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MINIREVIEW

Disulfide-Bond-Forming Pathways in Gram-Positive Bacteria

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Disulfide bonds are important for the stability and function of many secreted proteins. In Gram-negative bacteria, these linkages are catalyzed by thiol-disulfide oxidoreductases (Dsb) in the periplasm. Protein oxidation has been well studied in these organisms, but it has not fully been explored in Gram-positive bacteria, which lack traditional periplasmic compartments. Recent bioinformatics analyses have suggested that the high-GC-content bacteria (i.e., actinobacteria) rely on disulfide-bond-forming pathways. In support of this, Dsb-like proteins have been identified in *Mycobacterium tuberculosis*, but their functions are not known. *Actinomyces oris* and *Corynebacterium diphtheriae* have recently emerged as models to study disulfide bond formation in actinobacteria. In both organisms, disulfide bonds are catalyzed by the membrane-bound oxidoreductase MdbA. Remarkably, unlike known Dsb proteins, MdbA is important for pathogenesis and growth, which makes it a potential target for new antibacterial drugs. This review will discuss disulfide-bond-forming pathways in bacteria, with a special focus on Gram-positive bacteria.

Before the discovery of chaperones, it was generally accepted that protein folding was dependent upon primary amino acid sequences and the laws of thermodynamics. This notion was first challenged by Anfinsen's classical RNase A folding experiments in the 1960s. Following its denaturation, Anfinsen and colleagues (1) observed that RNase A could spontaneously refold *in vitro*, but the process was slow and prone to error. It was discovered that the formation of four disulfide bonds within RNase A was a limiting factor for its folding. *In vivo*, protein maturation is accelerated by protein disulfide isomerase (PDI) (2, 3). PDI is a multidomain thioredoxin-like enzyme that catalyzes disulfide bonds and reduces nonnative linkages in proteins secreted into the glutathione-based oxidizing environment of the endoplasmic reticulum (ER) (4).

Anfinsen's pioneering work revealed a major protein folding mechanism used by both eukaryotic and prokaryotic organisms. The formation of native disulfide bonds, often referred to as oxidative protein folding, is essential for the stability of many secreted polypeptides (5, 6). In Gram-negative bacteria, protein oxidation occurs in the extracytoplasmic periplasm and is regulated by a family of thioredoxin-like enzymes, DsbA to DsbG (7). DsbA and DsbB work in concert to catalyze disulfide bond formation in nascent proteins secreted into the periplasm by SecYEG (8). In contrast, the reduction or rearrangement of incorrectly formed disulfide bonds in this compartment is controlled by DsbC, DsbG, and DsbD (9–11).

Disulfide bond formation is not fully understood in Gram-positive bacteria, which lack traditional periplasmic spaces. Gram-positive cell envelopes are composed of a single membrane that is surrounded by thick layers of peptidoglycan. Although a space between these regions has been observed in a number of organisms by electron microscopy, it is not considered to be equivalent to those spaces found in Gram-negative bacteria (12, 13). Therefore, it is possible that proteins secreted by Gram-positive bacteria are exposed to oxidative stresses within the extracellular milieu, which can cause misfolding. It was proposed that Gram-positive bacteria avoid this potential stress by simply not catalyzing disulfide bonds in the exoplasm (14). An analysis of bacterial secretomes partially supported this hypothesis (14, 15). Low-GC bacteria, i.e., *Firmicutes*, were found to secrete few, if any, proteins with multiple Cys residues, which suggests that they lack disulfide bonds. Dsb-like proteins have been identified in some *Firmicutes*, like *Bacillus*, but substrates are unknown or arranged with their putative oxidoreductases in gene clusters (16–18). These current data suggest that *Firmicutes* do not rely on disulfide bond formation as a general tool to fold secreted proteins.

In contrast to *Firmicutes*, actinobacteria secrete an abundance of proteins with multiple Cys residues, which suggests that they possess oxidative folding pathways (14). Current efforts to elucidate disulfide bond formation in these organisms have focused heavily on *Mycobacterium tuberculosis*. Four Dsb-like factors, vitamin K epoxide reductase (VKOR), *M. tuberculosis* DsbA (Mt-DsbA), DsbE, and DsbF, have been identified in this bacterium (19–22). *In vitro* analyses of these enzymes have been extensive, but their biological functions are not clear. The ability to study these factors *in vivo* is probably hindered by the slow-growth phenotype of *M. tuberculosis* and a lack of facile genetic tools.

