supplemental Fig. S1 and Table 1).

Computational analysis was performed on the proteomes to separate them into the following subproteomes: lipoproteins, secreted proteins, TM proteins, and cytoplasmic proteins (Fig. 1*A* and supplemental Fig. S2). Because only cysteines with free thiols in mature secreted proteins are susceptible to oxidation, the signal peptides were removed and examined separately, and the lipoprotein lipid-linked N-terminal cysteines were excluded prior to analysis (supplemental Fig. S3) (38).

Bacteria with the capacity for disulfide bond formation have previously been shown to favor the incorporation of cysteines in pairs (*i.e.* an even-biased manner) in their substrate proteins (27, 29, 39). Thus, this characteristic can be used as a strong indication of disulfide bond formation. We first analyzed the average cysteine distribution in all of the predicted secreted proteins, lipoproteins, TM proteins, and signal peptides from aerobic and anaerobic Gram-positive and Gram-negative bacteria.

All but anaerobic Gram-positive bacteria favored an even number of cysteines in their secreted proteins and lipoproteins, and no obvious trends were observed in the signal peptides and TM protein loops (supplemental Fig. S3). However, when the exported protein (lipoprotein and secreted protein) subproteomes were analyzed separately, a large number of those from
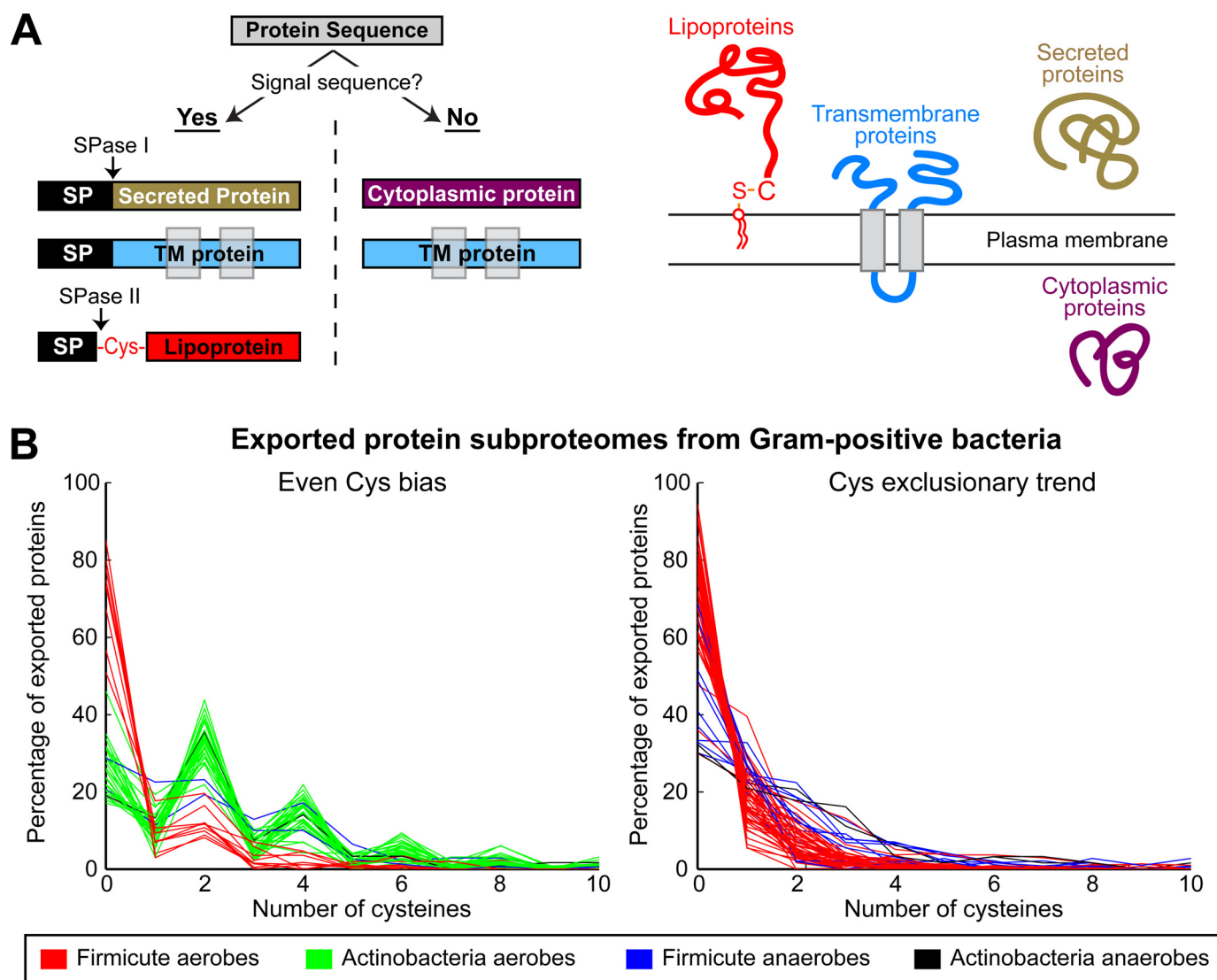
FIGURE 1. **Exported proteins from aerobic Firmicutes largely exclude cysteines, whereas those from Actinobacteria favor paired cysteines.** *A*, the schematics depict the protein characteristics used to classify the subproteomes. The SPase type I and II signal peptides (*SP*) and TM regions were used to identify and sort each proteome into its respective topology or subproteome. *B*, the exported proteins (secreted proteins and lipoproteins) from Actinobacteria show a profile suggestive of disulfide bond formation, whereas those from Firmicutes preferentially exclude cysteines. The subproteomes containing the exported proteins from Gram-positive bacteria were separated into two major cysteine incorporation patterns. All aerobic Gram-positive Actinobacteria possess an "even-numbered cysteine bias" (*left panel*) in their exported proteins, and the large majority of Gram-positive Firmicutes show a cysteine exclusion trend (*right panel*). Each *line* represents the cysteine distribution in the exported proteins from one bacterial proteome.

