

5-2015

Oxidative protein folding pathways in Gram-positive Actinobacteria

Melissa E. Robinson

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OXIDATIVE PROTEIN FOLDING PATHWAYS IN GRAM- POSITIVE ACTINOBACTERIA

A DISSERTATION

Presented to the Faculty of
The University of Texas
Health Science Center at Houston
and
The University of Texas
M. D. Anderson Cancer Center
Graduate School of Biomedical Sciences
in Partial Fulfillment
of the Requirements
for the Degree of
DOCTOR OF PHILOSOPHY

By
Melissa Elizabeth Robinson, B.S.

Houston, Texas

May 2015

ACKNOWLEDGEMENTS

First, I would like to thank my advisor and mentor Dr. Hung Ton-That for his patience and guidance in training. Dr. Ton-That and I first met in Connecticut when I was an undergraduate making the transition from humanities to science. At the time, I was an English major who had recently completed one Introduction to Microbiology course, and had zero laboratory experience. Still, he took a chance, and hired me to work as a research assistant for not one, but two summers. My positive experience inspired me to follow Dr. Ton-That to Texas for my graduate studies. I owe much of my success to his constant support and encouragement.

It takes a village to raise scientists. With that, I would also like to thank my committee members Joseph Alcorn, Peter Christie, Danielle Garsin, Ziyin Li, John Putkey, William Margolin, and Kevin Morano for their guidance and *constructive* criticism throughout my dissertation research. In addition, credit must be given to the post-doctoral fellows Chungyu Chang, Bo Chen, I-Hsiu Huang, Ju Huck Lee, Arunima Mishra, Beth Rogers, and Chenggang Wu. These wonderful scientists have been instrumental in the success of my day-to-day laboratory endeavors.

I would also like to thank my family. I am blessed to be surrounded with a supportive and loving network of parents, siblings, grandparents, uncles, aunts, cousins, and friends. Without you, none of this would be possible. Finally, I am grateful to my husband Caleb. You are, and continue to be my most favorite surprise in life.

OXIDATIVE PROTEIN FOLDING PATHWAYS IN GRAM-POSITIVE ACTINOBACTERIA

Melissa Elizabeth Robinson, B.S.

Advisory Professor: Hung Ton-That, Ph.D.

Disulfide bonds are important for the stability of many secreted proteins. These covalent linkages, which result from the oxidation of neighboring cysteine (Cys) residues, are often rate-limiting steps for protein folding and maturation. Disulfide bond formation is restricted to extracellular oxidizing compartments like the eukaryotic endoplasmic reticulum and Gram-negative bacterial periplasm. Protein oxidation has been well-studied in these organisms, but largely ignored in Gram-positive bacteria. Due to the absence of an outer membrane, these organisms are thought to lack compartments in which to catalyze oxidative protein folding.

This thesis reveals that Gram-positive Actinobacteria use disulfide bond formation to help fold secreted proteins in the exoplasm. Using the assembly of adhesive pili as a marker for disulfide bond formation in *A. oris* and *C. diphtheriae*, we found that protein oxidation is catalyzed by the membrane-bound MdbA. In *A. oris*, MdbA activity is maintained by VKOR, which is absent in *C. diphtheriae*. MdbA-catalyzed disulfide bond formation is required for the production of multiple virulence factors including diphtheria toxin. Therefore, mutations targeting *mdbA* have profound consequences for pathogenesis. *A. oris* mutants are defective in biofilm growth, while *C. diphtheriae* exhibits attenuated virulence in an animal model.

A major difference between disulfide bond forming enzymes expressed by Gram-negative and Actinobacteria is also revealed. Unlike the Gram-negative DsbA, MdbA is important for viability. The depletion of *A. oris mdbA*, and deletion of *C. diphtheriae mdbA* are associated with growth and division defects. We provide evidence that these phenotypes result because secreted growth factors like PBPs fail to form disulfide bonds. Remarkably, the

deletion of *C. diphtheriae* *mdbA* selects for a suppressor mutation that causes the overexpression of an oxidoreductase named TsdA.

In summary, this thesis shows that disulfide bond formation is a major pathway used by Gram-positive Actinobacteria to help fold secreted proteins. This work provides a better understanding of how proteins are folded within the Gram-positive exoplasm, and offers important considerations for developing antibacterial drugs that target oxidative folding pathways.

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Chapter 1:

Introduction

1.1 Anfinsen's discovery of oxidative protein folding chaperones.

Before the discovery of chaperones, it was generally accepted that protein folding was based on a primary amino acid sequence and the laws of thermodynamics. This notion was first challenged by Anfinsen's classical RNase A folding experiments in the 1960s. He denatured RNase A with β -mercaptoethanol and urea, and then measured the amount of time required for the protein to regain its activity. RNase A refolded in the presence of oxygen, but the process was slow and error-prone. Under optimal conditions, RNase A refolding took over 20 minutes (Anfinsen et al., 1961). Anfinsen found that the formation of four disulfide bonds within RNase A was a limiting factor for refolding. It was hypothesized that a factor was present *in vivo* to accelerate thiol oxidation. To test this, denatured RNase A was incubated with endoplasmic reticulum (ER) fractions isolated from rat and beef livers (Givol, Goldberger & Anfinsen, 1964; Goldberger, Epstein & Anfinsen, 1963). Remarkably, the half-time for RNase refolding was reduced four-fold. Protein Disulfide Isomerase (PDI) was later identified as the chaperone responsible for catalyzing the formation of disulfide bonds in this protein (Anfinsen, 1973).

PDI is a thioredoxin-like protein that oxidizes Cys residues within nascent proteins in the extracellular ER compartment (Tian et al., 2006). PDI is a U-shaped homodimer comprised of the catalytic α and α' domains, and the substrate-binding β and β' domains. The α and α' domains harbor canonical thioredoxin folds characterized by N-terminal $\beta\alpha\beta$ and C-terminal $\beta\beta\alpha$ motifs (Martin, 1995). The N-terminal α -helices contain a reactive disulfide bond formed between two Cys residues in a CxxC consensus sequence. This linkage is reduced to result in the formation of new disulfide bonds in unfolded substrates (Darby, Freedman & Creighton, 1994; Lyles & Gilbert, 1991). The reaction begins when a reduced Cys within a substrate attacks the disulfide bond within the CxxC linkage, which results in the formation of a mixed thiol intermediate (Fig. 1A). A new disulfide bond is formed when the intermediate is

resolved by another substrate Cys residue (Fig. 1B). In turn, the PDI CxxC motif is converted to a reduced form that rearranges nonnative Cys-linkages in other proteins (Fig. 1C). Thus, this folding chaperone serves two functions; it catalyzes and isomerizes disulfide bonds.

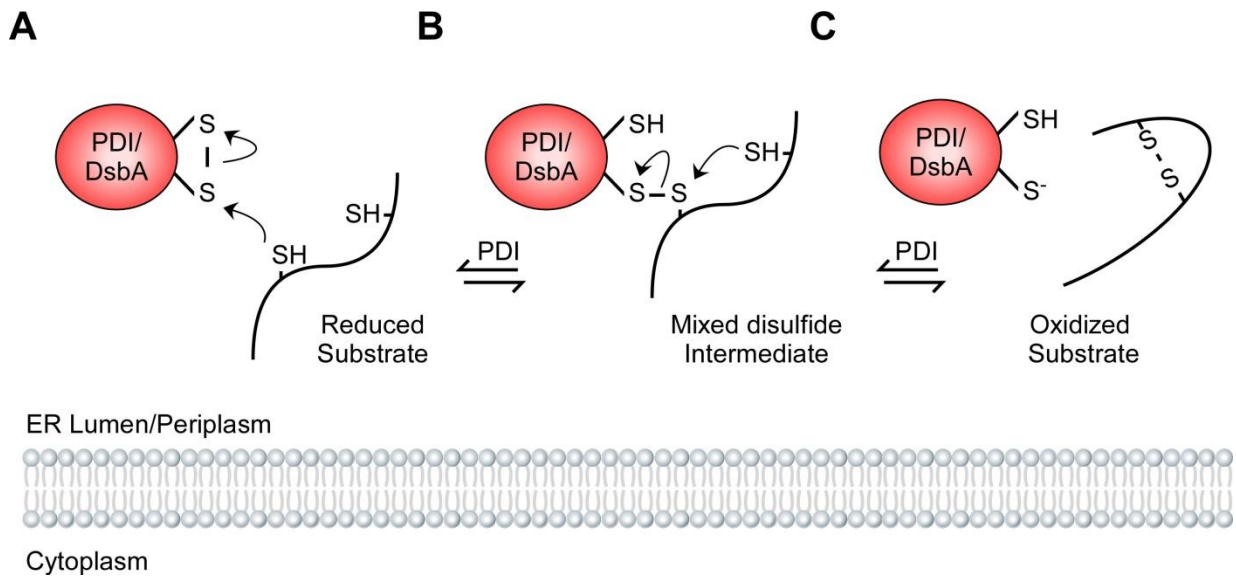


Figure 1. Disulfide bond formation is catalyzed in extracellular compartments of eukaryotic and prokaryotic organisms. (A) Eukaryotic PDI and *E. coli* DsbA donate reactive disulfide bonds within CxxC motifs to catalyze new thiol linkages in unfolded substrates. The reaction begins when a reduced substrate forms a mixed disulfide intermediate with the enzyme. **(B)** This intermediate is resolved by a second Cys residue within the substrate. **(C)** The substrate forms a disulfide bond, and the enzyme CxxC site is reduced. In its reduced form, PDI catalyzes disulfide bond isomerization in substrates with nonnative Cys-linkages (indicated by arrows).

Interestingly, although PDI is essential in yeast, its disulfide bond forming activity is not specifically required for cell viability. Laboissier et al. (1995) found that mutating the PDI CxxC sequence to SxxC was lethal, but a CxxS mutant was viable. Neither mutant could catalyze disulfide bond formation, but the CxxS variant retained a solvent-exposed Cys that still reduced nonnative disulfide bonds. Since the ER is an oxidizing environment, random disulfide bond formation can occur in the absence of PDI (Hwang, Sinskey & Lodish, 1992). However, spontaneous oxidation is not always accurate, so PDI may be more important for monitoring disulfide bond formation. It was concluded that the chaperone's oxidase activity is dispensable, but its isomerase activity is essential.

1.2 Oxidative protein folding in the Gram-negative bacterial periplasm

Beckwith's group identified the bacterial equivalent of PDI in *E. coli* nearly 30 years later (Bardwell, McGovern & Beckwith, 1991). This discovery was serendipitous since their experiments were originally designed to identify factors involved with inserting proteins into the bacterial membrane. To identify factors, β -galactosidase (β -gal) was fused to the N-terminus of the transmembrane protein MalF. Normally, β -gal is located in the cytoplasm, but its fusion to MalF causes it to be translocated into the periplasm. The C-terminus of β -gal attempts to re-enter the cytoplasm, but it becomes embedded in the membrane and is nonfunctional. It was reasoned that a mutation affecting protein translocation would trap β -gal in the cytoplasm where it could catabolize X-gal. Surprisingly, a functional β -gal mutant was found to harbor a mutation within a gene encoding a 21-kDa secreted thioredoxin-like protein. Under wild-type conditions, this factor oxidized the fusion protein in the periplasm. This caused it to misfold, thereby preventing the β -gal region from crossing the membrane to re-enter the cytoplasm. However, this mutant could not form disulfide bonds, so the MalF- β -gal fusion successfully translocated to the cytoplasm to metabolize X-gal. Beckwith's group named this factor Disulfide Bond Forming Protein A (DsbA).

E. coli DsbA is a monomeric protein that catalyzes disulfide bonds in unfolded proteins as they are secreted into the periplasm. Although DsbA does not display a higher-ordered structure like PDI, it does harbor the canonical N-terminal $\beta\alpha\beta$ and C-terminal $\beta\beta\alpha$ motifs (Martin, Bardwell & Kuriyan, 1993). These folds are separated by an extended α -helical region, which is not present in PDI. The DsbA active site is characterized by a CPHC consensus sequence that is surrounded by hydrophobic residues and abutted by a conserved cis-Proline (Martin et al., 1993). DsbA catalyzes oxidation in the same manner as PDI; it donates a disulfide bond within its CxxC motif to reduced substrates (Akiyama et al., 1992) (Fig. 1A). The reaction is initiated when a substrate Cys breaks the CxxC linkage resulting in a mixed disulfide with the DsbA N-terminal Cys (Darby & Creighton, 1995; Kadokura et al., 2004). Biochemical analysis has revealed that DsbA serves as a placeholder while the substrate folds around it (Kosuri et al., 2012). DsbA releases its substrate when a second Cys is positioned to form a new disulfide bond in the substrate (Fig. 1B). In turn, the DsbA CxxC motif is converted to a reduced form (Fig. 1C).

DsbA has an unusually high redox potential (-120 mV), which is defined as having a tendency to accept electrons (Zapun, Bardwell & Creighton, 1993). Due to this, oxidized DsbA is thermodynamically unstable, which makes the reduction of the CxxC motif highly favorable (Wunderlich, Jaenicke & Glockshuber, 1993). Bardwell and colleagues demonstrated that a His residue within the DsbA CPHC motif was required for this intrinsic property (Grauschopf et al., 1995). Upon its reduction, the N-terminal Cys residue becomes negatively-charged. This charge is stabilized by an electrostatic interaction with His (Guddat et al., 1997; Nelson & Creighton, 1994). Substituting this residue with a nonpolar or negatively-charged amino acid reduces the redox potential of DsbA (Grauschopf et al., 1995). The presence of vicinal His residues is a conserved feature of the CxxC motifs of disulfide bond forming enzymes. The positively charged amino acid is found in the CxxC motif of eukaryotic PDI and DsbA

equivalents in *Salmonella*, *Shigella*, *Pseudomonas*, and *Neisseria* (Kanehisa & Goto, 2000; Lu, Gilbert & Harper, 1992).

The high redox potential of DsbA presents a biological problem. Reduced DsbA must be re-oxidized before another disulfide bond can be catalyzed. However, the reduced form of the CxxC motif is more stable than the oxidized form (Wunderlich et al., 1993). Due to this, it is unlikely that this consensus sequence would spontaneously re-oxidize. How is DsbA activity recycled? *E. coli* DsbA is re-oxidized by DsbB, which was discovered simultaneously by Missiakas et al. (1993) and Bardwell et al. (1993). The first group found *dsbB* by screening a mutant library for increased sensitivity to DTT, while the latter used the MalF- β -gal fusion assay. DsbB is a 20 kDa integral membrane protein with two periplasmic loops that contain redox-active disulfide bonds (Cys⁴¹-Cys⁴⁴ and Cys¹⁰⁴-Cys¹³⁰) (Inaba et al., 2006; Jander, Martin & Beckwith, 1994). DsbB-catalyzed oxidation is initiated when the N-terminal Cys of the DsbA CxxC motif (shown as a thiolate ion in Fig. 2A) breaks the thiol linkage in the DsbB C-terminal periplasmic loop (Cys¹⁰⁴-Cys¹³⁰). This results in a mixed disulfide intermediate between DsbA Cys³⁰ and DsbB Cys¹⁰⁴ (Inaba & Ito, 2008). In turn, electrons are passed to DsbB Cys¹³⁰ (labeled with an asterisk) which attacks the DsbB N-terminal Cys⁴¹-Cys⁴⁴ linkage, resulting in a second mixed thiol intermediate between Cys¹³⁰ and Cys⁴¹ (Fig. 2A). This event transforms DsbB Cys⁴⁴ (labeled in green) into a thiolate ion, which becomes linked to a conjugated ubiquinone (Bader et al., 1999) (Fig. 2B). A free Cys within DsbA then triggers the resolution of these intermediates by reforming the linkage with Cys³⁰. This event releases Cys residues within DsbB to reform their native disulfide bonds (Fig. 2C). Ultimately, the electrons are deposited into the ubiquinone pool, and shuttled to the electron transport chain (Kobayashi et al., 1997).

The model of electron transfer between DsbA and DsbB is logical, but the redox potentials of the DsbB redox-active centers make it seemingly impossible (Inaba & Ito, 2002).

The DsbB Cys¹⁰⁴-Cys¹³⁰ linkage has a lower redox potential than DsbA Cys³⁰-Cys³³. This makes the flow of electrons between these enzymes unfavorable. How does DsbB catalyze the up-hill oxidation of DsbA? The crystal structure for a DsbA-DsbB complex revealed that the initial disulfide intermediate formed between DsbA Cys³⁰ and DsbB Cys¹⁰⁴ shifts DsbB Cys¹³⁰ towards the Cys⁴¹-Cys⁴⁴ redox center (Inaba et al., 2006) (Figs. 2A and 2B). This conformational change prevents DsbB Cys¹³⁰ from resolving the DsbA Cys³⁰- DsbB Cys¹⁰⁴ intermediate, thus forcing electrons to flow towards ubiquinone.

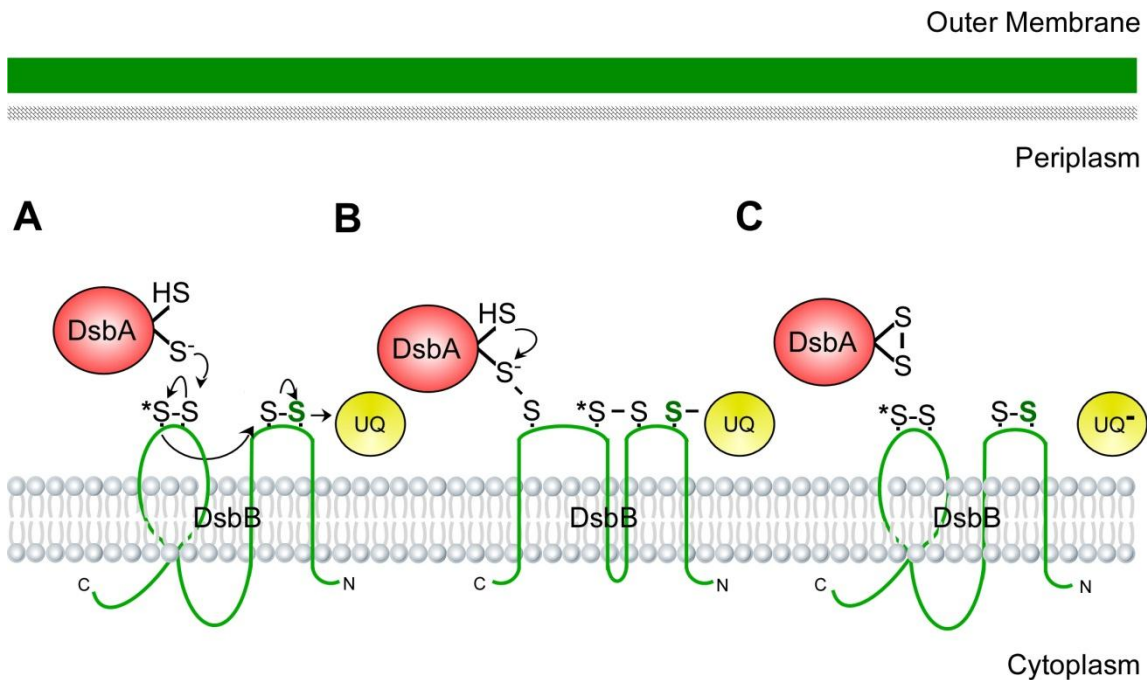


Figure 2. DsbB re-oxidizes DsbA. **(A)** The negatively-charged DsbA Cys³⁰ (shown as a thiolate ion) breaks a disulfide bond within the DsbB C-terminal periplasmic loop to form a mixed disulfide bond intermediate (DsbA Cys₃₀-DsbB Cys₁₀₄). This induces a conformational change, in which DsbB Cys₁₃₀ (labeled with an asterisk) shifts towards the N-terminal loop. Cys₁₃₀ breaks the Cys₄₁-Cys₄₄ linkage resulting in the formation of a second mixed intermediate between it and Cys₄₁. Cys₄₄ (shown in green) then links to ubiquinone **(B)** All three mixed intermediates are resolved upon reformation of the disulfide bond within the DsbA CxxC motif (Cys₃₀-Cys₃₃). **(C)** DsbA is re-oxidized, the native disulfide bonds within DsbB are reformed, and the electrons originating from DsbA are deposited with ubiquinone (Figure adapted from Kadokura and Beckwith, 2010).

1.3 Reductive pathways in the Gram-negative periplasm.

E. coli DsbA oxidizes Cys residues in the order that they emerge from the SecYEG translocon (Kadokura & Beckwith, 2009). This strategy for disulfide bond formation is flawed because not every secreted protein has consecutive disulfide bonds. *In vitro*, DsbA cannot reshuffle non-native Cys linkages in proteins (Zapun & Creighton, 1994). This suggested that DsbA is error-prone and unable to correct mistakes. Since the isomerase activity of PDI is important for protein folding in yeast, it was hypothesized that Gram-negative bacteria express an additional Dsb protein that reduces nonnative disulfide bonds. To identify this factor, an *E. coli dsbA* mutant was transformed with an overexpression library, and screened for clones with restored disulfide bond formation (Missiakas, Georgopoulos & Raina, 1994). The overexpression of a gene called *dsbC* rescued oxidative protein folding in the *dsbA* mutant. DsbC is a 26 kDa protein with a CGYC motif and C-terminal dimerization domain (McCarthy et al., 2000). Endogenous expression of *dsbC* is required for the stability of proteins with non-consecutive disulfide bonds, and resistance against oxidative stress (Hiniker & Bardwell, 2004; Hiniker, Collet & Bardwell, 2005). Unlike DsbA, DsbC also rearranges disulfide bonds in misfolded proteins *in vitro* (Zapun et al., 1995). Thus, the role of eukaryotic PDI is divided between two proteins in bacteria. DsbA catalyzes disulfide bond formation, while DsbC corrects Cys mispairing.

DsbC-driven reduction is essentially the reverse of DsbA. In its active form, the dimer's reactive CxxC motifs are reduced (Rietsch et al., 1997). An N-terminal Cys (shown as a thiolate ion in Fig. 3A) breaks nonnative disulfide bonds in substrates. This results in the formation of a mixed intermediate, which is resolved by the C-terminal Cys residue of the DsbC CxxC motif, or another Cys within the substrate (Kadokura & Beckwith, 2010). In the first scenario, the substrate is released in a reduced form, which is re-oxidized by DsbA. In

turn, the DsbC CxxC motif is oxidized. Re-reduction is catalyzed by the integral membrane protein DsbD. DsbD harbors three thioredoxin-like domains (α , β , γ) that participate in the transfer of electrons (Stewart, Katzen & Beckwith, 1999). Electrons derived from cytoplasmic thioredoxin are transported through these domains, and finally delivered to DsbC (Rietsch et al., 1996). If the DsbC substrate forms a new disulfide bond independent of DsbA, DsbC is released in an active, reduced state.

Similar to eukaryotic PDI, DsbC forms a dimer. This higher-ordered conformation prevents cross-talk between oxidative and reductive pathways in the periplasm. Specifically, the purpose of DsbC dimerization is to avoid oxidation by DsbB. Attempts to model an interaction between these two proteins revealed a steric clash between one DsbC protomer and the cytoplasmic membrane (Pan, Sliskovic & Bardwell, 2008). *In vivo*, DsbC mutants unable to form dimers rescue *dsbA* null phenotypes (Bader et al., 2001). As a monomer, the DsbC CxxC motif is oxidized by DsbB, which then allows it to catalyze disulfide bond formation.

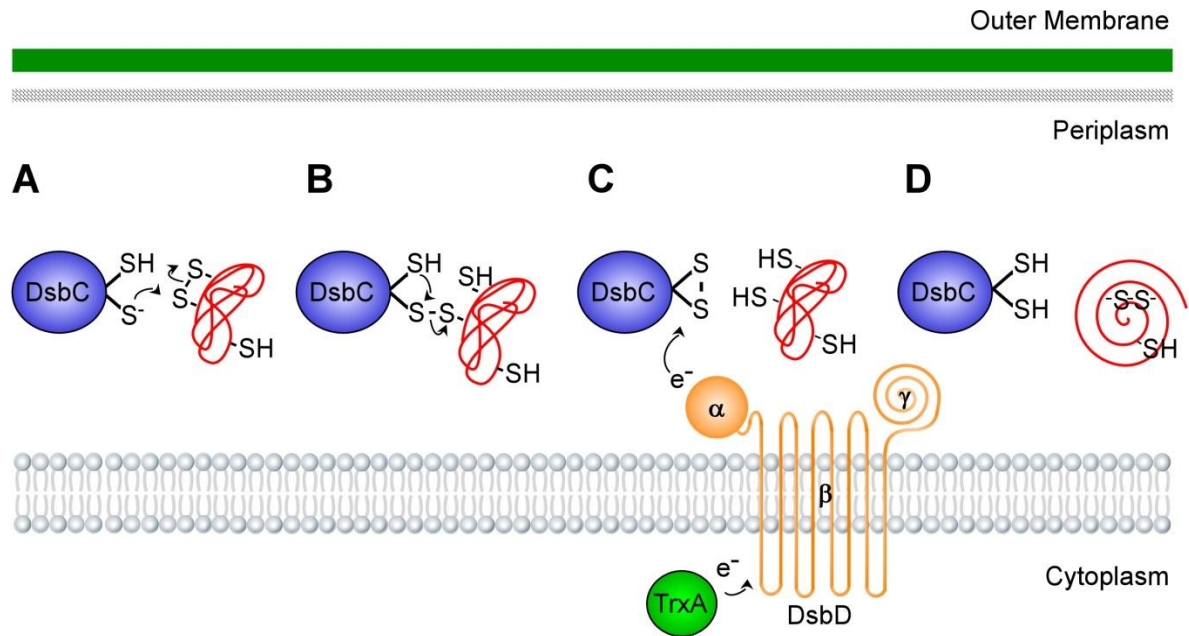


Figure 3. Disulfide bond isomerization is catalyzed by DsbC. **(A)** The N-terminal Cys residue (indicated by a thiolate ion) of the DsbC CxxC motif breaks nonnative Cys-linkages in proteins. **(B)** This results in a mixed disulfide intermediate, which is resolved by the C-terminal Cys of the CxxC motif. **(C)** This results in the formation of a disulfide bond between residues of the CxxC motif, and the release of the substrate in a reduced form. DsbC is then re-reduced by DsbD. Electrons are transmitted from cytoplasmic thioredoxin A (TrxA), to each DsbD redox domain (α , β , γ), and finally to DsbC. **(D)** DsbC is reduced and the substrate is re-oxidized to form its native Cys-linkages (Figure adapted from Kadokura and Beckwith, 2010).