Recently, the oral pathogens *Corynebacterium diphtheriae* and *Actinomyces oris* were introduced as alternative models to study disulfide bond formation in actinobacteria. Using adhesive pili and diphtheria toxin as model substrates, these bacteria were revealed to possess membrane-localized disulfide-bond-forming systems led by the oxidoreductase MdbA (23, 24). Remarkably, *mdbA* mutants exhibit severe morphological defects, indicating that unlike known Gram-negative Dsb proteins, MdbA is important for growth. In addition, the corynebacterial *mdbA* mutant is attenuated in virulence due to defective toxin production and pilus assembly. Thus, MdbA may serve as a powerful target for new bactericidal drugs. This review will discuss disulfide bond formation in bacteria, with a special focus on recent efforts to elucidate

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oxidative folding pathways in actinobacteria. For detailed mechanisms on disulfide bond formation in Gram-negative bacteria, excellent reviews can be found in references 25 and 26.

OXIDATIVE PROTEIN FOLDING IN THE GRAM-NEGATIVE PERIPLASM

DsbA/DsbB disulfide-forming pathway. The discovery of *E. coli* DsbA by Bardwell and colleagues (27) was serendipitous, since their experiments were originally designed to identify factors involved with membrane protein insertion. In this screen, cytoplasmic β-galactosidase (β-Gal) was fused to the N terminus of MalF, a known transmembrane protein. Under normal conditions, MalF–β-Gal is transported to the periplasm, but β-Gal attempts to reenter the cytoplasm and becomes embedded in the membrane (28). Blue/white screening was used to identify mutations preventing the initial translocation of this fusion protein. Surprisingly, a functional β-Gal was identified in a strain harboring a mutation in a secreted thioredoxin-like protein named DsbA. When functional, DsbA catalyzed nonnative disulfide bonds in β-Gal within the periplasm, which prevented its reentry into the cytoplasm. The loss of DsbA prevented these linkages from forming, which permitted the translocation of β-Gal.

E. coli DsbA is a 21-kDa monomeric protein that catalyzes the formation of disulfide bonds in unfolded proteins as they are secreted into the periplasm. DsbA harbors a canonical thioredoxin-like fold that is characterized by an N-terminal βαβ motif and a C-terminal ββα motif (29). However, unlike thioredoxin, these motifs are separated by an extended α-helical domain. The DsbA active site is composed of a reactive disulfide bond found in a CXXC (CPHC) consensus sequence that is located in the N terminus and abutted by a *cis*-proline (29). To catalyze new disulfide bonds, the CXXC linkage within DsbA is broken by a substrate Cys, resulting in the formation of a mixed intermediate (30, 31). In this state, DsbA may serve as a placeholder for substrate folding, as in the case of PDI (32). When folding is near completion, another substrate Cys is positioned to resolve the intermediate, which results in the formation of a new disulfide bond and release of the substrate. In turn, the DsbA CXXC motif is reduced (Fig. 1).

The ability of DsbA to form disulfide bonds is dependent on its high redox potential, or tendency to accept electrons (33). This intrinsic property is dependent on the pK_a of the solvent-exposed Cys in the CXXC motif and a positively charged His residue in the CXXC consensus sequence (CPHC). The pK_a of the solvent-exposed Cys is only 3.5, so it is negatively charged at physiological pH. This charge is stabilized by an electrostatic interaction with His (34, 35). His residues are strong indicators of the redox potential of CXXC motifs of thioredoxin-like proteins. Grauschopf et al. (36) found that substituting a nonpolar or negatively charged amino acid for His reduces the ability of DsbA to accept electrons *in vitro*. His residues are common features of other known disulfide-bond-forming enzymes. This amino acid is found in the CXXC motifs of eukaryotic PDI and DsbA equivalents in *Salmonella*, *Shigella*, *Pseudomonas*, and *Neisseria* species (37, 38). Although the strong oxidizing power of DsbA and DsbA-like proteins makes them effective disulfide-bond-forming enzymes, it presents a biological problem. These enzymes are more stable in their reduced forms, so it is unlikely that they spontaneously recycle their disulfide-bond-forming activity (39).

In *E. coli*, DsbA is regenerated by DsbB (Fig. 1), the discovery of which was simultaneously reported by Missiakas et al. (40) and Bardwell et al. (41). DsbB is a 20-kDa transmembrane protein that harbors two periplasmic loops with redox-active disulfide bonds. Using a series of disulfide exchanges, DsbB reoxidizes DsbA by shuttling electrons to a conjugated quinone, a component of the electron transport chain (42). Although the transfer of electrons between DsbA and DsbB is logical, their comparative redox potentials initially challenged this model. The redox potential of DsbB is lower than that of DsbA, which makes the flow of electrons between these enzymes unfavorable (43). An elegant structural study conducted by Inaba et al. (44) revealed that DsbB compensates for the difference in redox potential by a conformational change. Upon the transfer of DsbA's electrons to the C-terminal redox center, this region shifts toward the N-terminal redox site. This sterically hinders the flow of electrons back to DsbA, thus allowing the reaction to move forward.