aerobic Gram-positive bacteria (~60%) were found to average less than one cysteine/protein and to lack the even-numbered cysteine bias (supplemental Fig. S4). From these observations, we were able to classify the vast majority of all of the exported protein subproteomes by one of two patterns.

The first is an "even cysteine-biased" pattern defined by an up-and-down trend in the percentage of exported proteins that correspond to an even (up) and odd (down) number of cysteines (0 Cys > 1 Cys and 2 Cys > 1 Cys), and the second is a "cysteine exclusion trend" (0 Cys > 1 Cys > 2 Cys) (Table 1). Only 5% of the exported protein subproteomes did not follow either pattern and were labeled as "remaining," and those with fewer than 25 proteins were excluded from further analysis (supplemental Fig. S5 and Table 1). Following this separation, we could confirm a conclusion in a recent study that an obvious distinction

exists between the two major phyla of Gram-positive bacteria (27).

All exported protein subproteomes from aerobic Actinobacteria fell into the even cysteine-biased pattern, whereas those from aerobic Firmicutes followed mainly the cysteine exclusion trend (Fig. 1*B*, Table 1, and supplemental Fig. S6). The exported proteins from aerobic Firmicutes have a mean of ~0.5 Cys, and an astonishing 70% of them are devoid of cysteine residues, compared with only 33% in anaerobic Firmicutes (supplemental Fig. S7). In contrast, the exported proteins from aerobic Actinobacteria have a mean of ~2.2 Cys, only 25% of them lack cysteine residues, and about 60% contain even numbers of cysteines, which is significantly higher than for aerobic Gram-negative Proteobacteria (supplemental Figs. S3*A*, S6, and S7). Thus, the maturation of the exported proteins from aerobic Acti-

TABLE 1

Distribution of bacterial exported and cytoplasmic subproteomes to the indicated cysteine incorporation trends

The number of subproteomes that were classifiable into one of the two cysteine incorporation trends is displayed with respect to the total number of subproteomes that were analyzed from each class of bacteria. For the specific species distribution and those that were not classifiable by these trends, see the supplemental data.

	Phyla	Exported proteins		Cytoplasmic proteins	
		Even bias	Exclusion	Semi-normal	Exclusion
Gram-positive bacteria					
Aerobic	Actinobacteria	31/31		27/31	4/31
	Firmicutes	10/64	51/64	17/64	43/64
Anaerobic	Actinobacteria	1/3	2/3	2/3	1/3
	Firmicutes	2/20	11/20	16/20	2/20
Gram-negative Proteobacteria					
Aerobic	Alpha	49/52		50/52	2/52
	Beta	34/34		33/34	1/34
	Delta	3/5	2/5	4/5	
	Epsilon	12/12		8/12	1/12
	Gamma	80/86		83/86	1/86
Anaerobic	Alpha	5/13	4/13	12/13	1/13
	Beta	2/2		2/2	
	Delta	2/11	9/11	11/11	
	Epsilon	1/1		1/1	
	Gamma	5/6		5/6	

nobacteria likely involves disulfide bond formation, and aerobic Firmicutes tend to exclude cysteines from their exported proteins (27).