DsbD also shuttles electrons to the periplasmic proteins DsbG and DsbE (Bessette et al., 1999; Reid, Cole & Eaves, 2001). Similar to DsbC, DsbG was identified by screening an overexpression library for factors that rescued disulfide bond formation in a *dsbA* mutant (Andersen et al., 1997). DsbG also forms a dimer, and exhibits disulfide bond isomerase activity *in vitro* (Bessette et al., 1999). However, the function of this enzyme *in vivo* is not known. It is proposed that DsbG targets specific substrates, or that it is expressed during certain conditions (Bessette et al., 1999; Hiniker & Bardwell, 2004). *dsbG* is encoded immediately upstream of the antioxidant alkyl hydroperoxide reductase (*aphC*), so it is possible that its expression is activated in response to oxidative stress (Zhou & Rudd, 2013). Unlike other Dsb proteins, DsbE is not involved with protein folding in the periplasm. DsbE, also known as CcmG, is required for synthesis of cytochrome c, a component of the electron transport chain (Fabianek, Hennecke & Thony-Meyer, 1998). DsbE reduces Cys residues in apocytochrome to allow for heme attachment.

1.4 Oxidative protein folding is required for bacterial virulence.

Unlike in eukaryotic cells, bacterial oxidative folding pathways do not appear to be essential. *E. coli dsbA* mutants exhibit a slow growth phenotype if they are cultured in minimal media, but this may result from decreased uptake of glucose because the corresponding sugar transporters require disulfide bonds (Bessette et al., 2001). Disulfide isomerase pathways are also nonessential since *dsbC* null mutants display no growth defect (Vertommen et al., 2008). This is not due to redundancy between oxidative folding factors as a triple *dsbA⁻ dsbC⁻ dsbG⁻* mutant is still viable (Vertommen et al., 2008). *dsbD* null mutants are temperature-sensitive, but this is caused by the disruption in cytochrome c synthesis because DsbD is required to reduce DsbE (Missiakas, Schwager & Raina, 1995).

Although Dsb proteins are not required for survival, they are essential for pathogenesis (Heras et al., 2009). Bacteria secrete an arsenal of disulfide bond-containing

virulence factors that promote host colonization, dissemination, and death. *dsbA* mutants are often attenuated in virulence because these factors are misfolded and degraded (Heras et al., 2009). Type I fimbriae expressed by uropathogenic *E. coli* type I fimbriae is one well-studied example of a virulence factor requiring oxidative protein folding (Totsika et al., 2009). Type I fimbriae promote virulence by mediating adherence to the host uroepithelial lining. They are comprised of multiple subunits that are assembled by the periplasmic chaperone FimC and outer-membrane usher FimD (Busch, Phan & Waksman, 2015). These subunits are not assembled into fimbriae in a *dsbA* mutant (Heras et al., 2009). Crespo et al. (2012) reported that the formation of disulfide bonds within fimbrial subunits was a prerequisite for their assembly. *In vitro*, FimC only folded subunits with disulfide bonds. The inability of FimC to interact with reduced subunits was proposed to serve as a measure of quality control. FimC may not fold, and subsequently deliver reduced subunits to FimD because they cannot form stable fimbriae.

Following colonization, many bacteria secrete toxins to manipulate their hosts. DsbA catalyzes disulfide bond formation in toxins secreted by *E. coli*, *Bordetella pertussis*, *Pseudomonas aeruginosa*, and *Vibrio cholerae* (Heras et al., 2009). Secretin proteins of Type III secretion systems expressed by *E. coli*, *Salmonella enterica*, and *Yersinia pestis* also require disulfide bond formation (Jackson & Plano, 1999; Miki, Okada & Danbara, 2004; Miki et al., 2008). Secretin, which forms the outer membrane component of the Type III secretion systems, is required for the translocation of virulence factors into hosts. In the absence of *dsbA*, they are unstable. As a consequence, bacteria cannot translocate toxins to their cell targets. Finally, motility is important for pathogens to survive and disseminate within a host. DsbA is required for flagellar synthesis in *E. coli* (Dailey & Berg, 1993). FlgI, which forms the peptidoglycan layer or P-ring of the flagellar basal body, contains a disulfide bond. In the absence of *dsbA*, FlgI becomes degraded, which inhibits flagellar synthesis (Hizukuri et al., 2006).

Targeting bacterial pathogenesis is an attractive alternative for the development of new antimicrobials (Cascioferro, Totsika & Schillaci, 2014). Since *dsbA* is important for virulence, but not growth, inhibitors of disulfide bond formation could allow a host to clear an infection without providing a selective pressure to mutate. Adams et al. (2014) recently screened a chemical library for inhibitors of *E. coli* DsbA. One compound inhibited both DsbA activity *in vitro*, and *E. coli* motility *in vivo*. Importantly, it did not affect bacterial growth. Structural analysis revealed that this compound blocks substrate access to DsbA by binding to a hydrophobic groove near the active site. Although it is not clear whether this potential drug can inhibit disulfide bond forming proteins in other pathogens, it is an important first step towards the development of new antimicrobials.

1.5 Gaps in Understanding: Disulfide bond formation in Gram-positive bacteria.

Disulfide bond forming pathways are not understood in Gram-positive bacteria, which lack periplasmic spaces. Gram-positive cell envelopes are comprised of a single membrane surrounded by a thick layer of peptidoglycan. Although a space between these regions has been observed by Cryo-EM, it is not considered to be equivalent to the Gram-negative periplasm (Matias & Beveridge, 2005; Matias & Beveridge, 2006). Due to the diffusive nature of peptidoglycan, it is possible that this space is exposed to the extracellular milieu. Therefore, secreted unfolded proteins with multiple Cys residues could be damaged by environmental stress. It was proposed that Gram-positives prevent this potential folding stress by avoiding disulfide bond formation in the exoplasm (Daniels et al., 2010).

A survey of Gram-positive secreted proteomes partially supported this conjecture (Daniels et al., 2010; Dutton et al., 2008). The low GC Firmicutes, including *Bacillus*, *Staphylococcus*, *Lactobacillus*, and *Streptococcus* were found to secrete few, if any proteins with multiple Cys residues indicating that they lack disulfide bonds. Dsb-like enzymes have

been identified in some of these organisms, but, unlike Gram-negative bacteria, their genes are arranged in operons with specific substrates. *B. subtilis* harbors two gene clusters with the putative oxidoreductase enzymes *bdbA-D*. *bdbA* and *bdbB* belong to an operon that encodes sublancin, an antibiotic that harbors disulfide bonds (Dorenbos et al., 2002). *bdbC* and *bdbD*, which are contained in a competence gene cluster, are required for the stability of the disulfide bond-containing ComCG pseudopilus (Meima et al., 2002). *In vivo*, Bdb proteins are not fully interchangeable suggesting that their activity is limited to substrates within their respective operons (Dorenbos et al., 2002). Furthermore, *bdbCD* are regulated by *comX*, so it is unlikely that they are constitutively expressed (Meima et al., 2002). Together, these data suggested that Bdb proteins do not have a housekeeping role in oxidative protein folding.

In contrast to Firmicutes, Gram-positive Actinobacteria including *Corynebacterium*, *Streptomyces*, and *Mycobacterium* secrete an abundance of Cys-containing proteins, and encode Dsb-like enzymes (Daniels et al., 2010; Dutton et al., 2008). Disulfide bond forming pathways are yet to be explored in these organisms. The discovery of a novel redox protein Vitamin K epoxide reductase (VKOR) expressed by *M. tuberculosis* was recently reported (Dutton et al., 2008). VKOR is a transmembrane protein with two periplasmic loops containing redox-active disulfide bonds. Although expression of this enzyme can restore disulfide bond formation in an *E. coli dsbB* mutant, it is not a DsbB homolog (Wang et al., 2011). VKOR is related to mammalian VKOR, an enzyme involved in vitamin K recycling. In addition to VKOR, several other Dsb-like proteins have been identified in *M. tuberculosis* (Chim et al., 2010; Goulding et al., 2004). These proteins have been crystallized, and their redox activity has been examined *in vitro*, but cellular functions are unknown. To date, *M. tuberculosis* is the only established model to study oxidative protein folding in Actinobacteria. Although structural analyses of these proteins have been extensive, *in vivo* work is lacking. Do the identified oxidoreductases form or reduce disulfide bonds? From where do they receive this oxidizing,

or reducing power? Are these proteins involved in protein folding like DsbA and DsbC, or are they required for the synthesis of co-factor-containing proteins like DsbE? Do they target specific or multiple substrates? Finally, are they important for virulence? Better tools are needed to elucidate oxidative folding pathways in Actinobacteria.

1.6 Pilus assembly, a tool to study oxidative protein folding in Actinobacteria.

The Ton-That laboratory investigates the assembly of pili in Gram-positive bacteria using the Actinobacterial oral pathogens *Actinomyces oris* and *Corynebacterium diphtheriae* as models. Pili are important virulence factors that mediate adherence to host tissues, promote biofilm formation, and induce inflammation (Danne & Dramsi, 2012; Mandlik et al., 2008). Gram-positive pili are comprised of individual subunits that are covalently linked and anchored to the cell wall (Ton-That & Schneewind, 2004). The precursors of pilus subunits are characterized by an N-terminal signal peptide and pilin motif, and a C-terminal cell wall sorting signal (CWSS) that is comprised of an LPxTG motif, hydrophobic patch, and positively-charged tail (Ton-That, Marraffini & Schneewind, 2004). The N-terminal signal peptide targets pilus precursors for translocation via the SecYEG machinery. Unfolded precursors are secreted into the exoplasm where they are tethered to the membrane by the CWSS's hydrophobic patch. Here pilus subunits become folded and are assembled into pili by transpeptidase sortase enzymes (Fig. 4). A pilus-dedicated sortase cleaves its substrates at the LPxTG motif resulting in the formation of an acyl-enzyme intermediate between a substrate Thr and sortase Cys (Guttilla et al., 2009). This linkage is resolved by a nucleophilic lysine within the N-terminal pilin motif of a neighboring pilin-sortase complex (Ton-That et al., 2004). This results in the formation of an isopeptide bond between the Thr of one pilin and Lys of another (Budzik et al., 2008). This reaction occurs repeatedly to synthesize a pilus fiber in a bottom-up fashion. When assembly is complete, the pilus is transferred to a

housekeeping sortase, which links it to Lipid II precursor for incorporation into the cell wall (Swaminathan et al., 2007).

A. oris, a pioneer colonizer of the oral cavity, uses pili to facilitate the formation of dental plaque. This bacterium expresses two varieties of pili called type 1 and 2 fimbriae (Mishra et al., 2007). The majority of genes encoding pilus subunits and their pilus-specific sortases are arranged in operons. Type 1 fimbriae, which are comprised of the major subunit FimP and minor tip protein FimQ, are assembled by sortase C1 (SrtC1) (Mishra et al., 2007). These pili are required for the initial colonization of the oral cavity. Specifically, FimQ mediates this attachment by binding to proline-rich saliva deposits on the tooth surface (Wu et al., 2011). Type 2 fimbriae, which are comprised of the major subunit FimA and minor subunits FimB and CafA, promote the development of biofilm by mediating interactions with host cells and oral cavity co-colonizers (Mishra et al., 2007). SrtC2 is the designated sortase for assembly of these pili. The transpeptidase polymerizes FimA to form the fimbrial shaft, and cross-links CafA or FimB to the tip. CafA is a recently discovered component of the type 2 fimbriae (Reardon-Robinson et al., 2014). Unlike most genes that encode pilus subunits, *cafA* is not found in the type 2 fimbrial gene cluster. CafA specifically mediates interspecies interactions by binding to lactose moieties on surface of oral streptococci, and host epithelial and blood cells (Reardon-Robinson et al., 2014). FimB is proposed to mediate interbacterial interactions that promote oral biofilm development, but its binding partner(s) have yet to be identified.

C. diphtheriae, the causative agent of diphtheria disease, expresses three types of heterotrimeric pili called SpaA-, SpaD-, and SpaH-type pili (Gaspar & Ton-That, 2006; Ton-That & Schneewind, 2003). Out of the three, SpaA-type pili are well-studied. These adhesive factors, which are comprised of the major subunit SpaA and minor pilins SpaB, and SpaC, are assembled by SrtA. SpaA is polymerized to form the pilus shaft, while SpaB is positioned at

the base, and SpaC is cross-linked to the tip (Ton-That & Schneewind, 2003). The minor pilins SpaB and SpaC serve as adhesions to facilitate colonization of the pharyngeal epithelial lining, where symptoms of diphtheria disease first develop (Mandlik et al., 2007).

Prior to sortase-mediated assembly, pilins expressed by *A. oris* and *C. diphtheriae* are translocated across the cytoplasmic membrane in unfolded states. How are these precursors folded correctly in the Gram-positive exoplasm? A clue was revealed when the crystal structures for the major pilus subunits FimA, FimP, and SpaA were solved (Kang et al., 2009; Mishra et al., 2011; Persson et al., 2012). All three proteins contained typical elements of Gram-positive pilins including tandem Ig-like domains and intramolecular isopeptide bonds (Vengadesan & Narayana, 2011). However, unlike pilus proteins secreted by Firmicutes, these subunits also contained disulfide bonds. *A. oris* FimA and FimP were predicted to possess disulfide bonds in both the N- and C-termini, while *C. diphtheriae* SpaA was predicted to harbor a single linkage in its C-terminus. We hypothesized that these bonds formed *in vivo*, and that they were important for protein folding. Similar to Gram-negative bacteria, it appeared that Actinobacteria use disulfide bond formation to help fold secreted virulence factors. We proposed to use pilus assembly as a model to study disulfide bond formation in *A. oris* and *C. diphtheriae*.

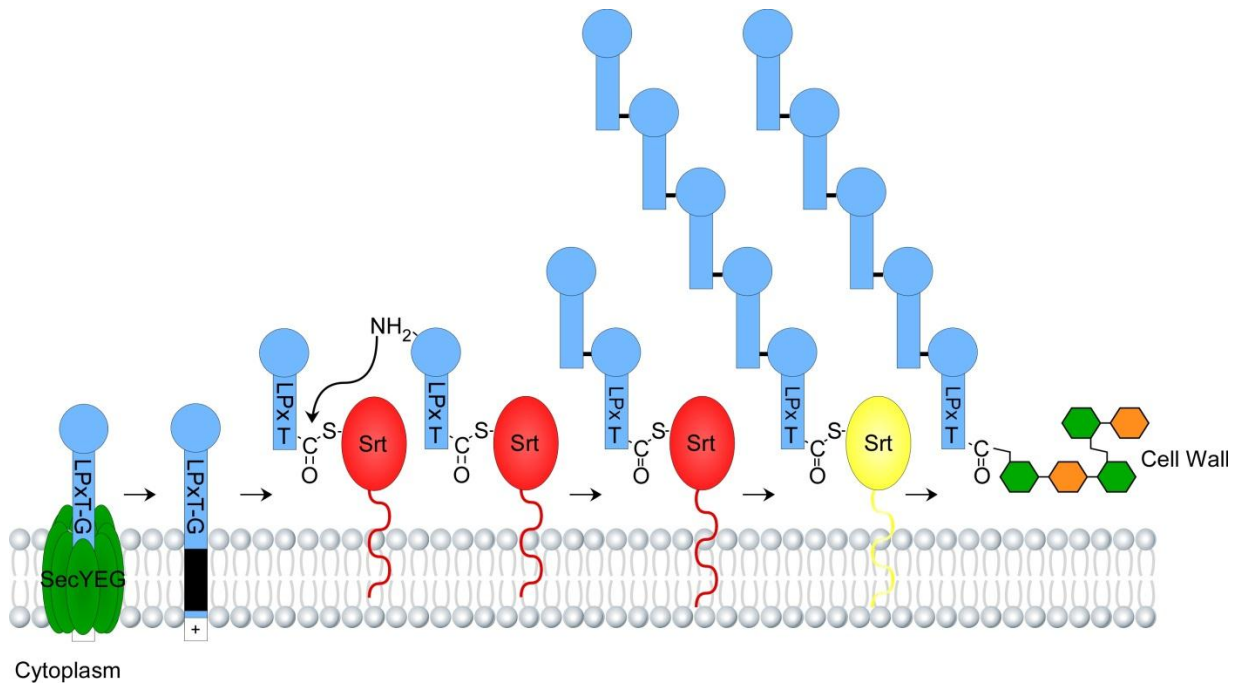


Figure 4. Sortase-mediated pilus assembly in Gram-positive bacteria. Precursors of pilus subunits (shown in blue) are characterized by an N-terminal signal peptide and pilin motif, and a C-terminal cell wall sorting motif (CWSS). The pilus precursors are translocated into the exoplasmic space where they are folded, and tethered to the cytoplasmic membrane by the CWSS hydrophobic segment and positively charged tail. The subunits are cleaved by a pilin specific sortase (shown in red) at the LPxTG motif resulting in the formation of an enzyme-acyl intermediate between the sortase and Thr of the LPxTG motif. Two pilins become cross-linked when a Lys residue within the pilin motif resolves the enzyme-acyl linkage of a neighboring pilin-sortase complex. This reaction occurs repeatedly to form the pilus fiber. When pilus assembly is complete, the fiber is transferred to a housekeeping sortase (shown in yellow), which anchors it to the cell wall.

1.7 Summary and significance

Gram-negative pathogens secrete proteins to colonize, manipulate, and kill host cells. In the absence of *dsbA*, many of these proteins are degraded, which causes virulence to become attenuated (Heras et al., 2009). Due to these observed phenotypes, it is proposed that Dsb proteins are excellent candidates for the development of new antimicrobial drugs. Since disulfide bond formation is not required for growth, it is proposed that bacteria would be less likely to develop resistance against drugs targeting this pathway (Cascioferro et al., 2014).

Gram-positive bacteria also secrete virulence factors. Unlike Gram-negatives, they are thought to lack periplasmic compartments in which to fold these proteins. Oxidative forms of stress present in the extracellular milieu could mis-oxidize Cys residues. As a consequence, many Gram-positives are believed to avoid this stress by not utilizing disulfide bond formation (Daniels et al., 2010; Dutton et al., 2008). However, Gram-positive Actinobacteria may be an exception. Elucidating disulfide bond forming pathways in these organisms will further our understanding of how secreted proteins are processed in the exoplasm, as well as expand the repertoire of available targets for drug development.

This thesis elucidates Actinobacterial oxidative folding pathways in *A. oris* and *C. diphtheriae* using pilus proteins as model substrates. MdbA, a membrane-bound oxidoreductase, catalyzes disulfide bond formation within secreted virulence factors like pili and diphtheria toxin. In *A. oris*, MdbA activity is dependent on VKOR, which is absent in *C. diphtheriae*. Remarkably, unlike Gram-negative DsbA, MdbA is also required for growth and division. The *C. diphtheriae* *mdbA* mutant selects for a suppressor mutation that induces overexpression of the oxidoreductase named *tsdA*, which restores disulfide bond formation.

This study highlights several important considerations for the development of antimicrobial agents against Gram-positive pathogens. First, oxidative protein folding pathways are not conserved among bacteria. The *in vivo* functions of newly identified oxidoreductases have been elucidated in this work. This can contribute to the development of disulfide bond forming inhibitors that target a broader spectrum of bacteria. Second, unlike Gram-negative Dsb proteins, MdbA is important for growth. We suspect that the essentiality of disulfide bond forming pathways in Actinobacteria is a common trait since the deletion of *M. tuberculosis vkr* also confers a growth defect (Dutton et al., 2008). Although drugs targeting these pathways in Actinobacteria would make powerful bactericides, they will provide a selective pressure to gain resistance. Future studies can circumvent this by investigating mechanisms by which Actinobacteria may acquire resistance.

In summary, due to the lack of a recognizable periplasm, it was once thought that Gram-positive bacteria did not rely on the formation of disulfide bonds to help fold secreted proteins. Here we demonstrate that Actinobacteria represent a special class of Gram-positives that utilize disulfide bond formation as a general folding tool. This work expands our understanding of how proteins fold in the exoplasm, and provides insight for the development of antimicrobial agents to target important Gram-positive pathogens.

Chapter II:

Materials and Methods

2.1 Bacterial strains, primers, plasmids, and media

Bacterial strains, primers, and plasmids used in this study are listed in Tables 2.1 and 2.2. *Actinomyces* and *Corynebacterium* strains were grown in Heart Infusion (HI) broth or on HI agar plates. Streptococci were grown in HI broth supplemented with 1% glucose. *E. coli* DH5 α , BL21, and S17 used for molecular cloning, protein purification, and gene deletions, respectively, were grown in Luria Broth (LB). Kanamycin (Kan) or ampicillin (AMP) was added at 50 $\mu\text{g mL}^{-1}$ or 100 $\mu\text{g mL}^{-1}$, respectively, when required. Polyclonal antibodies were raised against recombinant pilus proteins MdbA and TsdA in rabbits as previously described (Mishra et al., 2007).

2.2 Construction of recombinant plasmids-

pVKOR – To construct pVKOR, primers *vkor_F_NdeI* and *vkor_R_KpnI* were used to PCR amplify the promoter and coding regions of *A. oris vkor* along with flanking NdeI and KpnI sites. The resulting PCR product was digested within these enzymes, cloned into pJRD215 pre-cut with NdeI and KpnI, and then used to transform *E. coli* DH5 α . The resulting plasmid was electroporated into MR107 and MR108.

pMdbA_{AO} – The promoter and open-reading-frame (ORF) of *A. oris mdbA* were PCR amplified using primers *mdbA_{AO}_F_XbaI* and *mdbA_{AO}_R_EcoRI* designed with XbaI and EcoRI cut sites. The PCR product was digested with XbaI and EcoRI, and cloned into pJRD215 pre-cut with the same enzymes. The resulting plasmid was electroporated into MR108.

pAraC-MdbA_{AO} – Using the primers *mdbA_{AO}_F_ATG* and *mdbA_{AO}_R_EcoRI*, the ribosome binding site (RBS) and ORF of *A. oris mdbA* was PCR-amplified using Phusion polymerase® (NEB) to generate blunt ends. The resulting product was 5' phosphorylated with Polynucleotide Kinase (NEB) and then digested with EcoRI. Using pBad33 as a template, the

primers *araC_F_KpnI* and *araC_R* amplified *araC* and the corresponding arabinose-inducible promoter, which was then digested with KpnI. Finally, the PCR fragments were cloned into pJRD215 precut with EcoRI and KpnI, and the resulting plasmid was electroporated into MR111.

pMdbA_{cd} – Primers *mdbA_{cd}_BamHI_F* and *mdbA_{cd}_BamHI_R* were designed to PCR-amplify the promoter and coding regions of *C. diphtheriae mdbA* with appending BamHI sites. The DNA fragment was digested with the appropriate restriction enzyme, and cloned into pCGL0243 pretreated with alkaline phosphatase and BamHI. The resulting plasmid was electroporated into NJ2.

pMtbDsbA- The primers *dsbA_{Mtb}_F* and *dsbA_{Mtb}_R_HindIII* were used to PCR amplify the ORF for the putative *M. tuberculosis dsbA* using Phusion DNA polymerase ® (NEB) to generate blunt ends. This product was 5' phosphorylated and then cut with HindIII. The promoter and RBS for *A. oris mdbA* was amplified with primers *PmdbA_{AO}_F_KpnI* and *PmdbA_{AO}_R*, and then digested with KpnI. Both DNA fragments then were ligated with pJRD215 precut with KpnI and HindIII to construct the recombinant plasmid, which was electroporated into MR108.

pEcDsbA_{AO}- Using primers *dsbA_{Ec}_F* and *dsbA_{Ec}_R_EcoRI*, the regions encoding extracellular portions of *E. coli dsbA* was PCR amplified using Phusion DNA polymerase ® (NEB) to generate blunt ends. The PCR product was 5' phosphorylated and cut with EcoRI. Segments encoding the promoter, RBS, and cytoplasmic and transmembrane domains for *A. oris mdbA* was amplified with primers *PmdbA_{AO}_F_KpnI* and *PmdbA_{AO}TM_R*, and then digested with KpnI. Both DNA fragments then were ligated with pJRD215 precut with KpnI and HindIII to construct the recombinant plasmid which was electroporated into MR108.

pEcDsbA_{Cdi}- The primers *dsbA_{Ec}_F* and *dsbA_{Ec}_R_BamHI* were used to PCR amplify the extracellular regions of *E. coli dsbA* using Phusion DNA polymerase ® (NEB) to generate blunt ends. The resulting product was 5' phosphorylated and cut with BamHI. The segments encoding the promoter, RBS, and cytoplasmic and transmembrane domains for *C. diphtheriae mdbA* was amplified with primers *PmdbA_{Cd}_F_BamHI* and *PmdbA_{Cd}TM_R*, and then digested with BamHI. Both DNA fragments then were ligated with pCGL0243 precut with BamHI and treated with alkaline phosphatase (NEB) to generate the recombinant plasmid, which was electroporated into NJ2.

pJRD-MdbA_{Cd}- Using primers *mdbA_{Cd}_F* and *mdbA_{Cd}_R_HindIII*, the ORF for *C. diphtheriae mdbA* was PCR amplified using Phusion DNA polymerase ® (NEB) to generate blunt ends. The resulting product was 5' phosphorylated and cut with HindIII. The promoter and RBS of *A. oris mdbA* was amplified with primers *PmdbA_{AO}_F_KpnI* and *PmdbA_{AO}_R*, and then digested with KpnI. Both DNA fragments then were ligated with pJRD215 precut with KpnI and HindIII to construct the recombinant plasmid for electroporation into MR108.

pAraC-TsdA- The ribosome binding site and ORF for *C. diphtheriae tsdA* was PCR amplified with Phusion ® polymerase using *tsdA_RBS_F* and *tsdA_R_HindIII*, and the resulting product was digested with HindIII and 5' phosphorylated. An arabinose-inducible promoter was PCR-amplified from pBad33 using primers *araC_F_PstI* and *araC_R*. The resulting product was digested with PstI. The two fragments were then incubated with pCGL0243 pretreated with PstI and HindIII to form pAraC-TsdA, which was electroporated into NJ2.