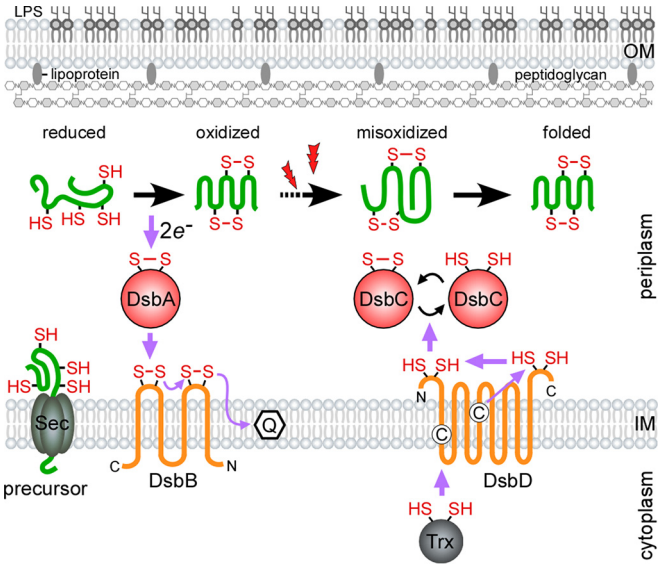

FIG 1 Oxidative protein folding in the Gram-negative periplasm. To catalyze oxidative protein folding, DsbA donates a reactive disulfide bond to reduced protein precursors as they are secreted into the oxidizing periplasm by SecYEG. Following catalysis, the DsbA active site is reoxidized by the transmembrane protein DsbB, which shuttles the gained electrons to the electron transport chain via a conjugated quinone. Extracellular oxidative stress (denoted by lightning bolts) or the lack of DsbA proofreading activity can cause substrates to become misoxidized. Aberrant disulfide bonds are reshuffled by the reductase DsbC. The reducing power of DsbC is maintained by the transmembrane DsbD, which receives electrons from cytoplasmic thioredoxin (adapted from reference 26). The purple arrows denote the direction of the electron flow, and the cysteine residues in the membrane domains of DsbD are shown as circled C's.

Disulfide-reducing pathways. *E. coli* DsbA favors the formation of consecutive disulfide bonds in substrates, i.e., oxidation of Cys residues in the order they emerge from the SecYEG (8). This strategy is flawed, since not all secreted proteins possess consecutive disulfide bonds. In these cases, DsbA will catalyze the formation of nonnative disulfide bonds, leading to misfolding. Due to its high redox potential, DsbA cannot reduce nonnative disulfide bonds to correct its errors (45).

Disulfide bond formation in the periplasm is monitored by DsbC (Fig. 1), a 26-kDa isomerase that harbors an N-terminal CXXC motif and C-terminal dimerization domain. This thioredoxin-like enzyme is required for the proper folding of secreted

proteins with nonconsecutive disulfide bonds and proteins damaged by oxidative stress (9, 11). In its active form, the DsbC CXXC motif is reduced, which allows the transfer of electrons to misoxidized substrates (46). In turn, DsbC becomes oxidized and is reduced by the transmembrane protein DsbD (Fig. 1). DsbD harbors three domains, the N-terminal IgG-like periplasmic α-loop, the hydrophobic core-β, and the C-terminal thioredoxin-like periplasmic γ-loop, which participate in the transfer of electrons (47–49). The active site with Cys103 and Cys109 is located at the IgG-like domain (50). Electrons derived from cytoplasmic thioredoxin are transported through these domains and then delivered to the CXXC site of DsbC (51) (Fig. 1).

DsbC dimerization is essential for function. Mutational analysis revealed that this higher-order structure prevents cross talk between oxidative and reductive pathways in the periplasm. Bader et al. (52) found that the disruption of DsbC dimerization enables it to rescue an *E. coli dsbA*-null strain. This suggests that dimerization is required to prevent oxidization by DsbB. In support of this, attempts to model an interaction between DsbB and DsbC revealed a steric clash between one DsbC protomer and the cytoplasmic membrane (53).

In addition to maintaining DsbC in a reduced state, DsbD and DsbD homologs also shuttle electrons to DsbG, DsbE, and peroxiredoxins in the periplasm (54–56). Although DsbG has no known substrates, *in vitro* data suggest that it is a disulfide isomerase (54). Unlike other Dsb proteins, DsbE is not involved in general secreted protein folding. DsbE, also known as CcmG, is required for the synthesis of cytochrome *c*, a component of the electron transport chain (57). The reduction of peroxiredoxin is important, as this redox protein scavenges peroxides in the periplasm to combat oxidative stress (56).