Exported Proteins from Firmicutes Are Significantly Longer—To understand how the exported proteins from Firmicutes have evolved in the absence of disulfide bond stabilization, we examined their length in comparison with that of their cytoplasmic proteins. In Firmicutes, Actinobacteria, and Gram-negative bacteria, the average length of their mature exported proteins is longer than that of their respective cytosolic proteins, which are ~325 amino acids in length (Fig. 2*A*). However, the mature exported proteins from aerobic Firmicutes have an average length that is ~70 amino acids longer than that of their cytoplasmic proteins. This difference is substantially greater than the 7- and 16-amino acid increases seen in aerobic Actinobacteria and Gram-negative Proteobacteria, respectively. Although different cell wall attachment motifs in the Firmicute exported proteins may contribute to the increase in length, it is also possible that these proteins require more extensive noncovalent structural interactions to stabilize their proteins in the absence of disulfide bonds.

Aerobic Firmicutes Exclude Cysteines from Their Cytoplasmic Proteins—Analysis of the cytoplasmic subproteomes revealed that aerobic Gram-positive bacteria encode cysteines with a low frequency, resulting in a unique distribution profile that peaks at 0 Cys compared with 2 Cys in Gram-negative Proteobacteria and Gram-positive anaerobes (Fig. 2*B* and supplemental Fig. S8). Upon investigating the population variations of the cytoplasmic subproteomes from Gram-positive bacteria, we were able to assign them to either a cysteine exclusion trend (0 Cys > 1 Cys > 2 Cys) or a "semi-normal cysteine trend" (1 Cys > 0 Cys). Using these parameters, the majority of aerobic Firmicutes (67%) were found to have a cysteine exclusion trend in their cytoplasmic proteins, whereas Actinobacteria and anaerobic Firmicutes had a strong inclination toward a semi-normal cysteine trend (Fig. 2*C* and Table 1). Thus, aerobic Firmicutes appear to possess a general tendency to exclude cysteines from their proteins in comparison with other bacteria.

Actinobacteria Possess Disulfide-bonded Proteins—To examine whether aerobic Actinobacteria possess disulfide-bonded

proteins, whole cell lysates from *C. glutamicum* were separated and resolved by two-dimensional nonreducing/reducing SDS-PAGE (35). In accordance with the predictive data, both intermolecular (Fig. 3, below the *diagonal*) and large intramolecular (above the *diagonal*) disulfide-bonded proteins were present in *C. glutamicum* and in the control lysate from *E. coli*. As expected, no obvious disulfide-bonded proteins were detected in the lysate from the Firmicute *S. aureus*. Because some exported Firmicute proteins do not bind to the cell envelope following secretion, concentrated conditioned LB media from *S. aureus* and other Firmicutes were examined, but only a few proteins were observed, and none of them showed mobility shifts attributed to disulfide bond formation (data not shown).

Firmicutes Are Resistant to Highly Reducing Conditions—Many proteins contain intramolecular disulfide bonds that do not cause a mobility shift when comparing their nonreduced and reduced states following separation by SDS-PAGE. Therefore, as a second test of our predictions, we investigated the ability of various bacteria to tolerate growth in rich media containing the disulfide bond-reducing agent DTT. We hypothesized that Firmicutes, whose exported proteins are predicted to lack disulfide bonds, would tolerate higher levels of DTT in comparison with the disulfide bond-forming Actinobacteria. Strikingly, 20 mM DTT had little effect on the liquid growth of various aerobic Firmicute species, and more impressively, they were all capable of growth in 100 mM DTT (Fig. 4 and supplemental Fig. S9). In contrast, the growth of numerous species from Actinobacteria and Gram-negative Proteobacteria was sensitive to DTT in a concentration-dependent manner, with 20 mM DTT almost entirely inhibiting growth.