Recombinant vectors using pMCSG7- To generate recombinant, His-tagged MdbA proteins, primers (See Table 2-2) were designed to amplify the extracellular-coding regions of *mdbA* within *A. oris* and *C. diphtheriae*. The resulting PCR products were cloned into pMCSG7 using ligation-independent cloning (Stols et al., 2002). Purified DNA fragments were

treated with LIC-competent T4 DNA polymerase (Novagen) and 2.5 μ M dCTP. Meanwhile, pMCSG7, precut with SspI, was treated with LIC-competent T4 polymerase and dGTP to generate complementary overhangs between the linearized vector and *mdbA* genes. The products were then incubated over a gradient of temperatures (3 min at 70°C, 2 min at 65°C, 2 min at 60°C, 2 min at 55°C, 1 min at 50°C, 1 min at 45°C, 1 min at 40°C, 1 min at 35°C, 1 min at 30°C, 5 min at 25°C) to promote annealing. The resulting plasmids were used to transform *E. coli* DH5 α and the insert was confirmed by DNA sequencing. The plasmids were then introduced into *E. coli* BL21 (DE3) for protein expression.

2.3 Site-directed mutagenesis of recombinant plasmids

To construct cysteine-to-alanine mutations within FimA, overlapping primers (Table 2-2) carrying the target mutations were used in PCR-amplification using pCR2.1-FimA (Mishra et al., 2011) as a template. The PCR products were digested overnight at 37°C with Dpn1 to remove the parental template, and the resulting DNA samples were used to transform DH5 α . The generated mutations were confirmed by sequencing, and *fimA* was removed from pCR2.1 by digestion with XbaI and EcoRI. The DNA fragment was cloned into pJRD508FimB precut with similar restriction enzymes. The resulting plasmids were electroporated into AR4 (Mishra et al., 2011).

To generate cysteine-to-alanine mutations within SpaA, VKOR, MdbA_{Cd}, and MdbA_{Ao}, inverse PCR was utilized using recombinant plasmids as templates (Table 2-1). Appropriate primers (Table S3) carrying the desired mutations were 5' phosphorylated and used to PCR the plasmid templates with Phusion HF DNA polymerase (NEB). The resulting linear products were purified products, treated with ligase to reform the circular plasmids, and used to transform *E. coli* DH5 α . DNA sequencing confirmed the desired mutations, and the plasmids were introduced to the appropriate strains (Table 2-1).

2.5 Gene deletions

For *A. oris*, nonpolar, in-frame deletion mutants were generated using the Galk counter-selection method previously established by Mishra et al. (2010). Briefly, approximately 1Kb-fragments up- and downstream of a target gene were amplified, fused, and then cloned into pCWU2, an integrative plasmid expressing Kan resistance and *galk* genes (Mishra et al., 2010). The resulting plasmid was electroporated into *A. oris* CW1, which lacks a functional *galk*. Co-integrants resulting from a single crossover event were selected for growth on Kan. To promote a recombination event, cells were grown in HI broth without Kan. The loss of the integrative plasmid was selected for growth on HI agar plates containing 0.2% 2-deoxygalactose (2-DG), which is converted to a toxic intermediate when the plasmid-derived *galk* is present. The generated mutants were identified by PCR and subsequently analyzed by western blotting and EM.

For *C. diphtheriae*, nonpolar, in-frame deletion mutants were generated using the SacB counter-selection protocol as reported by Ton-That and Schneewind (2003). Similar to *A. oris*, approximately 1Kb-fragments up- and downstream of the genes of interest were cloned into the integration plasmid pK19mobsacB expressing Kan resistance and *sacB* genes (Schafer et al., 1994). The resulting plasmid was introduced into *E. coli* S17-1 for conjugation with *C. diphtheriae*. Integration clones were selected for growth on Kan and nalidixic acid (to kill *E. coli*). To induce a double-crossover event leading to plasmid excision, *C. diphtheriae* were grown overnight without antibiotics. Loss of the integrated plasmid was selected for growth on HI agar plates containing 1% sucrose, which is toxic to cells expressing *sacB*. Gene deletions were identified by PCR and analyzed by western blotting.

2.4 Tn5 transposon mutagenesis of *A. oris*

A library of roughly 3,000 Tn5 mutants was created using the Tn5 transposon system recently developed for *A. oris* (Wu et al., 2014). To identify factors required for fimbrial assembly and bacterial coaggregation, we set up a cell-based screen that is dependent on type 2 fimbriae-mediated interaction with *S. oralis*. In this screen, *Actinomyces* Tn5 mutants grown in 96-well plates were mixed with equal cell numbers of *S. oralis* 34 in coaggregation buffer (Mishra et al., 2010). Coaggregation was visualized using an inverted microscope, and scored by comparing the strains to positive (*A. oris* MG-1 and *S. oralis* 34) and negative controls (*S. oralis* OC1 lacking RPS receptors or *A. oris* $\Delta fimA$). Four coaggregation-deficient mutants obtained from this screen were confirmed by further coaggregation and fimbrial assembly assays. Chromosomal DNA of these mutants was isolated, and the genes disrupted by Tn5 were identified by TAIL PCR (Liu & Whittier, 1995) and DNA sequencing.

2.6 Protein purification

Cultures of *E. coli* BL21 (DE3) harboring individual recombinant plasmids (Table 2.1; pMCSG7s) were grown at 37°C in LB until an OD₆₀₀ of approximately 0.7. Protein expression was induced by the addition of 1mM IPTG at 30°C for 3 hours. Cell pellets were harvested by centrifugation and re-suspended in EQ buffer (50mM Tris-HCl pH 7.5, 100mM NaCl). Cell lysis was achieved by using a French Press cell. Clear lysates obtained by centrifugation were subject to affinity chromatography, and purified His-tagged proteins were dialyzed in dialysis buffer (50mM Tris-HCl pH 7.5, 100mM NaCl, 10% glycerol) at 4°C and stored at -20°C.

2.7 Cell fractionation and western blotting-

Overnight cultures of *A. oris* and *C. diphtheriae* strains were used to inoculate fresh cultures (1:50 dilution). When appropriate, 50 µg mL⁻¹ Kan was added. Cells grown to early-

or mid-log phase, at 37°C for *A. oris* and 30°C or 37°C for *C. diphtheriae*, were normalized to an OD₆₀₀ of 1.0, and subject to centrifugation to separate the medium (S) and cell fractions. *A. oris* were washed and re-suspended in SMM buffer (0.5M sucrose, 10mM MgCl₂, 10mM maleate pH 6.8), and treated with *Mutanolysin* (*Sigma*), a cell wall hydrolase, at 37°C for 4 hours. *C. diphtheriae* were washed and re-suspended in hydrolase buffer (0.5M sucrose, 10 mm MgCl₂, phosphate buffered saline (PBS) pH 7.4), and then incubated with Dip0218, a *C. diphtheriae* prophage encoded cell wall hydrolase isolated by our lab, at 37°C for 3 hours. After treatment, the soluble cell wall fractions (W) were separated from the protoplasts (P) by centrifugation. P fractions were then washed in SMM buffer, re-suspended in PBS containing 0.1% Triton-114, and subjected to three freeze/thaw cycles using dry ice-ethanol and 100°C water baths. When needed, the cytoplasmic fraction (C) was isolated by ultracentrifugation of lysed protoplasts prior to TCA precipitation. The S, W, and C fractions were TCA precipitated and acetone washed. Protein samples were re-suspended in reducing or non-reducing SDS-loading buffer, heated at 60°C for 10 minutes, and separated on Tris-glycine gels. Proteins were detected with rabbit antisera diluted in 5% milk (1:5000 α -FimA, 1:20,000 α -SpaA, 1:20,000 α -MdbA_{Cd}, 1:20,000 α -MdbA_{AO}, 1:1000 α -DT, 1:10,000 α -FimP) followed by horseradish peroxidase (HRP) (1:10,000) conjugated goat anti-rabbit IgG for detection by chemiluminescence.

2.8 Electron microcopy

Bacteria grown on HIA plates or liquid media were suspended and washed in 0.1M NaCl, and then re-suspended in PBS. To view the cells by electron microscopy, 7 μ L of culture were placed onto carbon-coated nickel grids (Electron Microscopy Sciences) for 1 minute, washed 3 times with sterile water, and then stained with 1% uranyl-acetate for another minute. For immunogold labeling, 7 μ L of culture were placed onto the grids for 1 minute, and then washed in PBS 1% BSA three times. The grids were subsequently blocked in PBS 1%

BSA with 0.1% gelatin for 1 hour at room temperature. Pili were stained with primary antibodies diluted in PBS 1% BSA (1:100 α -FimA, 1:25 α -FimB, 1:100 α -FimP, 1:25 α -FimQ, or undiluted depleted α -SpaA) for 1 hour, and then washed 3 times in PBS 1% BSA. Grids were blocked again in gelatin for 30 minutes, washed once, and then stained with secondary antibody conjugated to 12 or 18nm gold particles diluted 1:20 in PBS 1% BSA for 1 hour. Finally, the samples were washed 5 times with sterile water, and stained with 1% uranyl-acetate for 1 minute. All samples were viewed using a Jeol Jem-1400 electron microscope.

2.9 Alkylation of pilus proteins

For *A. oris*, FimA monomers were isolated from *A. oris* AR4 pFimA-K198A, a mutant strain that expresses cell wall-anchored monomeric FimA (Mishra et al., 2011). Bacteria grown overnight on HI agar plates were washed and re-suspended in SMM buffer (500mM sucrose, 10mM MgCl₂, 10mM maleate, pH 6.8), and treated with 300U mL⁻¹ *Mutanolysin* for 3 hours at 37°C. The soluble cell wall fractions were isolated by centrifugation, TCA precipitated, and acetone washed. For *C. diphtheriae*, SpaA monomers were collected from the medium fraction of *C. diphtheriae* HT3, a mutant that secretes monomeric SpaA pilins into the culture medium because it lacks sortases (Swaminathan et al., 2007). SpaA were isolated from the medium fraction of mid-log phase grown bacteria by centrifugation, TCA precipitated, and acetone washed.

FimA and SpaA proteins were alkylated by similar methods. The proteins were reduced in DTT-containing buffer (100mM Tris-HCl, 1% SDS, 100mM DTT, pH 8) at room temperature for 1 h, followed by TCA precipitation and acetone wash to remove the DTT. The resulting pellets were treated with Methoxypolyethylene glycol maleimide (Mal-PEG) in alkylation buffer (100mM Tris-HCl pH 6.8, 1% SDS, 20mM Mal-PEG 2Kdal) at room temperature for 1 h, followed by TCA precipitation and acetone wash. Protein samples were

then dissolved in SDS-loading buffer and separated by 3-20% Tris-glycine gradient gels for immunoblotting with α -FimA or α -SpaA.

2.10 Alkylation of diphtheria toxin

Overnight cultures of *C. diphtheriae* were diluted 1:50 and grown at 30°C until reaching an OD₆₀₀ between 0.2 and 0.3. At this point, 10 $\mu\text{g mL}^{-1}$ of iron chelator ethylenediamine-di-(o-hydroxyphenylacetic) acid (EDDA) was added to the cell cultures to induce DT production for 2 h. DT was isolated from the culture medium by centrifugation, and then TCA precipitated and acetone-washed. Alkylation of DT by Mal-PEG was then performed as described in the previous section. DT protein was detected by immunoblotting with a monoclonal antibody generated against the A fragment of DT (Santa Cruz Biotechnology).

2.11 Coaggregation assays

Overnight cultures of *A. oris* and *S. oralis* were normalized to an OD₆₀₀ of 1.5, harvested by centrifugation, washed 3 times in TBS buffer (200mM Tris-HCl pH 7.4, 150mM NaCl, 0.1mM CaCl₂), and re-suspended in 500 μL of TBS. Equal cell volumes of *A. oris* and *S. oralis* suspensions were mixed in 12-well plates until co-aggregation was visible in the positive in control (WT *A. oris* mixed with *S. oralis*). Coaggregation was recorded using an Alpha Imager.

2.12 Biofilm assays

For biofilm growth, equivalent overnight cultures of *A. oris* were used to inoculate fresh cultures (1:100 dilution) in 24-well plates containing 1% sucrose at 37°C with 5% CO₂ for 48 h. The resulting biofilms were washed gently 3 times with PBS, air-dried overnight, and then stained with 0.5% crystal violet for 30 minutes. Cell growth was quantified by optical

density (OD₅₈₀) of crystal violet extracted by 80% ethanol. The results are presented as an average of three independent experiments performed in triplicate.

2.13 MdbA *in vitro* activity assays

Recombinant FimA was reduced overnight at room temperature in reduction buffer (100mM DTT in 50 mM Tris-HCl, pH 8.0). The next day, free thiols were acid-trapped by the addition of HCl, DTT was removed by centrifugation using 3-KDa Amicon centrifugal filters and exchanged with 50mM Tris-HCl, pH 3.5. 3 μ M of reduced FimA was incubated in redox buffer (100mM Tris-HCl, pH 8.0, 2mM EDTA, 0.2mM GSSG, 1mM GSH) in the presence of 1.8 μ M of wild-type or mutant MdbA at 37°C. Similar reactions without enzymes were used as controls. The reactions were stopped at time intervals 0, 5, 10, 15, and 30 minutes by the addition of Mal-PEG buffer (20mM Mal-PEG, 1% SDS, 100mM Tris-HCl, pH 6.8). After incubation at room temperature for 1 h, glycerol was added to a final concentration of 20% before SDS-PAGE using 3-20% Tris-glycine gels. FimA and MdbA were detected by Coomassie staining.

2.14 Animal model of infection

Mid-log phase cultures of *C. diphtheriae* cells grown in HI broth at 30°C were collected by centrifugation, washed and re-suspended in PBS. Groups of six Hartley guinea pigs (4-5 week-old) were infected via intraperitoneal injections with 2.5 X 10⁷ CFUs of each bacterial strain. Animal survival was monitored for seven days. Over the course of the experiment, animals that were severely moribund were humanly euthanized. The survival curves were analyzed using Mantel-Cox and chi-square tests.

2.6 Isolation of *C. diphtheriae* $\Delta mdbA$ suppressor strains

Overnight cultures of WT and *C. diphtheriae* $\Delta mdbA$ grown at 30°C were diluted 1:50, and shifted to 37°C for 24 hours. Serial dilutions of both strains were plated onto HI agar and incubated overnight at 37°C. To identify suppressors, plates were screened for $\Delta mdbA$ colonies similar in size to WT as $\Delta mdbA$ colonies are not normally visible at this temperature. Three colonies were isolated, and PCR analysis confirmed that these strains were *C. diphtheriae* $\Delta mdbA$. Genomic DNA of the suppressor mutants was isolated and sent for whole genome sequencing (Genome Research and Systems Biology Center for Biotechnology, Bielefeld University).

2.14 q-RT PCR

To prepare RNA, *C. diphtheriae* grown to log-phase was normalized to an OD₆₀₀ of 1.0, collected by centrifugation, re-suspended in 1mL Trizole®, and lysed with glass beads. To extract nucleic acid from the cell lysates, chloroform was added to the samples, and RNA was precipitated using cold isopropanol and 100mM sodium acetate. The RNA preparations were then incubated at -80°C for 20 minutes, and centrifuged at 12,000xg for 30 minutes. The resulting pellet was washed in cold 70% ethanol, dried, re-suspended in water, and treated with Dnase at 37°C for 1.5 hours. RNA was then extracted using phenol:chloroform:isoamyl alcohol (PCA), and then precipitated using cold ethanol, 0.5% glycogen, and 75mM sodium acetate. The RNA samples were incubated at -80°C for 20 minutes, spun at 12,000xg for 30 minutes, washed in 70% cold ethanol, dried, and then re-suspended in water. The SuperScript® III First-Strand Synthesis System (Invitrogen) was used to generate cDNA. First, a 10 µL solution of prepared RNA (1µg), Random Hexamer (50ng), and dNTP (1mM) was heated to 65°C for 5 minutes to denature the RNA, and then placed on ice for 1 minute. The samples were then diluted to a 20µL reaction volume by adding 5X RT buffer, DTT

(2.5mM), and SuperScript ® III (200 U). To anneal the random hexamer primers, the samples were incubated at 25°C for 25 minutes, followed by cDNA synthesis at 50°C for 1 hour. The reaction was then terminated by incubating the samples at 70°C for 15 minutes, which were then stored at -20°C.

Quantitative RT-PCR was performed using iTaq Universal Sybro®Green Supermix and primers designed to amplify a 163-base region inside the *tsdA* ORF. Transcriptional analysis was performed in 10uL reaction volumes containing 4uL cDNA (diluted 1:200), 0.5 uL of both primers (100pM), and 5uL of 2X Sybro®Green Supermix. The RT-PCR program consisted of 95°C for 30 seconds, and 40 cycles of 95°C for 10 seconds, and 53°C for 30 seconds. The amplification of 16S rRNA was used as a loading control. The relative mRNA levels were calculated using the $2e^{-\Delta\Delta CT}$ method. An unpaired T-test was used to measure significance. The results are presented as an average of three independent experiments performed in triplicate.

2.16 Van-FL Fluorescence microscopy

Overnight cultures of *C. diphtheriae* were diluted 1:50 and then grown at 30°C or 37°C until reaching log phase. A 1:1 mixture of vancomycin (Van) and Van-FL was added to cultures at a concentration of 1µg/mL, and incubated for 10 minutes with agitation at 37°C. The samples were then placed on agar pads and viewed by a fluorescence microscope using excitation/emission wavelengths 504nm/510nm.

2.17 Spot dilution assays

Spot dilutions of *C. diphtheriae* were performed using overnight cultures grown at 30°C. To test for temperature sensitivity, *C. diphtheriae* cultures were spotted (10^{-3} - 10^{-6}) onto HI agar plates, and then incubated at 37°C for 24 hours or 30°C for 48 hours. To test for

antibiotic sensitivity, *C. diphtheriae* were spotted (10^{-3} - 10^{-6}) into HI agar plates containing various concentrations of Ampicillin or Penicillin, and incubated at 30°C for 48 hours. The concentrations of antibiotics chosen were based on previous calculations of antimicrobial MICs for *C. diphtheriae* (Soriano, Zapardiel & Nieto, 1995).

Table 2-1: Bacterial strains and plasmids used in these studies

Strain or plasmid	Description	Reference
A. oris Strains		
MG-1	Parental Strain (WT)	(Mishra et al., 2007)
CW1	$\Delta galK$; an isogenic derivative of MG1	(Mishra et al., 2007)
AR4	$\Delta fimA$; an isogenic derivative of CW1	(Mishra et al., 2010)
AR4 pFimA	AR4; <i>fimA</i> complement	(Mishra et al., 2010)
AR4 pK198A	AR4 containing pFimA _{K198A}	(Mishra et al., 2010)
MR100	AR4 containing pFimA _{C116A}	This Study
MR101	AR4 containing pFimA _{C157A}	This Study
MR102	AR4 containing pFimA _{C394A}	This Study
MR103	AR4 containing pFimA _{C445A}	This Study
CWa	$\Delta fimPQA$; an isogenic derivative of CW1	This Study
MR104	CWa containing pFimA	This Study
MR105	CWa containing pFimA _{C394A}	This Study
MR106	CWa containing pFimA _{C445A}	This Study
MR107	MG1 <i>vkor::Tn5</i>	This Study
MR108	$\Delta vkor$; an isogenic derivative of CW1	This Study
MR109	MR108 containing pVKOR	This Study
MR110	MR108 containing pVKOR _{C175A}	This Study
MR111	MR108 containing pMdbA _{Ao}	This Study
MR112	MR108 containing pMdbA _{Ao C136A}	This Study
MR113	$\Delta mdbA_{Ao}$ pAraC-MdbA _{Ao}	This Study
MR114	MR108 containing pMtnDsbA	This Study
MR115	MR108 containing pEcDsbA _{Ao}	This Study

MR116	MR108 containing pJRD-MdbA _{Cd}	This Study
<i>C. diphtheriae</i> Strains		
NCTC13129	Parental Strain (WT)	(Ton-That & Schneewind, 2003)
HT11	$\Delta spaA$	(Ton-That & Schneewind, 2003)
HT11 pSpaA	HT11 containing pSpaA	(Ton-That & Schneewind, 2003)
HT11 pC383A	HT11 containing pSpaA _{C383A}	This Study
HT11 pC483A	HT11 containing pSpaA _{C443A}	This Study
MR117	HT11 containing pSpaA _{C383A, C443A}	This Study
NJ2	$\Delta mdbA_{Cd}$	This Study
NJ6	NJ2 containing pMdbA _{Cd}	This Study
NJ7	NJ2 containing pSpaA	This Study
NJ8	NCTC13129 containing pSpaA	This Study
MR118	NJ2 containing pEcDsbA _{Cd}	This Study
HT3	$\Delta srtA\Delta srtF$	(Swaminathan et al., 2007)
HT28	Δtox	This Study
XM5	$\Delta spaA-I$	This Study
MR119	NJ2 suppressor 1	This Study
MR120	NJ2 suppressor 2	This Study
MR121	NJ2 suppressor 3	This Study
MR122	$\Delta tsdA$	This Study
MR123	HT11 containing pAraC-TsdA	This Study

***S. oralis* Strains**

So34	Parental Strain	(Yoshida et al., 2006)
OC1	$\Delta wchA$; an isogenic derivative of 34	(Yoshida et al., 2006)

***E. coli* Strains**

MR126	BL21 containing pMCSG7-MdbA _{Ao}	This Study
MR127	BL21 containing pMCSG7-MdbA _{Ao C216A}	This Study
MR128	BL21 containing pMCSG7-MdbA _{Cd}	This Study
MR129	BL21 containing pMCSG7-MdbA _{Cd C91A}	This Study
ARU60	BL21 containing pMCSG7-FimA	(Mishra et al., 2010)

Plasmids

pJRD215	<i>Actinomyces/E. coli</i> shuttle vector, Kan [®]	(Yeung & Kozelsky, 1994)
pCGL0243	<i>Corynebacterium/E. coli</i> shuttle vector, Kan [®]	(Ankri et al., 1996)
pK19MobsacB	<i>Corynebacterium</i> integration plasmid	(Schafer et al., 1994)
pMCSG7	Ligation-independent cloning for protein expression	(Stols et al., 2002)
pCWU2	Integrative plasmid expressing <i>galk</i>	(Mishra et al., 2010)
pJRD508FimB	pJRD215 containing the <i>fimB</i> promoter	(Mishra et al., 2010)
pCR2.1_FimA	pCR2.1 containing <i>fimA</i> ORF	(Mishra et al., 2010)
pFimA	pJRD215 containing <i>fimA</i> under control of the <i>fimB</i> promoter	(Mishra et al., 2010)
pFimA _{C116A}	pFimA harboring a C116A mutation	This Study
pFimA _{C157A}	pFimA harboring a C157A mutation	This Study
pFimA _{C394A}	pFimA harboring a C394A mutation	This Study
pFimA _{C445A}	pFimA harboring a C445A mutation	This Study
pVKOR	pJRD215 expressing WT <i>vkor</i>	This Study

pVKOR _{C175A}	pVKOR harboring a C175A mutation	This Study
pMdbA _{Ao}	pJRD215 expressing wild-type <i>mdbA_{Ao}</i>	This Study
pMdbA _{Ao} C136A	pJRD215 expressing <i>mdbA_{Ao}</i> C136A	This Study
pAraC-MdbA _{Ao}	pJRD215 expressing <i>mdbA_{Ao}</i> under control of an arabinose-inducible promoter	This Study
pMtnDsbA	pJRD215 expressing a putative <i>M. tuberculosis dsbA</i> under control of the <i>mdbA_{Ao}</i> promoter	This Study
pEcDsbA	pJRD215 expressing <i>E. coli dsbA</i> under control of the <i>mdbA_{Ao}</i> promoter	This Study
pJRD-MdbA _{Cdi}	pJRD215 expressing <i>C. diphtheriae mdbA</i> under control of the <i>mdbA_{Ao}</i> promoter	This Study
pSpaA	pCGL0243 expressing WT <i>spaA</i>	(Ton-That & Schneewind, 2003)
pSpaA C383A	pSpaA harboring a C383A mutation	(Ton-That & Schneewind, 2003)
pSpaA C443A	pSpaA harboring a C443A mutation	(Ton-That & Schneewind, 2003)
pSpaA C383A C443A	pSpaA harboring C383A, C443A mutations	This Study
pMdbA _{Cd}	pCGL0243 expressing WT <i>mdbA_{Cd}</i>	This Study
pAraC_TsdA	pCGL0243 expressing WT <i>tsdA</i> under control of AraC	This Study
pK19mobsacB-MdbA _{Cd}	pK19mobsacB allelic replacement of <i>mdbA_{Cd}</i>	This Study
pK19mobsacB-TsdA	pK19mobsacB allelic replacement of <i>tsdA</i>	This Study
pCWU2-VKOR	pCWU2 allelic replacement of <i>vkor</i>	This Study
pMCSG7-FimA	For recombinant FimA expression	(Mishra et al., 2010)
pMCSG7-MdbA _{Ao}	For expression of recombinant MdbA _{Ao}	This Study

pMCSG7-MdbA _{Ao} C216A	For expression of recombinant MdbA _{Ao} harboring a AxxC mutation	This Study
pMCSG7-MdbA _{Cd}	For expression of recombinant MdbA _{Cd}	This Study
pMCSG7-MdbA _{Cd} C91A	For expression of recombinant cMdbA harboring a AxxC mutation	This Study

Table 2-2 Primers used in this study (Restriction sites are underlined).