Oxidative folding pathways in Gram-negative bacteria are nonessential but required for virulence. Disulfide-bond-forming pathways in Gram-negative bacteria do not appear to be essential (27). *E. coli dsbA* mutants are slow growing in minimal medium, but this is attributed to a defect in glucose uptake, because glucose transporters require disulfide bonds (58). Disulfide isomerase pathways are also nonessential, since *dsbC*-null mutants grow normally under nonstress conditions (59). This is not due to redundancy between oxidative folding factors, since a *dsbA dsbC dsbG* triple-deletion mutant is also viable (59). *dsbD*-null mutants are temperature sensitive, but this phenotype is caused by a disruption in DsbE-led cytochrome *c* synthesis (60).

Although Dsb proteins are not generally important for growth, they are essential for pathogenesis. In addition to *E. coli*, other important Gram-negative pathogens, including *Salmonella enterica*, *Shigella flexneri*, *Yersinia pestis*, *Bordetella pertussis*, and *Pseudomonas aeruginosa*, secrete an arsenal of disulfide-bond-containing virulence factors (61). DsbA- and DsbA-like proteins expressed by these organisms are required for the proper folding of virulence factors, like adhesive pili, secretion systems, flagella, and toxins. *dsbA* mutations are often associated with decreased virulence, because disulfide-bond-containing secreted proteins are misfolded and degraded. For an in-depth review of this topic, see reference 61.

GRAM-POSITIVE DISULFIDE-BOND-FORMING PATHWAYS

The *Firmicutes*. Although a space between the Gram-positive cytoplasmic membrane and cell wall has been observed by cryoelectron microscopy, it is not considered to be equivalent to that in the Gram-negative periplasm (12, 13). Due to the diffusive nature of peptidoglycan, it is possible that this space is exposed to the extracellular milieu. Therefore, environmental stress might cause aberrant oxidation in unfolded proteins with multiple Cys residues.

A survey of Gram-positive secreted proteomes revealed that many of these bacteria avoid oxidative folding stress by simply not utilizing disulfide bond formation (14, 15). The low-GC *Firmicutes*, including *Staphylococcus*, *Lactobacillus*, and *Streptococcus* spp., were found to secrete few, if any, proteins with two or more Cys residues, suggesting that these bacteria do not fold secreted proteins with disulfide bonds. In support of this, some *Firmicutes*, including *Streptococcus pneumoniae*, do not express any *dsb*-like genes (14, 15). One apparent exception to this trend is *Bacillus* (for a review, see reference 62). *Bacillus brevis* BdbA was the first DsbA-like protein discovered in this genus (16). Although BdbA has no known substrates, its overexpression was shown to rescue *E. coli* Δ*dsbA* phenotypes. While it is tempting to speculate that this protein is equivalent to DsbA, the overexpression of thioredoxin, DsbC, and DsbG, which are known to reduce proteins, in the periplasm rescues *dsbA*-null phenotypes (63–65). Therefore, one cannot conclude how *B. brevis* BdbA functions *in vivo* by expressing the protein in *E. coli*.

Bacillus subtilis harbors two gene clusters with the putative oxidoreductase-encoding genes *bdbA* to *bdbD*. *bdbA* and *bdbB* belong to an operon that encodes sublancin 168, an antibiotic with disulfide bonds (17). BdbA and BdbB, which are proposed to be analogous to DsbA and DsbB, respectively, catalyze disulfide bond formation in sublancin 168. *bdbC* and *bdbD* are contained within a competence gene cluster along with the disulfide-bond-containing ComCG pseudopilus (18). BdbC and BdbD, which are also proposed to be DsbB and DsbA equivalents, respectively, are required for production of the ComCG pseudopilus. Since *B. subtilis bdb* genes are genetically linked with their only known substrates, it is unlikely that they target other secreted proteins. In support of this, Bdb proteins in *B. subtilis* are not fully interchangeable (17). Furthermore, the transcription of *bdbCD* was found to be dependent on *comX*; although expression profiles of *bdbCD* have not been conducted, this observation suggests that the genes are transcribed in stationary phase. Therefore, BdbC and BdbD are probably not constitutively present to help fold the *Bacillus* secretome. It is more likely that the disulfide-bond-forming factors in *Bacillus* are exceptions that were acquired by horizontal gene transfer.