Actinobacteria Encode Two Dimeric Oxidoreductases Capable of Productive Disulfide Bond Formation—In Gram-negative Proteobacteria, the enzymes that facilitate disulfide bond formation are oxidoreductases or DsbA homologs that reside in the periplasm and are recharged by a membrane-bound DsbB homolog (12). Several studies have identified the presence of DsbA and DsbB homologs across bacteria and used them as an indication of disulfide bound formation (16, 27, 28). To examine whether *C. glutamicum* possesses Dsb homologs capable of catalyzing disulfide bond formation, we identified three puta-
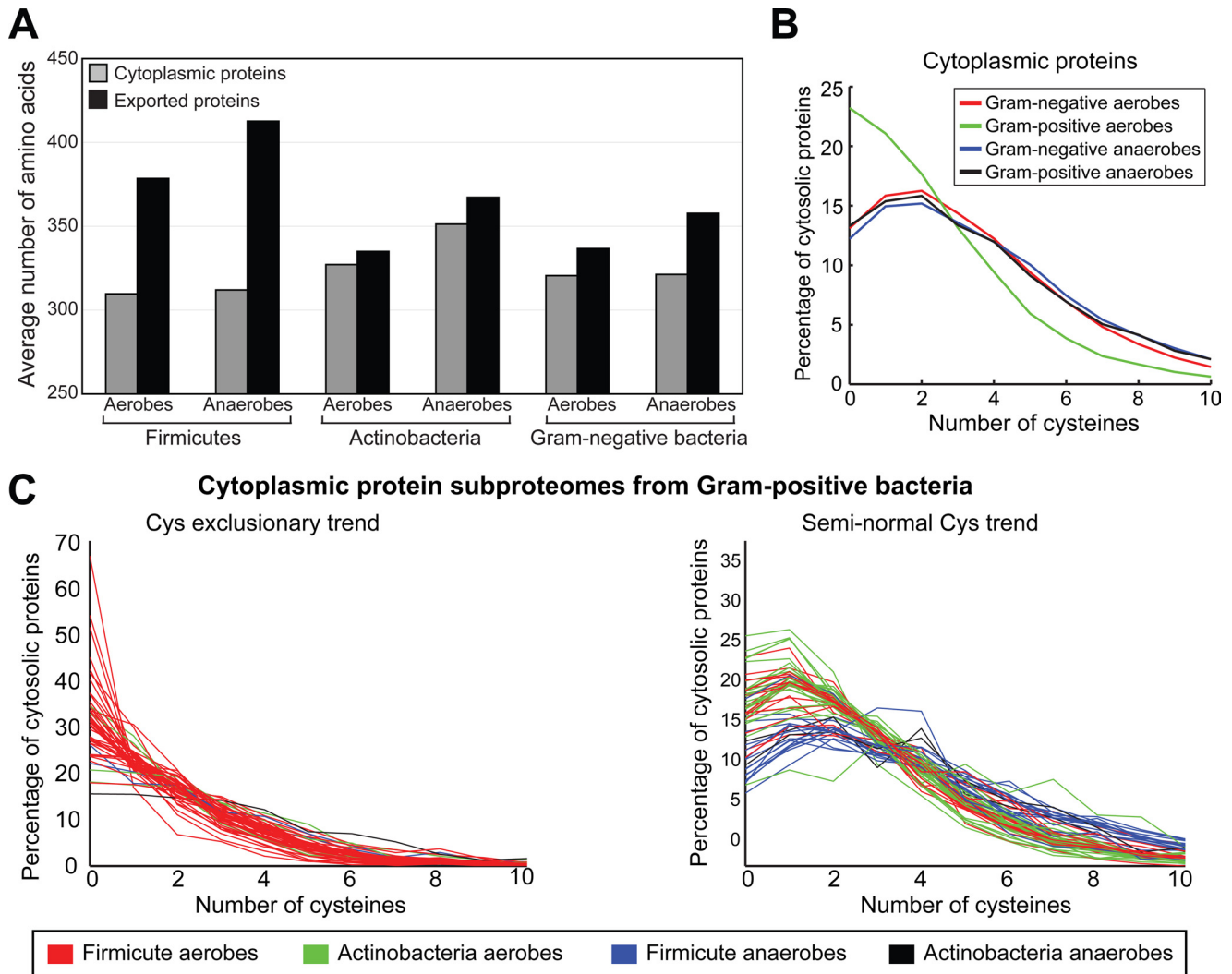
FIGURE 2. **Cysteine exclusion from the exported proteins, but not from the cytoplasmic proteins, of aerobic Firmicutes correlates with a substantial increase in protein length.** *A*, the average lengths of the predicted cytoplasmic and exported proteins from the indicated class of bacteria are displayed. The exported proteins from aerobic and anaerobic Firmicutes have an average length that is 22 and 32% longer than their respective cytoplasmic proteins, whereas those from aerobic and anaerobic Actinobacteria have average lengths that are only 2 and 5% longer, respectively. *B*, aerobic Gram-positive bacteria have a unique distribution in their cytoplasmic proteins with respect to cysteine content. Each *line* represents the percentage of cytoplasmic proteins from the different classes of bacteria that possess the indicated number of cysteine residues. *C*, the majority of aerobic Gram-positive Firmicutes show a cysteine incorporation pattern in their cytoplasmic proteins that follows the cysteine exclusion trend (*left panel*) *versus* the semi-normal cysteine distribution (*right panel*) favored by Actinobacteria and anaerobic Firmicutes.

tive secreted soluble Dsb-like proteins with Cys-*X*-*X*-Cys motifs (CG26, CG354, and CG2799) and analyzed their reductase and oxidase activities (Fig. 5).

In contrast to the DsbA homologs in Gram-negative bacteria, all three putative oxidoreductases from *C. glutamicum* exist as homodimers and not monomers (Fig. 5C). To test for reductase activity, equal molar amounts of each enzyme were examined for their ability to reduce insulin *in vitro* (37). CG26 reduced the highest amount of insulin, followed by DsbA from *E. coli* and CG2799, which showed weak activity. All of these enzymes functioned in a concentration-dependent manner, with the exception of CG354, which was inactive (Fig. 5D and supplemental Fig. S10).