Primer	Sequence ^(a)	Used for:
<i>fimA_F_C116A</i>	GGTGTGCCGGACAGCGCCGCTGCCAACCCCG CGCCCCC	pFimA _{C116A}
<i>fimA_R_C116A</i>	GGGGGCGGCGGGGTTGGCAGCGGCGCTGTC CGGCACACC	pFimA _{C116A}
<i>fimA_F_C157A</i>	G TTCAGGCCTACCTCGTGGCTGAGACCACGAC CCCTGGT	pFimA _{C157A}
<i>fimA_R_C157A</i>	ACCAGGGGTCTGTTGGTCTCAGCCACGAGGTAG GCCTGAAC	pFimA _{C157A}
<i>fimA_F_C394A</i>	AACGCCTACGCCAACACCGCTTCCAACGAGAA GGAGGGC	pFimA _{C394A}
<i>fimA_R_C394A</i>	GCCCTCCTTCTCGTTGGAAGCGGTGTTGGCGT AGGCGTT	pFimA _{C394A}
<i>fimA_F_C445A</i>	GTGAATGCCATGGAGCGCGCTTACGTCCTGGT TGAGACC	pFimA _{C445A}
<i>fimA_R_C445A</i>	GGTCTCAACCAGGACGTAAGCGCGCTCCATGG CATTAC	pFimA _{C445A}
<i>spaA_F_C383A</i>	GCCGAGGCCGACGGCAGCCTAGTCAAGTCCG AC	pSpaA _{C383A}
<i>spaA_R_C383A</i>	ACGATGCAGCTGGAACGTCGCGGTGCGATCG GC	pSpaA _{C383A}
<i>spaA_F_C443A</i>	GGCAAGGGAACCGAATTCGCCCTGGTAGAAAC A	pSpaA _{C443A}
<i>spaA_R_C443A</i>	GGCCCACGCGTCGGTATATTTTCATAACATTGG A	pSpaA _{C443A}
<i>vkor_A_HindIII_F</i>	AAAAGCTTACTGCAACCTCGATGTCATCGCC	pCWU2-VKOR
<i>vkor_B_R</i>	GAAGAGCCTGGCCAGATCTGTGGGCATGCGC GACAT	pCWU2-VKOR
<i>vkor_C_F</i>	CGCATGCCCACAGATCTGGCCAGGCTCTTCGG GTGA	pCWU2-VKOR

<i>vkor</i> _D_XbaI_R	AATCTAGACGTCGGTGTGCGGCTCAATGG	pCWU2-VKOR
<i>vkor</i> _F_NdeI	AACATATGACGCCTCGGTAACGGTGG	pVKOR
<i>vkor</i> _R_XbaI	AATCTAGATTCTTGGGCGCAGTCACC	pVKOR
<i>vkor</i> _F_C175A	GCTCCCTTCTGCATGGTCATCTGGTCCGTC	pVKOR _{C175A}
<i>vkor</i> _R_C175A	GAGCTTGCCGAAGGTCATGATGGACACGGA	pVKOR _{C175A}
<i>mdbA</i> _{Ao} _F_XbaI	AATCTAGACCACCCATCGGCCCATCCATCATG	pMdbA _{Ao}
<i>mdbA</i> _{Ao} _R_EcoRI	AAGAATTCTCAGCCTTGCTGAGTCGGCTGAGG	pMdbA _{Ao}
<i>mdbA</i> _{Ao} _F_ATG	ATGGTCGTCCGCTCGTCA	pAraC-MdbA _{Ao}
<i>mdbA</i> _{Ao} _C216A_F	GCCTCTCACTGCGCCCAGTTCGAG	pMdbA _{Ao} C216A, pMCSG7-MdbA _{Ao} C216A
<i>mdbA</i> _{Ao} _C216A_R	GGAGTAGTCGAAGTAGATGTCGAGAACGGG	pMdbA _{Ao} C216A, pMCSG7- MdbA _{Ao} C216A
<i>araC</i> _F_KpnI	AAGGTACCCTACTGTTTCTCCATACCCGTTT	pAraC-MdbA _{Ao}
<i>araC</i> _R	TACCAATTATGACAACTTGAC	pAraC-MdbA _{Ao} , pAraC-TsdA
<i>dsbA</i> _{Mtn} _F	GTGGCCGACAAATCCAAACGC	pMtnDsbA
<i>dsbA</i> _{Mtn} _R_HindIII	AA AAGCTTTCAGGATGTCGCGGTAGCAGCG	pMtnDsbA
<i>PmdbA</i> _{Ao} _F_KpnI	AAGGTACCCACCCATCGGCCCATCCATCATG	pMtnDsbA, pEcDsbA _{Ao} , pJRD-MdbA _{Cd}
<i>PmdbA</i> _{Ao} _R	GCGGTGCTCCTTGTGATGCGGTGAC	pMtnDsbA
<i>dsbA</i> _{Ec} _F	GCGCAGTATGAAGATGGTAAACAG	pEcDsbA _{Ao} , pEcDsbA _{Cd}
<i>dsbA</i> _{Ec} _R_EcoRI	AAGAATCCGCCCCGTGAA TATTCA CGGGCTT	pEcDsbA _{Ao}
<i>PmdbA</i> _{Ao} TM_R	GCCGAGGTAGACCAGGTAGCC	pEcDsbA _{Ao} , pJRDMdbA _{Cd}
<i>mdbA</i> _{Cd} _A_F_HindIII	AAGCTTAATGGCACCGTATGGTCGACT	pK19mobsacB-MdbA _{Cd}
<i>mdbA</i> _{Cd} _B_R	CCCATCCACTAACTTAAACACCTAGAACCAGC GTTTTTACT	pK19mobsacB-MdbA _{Cd}

<i>mdbA_{Cd}_C_F</i>	TGTTTAAGTTT TAGTGGATGGGTGGGTTGAGCA AGCAACC	pK19mobsacB- MdbA _{Cd}
<i>mdbA_{Cd}_D_R_XbaI</i>	<u>TCTAGA</u> AGAACTCACTGACACAAGCCC	pK19mobsacB- MdbA _{Cd}
<i>mdbA_{Cd}_BamHI_F</i>	AAAGGATCCCGCCTTCGCACGGTTCTTCAT	pMdbA _{Cd} , pEcDsbA _{Cd}
<i>mdbA_{Cd}_BamHI_R</i>	AAAGGATCCTTAGTGATGGTG	pMdbA _{Cd}
<i>mdbA_{Cd}_C91A_F</i>	TCGGCACCACATTGCGCCGAGCTTGGC	pMCSG7- MdbA _{Cd} C91A
<i>mdbA_{Cd}_C91A_R</i>	GAAGTCCTCGTAGAAGTCGATCTTCTT	pMCSG7-MdbA _{Cd} C91A
<i>PmdbA_{Cd}_TM_R</i>	CACAACAACATAGGTCACAAC	pEcDsbA _{Cdi}
<i>mdbA_{Cd}_F</i>	CAGGGCAAAGCACACCAAGCTAA	pJRD-MdbA _{Cd}
<i>mdbA_{Cd}_R_HindIII</i>	AAAAAGCTTTTAGTGATGGTG	pJRD-MdbA _{Cd}
<i>tsdA_F_BamHI</i>	AAGGATCCAGAGTGTCTCGTTGGGTTCG	pAca-TsdA
RTPCR_ <i>tsdA_F</i>	TAGCGGTAAGGCGGGTTCG	RT PCR
RTPCR_ <i>tsdA_R</i>	GATCTTTCGCGTTACGACGGTG	RT PCR
<i>tsdA_A_XbaI</i>	AAATCTAGAGTTTGAGGAAAGCGGTTT	pK19mobsacB-TsdA
<i>tsdA_B_R</i>	CCCATCCACTAACTTAAACACTTCTGCATGAA GTACAT	pK19mobsacB-TsdA
<i>tsdA_C_F</i>	TGTTTAAGTTT TAGTGGATGGGTGATTGAGTCG GAGCTGA	pK19mobsacB-TsdA
<i>tsdA_D_XbaI</i>	AAATCTAGAGAACTCGCCGCCAGCGAA	pK19mobsacB-TsdA
<i>ParaC_F_PstI</i>	AAACTGCAGTTATGACAACTTGACGGCTACATC ATTCAC	pAraC-TsdA
<i>Lic_mdbA_{Ao}_DAK_F</i>	TACTTCCAATCCAATGCAGACGCCAAGAAGAA CCCCA	pMCSG7- MdbA _{Ao}
<i>Lic_mdbA_{Ao}_VQG_R</i>	TTATCCACTTCCAATGTCAGCCTTGCTGAGTCG GC	pMCSG7-MdbA _{Ao}
<i>Lic_mdbA_{Cd}_ANK_F</i>	TACTTCCAATCCAATGCAGTGCAGGGCAAAGC ACAC	pMCSG7- MdbA _{Cd}

Lic_ *mdbA*_{Cd}_ATS_R TTATCCACTTCCAATGTTAAGAGGTTGCTTGCT pMCSG7- *MdbA*_{Cd}
CAACCC

Chapter III:

Gram-positive pilus assembly requires disulfide bond formation

3.1 Introduction

Bacterial pathogens secrete an arsenal of virulence factors to colonize and manipulate hosts. Among these are adhesive pili or fimbriae, which mediate attachment, promote biofilm formation, and modulate host immunity (Hendrickx et al., 2011; Proft & Baker, 2009). Pili expressed by Gram-positive bacteria are comprised of covalently-linked subunits that are anchored to the surface peptidoglycan. The assembly pathway for these virulence factors was first elucidated using *C. diphtheriae* SpaA-type pili, and later found to be conserved among Gram-positive bacteria (Danne & Dramsi, 2012; Ton-That et al., 2004; Ton-That & Schneewind, 2003)

Pilus subunits harbor a number of conserved features including an N-terminal signal peptide and pilin motif, and C-terminal CWSS, which is comprised of an LPxTG motif, hydrophobic patch, and positively-charged tail. The N-terminal signal peptide targets the subunits for secretion via the Sec machinery. When they reach the exoplasm, the proteins are tethered to the membrane by the C-terminal hydrophobic patch and positively-charge cytoplasmic tail. Here, a series of transpeptidase sortase enzymes assemble the subunits into pili (Fig. 4). A pilus-specific sortase cleaves a subunit at its LPxTG motif, which results in the formation of an acyl-enzyme intermediate between the substrate Thr and sortase Cys (Mazmanian et al., 1999). The complex is resolved by a nucleophilic lysine in the pilin motif of a neighboring sortase-subunit complex. This results in two subunits becoming cross-linked by a Lys-Thr isopeptide bond (Ton-That et al., 2004) (Fig. 4). This transpeptidase reaction occurs repeatedly to form a pilus polymer by a bottom-up mechanism. Upon completion, the pilus is transferred to a housekeeping sortase, which anchors it to lipid II precursor (Perry et al., 2002; Swaminathan et al., 2007).

SecYEG substrates are translocated into the exoplasm in unfolded states. How are Gram-positive pilins properly folded outside the cell if these organisms lack periplasmic

spaces? The crystal structures of the major pilus subunits FimA and SpaA expressed by Actinobacterial oral pathogens *Actinomyces oris* and *Corynebacterium diphtheriae*, respectively, revealed a clue. Both pilins were predicted to contain disulfide bonds suggesting that they undergo oxidative folding (Kang et al., 2009; Mishra et al., 2011). *A. oris* FimA, which forms the shaft of type 2 fimbriae, was predicted to harbor Cys linkages in both the N- and C-termini. The crystal structure for *C. diphtheriae* SpaA, the major subunit of SpaA-type pili, was shown to contain a disulfide bond in its C-terminus (Kang et al., 2009).

Disulfide bond formation is important for the proper folding of many secreted proteins. These linkages stabilize protein structure and protect against degradation. For example, bovine pancreatic trypsin inhibitor (BPTI) requires disulfide bond formation to maintain its tertiary structure. This protein can be denatured by simply exposing it to reducing agents (Braakman & Bulleid, 2011). Inhibiting disulfide bond formation often leads to increased susceptibility to proteolysis (Heras et al., 2009). *E. coli* FlgI, a component of the bacterial flagellar motor, requires a single disulfide bond in its C-terminus. Reduced FlgI is heavily degraded, thereby preventing the flagellar synthesis (Hizukuri et al., 2006). Disulfide bonds have also been shown to guide protein folding pathways. For example, patterns of non-native disulfide bond formation have been detected in folding intermediates of Rnase A and LDL receptor (Jansens, van Duijn & Braakman, 2002; Ruoppolo et al., 1996). In other cases, disulfide bond formation is a prerequisite for folding. Uropathogenic *E. coli* FimC, a specific chaperone for type 1 fimbriae, will not catalyze substrate folding unless its targets have formed native disulfide bonds (Crespo et al., 2012).

Here, we show that FimA and SpaA Cys residues are oxidized to form thiol linkages *in vivo*. The formation of these disulfide bonds is important for protein folding and stability. Cys-to-Ala mutations targeting these regions abolishes assembly of type 2 fimbriae and SpaA-type pili on cell surfaces. Instead, an abundance of low molecular weight (LMW) FimA and SpaA

products are secreted into the culture medium. In *A. oris*, we found that the failure to form type 2 fimbriae is associated with the absence of biofilm growth and co-aggregation with its oral cavity co-colonizer *S. oralis*. This work expands our understanding of how pilus assembly occurs in Gram-positive bacteria, and reveals a potential folding pathway for virulence factors secreted into the exoplasm. We hypothesize that *A. oris* and *C. diphtheriae* possess machinery to catalyze disulfide bond formation in proteins like pili, and have identified putative oxidoreductase enzymes in both organisms.

3.2 Results

3.2.1 *A. oris* FimA and *C. diphtheriae* SpaA form disulfide bonds *in vivo*.

Structural studies predicted that Cys residues within pilus subunits like FimA and SpaA are oxidized to form disulfide bonds (Kang et al., 2009; Mishra et al., 2011; Persson et al., 2012). FimA is predicted to form the disulfide bonds C116-C157 in the N-terminus and C394-C445 in the C-terminus, while SpaA is predicted to harbor a single C383-C443 linkage in its C-terminus (Fig. 5). To test if these bonds form *in vivo*, we turned to alkylation using Mal-PEG, a 2 kDa agent that reacts with free sulfhydryl groups to form stable thioether bonds (Makmura et al., 2001). FimA was collected from *A. oris* by muramidase treatment, TCA precipitated, and acetone washed. The resulting pellets were re-suspended in buffer with or without DTT, and then incubated with Mal-PEG. Mal-PEG was then removed by TCA precipitation, and FimA was detected by western blotting. The migration of FimA incubated with Mal-PEG was unchanged when compared to the untreated samples (Fig. 5A; compare lanes 1 and 3). However, pretreatment with reducing DTT produced a visible up-shift signifying the modification of free sulfhydryl groups (lane 4). Similar to FimA, Mal-PEG modification of SpaA occurred following DTT treatment (Fig. 5B; compare lanes 3 and 4). Since DTT treatment was required prior to MAL-PEG modification in both proteins, we conclude that disulfide bonds are present within FimA and SpaA *in vivo*.

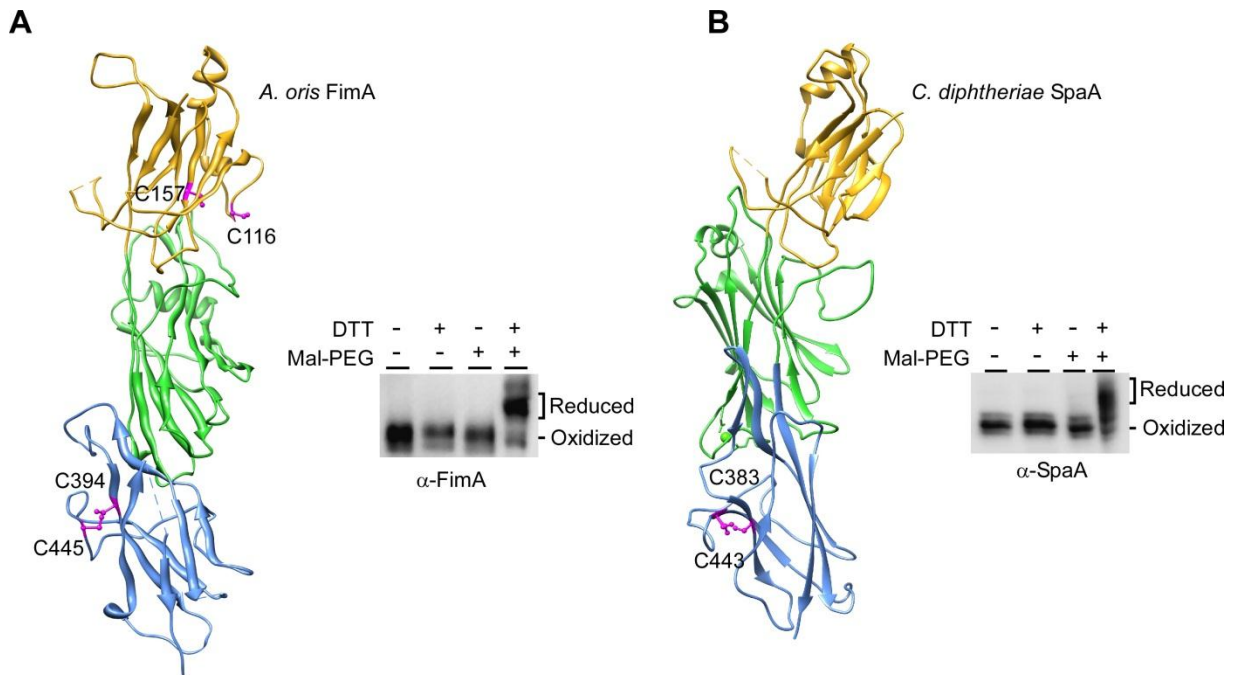


Figure 5. *A. oris* FimA and *C. diphtheriae* SpaA contain disulfide bonds. (A) The FimA crystal structure (Mishra et al., 2011) revealed a possible disulfide bond between residues C394 and C445. The modeled IgG-fold domain (yellow) at the N-terminus, absent from the original structure, contained two Cys residues (C116 and C157). FimA monomers isolated from the cell wall were treated or mock-treated with DTT, followed by Mal-PEG. The protein samples were analyzed by immunoblotting with antibodies against FimA (α -FimA). The reduced and oxidized forms of FimA are indicated. **(B)** The SpaA crystal structure predicts the formation of a single disulfide bond formed between residues C383 and C443 within the C-terminal IgG-like domain (Kang et al., 2009). The presence of the C-terminal disulfide bond in SpaA monomers was demonstrated by Mal-PEG alkylation as described (A). The reduced and oxidized forms of SpaA are indicated.

3.2.2 Disulfide bond formation is required for *A. oris* pilus assembly, biofilm formation, and interbacterial interactions.

We wondered if disulfide bonds were important for pilus assembly. Using pFimA as a template, Cys residues were mutated to alanine and the resulting plasmids were used to transform *A. oris* $\Delta fimA$. Medium (M) and cell wall (W) fractions were isolated, boiled in SDS, separated using a 3-12% Tris-glycine gel, and blotted with α -FimA. Polymerized FimA (P) was detected in both M and W fractions collected from the parental MG1 (WT) along with the FimA monomer (60 kDa) (Fig. 6; lanes 1-2). No protein was detected in $\Delta fimA$ (lanes 3-4). Fimbrial assembly was restored upon complementation with pFimA, pC116A, or pC157A (lanes 5-10). Remarkably, cells expressing pC394 and pC445A produced no fimbriae. Instead, traces of FimA dimers, along with an abundance of low-molecular-weight (LMW) products were detected in the M fractions (lanes 11 and 13). To examine pilus assembly on the cell surface we turned to immunogold electron microscopy (IEM). To identify type 2 fimbriae, bacteria were blotted with α -FimA or α -FimB, followed by secondary antibodies conjugated to gold particles. FimA polymers were visible on the surface of bacteria expressing pFimA (Fig. 6C). FimB, which cross-links to the tip of FimA polymers, was detected around the cell surface (Fig. 6G). No fimbriae were observed on the surface of $\Delta fimA$, pC394A or pC445A mutants, but FimB was present on the cell surface (Fig. 6D-I).

We also tested whether the Cys-to-Ala mutations affected *A. oris* biofilm formation and co-aggregation with *Streptococcus oralis*, two processes known to require type 2 fimbriae (Mishra et al., 2010). To grow biofilm, *A. oris* were grown in rich media supplemented with 1% sucrose at 37°C with 5% CO₂. After 48 hours, the resulting biofilms were washed with PBS, dried, and stained with crystal violet. *A. oris* MG1 formed a robust biofilm, while $\Delta fimA$ produced nothing (Fig. 6J). Complementation with pFimA, pC116A, or pC157A restored growth, but pC394A and pC445A did not. Finally, co-aggregation between *A. oris* and *S.*

oralis was tested (Fig. 6K). Stationary phase cultures of *A. oris* and *S. oralis* were collected, washed in TBS, and mixed together. Co-aggregation between MG1 and *S. oralis* So34 was visible, but not detected when MG1 was combined with *S. oralis* OC1, a mutant that lacks the receptor for type 2 fimbriae (Mishra et al., 2010). The $\Delta fimA$ mutant did not co-aggregate with So34, but complementation with pFimA, pC116A, or p157A restored the interaction. Identical to $\Delta fimA$, pC394A and pC445A also failed to co-aggregate. Altogether, these data show that the C-terminal C394-C445 disulfide bond is essential for the assembly and function of type 2 fimbriae, while the N-terminal C116-C157 linkage is dispensable.

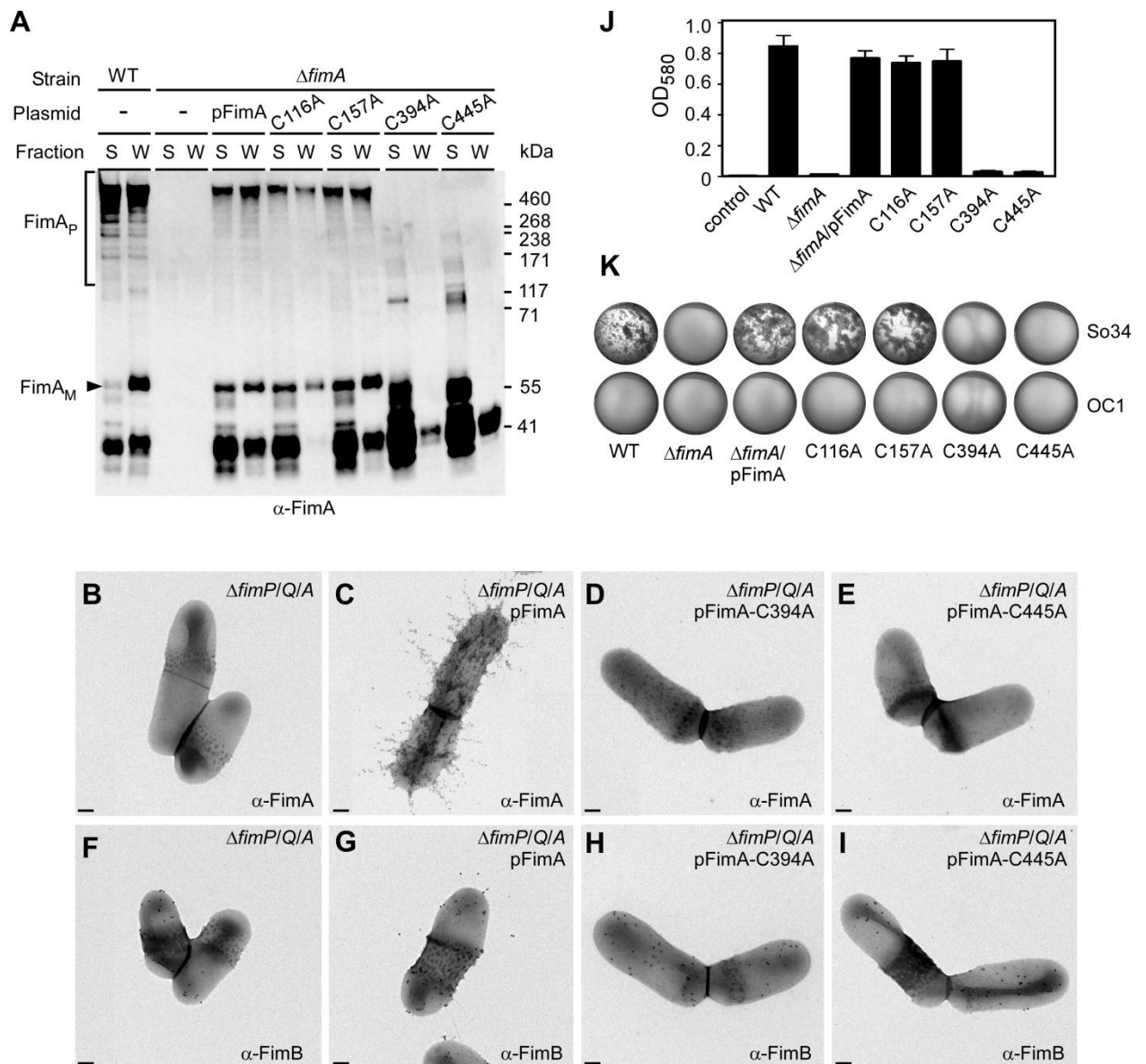


Figure 6. Disulfide bond formation is required for FimA polymerization, biofilm formation, and interbacterial interactions in *A. oris*. (A) Culture medium (M) and cell wall (W) fractions were collected from the *A. oris* parental strain (MG1) and its isogenic derivatives. Equivalent protein samples harvested by TCA precipitation were analyzed by immunoblotting with α -FimA. Monomeric and polymeric forms of FimA, as well as molecular mass markers (kDa) are indicated. (B-I) Overnight cultures of *A. oris* were immobilized on nickel grids, and stained with α -FimA (D-G) or α -FimB (H-K) followed by secondary

antibodies conjugated to 12 or 18nm gold particles, respectively. The samples were stained with 1% uranyl acetate and viewed by a transmission electron microscope. Scale bars indicate 0.5µm. Note that *A. oris* lack type 1 fimbriae to eliminate background. **(J)** *A. oris* biofilms were cultivated in 12-well plates at 37°C with 5% CO₂ for 48 hours. The resulting biofilms were stained with crystal violet, and quantified by measuring absorbance at 580nm. **(K)** To assay an interspecies interaction, *A. oris* and RPS-positive *S. oralis* (So34) cells were mixed in equal numbers and imaged. *S. oralis* OC1 strain lacking RPS was used as a negative control.