DsbA-like genes have also been identified in *Staphylococcus aureus* and *Streptococcus gordonii* (66, 67). *S. aureus* DsbA (SaDsbA) is a membrane-bound lipoprotein that was shown *in vitro* to have thiol-disulfide oxidoreductase activity (68). The deletion of *S. aureus dsbA* is associated with decreased levels of only the ComCG pseudopilus, indicating it may be functionally similar to *Bacillus* BdbD (69). Interestingly, biochemical analysis has revealed that the SaDsbA CXXC active site is equally stable in its oxidized and reduced forms, which indicates that it does not appear to require a DsbB-like partner (66). This characteristic is highly unusual, since known disulfide-bond-forming factors derive their oxidizing power from an unstable disulfide bond (39). If ComCG is one of few proteins secreted by *S. aureus* that require disulfide bond formation, it may not be necessary for SaSdbA to be a strong oxidizer. Further analysis is needed to elucidate the role of SaDsbA *in vivo*. Finally, a single DsbA-like factor has also been identified in *S. gordonii* (67). Similar to *B. subtilis* and *S. aureus*, this putative oxidoreductase has been implicated in competence

development. Based upon the current bioinformatics data and limited *in vivo* evidence, while some *Firmicutes* appear to encode a few oxidoreductases for specific substrates, their secretomes generally do not rely on disulfide bonds for folding.

The *Actinobacteria*. In contrast to the *Firmicutes*, Gram-positive actinobacteria, like *Corynebacterium*, *Streptomyces*, and *Mycobacterium* spp., secrete an abundance of Cys-containing proteins and encode redox proteins (14, 15). The exploration of disulfide bond formation within these organisms is a relatively recent endeavor and has heavily focused on *M. tuberculosis*. Disulfide bond formation is hypothesized to be a major folding pathway for this bacterium, as 60% of its secreted proteins contain two or more Cys residues (70). The first novel secreted oxidoreductase discovered in this bacterium was VKOR (15). VKOR is a quinone-conjugated transmembrane protein with two periplasmic loops containing redox-active disulfide bonds (22). Although expression of this enzyme can restore disulfide bond formation in an *E. coli dsbB* mutant, it is not a DsbB homologue (71). Rather, VKOR is related to mammalian VKOR, an enzyme involved in vitamin K recycling. Due to its functional similarity to *E. coli* DsbB, VKOR is predicted to reoxidize a DsbA-like enzyme, but its protein folding role *in vivo* has not been demonstrated.

In addition to *M. tuberculosis* VKOR, three secreted oxidoreductases, Mt-DsbA, Mt-DsbE, and Mt-DsbF, have also been identified. The structural and biochemical analyses of these proteins have been extensive. Although they are not identical to *E. coli* DsbA, all three factors display a canonical thioredoxin-like fold, N-terminal CXXC motif, and extended α-helical domain (19–21). Mt-DsbA, a membrane-anchored oxidoreductase, is encoded in an operon with the VKOR gene. Mt-DsbA has been shown to interact with VKOR-derived peptides *in vitro*, suggesting that the two factors form a redox pair (72). Since VKOR is a DsbB analogue, it is logical that Mt-DsbA would be equivalent to *E. coli* DsbA. In support of this, the redox potential of Mt-DsbA (−99 mV) is quite high, and the protein might oxidize a substrate *in vitro*, although it failed to reshuffle disulfide bonds in scrambled RNase A (72). All three traits are reminiscent of *E. coli* DsbA. However, a conflicting study reported that recombinant Mt-DsbA did not oxidize hirudin but successfully unscrambled RNase A (19). These discrepancies underscore the importance of studying Mt-DsbA in *vivo*. The identification of Mt-DsbA substrates would further enhance our understanding of its role in *M. tuberculosis*.

Efforts to study Mt-DsbA thus far have depended on the expression of the protein in *E. coli*. Interestingly, this factor cannot rescue a flagellar defect associated with the deletion of *E. coli dsbA* (72). The Mt-DsbA crystal structure revealed that the enzyme has a less flexible and hydrophobic catalytic binding site than that of *E. coli* DsbA (72). A restrictive binding cleft might have interfered with Mt-DsbA binding to *E. coli* DsbB for reoxidation. However, this possibility was not explored by testing if the expression of Mt-*dsbA* and the VKOR gene could restore oxidative protein folding in an *E. coli dsbA dsbB* double mutant. It was alternatively proposed that the Mt-DsbA binding cleft is optimized for specific substrates in *M. tuberculosis*, so it could not recognize the *E. coli* secretome (72). It is noteworthy that since the majority of proteins secreted by *M. tuberculosis* are predicted to contain disulfide bonds, a DsbA equivalent in this organism should be able to target an array of substrates.