Oxidation activity was determined by the ability of the enzymes to properly oxidize reduced RNase A (36). Both CG26 and CG2799, but not CG354, could productively oxidize reduced RNase A in a concentration-dependent manner (Fig.

5E and supplemental Fig. S11). Homology searches revealed that CG26 is a conserved secreted enzyme with its closest homologs in a diverse number of Actinobacteria (supplemental Fig. S12). In contrast, CG2799 has only two homologs, indicating that it likely performs a specific function for Corynebacteria (supplemental Fig. S13). Together, these data indicate that Actinobacteria also possess a functional DsbA-like homolog (A-DsbA) that potentially acts as a homodimer to oxidize their exported proteins during maturation.

DISCUSSION

Although several mechanisms are known for how Gram-negative bacteria and their proteins have adapted to the challenges posed by oxidative environments, very little is known about how Gram-positive bacteria have evolved to overcome these problems. To address this issue, we predicted the subcellular location of ~1,000,000 bacterial proteins by computa-

Gram-negative Proteobacteria
Escherichia coli

Gram-positive Actinobacteria
Corynebacterium glutamicum

Gram-positive Firmicute
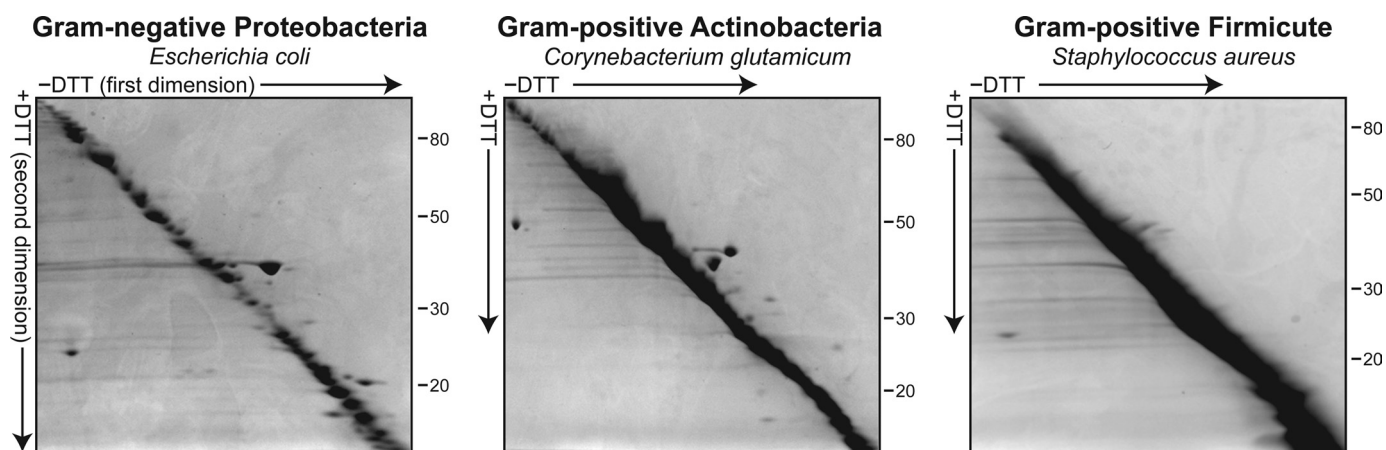*Staphylococcus aureus*

FIGURE 3. **Actinobacteria possess disulfide-bonded proteins.** Actinobacteria (*C. glutamicum*) and Gram-negative Proteobacteria (*E. coli*) express proteins with both inter- and intramolecular disulfide bonds. Whole cell lysates from the indicated bacteria were separated by two-dimensional nonreducing/reducing SDS-PAGE and visualized by Coomassie Blue staining. Intramolecular disulfide-bonded proteins lie above the *diagonal*, and intermolecular disulfide-bonded proteins lie below the *diagonal*.
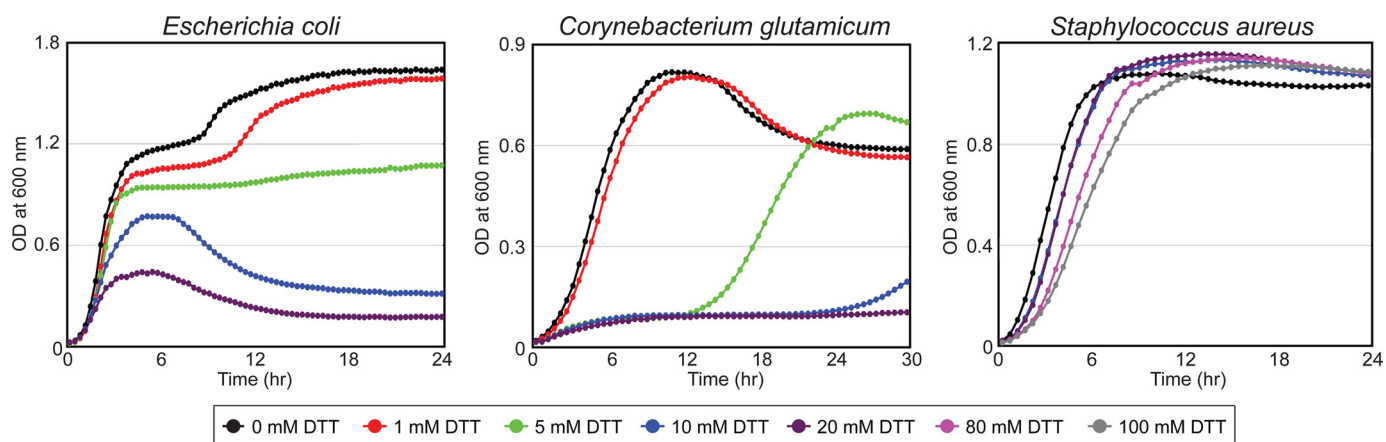