3.2.3 *C. diphtheriae* SpaA-type pilus assembly requires disulfide bond formation.

To test if the SpaA C383-C443 linkage is also important for pilus assembly, Cys-to-Ala mutations were generated within pSpaA, and the resulting plasmids were used to transform *C. diphtheriae* $\Delta spaA$. M and W fractions were isolated, boiled in SDS, separated by SDS-PAGE, and blotted with α -SpaA. SpaA polymers were readily detected in the W fraction of wild-type cells with some secreted into the M fraction (Fig 7A; lanes 1-2). Protein detection was abolished in $\Delta spaA$ (lanes 3-4), but restored upon plasmid complementation (lane 6). Similar to *A. oris*, SpaA polymers were not detected in pC383A or pC443A, but LMW SpaA products were secreted into the culture media, along with a potential SpaA dimer in the M fraction of pC383A (lanes 7 and 9). SpaA assembly was also examined using IEM. *C. diphtheriae* was immobilized on nickel grids, and blotted with α -SpaA, followed by secondary antibody conjugated to gold particles. The complementation of $\Delta spaA$ with pSpaA produced long pili that covered the bacterial surface. Similar to western blotting, SpaA polymers were not found on the surfaces of cells expressing pC383A or pC443A (Fig. 7C-D). Pilus assembly was also defective in a double Cys mutant, but one or two short pili were visible on the cell surface (Fig. 7E). Together, these results show that the C383-C443 linkage is essential for pilus assembly.

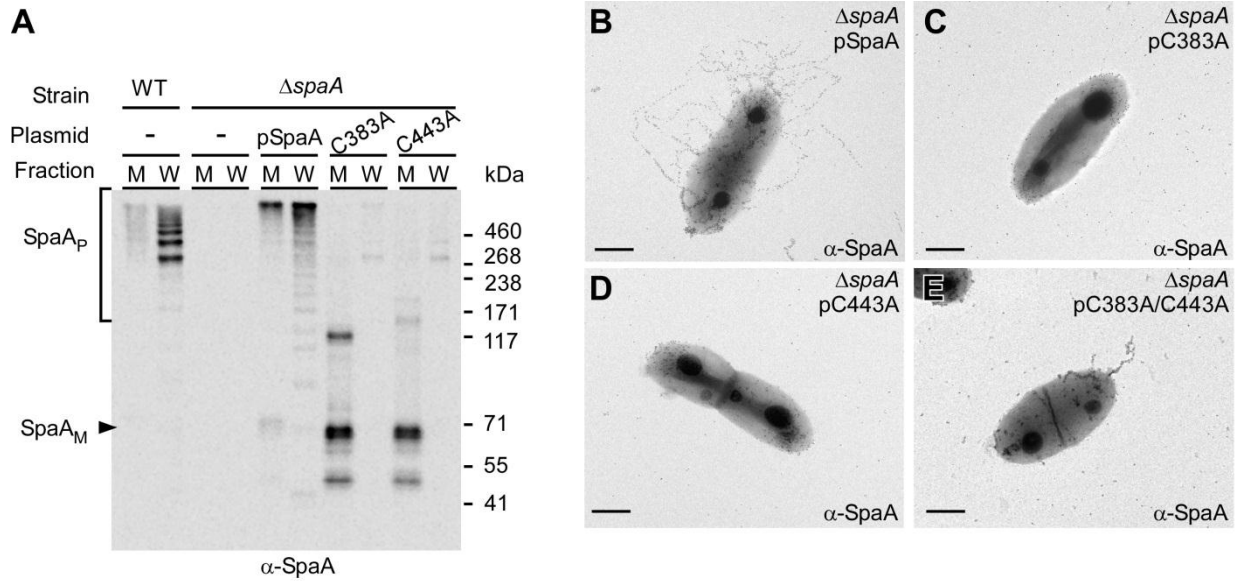


Figure 7. *C. diphtheriae* SpaA-type pilus assembly requires disulfide bonds. (A) M and W fractions were collected from *C. diphtheriae*, separated on a 3-12% Tis-Glycine gel, and immunoblotted with α -SpaA. **(D)** *C. diphtheriae* were prepared as described in Fig. 6, but cells were stained with α -SpaA followed by secondary antibody conjugated to 12nm gold particles. Scale bars indicate 0.5 μ m.

3.2.4 *A. oris* and *C. diphtheriae* encode putative oxidoreductase enzymes.

In Gram-negative bacteria, disulfide bond formation is catalyzed in the extracellular periplasm by Dsb enzymes (Kadokura & Beckwith, 2010). Due to the lack of an outer membrane, it is generally accepted that Gram-positives do not possess similar compartments. Interestingly, this correlates with a trend in which many Gram-positives secrete little, if any, proteins with multiple Cys residues (Daniels et al., 2010; Dutton et al., 2008). It was suggested that the avoidance of disulfide bond formation is an adaptation to lacking a periplasmic space. Recently, it was revealed that Gram-positive Actinobacteria may be exceptions to this rule (Daniels et al., 2010; Dutton et al., 2008).

We wondered if oxidoreductases were present in the exoplasm to catalyze intramolecular disulfide bonds in the *A. oris* and *C. diphtheriae* pilus subunits. All known disulfide bond forming enzymes are members of the thioredoxin superfamily (Kadokura & Beckwith, 2010). To identify potential Dsb-like proteins, *E. coli* thioredoxin was BLASTed against the *A. oris* and *C. diphtheriae* proteomes. Four candidates in *A. oris* and five in *C. diphtheriae* were identified (Chen et al., 2005; Kanehisa & Goto, 2000) (Fig. 8). All nine proteins were predicted to be membrane-bound and harbor extracellular CxxC motifs. Interestingly, although *A. oris* Ana_1994 and *C. diphtheriae* Dip_1880 share only 15% and 23% sequence similarity to *E. coli* DsbA, respectively, all three proteins harbor a His residue in their CxxC motifs. This positively-charged residue contributes to the high redox potential of many known disulfide bond forming proteins (Grauschopf et al., 1995; Lundstrom, Krause & Holmgren, 1992). It is possible that some or all of these candidates regulate FimA and SpaA oxidation in the exoplasm.

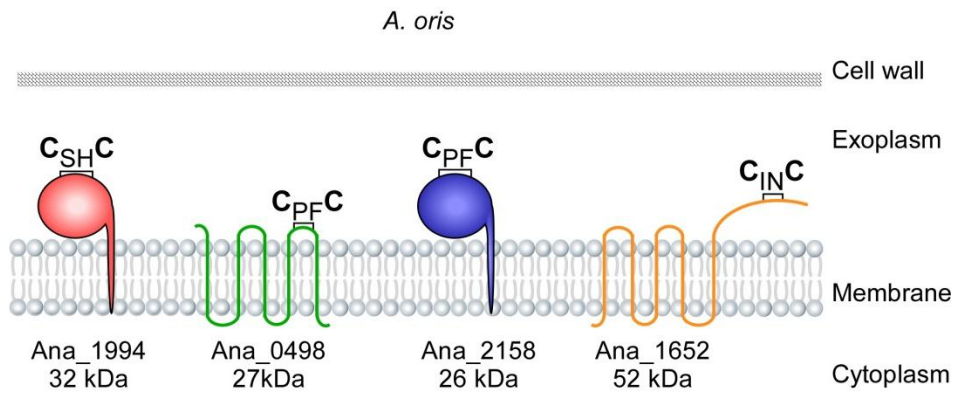
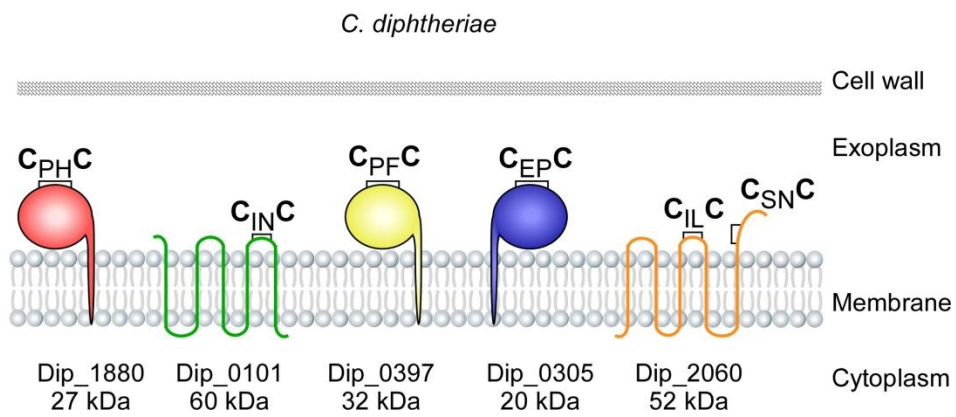
A**B**

Figure 8. Putative thiol-oxidoreductases in *A. oris* and *C. diphtheriae*. BLAST analysis revealed potential disulfide bond forming proteins in *A. oris* **(A)** and *C. diphtheriae* **(B)**. All proteins are membrane-bound and possess extracellular CxxC motifs typical of thioredoxin-like enzymes.

3.3 Discussion

Adhesive pili are valuable virulence determinants for bacteria because they promote host colonization, inflammation, and biofilm formation (Danne & Dramsi, 2012). *A. oris* FimA and *C. diphtheriae* SpaA are major subunits of cell wall-anchored pili required for initial colonization of the oral cavity (Mandlik et al., 2007; Mishra et al., 2010). Prior to their assembly, FimA and SpaA are synthesized in the cytoplasm, and transported in unfolded states to the exoplasm (Mazmanian, Ton-That & Schneewind, 2001). Since Gram-positive bacteria lack periplasmic compartments, we wondered how these virulence factors are folded in a seemingly unregulated environment. A clue was provided upon solving the crystal structures for the proteins, which predicted the formation of disulfide bonds (Kang et al., 2009; Mishra et al., 2011). Using alkylation, we confirmed that these bonds were present *in vivo* (Fig. 5). Mutational analysis revealed that disulfide bonds in the C-termini of FimA and SpaA were essential for pilus assembly (Figs. 6 and 7). The failure to form these linkages was associated with the secretion of LMW FimA and SpaA products into the culture media.

It is unlikely that the FimA and SpaA Cys mutants formed pili, which then collapsed into the observed LMW products that were secreted into the culture medium. Pilus subunits are held together by isopeptide bonds that link the N-terminus of one pilin to the C-terminus of another (Budzik et al., 2008) (Fig. 4). These linkages should be sufficient to hold the subunits together with or without disulfide bonds. If disrupting disulfide bond formation caused FimA or SpaA polymers to fall apart, multiple lengths of pili should have been detected in the M fractions. However, only one HMW band of Cys-mutated FimA and SpaA (around 120 kDa) was dominant in these fractions. Since monomeric FimA and SpaA are approximately 60 kDa, we predict that these species represent dimers formed by intermolecular disulfide bonds. In support of this, the HMW bands were not detected when β -mercaptoethanol was added to the samples prior to separation by SDS-PAGE (data not shown).

It is more likely that the FimA and SpaA mutants were degraded before they could be assembled into pili. Many bacterial virulence factors are susceptible to proteolysis when oxidative protein folding is disrupted (Heras et al., 2009). For example, *E. coli* FlgI, which makes up the flagellar P-ring, requires a disulfide bond in its C-terminus (Dailey & Berg, 1993). Hizukuri et al. (2006) showed that reduced FlgI was still functional, but highly susceptible to proteolysis. In support of this, overexpressing FlgI harboring Cys-to-Ala mutations partially restored P-ring synthesis. This may explain why a few SpaA fibers were visible on the surface of *C. diphtheriae* expressing a double Cys mutant (Fig. 7E). It is possible that the substituted Ala residues formed a nonpolar interaction that is absent in pilins harboring single Cys mutations. This interaction may have conferred some proteolytic stability to permitted low levels of SpaA polymerization.

It is interesting that the C-terminal FimA disulfide bond was required for type 2 fimbriae assembly, while the N-terminal linkage was dispensable. Since sortase processes pilus subunits via the C-terminus, it is conceivable that pilus assembly is dependent on the proper folding of this region (Mazmanian et al., 1999; Ton-That et al., 2004). Many Gram-positive pilins contain a C-terminal folding element called the E box (Mandlik et al., 2008). The E-box contains a conserved Glu residue that catalyzes an intramolecular isopeptide bond between Lys and Asn residues (Kang et al., 2007; Mishra et al., 2011). This linkage is essential for protein stability. Cozzi et al. (2012) examined the role of the E box in pili expressed by Group B *Streptococcus* using NMR spectrometry and computer simulations. NMR spectrometry showed that a Glu-to-Ala mutation within this region caused pilus subunits to unfold. Furthermore, a molecular dynamics simulation predicted that more water molecules were present in the interior of the pilin mutant compared to wild-type indicating that the integrity of the hydrophobic core was lost. Thus, it was concluded that the E-box is required for the proper folding of the C-terminus of the tested pilins. Disulfide bonds within similar regions of FimA and SpaA may serve a similar purpose. We predict that sortase cannot

recognize subunits lacking their disulfide bonds. As a consequence, FimA and SpaA are discarded into the culture media. Crystal structures of the FimA and SpaA Cys mutants would be beneficial for investigating how these thiol linkages contribute to protein folding.

Protein oxidation has not been well-explored in Gram-positives, which lack periplasmic spaces. It has been suggested these bacteria avoid disulfide bond formation to protect secreted proteins from aberrant oxidation (Daniels et al., 2010). Recently, Actinobacteria have been identified as a subset of Gram-positive bacteria that may use thiol oxidation (Daniels et al., 2010; Dutton et al., 2008). Pilus assembly in *A. oris* and *C. diphtheriae* will serve as a useful model to study disulfide bond formation in these organisms. We have identified several putative oxidoreductases that could be involved with disulfide bond catalysis. Future experiments will focus on determining if FimA and SpaA oxidation is dependent on any these factors.

In summary, the virulence factors FimA and SpaA expressed by *A. oris* and *C. diphtheriae*, respectively, contain disulfide bonds. The formation of C-terminal Cys linkages within these subunits is essential for pilus assembly. This work adds another layer to our understanding of pilus assembly in Gram-positive bacteria, and provides a useful tool to study disulfide bond formation in Actinobacteria.

Chapter IV:

Oxidative folding of virulence determinants in Gram-positive bacteria

4.1 Introduction

Disulfide bonds are important for protein stability and function. These linkages, formed when cysteine (Cys) residues in nascent polypeptides become oxidized, are often rate-limiting steps for folding and maturation (Creighton, Zapun & Darby, 1995; Molinari & Helenius, 1999). With the exception of Archaea, disulfide bond formation is limited to extracytoplasmic compartments like the eukaryotic endoplasmic reticulum (ER), inner membrane space of mitochondria, and bacterial periplasm (Pedone, Limauro & Bartolucci, 2008). Eukaryotic protein disulfide isomerase (PDI) was the first disulfide bond forming enzyme identified by Anfinsen and colleagues during the 1960s (Goldberger et al., 1963; Goldberger, Epstein & Anfinsen, 1964). This multi-domain chaperone possesses two redox-active CxxC motifs, which catalyze disulfide bond formation, and reduce aberrant Cys-linkages in nascent proteins.

Beckwith's group discovered *Escherichia coli* DsbA, the archetype of Gram-negative disulfide bond forming proteins, nearly 30 years later (Bardwell et al., 1991). DsbA is a periplasmic protein that, similar to PDI, harbors a reactive disulfide bond in a CxxC motif. DsbA donates this bond to reduced Cys residues in nascent polypeptides secreted into the periplasm by SecYEG (Kadokura & Beckwith, 2009). In turn, DsbA is reduced and requires re-oxidation by the membrane-bound DsbB (Bardwell et al., 1993; Missiakas, Georgopoulos & Raina, 1993). DsbB resets the oxidative folding pathway by shuttling electrons from DsbA to quinone, a component of the electron transport chain (Kobayashi & Ito, 1999). Disulfide bond isomerization is catalyzed by the redox pair DsbC/DsbD, which reduce nonnative disulfide bonds in the periplasm (Missiakas et al., 1994; Missiakas et al., 1995). Although Dsb proteins expressed by Gram-negative bacteria are not required for growth, they are essential for virulence. Pathogens *E. coli*, *Shigella flexneri*, *Pseudomonas aeruginosa*, *Bordetella pertussis*, *Neisseria meningitidis*, and *Salmonella enterica* secrete an arsenal of disulfide-

bond containing virulence factors like flagella, adhesive pili, and toxins (Heras et al., 2009). Thus, DsbA is an important virulence factor.

Oxidative protein folding pathways in Gram-positive bacteria have not been well-explored. The Gram-positive cell envelope is comprised of a single cytoplasmic membrane that is surrounded by a thick layer of peptidoglycan. Although inner wall zones (IWZ) between these layers have been observed in *Bacillus subtilis* and *Staphylococcus aureus*, these organisms are not predicted to possess periplasmic spaces (Matias & Beveridge, 2005; Matias & Beveridge, 2006). Peptidoglycan is considered to be porous, so it is possible that Sec machinery secretes nascent proteins into an environment that is exposed to the extracellular milieu. The translocation of unfolded, Cys-containing proteins to this region may be hazardous because exposure to oxygen can cause aberrant oxidation (Anfinsen et al., 1961). Therefore, oxidative protein folding pathways within these organisms could be deleterious. Many Gram-positive bacteria appear to prevent misfolding of secreted proteins by avoiding disulfide bond formation. Recent bioinformatics analysis of the Cys-content of Gram-positive proteomes partially supported this conjecture (Daniels et al., 2010; Dutton et al., 2008). It was revealed that Firmicutes, including *B. subtilis* and *S. aureus*, tend to exclude Cys residues from secreted proteins. Dsb-like proteins have been identified in these organisms, but, unlike the Gram-negative bacteria, their genes are arranged in operons with specific substrates (Dorenbos et al., 2002; Meima et al., 2002; van der Kooi-Pol et al., 2012). This suggests that they do not participate in general oxidative folding. In contrast, Actinobacteria, such as *Corynebacterium*, *Streptomyces*, and *Mycobacterium*, secrete many proteins with multiple Cys residues, and encode extracellular thiol-oxidoreductase enzymes (Daniels et al., 2010). Intriguingly, in *Mycobacterium tuberculosis*, a membrane protein annotated as Vitamin K epoxide reductase (VKOR) is proposed to be a DsbB analog since its expression rescues disulfide bond formation in an *E. coli dsbB* mutant (Dutton et al., 2008). In

addition, several secreted DsbA-like proteins have been identified in *M. tuberculosis* and crystallized (Chim et al., 2013; Chim et al., 2010; Goulding et al., 2004). The current data suggest Actinobacteria use disulfide bond formation to help fold secreted proteins, but oxidative folding pathways have not been elucidated *in vivo*.

We previously reported that pilus proteins FimA and SpaA, expressed by *Actinomyces oris* and *Corynebacterium diphtheriae*, respectively, contain disulfide bonds (Kang et al., 2009; Mishra et al., 2011). Using FimA and SpaA as model substrates, this study elucidates disulfide bond forming pathways in two Actinobacterial models. We reveal that oxidative protein folding is catalyzed by a membrane-bound thiol-oxidoreductase enzyme named MdbA (*mdb* for monoderm disulfide bond-forming). Re-oxidation of MdbA in *A. oris* requires a second membrane-spanning thiol-oxidoreductase called VKOR. Importantly, MdbA is not limited to pilins. We show that it is required for production and stability of diphtheria toxin secreted by *C. diphtheriae*. Given that the majority of signal peptide-containing proteins (more than 60%) expressed by these bacteria have two or more Cys residues; we propose that disulfide bond formation is a major folding pathway for Actinobacteria (Chen et al., 2005; Kanehisa & Goto, 2000). This work provides the most comprehensive analysis of an oxidative protein folding pathway in Gram-positive bacteria to date. Importantly, our results have important implications for the development of new antimicrobials targeting important Actinobacterial pathogens including *Mycobacterium tuberculosis*.

4.2 Results

4.2.1 Identification of a disulfide bond-forming machine in *A. oris*.

Since *A. oris* co-aggregation with oral co-colonizer *S. oralis* is linked to FimA assembly, we aimed to identify factors involved with disulfide bond formation by screening a Tn5 transposon library of ~ 3,000 clones for co-aggregation mutants. Four mutants were

identified and mapped by TAIL-PCR (Liu & Whittier, 1995) and DNA sequencing. We revealed insertions within *fimA* and *srtC2* of the type 2 fimbrial gene locus, which validated the screen. An insertion was also identified in a gene encoding vitamin K epoxide reductase (VKOR). First identified in *Mycobacterium tuberculosis*, VKOR is hypothesized to serve as a DsbB analogue for some Gram-positive bacteria (Dutton et al., 2008). *A. oris* VKOR is a 27 kDa protein predicted to have five transmembrane helices, and a CxxC motif in an N-terminal exoplasmic loop. To confirm the phenotype of the Tn5::*vkor* mutant, an unmarked, non-polar deletion mutant of *vkor* was generated. This mutant also failed to co-aggregate with *S. oralis*, but the defect was rescued by expressing *vkor* from a plasmid (Fig. 9A).

To determine whether the coaggregation defect of the $\Delta vkor$ mutant is a result of a defect in pilus assembly, type 2 fimbriae was examined by IEM using antibodies against the shaft FimA and tip FimB fimbrillins. Compared to the parental MG1, the detection of type 2 fimbriae was severely diminished in the $\Delta vkor$ mutant, but restored upon expression of *vkor* from a plasmid (Fig. 9B-G). The $\Delta vkor$ mutant harboring a plasmid expressing VKOR with one of the Cys residues of the CXXC motif changed to Ala (C175A) remained defective in pilus assembly suggesting that VKOR is an oxidoreductase (Fig. 9H-I). To confirm this, $\Delta vkor$ cultures were supplemented with cystine, an exogenous oxidizing agent (Hizukuri et al., 2006). Remarkably, cystine restored type 2 fimbriae to wild-type levels (Fig. 9J-K). Together, these results demonstrate that VKOR contributes to pilus assembly via disulfide bond formation.

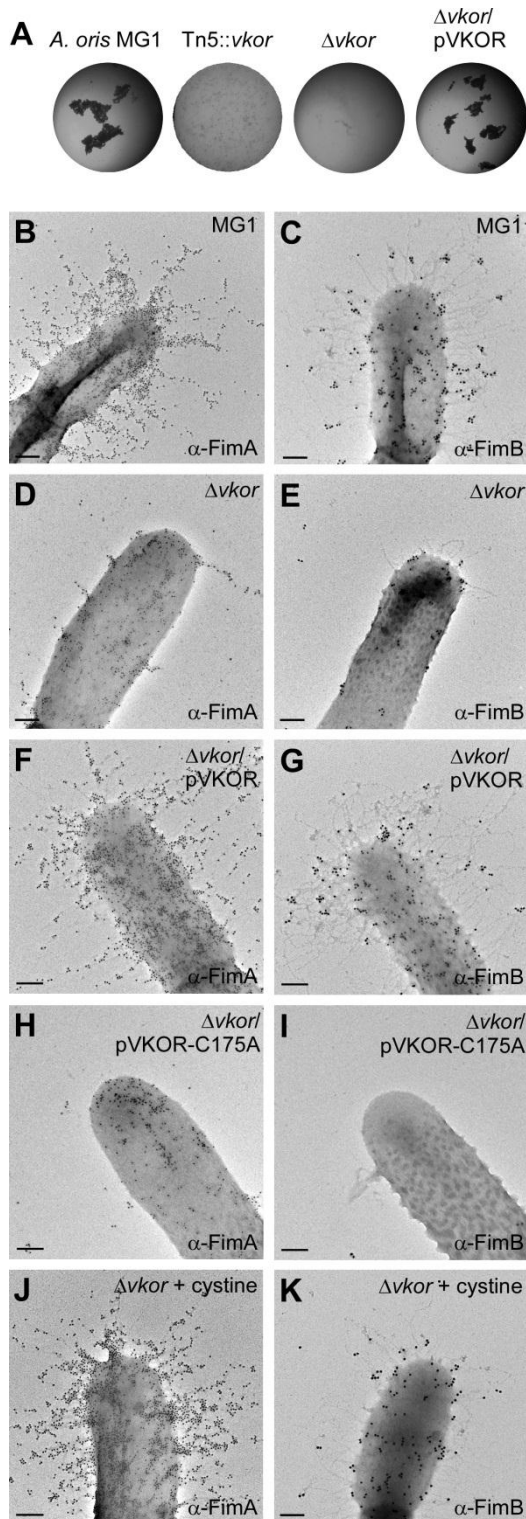


Figure 9. *A. oris* VKOR is required for type 2 fimbrial assembly. (A) For interspecies interaction, *A. oris* and RPS-positive *S. oralis* (So34) cells were mixed in equal numbers and imaged. (B-K) Overnight cultures of *A. oris* were immobilized on nickel grids, and stained with α -FimA (left column) or α -FimB (right column) followed by secondary antibodies conjugated to 12 or 18nm gold particles, respectively. The samples were stained with 1% uranyl acetate and viewed by a transmission electron microscope. Scale bars indicate 0.5 μ m.