If Mt-DsbA is limited in substrate recognition, *M. tuberculosis* expresses two additional oxidoreductases (Mt-DsbE and Mt-DsbF) that might participate in oxidative folding. Unlike Mt-DsbA, these factors are predicted to be secreted into the bacterial exoplasm (19). Mt-DsbE is hypothesized to oxidize proteins due to its relatively high redox potential (−128 mV) (20). The observation that Mt-DsbE can oxidize hirudin *in vitro* but exhibits no isomerase activity corroborates this conjecture. However, it should be noted that the redox potential of Mt-DsbE is similar to that of *E. coli* DsbC, which functions as a reductase (−130 mV) (73). The identification of an Mt-DsbE mutant phenotype is vital for determining its role in *M. tuberculosis*. Finally, Mt-DsbF has a higher calculated redox potential (−87 mV) than both Mt-DsbA and Mt-DsbE (21). This suggests that Mt-DsbF is the best suited for oxidizing secreted proteins. However, the gene encoding this factor is adjacent to a putative peroxiredoxin gene. Microarray data showed that these genes have similar expression profiles, and an *in vitro* pulldown experiment demonstrated that these proteins interact (21). It is possible that Mt-DsbA possesses a novel mechanism for disulfide bond formation or that it belongs to a different redox pathway in the cell. In summary, given the unknown biological functions of Mt-DsbA, Mt-DsbE, and Mt-DsbF, oxidative folding pathways in *M. tuberculosis* remain to be investigated.

ADHESIVE PILUS PROTEINS REVEAL OXIDATIVE PROTEIN-FOLDING PATHWAYS IN THE ACTINOBACTERIA *A. ORIS* AND *C. DIPHTHERIAE*

Recent investigations of pilus assembly in the oral pathogens *A. oris* and *C. diphtheriae* have advanced our understanding of disulfide bond formation in actinobacteria. Adhesive pili expressed by Gram-positive bacteria are host colonization factors composed of individual subunits that are covalently linked together and anchored to the cell surface by sortase enzymes (74). Prior to their assembly, pilus precursors are translocated to the exoplasm in unfolded states. Due to the lack of a recognizable periplasm, it was not known how these proteins attained their native conformations outside the cell. Structural studies of the major pilus proteins FimA and FimP expressed by *A. oris* and SpaA and SpaD of *C. diphtheriae* provided a clue (75–78). The presence of disulfide bonds in the crystal structures of all three pilins suggested that they required oxidative protein folding.

In vivo, these linkages are essential for pilus assembly. The failure to form disulfide bonds is associated with the absence of pili and the secretion of degradation products into the culture medium (23, 24). This suggests that the covalent linkages are important for the proper folding and/or stability of pilus precursors in the exoplasm. The discovery of disulfide bonds in FimA, FimP, and SpaA conferred a great advantage for studying protein oxidation in actinobacteria. Using *A. oris* and *C. diphtheriae* pilus proteins as model substrates, disulfide-bond-forming pathways vital for both virulence and growth were identified.

Using a combination of genetics, X-ray crystallization, and biochemical methods, the thiol-disulfide oxidoreductase enzyme called MdbA was identified in *A. oris* and *C. diphtheriae* (23, 24). In these organisms, it was shown that pilin precursors are oxidized by the membrane-bound oxidoreductase. In *A. oris*, MdbA activity is recycled by a VKOR homologue (23), while *C. diphtheriae* MdbA may be reoxidized by an unknown factor (24) (Fig. 2). MdbA shares low sequence homology with *E. coli* DsbA but is structurally similar. Crystal structures have revealed that both MdbA factors display thioredoxin-like folds and extended α-helical domains that most closely resemble *B. subtilis* BdbD and *M.*