FIGURE 4. **Firmicutes have a unique tolerance for high levels of reductant.** The reductant DTT severely impaired the growth of aerobic Gram-negative Proteobacteria and Actinobacteria, whereas aerobic Firmicutes were capable of growth even at high DTT concentrations (for additional species, see supplemental Fig. S9). Representative growth curves from three independent experiments are displayed as changes in $OD_{600\,nm}$. The indicated bacterial strains were grown in brain-heart infusion medium in the presence of 0, 1, 5, 10, and 20 mM DTT (*E. coli* and *C. glutamicum*) or 0, 10, 20, 80, and 100 mM DTT (*S. aureus*).

tional analysis and examined their respective cysteine incorporation patterns to identify potential strategies these bacteria have evolved in response to oxidative pressure on their proteins. We further determined the sensitivity of a range of bacterial species to the reductant DTT, looked for disulfide-bonded proteins by two-dimensional nonreducing/reducing SDS-PAGE, and identified two dimeric actinobacterial Dsb homologs capable of productive oxidation. Together, our predictive and experimental results, combined with those from previous studies (16, 27, 28), strongly suggest that aerobic Gram-positive Actinobacteria possess the capacity for regulated disulfide bond formation, whereas Firmicutes do not. Instead, aerobic Firmicutes largely exclude cysteine residues from both their exported and cytoplasmic proteins.

The complexity of the folding process is largely dependent on the environment and the amino acid composition of a protein. Oxidative environments present a challenge for proteins that contain cysteines due to their susceptibility to attack by reactive oxygen species (15, 40, 41). To alleviate this problem, Gram-negative bacteria are known to maintain their cytoplasm in a reducing state by the thioredoxin and glutaredoxin systems (42,

43). In their periplasm, they localize a soluble enzyme (DsbA) that functions together with the plasma membrane-bound DsbB to catalyze disulfide bond formation in their exported proteins, which increases their stability and protects them from deleterious oxidation (16, 17).

Two different strategies were found for how Gram-positive bacteria address the environmental pressure that accompanies the localization of their proteins. As noted previously by Dutton *et al.* (27), each maturation strategy correlates with a distinct cysteine incorporation pattern that depends on whether the proteomes originate from the Firmicute or Actinobacteria phyla. Firmicutes appear to use a simple and unique method to cope with the oxidative pressure on their exported proteins that we term "cysteine exclusion."