4.2.2 VKOR is required for type 1 fimbrial assembly.

FimB was detected on the surface of $\Delta fimA$ cells (Fig. 6F), but rarely observed on the surface of the $\Delta vkor$ mutant (Fig. 9E). This suggests that FimB, which contains 12 Cys residues, may also require disulfide bonds. This is consistent with our recent finding that a pilus-associated coaggregation factor named CafA requires Cys residues for stability (Reardon-Robinson et al., 2014). We predicted that VKOR targets multiple pilus substrates. To explore this, we examined the assembly of type 1 fimbriae by IEM. Similar to type 2 fimbriae, type 1 fimbrial structures were barely visible on the *vkor* mutant, but the defects were rescued by *vkor* complementation (Fig.10). Altogether, these results support that oxidoreductase activity of VKOR is important for general pilus assembly.

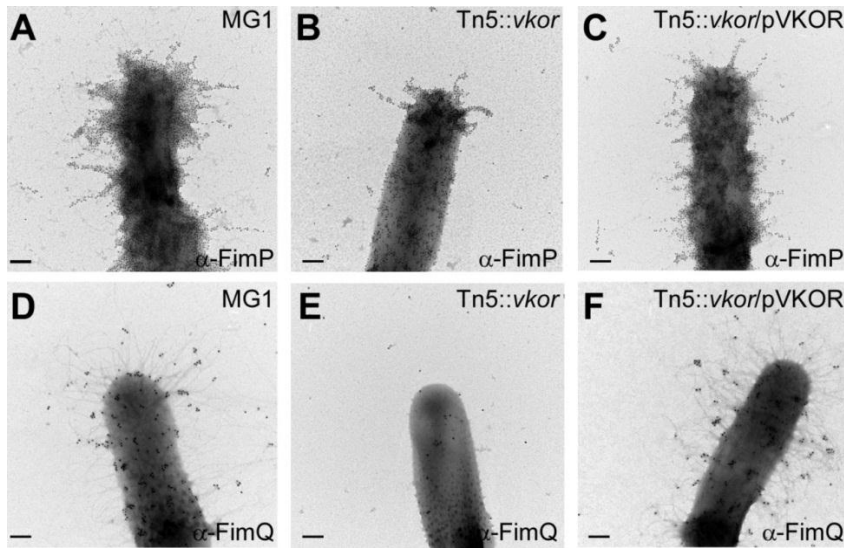


Figure 10. *A. oris* VKOR is required for type 1 fimbrial assembly. (A-F) *A. oris* were immobilized on nickel grids and stained with α -FimP or α -FimQ followed by a secondary antibody conjugated to 12 or 18nm gold particles, respectively. Note that unstained type 2 fimbriae are visible on some bacteria. Scale bars indicate 0.2 μ m.