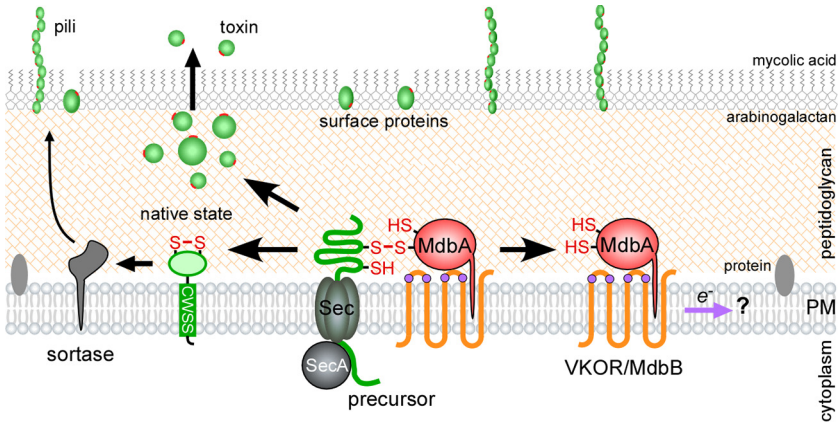

FIG 2 Oxidative protein folding in the actinobacterial exoplasm. Using pilus proteins and diphtheria toxin as model substrates, oxidative protein-folding pathways have been proposed in the actinobacterial pathogens *A. oris* and *C. diphtheriae*. Unfolded pilin precursors are oxidized by the membrane-tethered thiol-disulfide oxidoreductase MdbA. Proper folding is a prerequisite for sortase-mediated assembly of pili on the cell surface. *A. oris* MdbA is reoxidized by the transmembrane VKOR, while *C. diphtheriae* MdbA is hypothesized to be recycled by an unidentified factor called MdbB. It is not clear how VKOR or MdbB is reoxidized. The catalytic cysteine residues of VKOR are shown as purple circles, disulfide bonds formed in mature proteins are shown as red lines, and the purple arrow indicates the presumed direction of the electron flow (modified after references 23 and 24).

tuberculosis Mt-DsbA (23, 24). *A. oris* and *C. diphtheriae* MdbA enzymes also contain His residues in their CXXC motifs and possess neutral surface potentials near the predicted sites for substrate binding (23, 24). These observations suggest that MdbA exhibits strong redox potential and broad substrate specificity. Although these findings are supported by *in vivo* evidence, they have yet to be biochemically tested.

Since >60% of proteins secreted by *A. oris* and *C. diphtheriae* are predicted to contain at least one disulfide linkage (38, 79), it was hypothesized that MdbA proteins target multiple substrates. The disulfide-bond-containing diphtheria toxin (DT) was chosen as an additional model substrate. The deletion of *mdbA* was associated with the release of reduced and degraded DT. This phenotype, along with the lack of adhesive pili, had profound consequences on *C. diphtheriae* pathogenesis, as the *mdbA* mutant was attenuated in a guinea pig model of infection (24). The combined data indicated that MdbA is important for the general folding of secreted virulence factors with disulfide bonds.

Unlike *E. coli* DsbA and known Gram-negative DsbA-like enzymes, MdbA is also required for proper growth and division. This remarkable feature was discovered when multiple attempts to generate an *A. oris mdbA* deletion mutant failed. A conditional *mdbA* deletion mutant was created as an alternative by placing *mdbA* under the control of an inducible promoter. Decreased *mdbA* expression was associated with a cell-chaining and altered morphology (23). This surprising phenotype suggested that MdbA has a much broader role in protein folding (i.e., it targets secreted proteins involved with cell division). In contrast, a *C. diphtheriae mdbA* deletion mutant was successfully generated but resulted in a severe temperature-sensitive defect. The bacteria were observed to grow normally at 30°C but became coccoid, chained, and eventually stopped growing when the temperature was shifted to 37°C (24).

The misfolding of secreted penicillin-binding proteins (PBPs), which contain multiple Cys residues, is one potential basis for the *mdbA* growth phenotypes. PBPs synthesize peptidoglycan, a determinant of cell shape (80). The inhibition of cell wall synthesis and removal of existing peptidoglycan are known to transform rod-shaped bacteria, like *E. coli*, *Corynebacterium glutamicum*, and *Bacillus*, into cocci (81–84). It is proposed that *C. diphtheriae* and *A. oris* PBPs misfold in the absence of MdbA, which prevents normal growth. In support of this, the *C. diphtheriae* Δ*mdbA* mutant is more susceptible to antibiotics that target PBP function than the wild-type strain and exhibits abnormal vancomycin BODIPY-FL (Van-FL) staining (24). The reason why *A. oris mdbA* is essential while *C. diphtheriae mdbA* is important only for growth at 37°C is not known. It is possible that *C. diphtheriae* possesses some capability for background protein oxidation that is sufficient for growth at lower temperatures.

Finally, the housekeeping role of disulfide-bond-forming enzymes in actinobacteria is not limited to *A. oris* and *C. diphtheriae*. An *M. tuberculosis* transposon library generated by Sassetti et al. (85) revealed a low insertion frequency within the Mt-DsbA and VKOR genes, suggesting that the genes are important for growth. Consistent with this, slow-growth phenotypes of strains with VKOR gene deletions were observed in *M. tuberculosis* and *Mycobacterium smegmatis* (15, 71). These findings suggest that the bacterial thiol-disulfide oxidoreductases would provide excellent targets for the development of antimicrobials. Since *dsbA* mutants are attenuated in rodent models of infection (86, 87), efforts have recently been made to identify inhibitors of DsbA/DsbB as antivirulence agents. By screening a library of 1,123 fragments for compounds that bind to oxidized DsbA, Adams and colleagues (88) identified a small set of molecules that exhibit high binding affinity to DsbA and inhibit its activity *in vitro*. One of the compounds was shown to reduce bacterial motility, but it did not affect cell growth (88). Using a virtual screening approach, Duprez et al. (89) identified a set of noncovalent inhibitors of DsbA that exhibit inhibitory activity at millimolar concentrations (89). While the first two approaches are aimed at DsbA, the Beckwith group (90) employed a cell-based screening method to find compounds that target DsbB and revealed several inhibitors with a pyridazinone core. With further modification using a medicinal chemistry approach, they selected more potent inhibitors that have a broad spectrum for inhibition of DsbB in many other Gram-negative bacteria. Finally, by interfering with the DsbB-