Cysteine exclusion could have evolved by a selective growth advantage; however, its implementation as a protective strategy is evident based on the following findings. (i) Cysteine incorporation patterns are different between aerobic and anaerobic Firmicutes; (ii) compared with Actinobacteria and Gram-negative bacteria, Firmicutes have a high tolerance for reductant in the growth medium; and (iii) conserved cysteines that form disul-
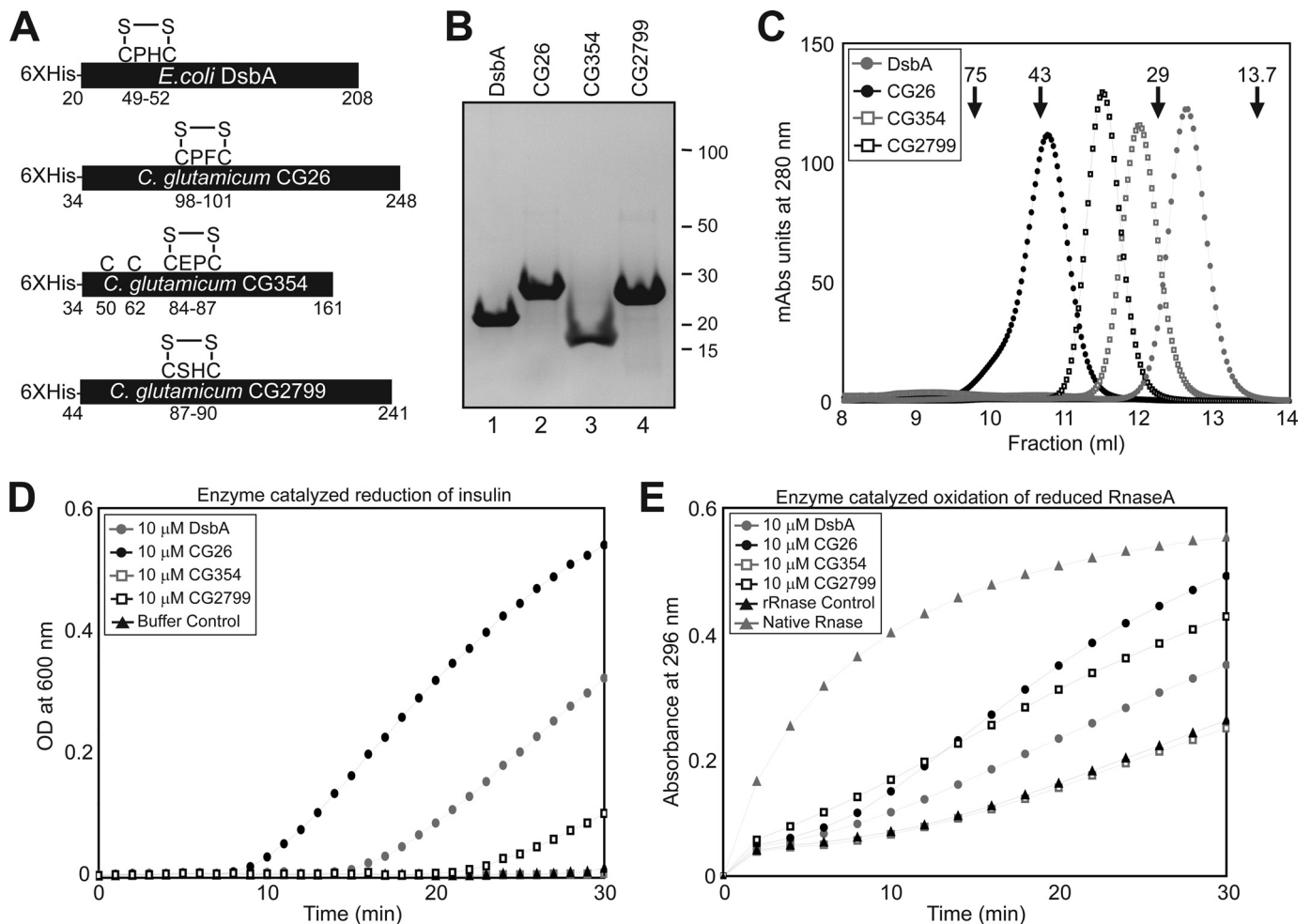
FIGURE 5. **The Actinobacterium C. glutamicum possesses two secreted dimeric oxidoreductases.** *A*, schematic representations and characteristics of the three putative oxidoreductases from *C. glutamicum* and DsbA from *E. coli*. The recombinant proteins were expressed with an N-terminal His tag replacing the signal peptide. The amino acid numbering refers to the unprocessed wild-type proteins, and the active-site Cys-*X*-*X*-Cys motifs are displayed. *B*, Coomassie Blue-stained gel of the recombinant proteins that were purified following expression in *E. coli*. *C*, size exclusion profiles of the purified proteins separated on a Superdex 75 column and monitored by the absorbance at 280 nm. The relative size of each standard is indicated with an *arrow*. *mAbs*, milli-absorbance. *D*, the actinobacterial enzyme CG26 from *C. glutamicum* efficiently reduces insulin *in vitro*, whereas CG2799 shows slight activity. Each enzyme was analyzed for the capacity to reduce insulin at 27 °C by monitoring precipitation of the reduced B chain observed by changes in $OD_{600\ nm}$. *E*, CG26 and CG2799 can correctly oxidize reduced RNase A *in vitro*. The ability of each enzyme to properly oxidize reduced RNase A (*rRnase*) in a glutathione redox buffer was assayed at 27 °C by monitoring the change in the absorbance at 296 nm that results from cleavage of cCMP by RNase A. The activities of native RNase A and reduced RNase A were included as controls.

fide bonds in class A β-lactamases and alkaline phosphatases from Gram-negative bacteria are not present in many of the aerobic Firmicute homologs (27, 44–47). Thus, the exported proteins in aerobic Firmicutes that contain cysteines, such as lipoproteins and the sortase enzymes involved in pilus biogenesis, do so as a functional requirement; otherwise, they would have been excluded.