4.2.3 Thiol-oxidoreductase MdbA rescues type 2 fimbrial assembly in a $\Delta vkor$ mutant and oxidizes FimA *in vitro*.

Since expression of *M. tuberculosis vkor* rescues an *E. coli dsbB* mutant, we suspected that *A. oris* VKOR does not directly catalyze disulfide bond formation (Dutton et al., 2008). Instead, the observed pilus-defect phenotype may result because VKOR is required to maintain the activity of another thiol-oxidoreductase. We hypothesized that VKOR oxidizes the CxxC active site of a DsbA-like enzyme. To identify putative thiol-oxidoreductases, we surveyed the *A. oris* genome for secreted thioredoxins (<http://genome.brop.org/>). We identified ANA_1994, a 32 kDa membrane-bound protein (Fig. 8A). Although MdbA is only 15.4% identical to *E. coli* DsbA, it shares the conserved N-terminal CxxC motif (Fig. 11). We proposed to name this factor MdbA for Monoderm disulfide bond forming protein A.

```

Ec DsbA -----MKK-----TWLALAGLVLAFSASAAQYEDGK-
Ao MdbA MVRSSPHHKEHRVSSNQPRQTKAQRREAARLKAKELREAEARRARRNTIARRSFTGAAGASVVGGLGYLVYLGVDARK
Cd MdbA -----MSKNAGSRKIQNPTKSNGLWALLALLVVVVAVVTVVVVQGA

Ec DsbA ----QYTTLEKP-----VAGAPQVLEFFS-FFCPHCYQFEEVLHISDNVK
Ao MdbA NP--KSSKFPAPSEGLATAKANQGGIPKQVLSDASWTYGEAALDTVAASAPVLDIYFDYSCSHCAQFEGLHTQ--EIN
Cd MdbA HQANKYADYDKESVSFTGSVTDSAIVLKAVN-----AKKDAKKIDFYEDFSCPHCAELGEVTDG--PMT

Ec DsbA KKLPEG-VKMTKYHVNFMGGDLGKDLTQAWAVAMALGVEDKVTVP--LFEGVQKTQTIRSASDIRDVFINAGIKGE---
Ao MdbA QLLSDKKITLALHPCKLLQQEWTSVVMNAMGVVLDEAPAQSLSFHNAAFEIFSQAIQTKNQSNMTVEGLVAAAANKVNP
Cd MdbA KATENGDIVVNLRIILNFLDRD-GDDGNSTKAGAAALAVAQSGDWE--TYWNYR-ALLMKEQKNITYGKWGDN-----

Ec DsbA -FYDAAWNSFVVKSLVAQQEKAAADVQLRGVPAMFVNGKYQLNPQGMDSNMDVFVQYADTVKYLSEKK-----
Ao MdbA KEVSAKFKAADVSDKYGKWVVLGDEAFKARELEGTPTVFFKGEKVDLNLKLTPTSLTELVTGSTPTAQSPSPQPTQQG
Cd MdbA -DFADVAKSLGASDEVTKIREGGAKEDFRKFAEANSKKLEKDGGSVSSPRVFIDGKEVKNGIETWVEQATS-----

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Figure 11. Sequence alignment of *E. coli* DsbA and MdbA enzymes. The primary amino acid sequences for *E. coli* DsbA (*Ec* DsbA), *A. oris* MdbA (*Ao* MdbA) and *C. diphtheriae* MdbA (*Cd* MdbA) were aligned. Black represents absolutely conserved residues, dark grey represents conserved residues, and light grey denotes similar residues.

Unfortunately, multiple attempts to delete *mdbA* were unsuccessful. To circumvent this, $\Delta vkor$ was transformed with a multi-copy plasmid carrying *mdbA*. If *mdbA* functions downstream of *vkor*, we hypothesized that MdbA overproduction would rescue pilus assembly. Remarkably, the assembly of type 2 fimbriae was restored to wild-type levels (Fig. 12C). In contrast, transforming $\Delta vkor$ with *mdbA* harboring a C139A mutation within the CXXC motif did not rescue assembly (Fig. 12D). This suggested that polymerization of FimA is dependent on the redox activity of MdbA.

We also tested the ability of MdbA to oxidize FimA *in vitro*. To reduce disulfide bonds, recombinant FimA was incubated overnight with DTT. The next day, free sulfhydryl groups were acid-trapped, and DTT was removed by filter centrifugation. FimA was then incubated with *A. oris* MdbA, no enzyme, or catalytically inactive MdbA (C139A) in glutathione redox buffer at 37°C for 30 min. At specific time points, the reactions were stopped by addition of Mal-PEG. The samples were then separated with 3-20% Tris-glycine gels, and FimA and MdbA were detected by Coomassie staining. When reduced FimA was incubated with wild-type *A. oris* MdbA, a faster migrating species of FimA (i.e. not modified by Mal-PEG), was visible within five minutes (Fig. 12E; lane 2). This signified that FimA Cys residues had become oxidized. After 30 min, the faster migrating species represented the majority of detected FimA (lane 5). Significantly, FimA remained reduced (i.e. modified by Mal-PEG) in the reactions containing MdbA C139A, or no enzyme (lanes 6-13). We concluded that *A. oris* MdbA possess oxidoreductase activity.

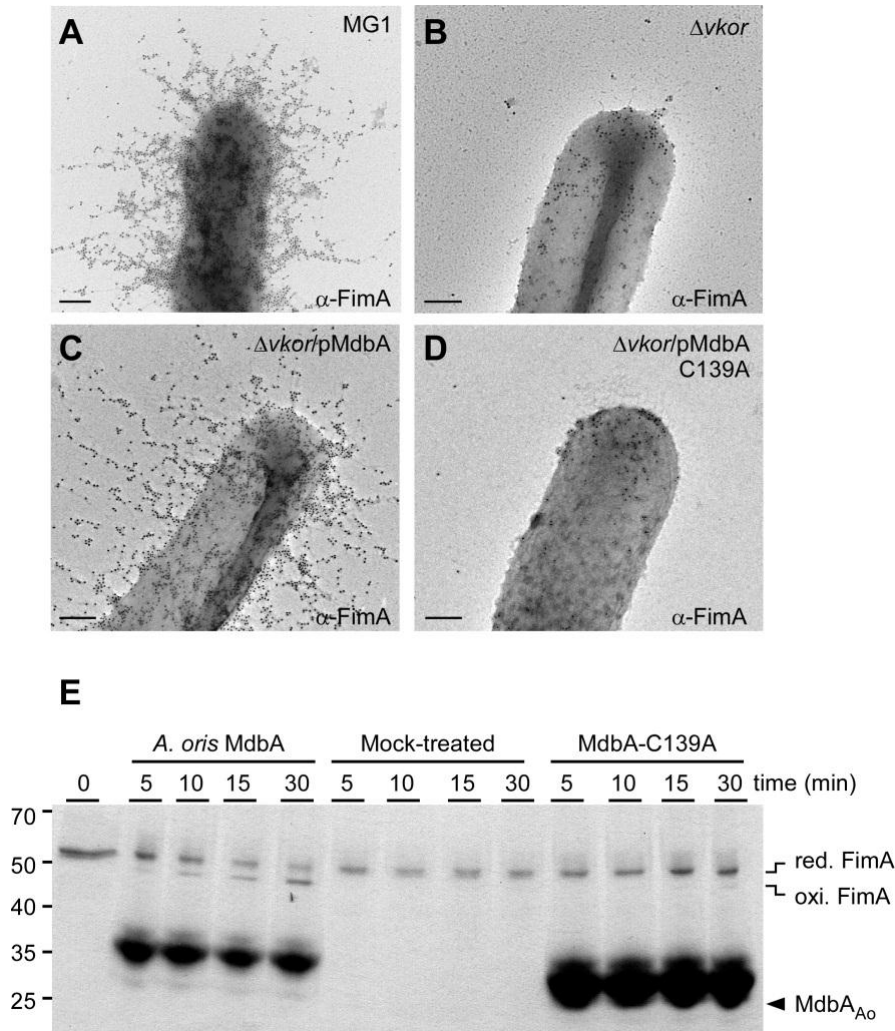


Figure 12. *A. oris* MdbA rescues type 2 fimbrial assembly in a $\Delta vkor$ mutant and oxidizes FimA *in vitro*. (A-D) Overnight cultures of *A. oris* were immobilized on nickel grids, and stained with α -FimA followed by secondary antibodies conjugated to 12nm gold particles. The samples were stained with 1% uranyl acetate and viewed by a transmission electron microscope. Scale bars indicate 0.1 μ m. (E) Recombinant FimA was reduced overnight in 100mM DTT. Free thiol groups were acid-trapped, and DTT was removed by filter centrifugation. FimA was left untreated or combined with wild-type, or mutant *A. oris* MdbA. The reactions were stopped by the addition of Mal-PEG, samples were separated on 3-20%

Tris-glycine gels, and proteins were detected by Coomassie blue staining. Oxidized and reduced forms of FimA and MdbA are indicated.

4.2.4 *A. oris* VKOR is required to oxidize the MdbA CxxC site.

We hypothesized that VKOR recycles MdbA activity by oxidizing its CxxC catalytic site. To investigate this, we examined the redox status of MdbA within wild-type and $\Delta vkor$ backgrounds. Whole cell lysates of *A. oris* were prepared by mechanical disruption with glass beads, and the protein samples were collected by TCA precipitation. The resulting pellets were re-suspended in buffer containing Mal-PEG, followed by TCA precipitation for SDS-PAGE analysis, and immunoblotting with *A. oris* MdbA antibodies (α -MdbA_{AO}). In wild-type samples, alkylation with Mal-PEG resulted in slight up-shift in MdbA migration due likely to the modification of the nonreactive C169 (Fig.13A). However, Mal-PEG treatment of the $\Delta vkor$ lysates caused a greater up-shift (Fig. 13B), indicating that Cys residues within the CxxC motif of MdbA were modified (i.e. present in a reduced state). These data support that VKOR is required for oxidation of the MdbA catalytic site. We conclude that *A. oris* MdbA and VKOR form a redox pair to catalyze disulfide bond formation.

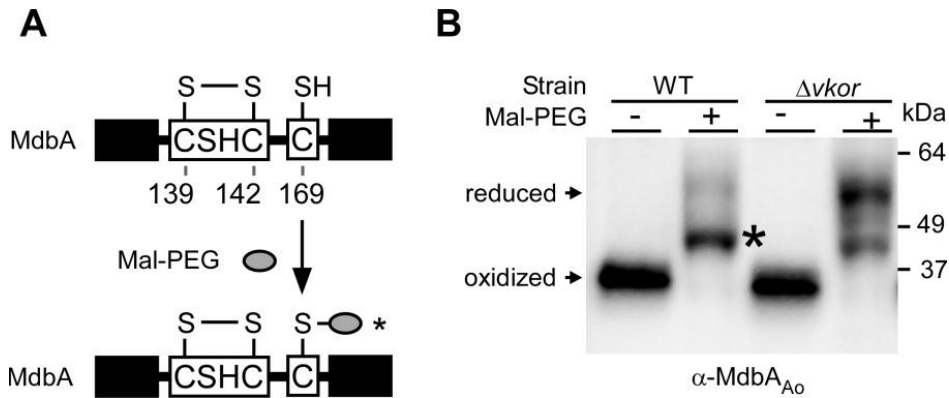


Figure 13. A. *oris* VKOR is required for oxidation of the MdbA CxxC motif (A) The primary sequence for MdbA contains 3 Cys residues. Since C139 and C142 are predicted to form a disulfide bond, alkylation with Mal-PEG should only result in the modification of C169 under WT conditions. **(B)** Whole cell lysates of wild-type *A. oris* and $\Delta vkor$ were treated with Mal-PEG, boiled in SDS, separated on a 3-20% Tris-Glycine gradient gel, and detected by immunoblotting with α -MdbA_{A0}. Reduced and oxidized forms of MdbA are indicated. Molecular mass markers are indicated.

4.2.5 Disulfide bond-forming machinery is conserved in Actinobacteria.

Previous bioinformatics suggested that Gram-positive Actinobacteria employ oxidative protein folding, while Firmicutes may not (Daniels et al., 2010; Dutton et al., 2008). We wondered if disulfide bond formation is a general tool to oxidize pilus precursors within the Gram-positive exoplasm. We chose *C. diphtheriae* SpaA, which contains a disulfide bond in its C-terminus (Chapter 3), as an additional model. Using bioinformatics analysis, we identified a potential thiol-oxidoreductase (DIP_1880; see Fig. 8B), which we also named *mdbA*. *C. diphtheriae mdbA* encodes a 27 kDa extracellular protein with an N-terminal membrane anchor. Similar to *A. oris* MdbA, *C. diphtheriae* MdbA displays low sequence identity to *E. coli* DsbA (23%), but harbors the CxxC consensus sequence (Fig. 11). Cell fractionation using antibodies generated against MdbA (α -MdbA_{Cd}) confirmed that the protein was membrane localized (Fig. 14A). Unlike *A. oris*, a *C. diphtheriae mdbA* deletion mutant was successfully generated. Pilus polymerization was examined by immunoblotting culture medium (M) and cell wall (W) fractions with α -SpaA. Compared to wild-type corynebacteria, the $\Delta mdbA$ mutant was not able to produce SpaA polymers, but complementation with plasmid-borne *mdbA* rescued this defect (Fig. 14B). Pilus polymerization was also examined using immuno-gold labeling EM. To visualize pili, wild-type and $\Delta mdbA$ strains were transformed with a multi-copy plasmid carrying *spaA*. Overexpression of *spaA* in wild-type *C. diphtheriae* produced long pili that covered the cell surface, but few were detected on $\Delta mdbA$ (Fig. 14C-D). To test whether *C. diphtheriae* MdbA functions as an oxidoreductase, we assayed its ability to oxidize *A. oris* FimA *in vitro* (Fig. 14E). Reduced FimA was incubated with wild-type *C. diphtheriae* MdbA, no enzyme, or MdbA C91A. Remarkably, a faster migrating species of FimA was detected after only 5 minutes of incubation with wild-type MdbA indicating that Cys residues had become oxidized (i.e. inaccessible to Mal-PEG) (lane 2). FimA remained reduced (i.e. modified by Mal-PEG) throughout the course of the

experiment if it was incubated with the catalytically inactive MdbA C91A or no enzyme (lanes 6-13). Together, these data show that SpaA pilus assembly is dependent on disulfide bond formation catalyzed by *C. diphtheriae* MdbA.

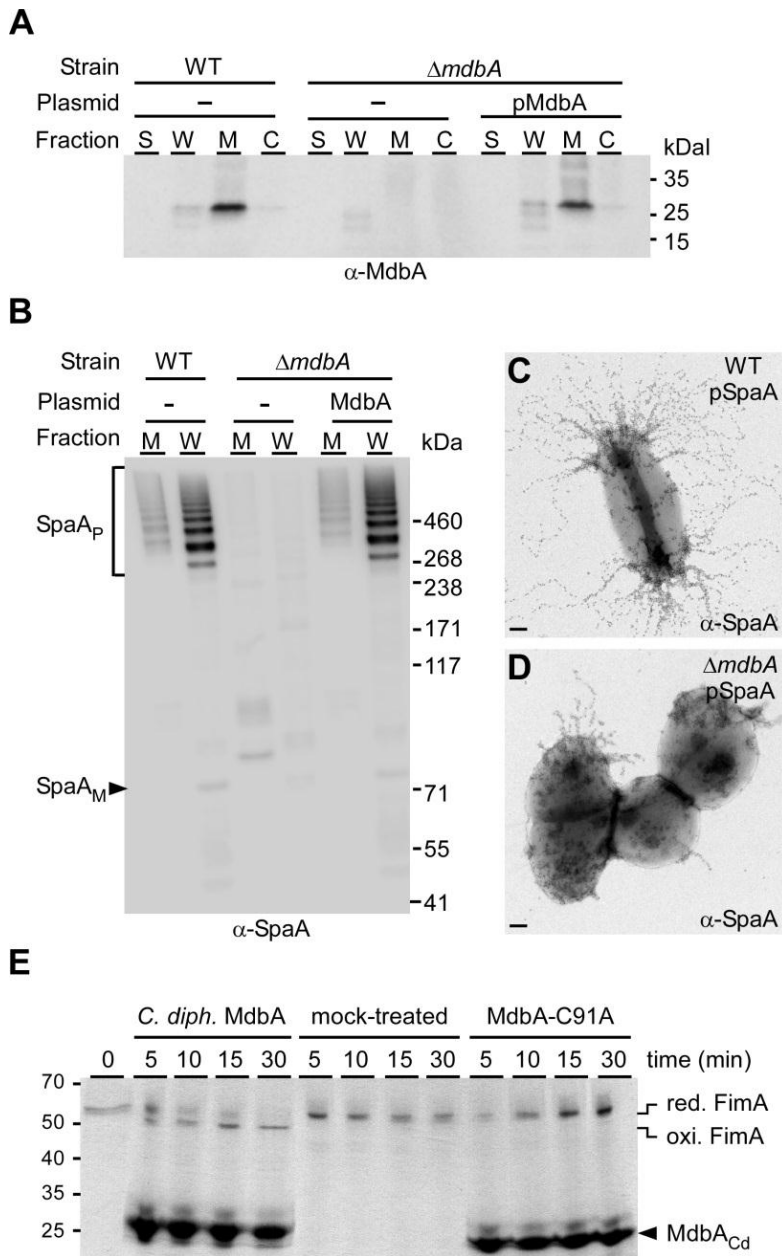


Figure 14. *C. diphtheriae* MdbA is required for pili assembly and exhibits oxidoreductase activity *in vitro*. (A) *C. diphtheriae* S, W, M, and C fractions were separated by SDS-PAGE, and immunoblotted with α -MdbA_{Cd} (B) *C. diphtheriae* M and W fractions were separated on a 3-12% gradient gel, and then immunoblotted with α -SpaA. (C-D) Immunoelectron microscopy of *C. diphtheriae* SpaA pilus assembly were performed as described in Fig. 7 using antibodies against SpaA and IgG-conjugated gold particles of 12nm. Scale bars

indicate 0.5 μ m. **(E)** Reduced *A. oris* FimA was left untreated or treated with recombinant wild-type or catalytically inactive MdbA_{Cd}. At timed intervals, the reactions were stopped by addition of Mal-PEG, and the samples were analyzed SDS-PAGE, followed by Coomassie staining. Oxidized and reduced forms of FimA and MdbA are indicated.

4.2.5 *C. diphtheriae* MdbA is required for disulfide bond formation in diphtheria toxin.

Since more than 60% of proteins secreted by *A. oris* and *C. diphtheriae* contain two or more Cys residues (Chen et al., 2005; Kanehisa & Goto, 2000), we hypothesized that MdbA targets additional secreted virulence factors. To investigate this possibility, we chose diphtheria toxin (DT) as a non-pilus protein model. DT, a 60 kDa A-B toxin secreted by *C. diphtheriae* upon encountering low iron conditions, contains two disulfide bonds (Boquet & Pappenheimer, 1976) (Fig. 15A). DT was induced by adding a metal chelator to the culture medium, collected by centrifugation, and then detected by western blotting. Wild-type DT was detected as a single band that ran near the 64 kDa marker (Fig. 15B). Overall less DT and more LMW products were secreted by the *mdbA* deletion mutant. However, complementation with *mdbA* on a plasmid rescued the defect.

To test whether *mdbA* is required for disulfide bond formation in DT, we again turned to alkylation by Mal-PEG. DT was isolated by TCA precipitation, suspended in buffer with or without DTT, and then treated with Mal-PEG. The toxin was then detected by western blotting analysis using antibodies derived against the DT A domain. DT obtained from WT cells showed no change in mobility unless samples were first treated with DTT (Fig. 15C; first 4 lanes). This demonstrated that the protein is released from bacteria with disulfide bonds. DT secreted by the $\Delta mdbA$ mutant was readily modified by Mal-PEG indicating that it was secreted in a reduced state (lane 6). We also noted that the level of secreted DT in the $\Delta mdbA$ mutant was significantly reduced, consistent with the observation described in Fig. 15C. These results support that MdbA is required for disulfide bond formation in both Actinobacterial pilus and non-pilus virulence factors.

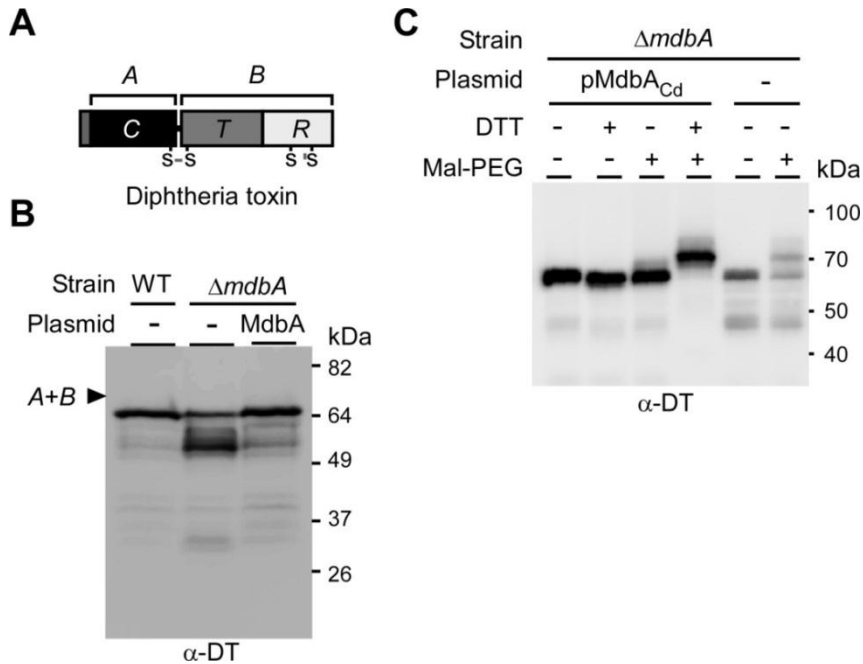


Figure 15. *C. diphtheriae* MdbA is required for disulfide bond formation in Diphtheria toxin. **(A)** A graphic presentation DT is shown highlighting its disulfide bonds. **(B)** DT production was induced by addition of the iron chelator EDDA to culture media. Secreted DT was separated from cells by centrifugation, and protein samples were analyzed by western blotting with monoclonal antibodies against domain A (α -DT). **(C)** DT collected from the culture medium of *C. diphtheriae* expressing or lacking MdbA was treated or mock-treated with DTT, followed by Mal-PEG alkylation. The protein samples were analyzed by immunoblotting with α -DT.

4.2.6. *C. diphtheriae* MdbA is required for virulence *in vivo*.

C. diphtheriae MdbA is required for assembly of SpaA-type pili and secretion of DT. Therefore, we hypothesized that MdbA is an important virulence factor. To examine this *in vivo*, we employed a guinea pig model of diphtheritic toxemia (Pappenheimer, Uhr & Yoneda, 1957). Groups of six animals were injected via the intraperitoneal route (IP) with 2.5×10^7 CFU of wild-type, Δtox (no DT), $\Delta spaA-I$ (no pili), or $\Delta mdbA$, and animal survival was monitored over seven days (Fig. 16). Within two days, 80% of guinea pigs inoculated with wild-type corynebacteria succumbed to infection. The Δtox mutant, which does not produce diphtheria toxin, did not cause a lethal infection. Remarkably, strains that were devoid of MdbA or pili were significantly attenuated in virulence as most of the animals survived. Of note, compared to the non-virulence phenotype of the Δtox mutant, there was no significant difference between this strain and the former two. The data supports that MdbA is an important virulence factor for *C. diphtheriae*.

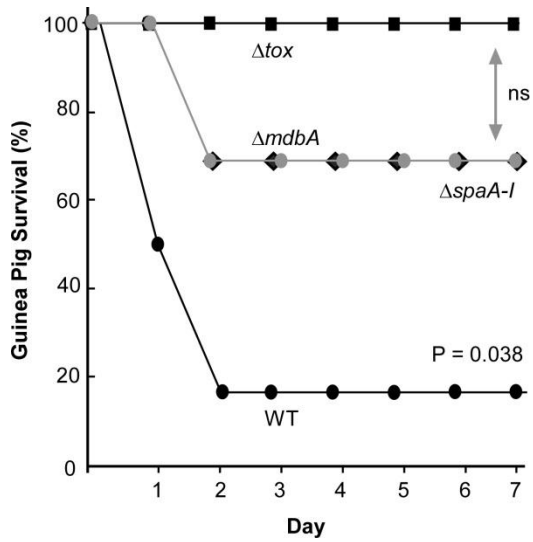


Figure 16: *C. diphtheriae* MdbA is required for virulence in a guinea pig model of acute toxemia. Six 4-week old guinea pigs were injected via IP with $\sim 2.5 \times 10^7$ corynebacteria of the wild-type (filled circles), pilus-less $\Delta spaA-I$ (filled diamonds), toxin-less Δtox (filled squares) or $\Delta mdbA$ (open diamonds) strains. Animal survival was monitored for 7 days; ns denotes not significant (n= 6).

4.2.6 Bacterial disulfide bond forming proteins are interchangeable.

To provide further evidence that MdbA proteins catalyze disulfide bond formation *in vivo*, we performed thiol- oxidoreductase swapping experiments. Since overexpression of *A. oris* *mdbA* rescues pilus assembly in a Δvkr mutant (Fig. 12), we reasoned that other disulfide bond forming proteins could produce a similar effect. *A. oris* Δvkr was transformed with plasmid constructs carrying the ORFs of *C. diphtheriae* *mdbA*, a *dsbA*-like gene encoded downstream of *M. tuberculosis* *vkor*, or *E. coli* *dsbA*. Since *E. coli* is a soluble periplasmic protein, it was fused to the N-terminal membrane anchor of *A. oris* MdbA. These strains were examined for FimA assembly using immunogold-EM. The overexpression of *C. diphtheriae* *mdbA* restored FimA polymers on the Δvkr cell surface (Fig. 17D). Pili were also detected on bacteria expressing *M. tuberculosis* and *E. coli* *dsbA*, but their levels appeared to be lower than Δvkr expressing *A. oris* or *C. diphtheriae* *mdbA* (Fig. 17E-F).

To investigate whether these strains could grow biofilm, *A. oris* were grown in rich media supplemented with 1% sucrose at 37°C with 5% CO₂. After 48 hours, the resulting biofilms were washed with PBS, dried, and stained with crystal violet (Fig. 17H). *A. oris* MG1 formed a robust biofilm, while $\Delta fimA$ failed to grow on its solid surface. The deletion of *vkor* severely diminished growth, while the overexpression *A. oris* and *C. diphtheriae* *mdbA* restored biofilm. Surprisingly, although relatively fewer pili were produced by Δvkr cells expressing *E. coli* or *M. tuberculosis* *dsbA*, these strains produced sufficient biofilm.

We also tested whether non-native oxidoreductases could restore pilus assembly in *C. diphtheriae* $\Delta mdbA$. This mutant was transformed with a multi-copy plasmid carrying *E. coli* *dsbA* fused to the *C. diphtheriae* *mdbA* promoter and N-terminal membrane anchor. Pilus assembly was examined by immunoblotting M and W fractions with α -SpaA. Remarkably, expression of the known disulfide bond forming enzyme restored pilus assembly to wild-type

levels (Fig. 17I; lanes 5-6). The observation that *E. coli* DsbA rescued pilus assembly in both *A. oris* Δvkr and *C. diphtheriae* $\Delta mdbA$ provides strong evidence that both proteins are required for protein oxidation *in vivo*. These data also show that disulfide bond forming proteins are interchangeable between species.

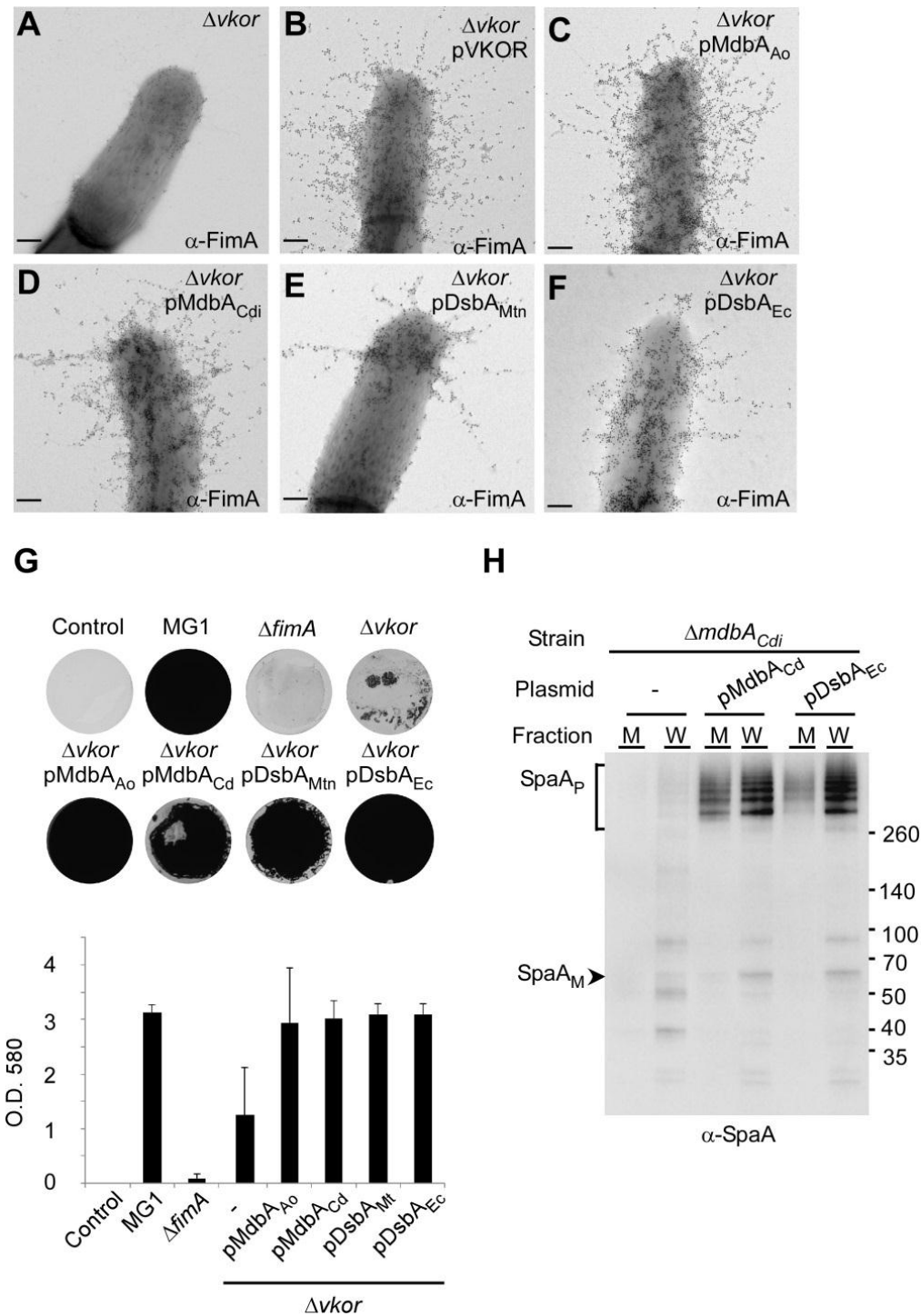


Figure 17. Disulfide bond forming proteins are interchangeable between bacterial species. (A-F) Immunogold EM of *A. oris* FimA pilus assembly were performed as described in Fig. 11 using antibodies against FimA and IgG-conjugated 12nm gold particles. Scale bars indicate 0.5 μ m. **(G)** *A. oris* biofilms were cultivated in 12-well plates at 37°C with 5% CO₂ for

48 hours. The biofilms were washed with PBS, dried, and stained with crystal violet. Pictured are representative images of three-independent experiments performed in triplicate. Growth was quantified by measuring absorbance at 580nm. **(H)** Polymerization of *C. diphtheriae* SpaA was analyzed by immunoblotting M and W fractions with α -SpaA.

4.3 Discussion

Disulfide bond formation is often a rate limiting step for protein folding (Creighton et al., 1995). Although oxygen can induce random thiol oxidation, the rate at which this occurs is not sufficient to meet cellular demands (Givol et al., 1964). Therefore, it was hypothesized that oxidative protein folding was an active cell process. Investigations led by Anfinsen and colleagues led to the discovery of eukaryotic PDI, which catalyzes disulfide bond formation in the ER (Goldberger et al., 1963; Goldberger et al., 1964). Thiol oxidation was later found to be catalyzed in the Gram-negative periplasm by DsbA (Bardwell et al., 1991).

Gram-positive disulfide bond forming pathways have not been well- explored because they are thought to lack extracellular compartments to regulate the folding of secreted proteins. Recent bioinformatics analysis of Gram-positive proteomes revealed that Actinobacteria may represent a subset of these organisms that use oxidative protein folding in the exoplasm (Daniels et al., 2010; Dutton et al., 2008). This work has elucidated disulfide bond forming pathways in the Actinobacterial pathogens *A. oris* and *C. diphtheriae*. Disulfide bond formation is catalyzed within Sec-translocated proteins by the membrane-bound MdbA, which requires re-oxidation by VKOR in *A. oris*, and an unidentified factor in *C. diphtheriae* (Fig. 18).

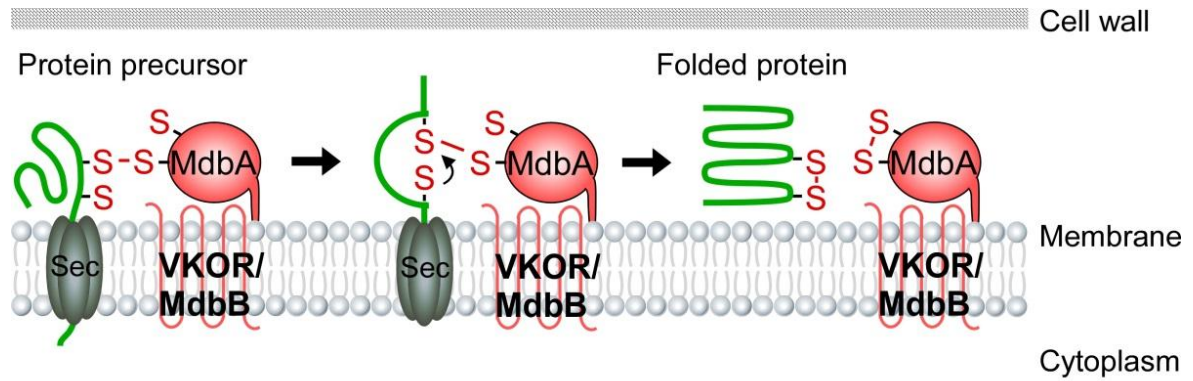


Figure 18. A model for disulfide bond formation in *A. oris* and *C. diphtheriae*. Disulfide bond formation is catalyzed within Sec-translocated proteins by the membrane-bound MdbA. Following catalysis, the CxxC active site of MdbA is re-oxidized by VKOR in *A. oris* and an unidentified factor (shown as MdbB) in *C. diphtheriae*.

We previously showed that the pilus subunits FimA and SpaA expressed by *A. oris* and *C. diphtheriae*, respectively, require disulfide bonds (Chapter 3). Since our lab has established tools to study pilus assembly, we proposed to use these virulence factors as models to study disulfide bond formation in Actinobacteria. To identify factors involved with FimA oxidation, we screened an *A. oris* transposon library for mutants that were defective in co-aggregation with *S. oralis*. This identified VKOR, which was first reported in *M. tuberculosis* (Dutton et al., 2008). We showed that the deletion of *vkor* abolished the assembly of type 2 fimbriae (Fig.9). In line with this, the $\Delta vkor$ mutant was defective in co-aggregation with *S. oralis*, and biofilm formation (Fig. 17) indicating that it may have diminished capability to colonize the oral cavity. Until now, a role for bacterial VKOR *in vivo* had not been demonstrated. The addition of cystine, an exogenous oxidizing agent, restored assembly demonstrating that VKOR has a role in disulfide bond formation. Excitingly, deletion of *vkor* also produced defects in the synthesis of type 1 fimbriae suggesting that it is required for the oxidation of multiple virulence factors (Fig. 10).

The expression of *M. tuberculosis vkor* rescues an *E. coli dsbB* mutant (Dutton et al., 2008). Therefore, we hypothesized that phenotypes associated with $\Delta vkor$ resulted from the failure to recycle the activity of a disulfide bond forming enzyme. A survey of the *A. oris* genome revealed *mdbA*, (Fig. 8) but multiple attempts to delete it were unsuccessful. To study *mdbA* activity *in vivo*, the *vkor* mutant was transformed with an extra copy of the gene on a plasmid. Overexpression of *mdbA* strain restored the FimA polymerization demonstrating that it functioned downstream of *vkor* (Bardwell et al., 1993) (Fig. 12). To test if this phenotype was dependent on MdbA redox activity, $\Delta vkor$ was also transformed with a construct of *mdbA* harboring a mutation within its CxxC motif. As expected, this mutant failed to restore FimA assembly (Fig. 12D). The overexpression of *E. coli dsbA*, a known disulfide bond forming enzyme, also restored pilus assembly in *A. oris* $\Delta vkor$. This further supported

the enzyme's role in disulfide bond formation (Fig. 17F and G). Finally, MdbA protein was shown to oxidize Cys residues within FimA *in vitro* (Fig. 12E). Altogether, these results demonstrate that *A. oris* MdbA catalyzes disulfide bonds.

It was hypothesized that *A. oris* MdbA and VKOR work in concert to catalyze disulfide bonds in secreted proteins. To show that VKOR maintained oxidized (i.e. active) MdbA, the protein was subjected to alkylation in the presence and absence of *vkor* (Fig. 13). Mal-PEG failed to modify the MdbA CxxC motif isolated from MG1 indicating that the Cys residues were linked by a disulfide bond. In contrast, the same residues were alkylated in $\Delta vkor$ lysates. These results directly showed that VKOR is required to recycle MdbA.

Multiple failed attempts to delete *A. oris mdbA* lead us to suspect the gene is essential. These observations suggest that the importance of MdbA extends beyond the proper folding of secreted virulence factors. An *A. oris mdbA* depletion mutant will need to be constructed to test what affect, if any, this protein has on bacterial growth or survival. Since VKOR is required for MdbA activity, it is unusual that we were able to delete *vkor*, but not *mdbA*. Under laboratory conditions (i.e. grown in rich medium with oxygen), it is possible that some MdbA can become randomly oxidized without VKOR. In support of this, a small portion of MdbA isolated from the *vkor* mutant was not alkylated by Mal-PEG indicating some protein harbored an oxidized CxxC motif (Fig. 13; lane 4). In Gram-negative bacteria, DsbA activity is not completely inhibited by the deletion of *dsbB*. A recent study of disulfide bond forming proteins in *Francisella tularensis* detected mixed populations of reduced and oxidized DsbA in a *dsbB* mutant (Ren, Champion & Huntley, 2014). This indicates that DsbA is at least partially functional without DsbB. Therefore, we speculate that enough MdbA is oxidized in *A. oris* $\Delta vkor$ to keep bacteria viable. It would be interesting to test if the *vkor* mutant can survive in growth conditions that do not favor random oxidation of MdbA (i.e. minimal medium and/or no