catalyzed recycling of DsbA, Halili and coworkers (91) synthesized analogues of ubiquinone and found a couple of dimedone derivatives with high inhibitory activity (50% inhibitory concentration [IC_{50}], ~1 μM) to *E. coli* DsbA, but none of these compounds inhibited human thioredoxin (91). While the identification of disulfide-bond-forming inhibitors is encouraging, it remains to be seen if these compounds have any antivirulence activity *in vivo*.

PERSPECTIVES

It was previously proposed that Gram-positive bacteria do not possess periplasmic compartments to regulate the folding of secreted proteins. While this may be true for *Firmicutes*, actinobacteria may be exceptions. Corynebacteria and mycobacteria exhibit unique cell envelope architectures in which peptidoglycan is cross-linked to arabinogalactan, which is esterifed by mycolic acid (92). Mycolic acid, a type of long-chain fatty acid, forms a hydrophobic surface layer that is visible by thin-section electron microscopy (EM) (93). This layer contributes to the high impermeability of corynebacteria and is known to form liposomes when cells are treated with detergent (93, 94). This layer of fatty acids is proposed to form a so-called mycomembrane, which may be analogous to the Gram-negative outer membrane. It is possible that some Gram-positives, like corynebacteria, contain enclosed compartments to protect thiol-containing secreted proteins from aberrant oxidation.

A. oris is not known to produce mycolic acid. It is possible that this organism possesses an outer lipid layer, but its ultrastructure has not been examined. Alternatively, it is also likely that its environment contributes to its oxidation of proteins in the exoplasm. *A. oris*, a pioneer colonizer of the oral cavity, inhabits the anaerobic layers of mature biofilm (95, 96). The absence of oxygen in this niche may allow the bacterium to avoid random oxidation of secreted proteins.

Finally, actinobacteria might avoid random Cys oxidation by coordinating translocation and folding events. In *C. diphtheriae*, the secretion and assembly of pilus subunits is thought to be a tightly coupled process. This is supported by thin-section microscopic data showing that Sec and sortase machinery colocalize (97). In addition, pilin precursors missing their C-terminal membrane anchors are still incorporated into pilus structures, suggesting that the subunits were processed by sortase during or immediately after translocation (98, 99). If MdbA must fold pilins, such as FimA, FimP, SpaA, and SpaD, before sortase-catalyzed assembly, it must also colocalize with Sec machinery. The ability of MdbA to oxidize substrates as they emerge from the cytoplasm might serve as an adaptation to the secretion of proteins into unfavorable environments. Coupling translocation with protein folding may increase the likelihood that disulfide bonds are catalyzed by cellular machinery rather than the extracellular milieu (Fig. 2).

In summary, disulfide bond formation is an important mechanism to fold many secreted proteins. Oxidative protein-folding pathways have been widely studied in Gram-negative bacteria. However, due to the lack of a periplasmic space, disulfide bond formation largely has been unexplored in Gram-positive bacteria. Observations that disulfide-bond-containing proteins are rare and often found in operons with putative oxidoreductases suggest that these bacteria do not rely on disulfide-bond-forming pathways. Bioinformatics analyses proposed that while many Gram-positive bacteria do not use protein oxidation, actinobacteria may be exceptions (14, 15). However, elucidation of the disulfide-bond-forming pathways in these organisms was impeded by a lack of *in vivo* work. Recent investigations of virulence factors secreted by the oral pathogens *A. oris* and *C. diphtheriae* have advanced our understanding of protein oxidation in actinobacteria. Not only are disulfide-bond-forming factors required for virulence in these organisms, but they are also required for proper growth and division. This unexpected discovery might pave the way for new antimicrobial drugs targeting important actinobacterial pathogens, like *M. tuberculosis*. Furthermore, oxidative protein folding appears to be an Achilles heel of antibiotic resistance in these pathogens, as a mutant defective in disulfide bond formation is highly sensitive to β-lactam antibiotics (24). Perhaps a combination of disulfide-bond-forming inhibitors and known antibiotics would enhance the efficacy of antibiotics and curtail antibiotic resistance.

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