Next, we characterized two disulfide bond-forming proteins in *C. glutamicum* and found that one (CG26) is conserved within the phylum of Actinobacteria. Like the oxidoreductase in the Actinobacterium *Mycobacterium tuberculosis,* which was identified by DsbE homology (48), these enzymes are not entirely homologous to the classical DsbA from Gram-negative bacteria. The differences in sequence ($\sim$15% identity) and oligomeric state from the *E. coli* DsbA suggest that, as a group, these enzymes should be classified as actinobacterial or A-DsbA. Together, these findings indicate that, although homology searches are useful to identify potential disulfide

bond-forming enzymes (16, 27, 28), experimental validation is essential to confirm that these proteins function as predicted (41).

The fact that A-DsbA enzymes exist as a dimer and not a monomer like the *E. coli* DsbA has several functional implications. The dimerization of A-DsbA potentially creates specificity for its membrane-bound partner, and it also places two Cys-*X*-*X*-Cys active sites in close proximity, which mimics the eukaryotic protein-disulfide isomerases that reside in the endoplasmic reticulum (49). The major difference is that protein-disulfide isomerases are monomeric proteins with two Cys-*X*-*X*-Cys active sites. It has recently been proposed that homologs of the vitamin K epoxide reductase enzyme recharge actinobacterial DsbA at the plasma membrane (27, 50). The vitamin K epoxide reductase enzyme that has been proposed to recycle A-DsbA also has potential analogy to eukaryotic oxidation, as homologs exist in the endoplasmic reticulum (27, 50). Thus, disulfide bond formation in Actinobacteria may be more evo-

lutionarily related to eukaryotes than to Gram-negative bacteria, suggesting that eukaryotic vitamin K epoxide reductase homologs may be responsible for recharging protein-disulfide isomerase.

One intriguing concept that arose from this study is that Firmicutes also favor cysteine exclusion in their cytoplasmic proteins. This suggests that aerobic Firmicutes may have a high level of oxidative pressure on their cytoplasmic proteins as well. A possible explanation for this is that the outer membrane, which is absent in Firmicutes, provides an additional permeability barrier that aids in the regulation of the cytoplasmic redox conditions.

These findings indicate that aerobic Actinobacteria likely possess a regulated compartment that contains the machinery for disulfide bond formation (27, 48). Supporting this, cryo-electron tomography studies have shown that the mycolic acids covalently linked to the peptidoglycan of Actinobacteria create an outer membrane-like structure that is absent in Firmicutes (51, 52). Thus, the presence of an outer membrane appears to be a strong driving force for evolving a system that facilitates disulfide bond formation by creating a regulated folding environment that co-localizes the enzymes and substrates.

Firmicutes such as *B. subtilis, S. aureus, E. faecalis*, and *Listeria monocytogenes* have previously been reported to encode homologs of DsbA from Gram-negative bacteria (16, 27, 28). Of these, *B. subtilis* is the only Firmicute that also encodes DsbB and for which substrates have been identified, but neither of these two substrates are involved in essential processes (53–57). In the case of *S. aureus*, where no disulfide-bonded substrates have been identified, the lipoprotein homolog of DsbA is thought to function independently by utilizing extracellular oxidants for its recycling in the absence of DsbB (28, 58, 59). Our analysis, combined with the existence of only two validated substrates that possess enzyme-catalyzed disulfide bonds, accentuates that Firmicutes generally exclude cysteines from their exported proteins. Thus, the few Firmicutes that contain DsbA and DsbB homologs likely utilize them for disulfide bond formation in proteins with nonessential niche functions.

From an evolutionary perspective, cysteine exclusion likely provides a selective advantage in extreme redox environments, potentially explaining why Firmicutes are over-represented among the bacteria that colonize and infect oxygen-exposed tissues such as the epidermis and the respiratory tract (60). This is supported by our finding that Firmicutes tolerate strong reductants and by the fact that Firmicute pathogens such as *Streptococcus pneumoniae* actively produce high levels of the powerful oxidant hydrogen peroxide (61). Moreover, the simple strategy of cysteine exclusion may provide Firmicutes with some protection against the oxidation-based immune defenses employed at the host-pathogen interface by neutrophils and macrophages. On the negative side, the inability of Firmicutes to cope with cysteine residues could create a barrier for lateral gene transfer, reducing the variability of their exported proteins, which constitute the majority of bacterial virulence factors. However, recent studies have demonstrated that Firmicutes can also maintain the structural stability of their exported proteins by utilizing alternative covalent bonds, which likely contributes to their ability to exclude cysteines (62). Although it

is clear that Firmicutes in general do not possess the capacity for disulfide bond formation, further investigation into whether they protect their few exported proteins with single cysteines by a reductase system that is functionally analogous to the one recently described in *E. coli* is warranted (19).

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