oxygen). It is also possible that *A. oris* expresses a second factor that re-oxidizes MdbA at lower levels, or that MdbA possesses an essential function that is independent of VKOR.

To explore the conservation of oxidative folding in Actinobacteria, we expanded our studies to *C. diphtheriae* SpaA, which requires the formation of a disulfide bond in its C-terminus. The deletion of *C. diphtheriae* *mdbA* abolished SpaA-type pilus assembly (Fig. 14). However, SpaA polymerization was restored if the mutant was complemented with endogenous *mdbA*, or *E. coli* *dsbA*, a known disulfide bond-forming enzyme (Fig. 17). Furthermore, purified *C. diphtheriae* MdbA oxidized FimA *in vitro* (Fig. 14). Altogether, this is strong evidence that *C. diphtheriae* MdbA catalyzes thiol oxidation.

More than 60% of proteins secreted through the *A. oris* and *C. diphtheriae* Sec translocon harbor two or more Cys residues (Kanehisa & Goto, 2000). Therefore, we hypothesized that MdbA targets multiple substrates. DT, a secreted virulence factor with two disulfide bonds, was chosen as an additional model of study (Choe et al., 1992). The production of full-length DT was significantly reduced in the Δ *mdbA* mutant with increased levels of LMW products (Fig.15). An alkylation assay demonstrated that *mdbA* mutants secrete reduced DT, irrefutable evidence that it requires MdbA-catalyzed oxidation. Since *C. diphtheriae* Δ *mdbA* is defective in the production of virulence factors like adhesive pili and DT, we hypothesized that MdbA is required for pathogenesis. A survival assay using a guinea pig model of acute toxemia showed that *C. diphtheriae* Δ *mdbA* is attenuated in virulence (Fig. 16). Together with *A. oris*, the data demonstrates that disulfide bond formation is a conserved tool to help fold secreted virulence factors in the Actinobacterial exoplasm.

We were not successful in identifying a factor that was required for *C. diphtheriae* MdbA recycling. BLAST analysis revealed several extracellular proteins with CxxC motifs (Fig. 8), but only deletion of *mdbA* was associated with a pilus assembly defect. To date, we have not identified additional candidates. Interestingly, a DsbA-like protein found in *S. aureus*

was found not to possess a DsbB-like partner (Dutton et al., 2008). *In vitro* analysis revealed that this protein exhibits a low redox potential, and is equally stable in oxidized and reduced forms (Heras et al., 2008). Although it is possible that *C. diphtheriae* also lacks an enzyme to re-oxidize MdbA, we doubt this is the case. *S. aureus* is not predicted to utilize oxidative protein folding within the exoplasm (Daniels et al., 2010; Dutton et al., 2008). Therefore, maintaining its DsbA-like enzyme in an oxidized state (i.e. primed to catalyze disulfide bond formation) may not be important if it does not have many (if any) substrates to oxidize. Due to the presence of a His residue in its CxxC motif, and its observed oxidoreductase activity, we predict that MdbA exhibits a high redox potential. This would prevent the enzyme from spontaneously re-oxidizing after catalysis (Grauschopf et al., 1995; Wunderlich et al., 1993). It is more likely *C. diphtheriae* MdbA's redox partner has not yet been found, or that its CxxC motif is oxidized by multiple oxidoreductases.

In summary, this work has elucidated disulfide bond forming pathways in Actinobacteria using *A. oris* and *C. diphtheriae* as models. To our knowledge, this is the first description of oxidative protein folding pathways in Actinobacteria. We showed that MdbA enzymes are required for the production of important secreted virulence factors like adhesive pili and toxin. Given the high number of Cys residues within secreted proteins, we predict that MdbA targets other substrates, and thus may serve a housekeeping role in protein folding. This study has significant implications for the development of antimicrobial drugs against important Actinobacterial pathogens.

Chapter V:

Deletion of Actinobacterial MdbA confers a growth defect and selects for a suppressor mutation that restores disulfide bond formation.

5.1 Introduction

Protein disulfide bonds in proteins result when neighboring Cys residues are oxidized. With the exception of Archaea, disulfide bond formation is restricted to extracytoplasmic compartments like the eukaryotic ER and bacterial periplasm. Disulfide bonds are catalyzed by oxidoreductase enzymes that donate a thiol linkage found in a conserved CxxC sequence to reduced substrates (Kadokura & Beckwith, 2010). The archetype of disulfide bond forming proteins in eukaryotes is PDI. In Gram-negative bacteria this task is performed by DsbA. Although these enzymes are functionally similar, they are not identical. PDI also reduces non-native Cys linkages in proteins, while DsbA is limited to disulfide bond formation (Zapun & Creighton, 1994). The ability of PDI to unscramble disulfide bonds is required for cell viability (Laboissiere, Sturley & Raines, 1995). Protein oxidation can occur randomly without PDI, but it is required to monitor the accuracy of Cys pairings. Since DsbA does not share this function, it is not surprising that it is nonessential. However, in general, bacterial viability does not appear to be dependent on Dsb enzymes. In Gram-negatives, disulfide bond isomerization is catalyzed by DsbC and DsbG (Kadokura & Beckwith, 2010). The genes encoding these enzymes are also not essential (Bessette et al., 1999; Missiakas et al., 1994; Rietsch et al., 1996). This is not due to redundancy because a triple *dsbA dsbC dsbG* null mutant is still viable (Vertommen et al., 2008). Recent attempts to identify additional factors that contribute to disulfide bond formation in a similar background have not revealed new Dsb proteins (Chng et al., 2012a).

Although disulfide bond formation is not important for growth, DsbA is required for pathogenesis. This functional characteristic makes the oxidoreductase an attractive target for new antimicrobial drugs. Numerous secreted virulence factors including adhesive pili, components of secretion systems, toxins, and flagella require disulfide bonds for proper folding and stability (Heras et al., 2009). Bacterial pathogens harboring *dsbA* mutations are

often attenuated in virulence because these factors are misfolded and/or degraded. It is proposed that drugs designed to target DsbA will not generate a selective pressure to mutate because they will target pathogenesis rather than growth (Adams et al., 2014; Cegelski et al., 2008; Halili et al., 2015).

Disulfide bond forming pathways in Gram-positive bacteria have been largely ignored. Since these organisms lack outer membranes, they are not thought to possess protective outer compartments for regulating protein oxidation. Therefore, it is possible that their exoplasm is exposed to the outer milieu. This makes the secretion of proteins with reduced Cys residues hazardous because extracellular forms of stress can cause aberrant oxidation. It has been suggested that many Gram-positives avoid this potential stress by excluding Cys residues from translocated polypeptides (Daniels et al., 2010). However, bioinformatics have revealed that Gram-positive Actinobacteria may be exceptions (Daniels et al., 2010; Dutton et al., 2008).

Using the oral pathogens *Actinomyces oris* and *Corynebacterium diphtheriae* as models, we recently elucidated oxidative folding pathways in Actinobacteria (Chapter 4). Disulfide bond formation is catalyzed by an exoplasmic, membrane-bound oxidoreductase called MdbA. Similar to other thioredoxin-like proteins, MdbA donates a disulfide bond found in a CxxC motif to form new linkages in unfolded substrates. In *A. oris*, MdbA activity is recycled by VKOR, while *C. diphtheriae* MdbA is re-oxidized by an unidentified factor. Since MdbA is important for the production of virulence factors like adhesive pili and toxin, it is important for pathogenesis in both organisms.

This work reveals a major difference between Gram-negative and Gram-positive disulfide bond forming pathways. Unlike DsbA, MdbA is important for viability. The depletion of *A. oris mdbA* and deletion of *C. diphtheriae mdbA* are associated with severe morphological defects. This suggests that growth factors secreted by these bacteria require

disulfide bonds. In support of this, we provide evidence that Penicillin binding proteins (PBPs) are MdbA substrates. Remarkably, the phenotypes associated with *C. diphtheriae* $\Delta mdbA$ select for a suppressor mutation that restores disulfide bond formation to wild-type levels. A single T-to-G substitution results in the overproduction of an oxidoreductase we have named TsdA. Our results reveal a unique function for bacterial disulfide bond forming proteins, and provide important considerations for the development of antimicrobials that target oxidative protein folding in Actinobacteria.

5.2 Results

5.2.1 Depletion of *A. oris* *mdbA* causes a division defect.

We suspected that *A. oris* *mdbA* is essential because we failed to excise the gene from the chromosome unless a second copy was provided on a plasmid. To study *mdbA* *in vivo*, we generated a depletion mutant by placing the plasmid-derived *mdbA* under control of an arabinose-inducible promoter. To test *mdbA* depletion, overnight cultures of the conditional mutant grown in 2% arabinose was washed in HIB, diluted in fresh media with or without inducer, and then incubated at 37°C until mid-log phase. Equal numbers of bacteria were harvested, and the medium (S), cell wall (W), membrane (M), and cytoplasmic (C) fractions were isolated. The samples were separated using SDS-PAGE, and then immunoblotted with antibodies against *A. oris* MdbA (α -MdbA_{AO}). As expected, MdbA was detected in the M fraction of MG1, and migrated near its predicted molecular mass (32 kDa) (Fig. 19A; lane 3). Although still present, MdbA signal was greatly diminished when arabinose was absent, but detection was restored when the inducer was added to the culture medium (Fig. 19A; compare lanes 7 and 11).

We also examined the depletion of *A. oris* *mdbA* by immunogold EM. Bacteria grown on HIA plates were immobilized on nickel grids, and blotted with α -FimA followed by

secondary antibodies conjugated to gold particles. FimA polymers were visible on the surface of the parental *A. oris* MG1 (Fig. 19B), but not on the conditional mutant grown without arabinose (compare Figs. 19C and D). This directly shows that *mdbA* is required for pilus assembly. Remarkably, the depletion of *mdbA* was also associated with morphological abnormalities. While MG1 and *mdbA*-expressing *A. oris* were rod-shaped, symmetrically dividing cells, the depletion mutant formed chains of bacteria that were sometimes observed to bend at odd angles indicating a defect in cell growth.

A

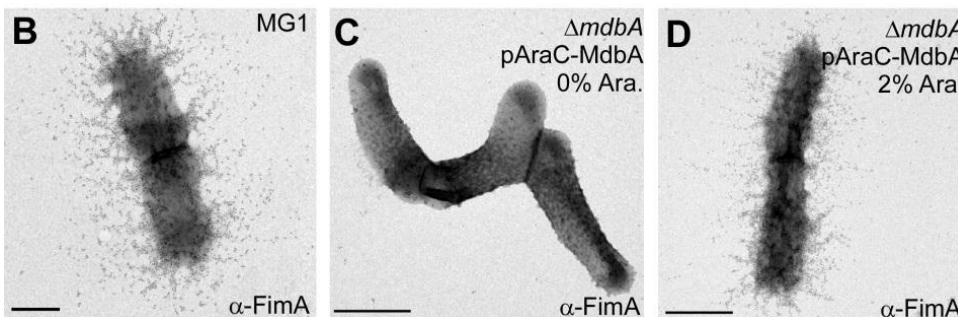
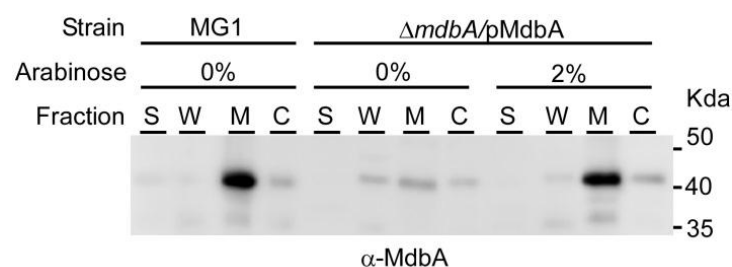


Figure 19. Depletion of *A. oris* *mdbA* abolishes pilus assembly and normal cell morphology. (A) Medium (S), cell wall (W), membrane (M), and cytoplasmic (C) fractions of *A. oris* were collected, separated by a 12% Tris-glycine gel, and immunoblotted with α -MdbA_{A0}. **(B-D)** Overnight cultures of *A. oris* grown on HIA plates were immobilized on nickel grids, and stained with α -FimA followed by secondary antibodies conjugated to 12nm gold particles. The samples were stained with 1% uranyl acetate and viewed by a transmission electron microscope. Scale bars indicate 0.2 μ m.

5.2.2 *C. diphtheriae* $\Delta mdbA$ is temperature-sensitive.

Unlike *A. oris*, a *C. diphtheriae* *mdbA* deletion was successfully generated, but this resulted in a temperature-sensitive phenotype. *C. diphtheriae* $\Delta mdbA$ growth was similar to wild-type at 30°C, but it failed to proliferate at 37°C (Fig. 20A). Normal growth was restored by complementation of *mdbA* on a plasmid. To examine cell morphology, overnight cultures of *C. diphtheriae* grown at 30°C were diluted into fresh media, and incubated at 30°C or 37°C until log phase. The cultures were then immobilized on nickel grids, strained with uranyl acetate, and viewed by EM. *C. diphtheriae* $\Delta mdbA$ grown at 30°C were indistinguishable from wild-type (compare Figs. 20C and 20D). However, a dramatic change was observed when the bacteria were shifted to 37°C. The *mdbA* mutant became round, and formed chains or clumps (Fig. 20E). Again, normal growth at 37°C was restored by plasmid complementation (Fig. 20F). Together with *A. oris*, these data suggest that Actinobacterial MdbA is required for proper peptidoglycan synthesis and division.

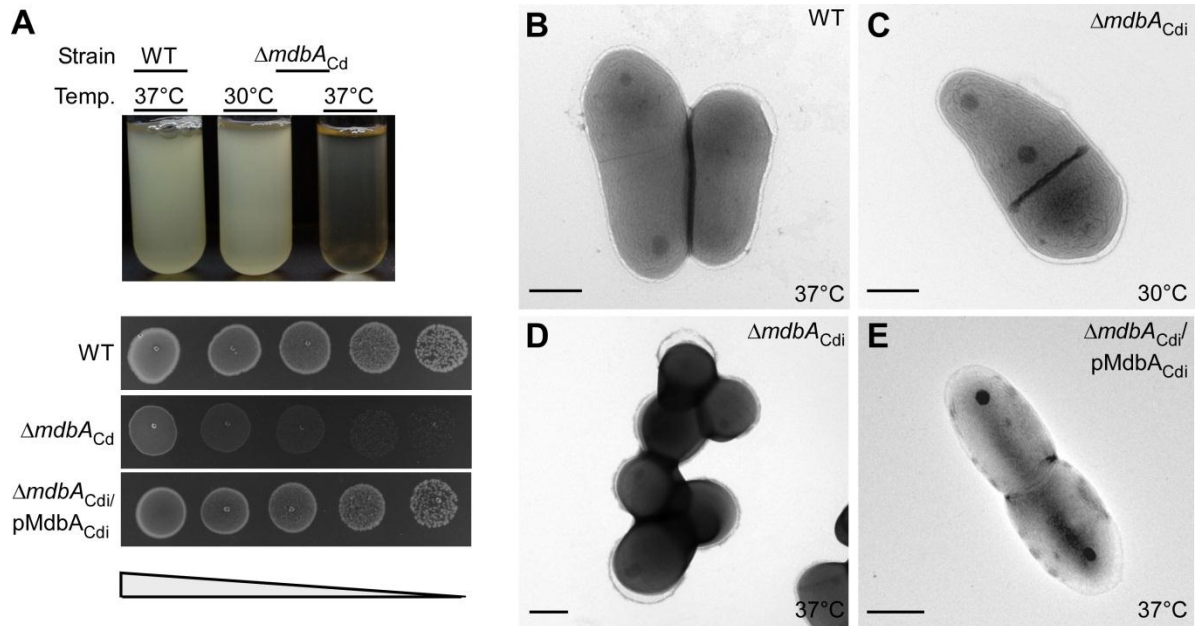


Figure 20. *C. diphtheriae* $\Delta mdbA$ is temperature sensitive. (A) HI medium was inoculated with single colonies of *C. diphtheriae* and incubated overnight at 37°C or 30°C (top panel). Stationary cultures of *C. diphtheriae* grown at 30°C were spot diluted on HI agar plates, and then incubated overnight at 37°C (lower panel). (B-E) Stationary cultures of *C. diphtheriae* grown at 30°C were diluted in fresh media and then incubated at 30°C or 37°C for 5 hours. The liquid cultures were immobilized on nickel grids, stained with 1% uranyl acetate, and viewed by a transmission electron microscope. Scale bars indicate 0.5μm.

5.2.3 *C. diphtheriae* $\Delta mdbA$ exhibit a cell wall synthesis defect.

Cell rounding of normally rod-shaped bacteria often indicates problems with cell wall synthesis (Botta & Buffa, 1981; Valbuena et al., 2007; Wei et al., 2003). We hypothesized that *C. diphtheriae* $\Delta mdbA$ may be defective in cell wall synthesis at 37°C. To investigate this, bacteria were stained with Van-FL, a fluorescent derivative of vancomycin that binds to the D-Ala, D-Ala moiety of nascent peptidoglycan. *C. diphtheriae* is known to grow at the cell poles, and divide at its center (Margolin, 2009). In line with this, new peptidoglycan was detected at the tips and septa of wild-type bacteria grown to log phase (Fig. 21A). Van-FL staining was remarkably different in the $\Delta mdbA$ strain. Nascent cell wall surrounded the cell circumferences, and septa were not visible in rounded cells.

In *C. glutamicum*, the deletion of *pbps*, which encode PBPs that catalyze cell wall synthesis, causes cell rounding (Valbuena et al., 2007). Based on this, we suspected that PBPs expressed by *C. diphtheriae* may be MdbA substrates. A survey of the *C. diphtheriae* proteome revealed that most identified PBPs harbor at least two Cys residues (Fig. 21B). To examine PBP function, we measured *C. diphtheriae* susceptibility to the β -lactams ampicillin and penicillin. If MdbA was required for the proper folding of PBPs, we expected that the deletion mutant would be more sensitive to antibiotics. Indeed, growth of the *mdbA* mutant was inhibited at lower concentrations of antibiotic than wild-type (Fig. 21C).

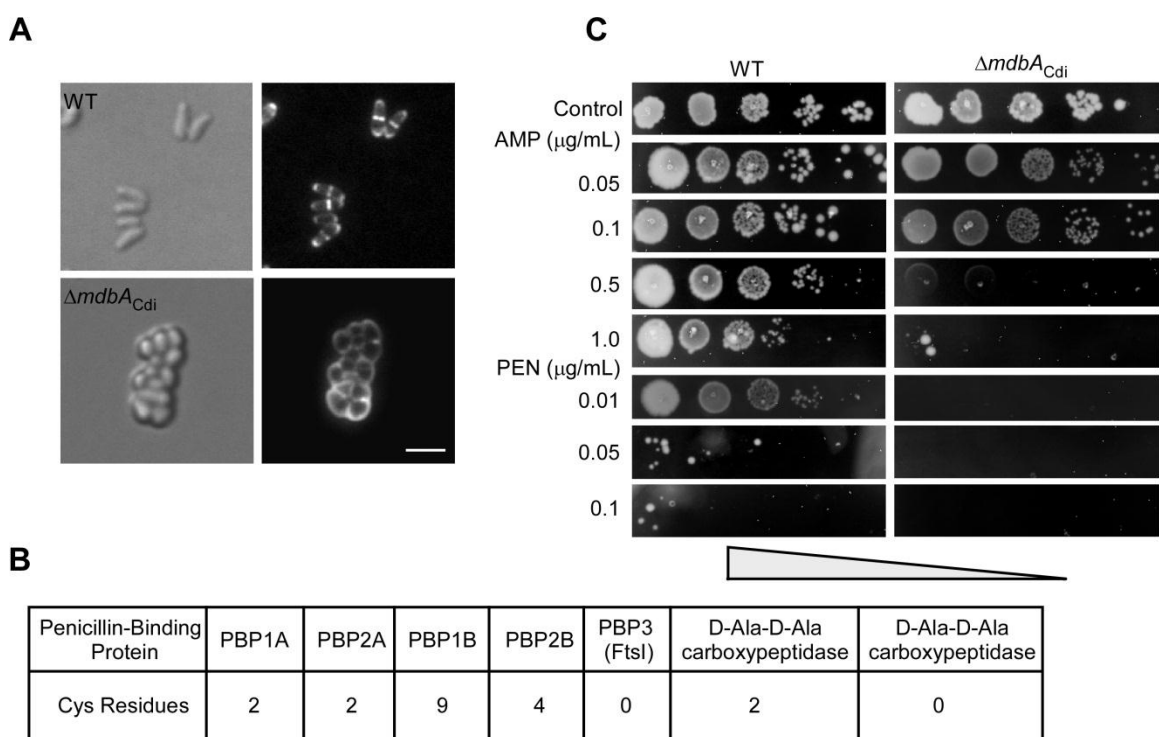


Figure 21. *C. diphtheriae* $\Delta mdbA$ exhibits abnormal Van-FL staining and increased sensitivity to β -lactams. (A) Overnight cultures of *C. diphtheriae* grown at 30°C were diluted into fresh media, and then incubated at 37°C until wild-type cells reached log phase. A 1:1 mixture of Van and Van-FL was added to cultures, and incubated for 10 minutes. The cells were then placed directly on agar pads, and viewed by a fluorescence microscope. Scale bars indicate 2.5 μ m. **(B)** The majority of identified PBPs contain multiple Cys residues indicating they may require disulfide bonds. **(C)** Overnight cultures of *C. diphtheriae* grown at 30°C were spotted (10^{-3} - 10^{-7}) on HIA plates containing various concentrations of ampicillin or penicillin, and incubated at 30°C for 48 hours.

5.2.4 Heat stress selects for *C. diphtheriae* $\Delta mdbA$ suppressor mutants.

Since *C. diphtheriae* is important for growth, we wondered if the deletion mutant could select for suppressor mutations. To test this, stationary cultures of the *mdbA* mutant grown at 30°C were diluted into fresh medium, and incubated overnight at 37°C. The next morning, the cultures were spread onto HI agar plates, which were also incubated at 37°C. After 24 hours, the plates were screened for colonies that formed at the non-permissive temperature. Three colonies named S1, S2, and S3 were isolated and confirmed to grow like wild-type at 37°C (Fig. 22A). To verify that these bacteria did not contain *mdbA*, genomic DNA was isolated, and PCR was used to detect the presence or absence of the gene. Similar to the parental strain $\Delta mdbA$, S1, S2, and S3 lacked the gene in question (Fig. 22B). MdbA protein was also not detected in the membrane fraction of any of the suppressors (Fig. 22C).

We previously showed that *mdbA* is required for the formation of disulfide bonds in secreted virulence factors like SpaA (Chapter 4). To test if SpaA polymerization was altered in the suppressor mutants, medium (M) and cell wall (W) fractions were isolated, separated by SDS-PAGE, and immunoblotted with α -SpaA. SpaA polymers were detected in the W fraction of wild-type cells with some secreted into the M fraction (Fig. 22D; lanes 1 and 2). No pili were observed in fractions collected from the *mdbA* mutant, but degradation products of SpaA were visible in the M fraction (lane 3). Remarkably, pilus assembly in S1, S2, and S3 was indistinguishable from wild-type (lanes 5-10).

The production of diphtheria toxin (DT), another MdbA substrate, was also examined. DT was induced by the addition of a metal chelator to the culture media. Equal numbers of bacteria were collected, DT was isolated from the cultures by centrifugation, and detected by western blotting. DT isolated from wild-type bacteria was readily detected, but absent in a strain lacking the toxin gene (*tox*) (Fig. 22E; lane 2). Consistent with previous results, the

detection of DT was diminished in the *mdbA* mutant (Chapter 4), but was restored in the suppressor mutants (compare lane 3 with lanes 4-6).

We next examined the cell morphologies of the suppressor mutants. Stationary cultures of *C. diphtheriae* grown at 30°C were diluted into fresh media, and shifted to 37°C until wild-type cells reached mid-log phase. The bacteria were then collected by centrifugation, washed in PBS, immobilized on nickel grids, stained with uranyl acetate, and viewed with a transmission electron microscope (Fig. 23). Again, *C. diphtheriae* $\Delta mdbA$ were indistinguishable from wild-type when grown at 30°C (Figs. 23 and B), but became coccoid, chained, and clumped at the nonpermissive temperature (Fig. 23C). Amazingly, S1, S2, and S3 exhibited no growth defects when shifted to 37°C (Fig. 23D-F). The combined data demonstrate that the suppressor strains are phenotypically identical to wild-type.

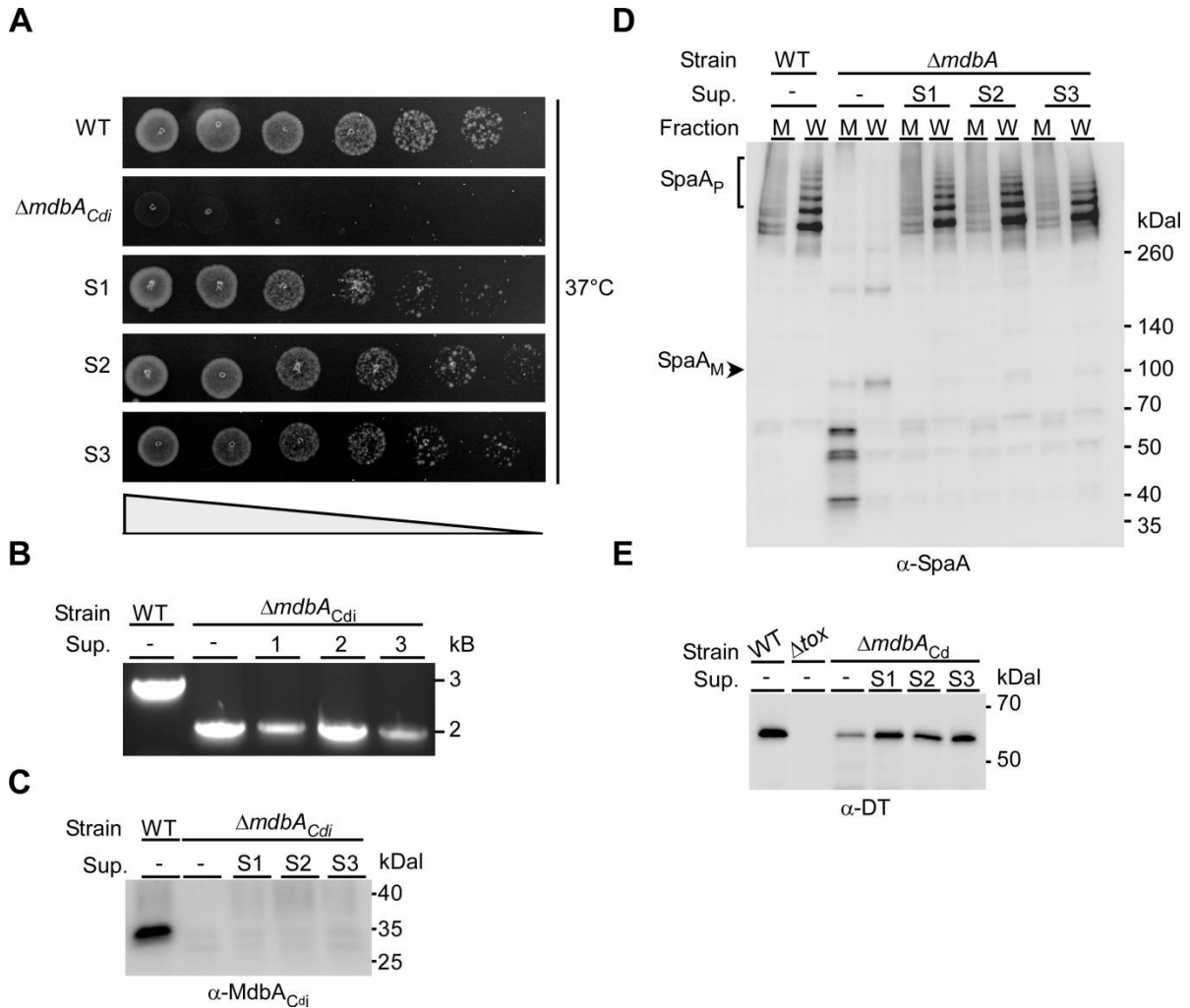


Figure 22. Heat stress selects for *C. diphtheriae* $\Delta mdbA$ suppressor mutants. (A) Stationary cultures of *C. diphtheriae* grown at 30°C were spot diluted on HI agar plates (10^{-3} - 10^{-8}), and incubated overnight at 37°C. **(B)** Chromosomal DNA isolated from *C. diphtheriae* and used to PCR-amplify 1 kb regions up-and downstream of *mdbA*. **(C)** Membrane fractions of *C. diphtheriae* were isolated, separated using SDS-PAGE, and immunoblotted with α -MdbA_{Cdi}. **(D)** Medium (M) and cell wall (W) fractions were isolated from *C. diphtheriae*, separated on a 4-20% gradient gel, and immunoblotted with α -SpaA. **(E)** DT was induced by the addition of metal chelator to liquid cultures, collected by centrifugation, separated by SDS-PAGE, and detected by α -DT.

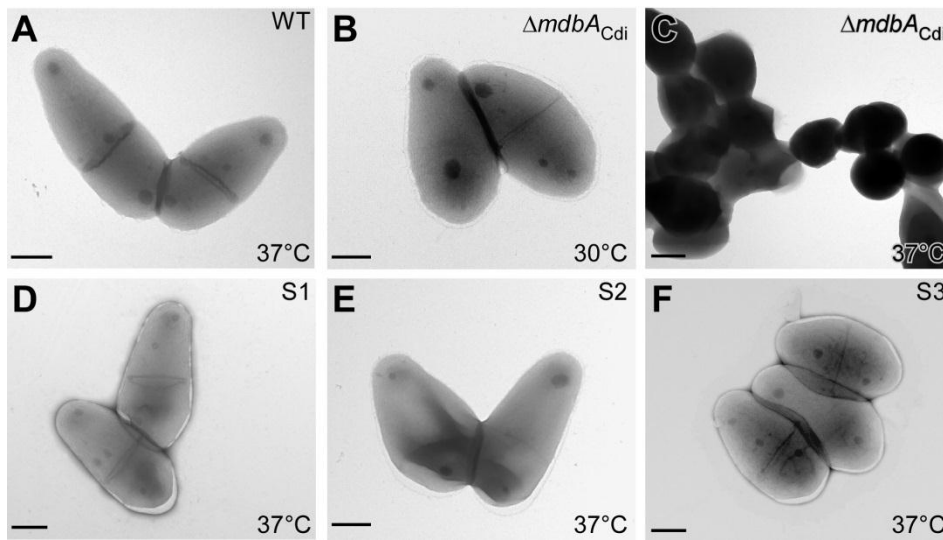


Figure 23. *C. diphtheriae* $\Delta mdbA$ suppressor mutants exhibit normal morphology at the non-permissive temperature. (A-F) Stationary cultures of *C. diphtheriae* grown at 30°C were diluted into fresh media and incubated at 30°C or 37°C until wild-type reached log phase. The bacteria were collected from liquid cultures by centrifugation, washed in PBS, immobilized on nickel grids, stained with 1% uranyl acetate, and viewed by a transmission electron microscope. Scale bars indicate 0.5μm.

5.2.5 The *mdbA* suppressor mutants harbor a single nucleotide change within the promoter region of *tsdA*.

To identify the change(s) that permitted *C. diphtheriae* to compensate for the deletion of *mdbA*, chromosomal DNA was isolated and subjected to whole genome sequencing. All three strains harbored a single T-to-G mutation within the predicted -10 box for the promoter of *cdi_0397*, which encodes a putative extracellular oxidoreductase with a CPFC motif (Fig. 24A). Additionally, S2 was found to harbor an Arg-to-His mutation within a predicted peptidase. S3 was identical to S1, so it was eliminated from subsequent experiments.

cdi_0397, which we renamed *temperature-sensitive disulfide bond forming protein A* (*tsdA*), was identified during our initial search for MdbA (Chapter 3). However, deletion of this gene produced no defect in pilus assembly, so it was not studied further. q-RT PCR was used to analyze whether the T-to-G mutation altered TsdA expression levels. Whole cell RNA was collected from *C. diphtheriae* grown to log phase at 30°C, converted to cDNA, and analyzed for *tsdA* transcripts. Remarkably, *tsdA* expression in S1 and S2 was approximately 30-times higher than wild-type and the parental $\Delta mdbA$ mutant (Fig. 24B). The expression of *tsdA* was slightly higher in the $\Delta mdbA$ mutant than wild-type, but the difference was not significant. Protein levels of TsdA were also examined in these strains. Membrane fractions of *C. diphtheriae* were isolated, separated by SDS-PAGE, and immunoblotted with TsdA antibodies (α -TsdA). A faint band indicative of the 32 kDa protein was detected in the membrane of both the wild-type and $\Delta mdbA$ (Fig. 24D; lanes 1 and 3), but this signal was not detected in the *tsdA* deletion mutant (lane 2). In accordance with the qRT-PCR, significantly more TsdA was detected in S1 and S2 than wild-type, or $\Delta mdbA$. Altogether, the data demonstrate that *tsdA* is overexpressed in the suppressor mutants.

To test if the basal expression of *tsdA* can compensate for the loss of *mdbA* at 30°C, we attempted to generate a double deletion mutant. Unfortunately, multiple attempts to construct this strain were fruitless. As an alternative, we overexpressed *tsdA* in *C. diphtheriae* Δ *mdbA* by transforming the strain with a plasmid carrying an extra copy of the gene under control of an arabinose-inducible promoter (pAra-TsdA). SpaA polymerization was used as a marker to test for the restoration of disulfide bond formation. M and W fractions of *C. diphtheriae* were collected, separated by SDS-PAGE, and immunoblotted with α -SpaA. SpaA polymers were detected in both the M and W fractions of wild-type *C. diphtheriae* (Fig. 24; lanes 1 and 2). SpaA polymerization in the *tsdA* deletion strain was no different from wild-type (lanes 3 and 4). Pili were noticeably absent in the *mdbA* deletion mutant (lanes 5 and 6), but restored when the strain was transformed with pMdbA (lanes 7 and 8). Similar to the complemented strain, SpaA polymers were detected in the W fraction of Δ *mdbA* harboring pAra-TsdA (lanes 9 and 10). Altogether, these results show that *tsdA* overexpression is sufficient to restore disulfide bond formation in the *mdbA* mutant.

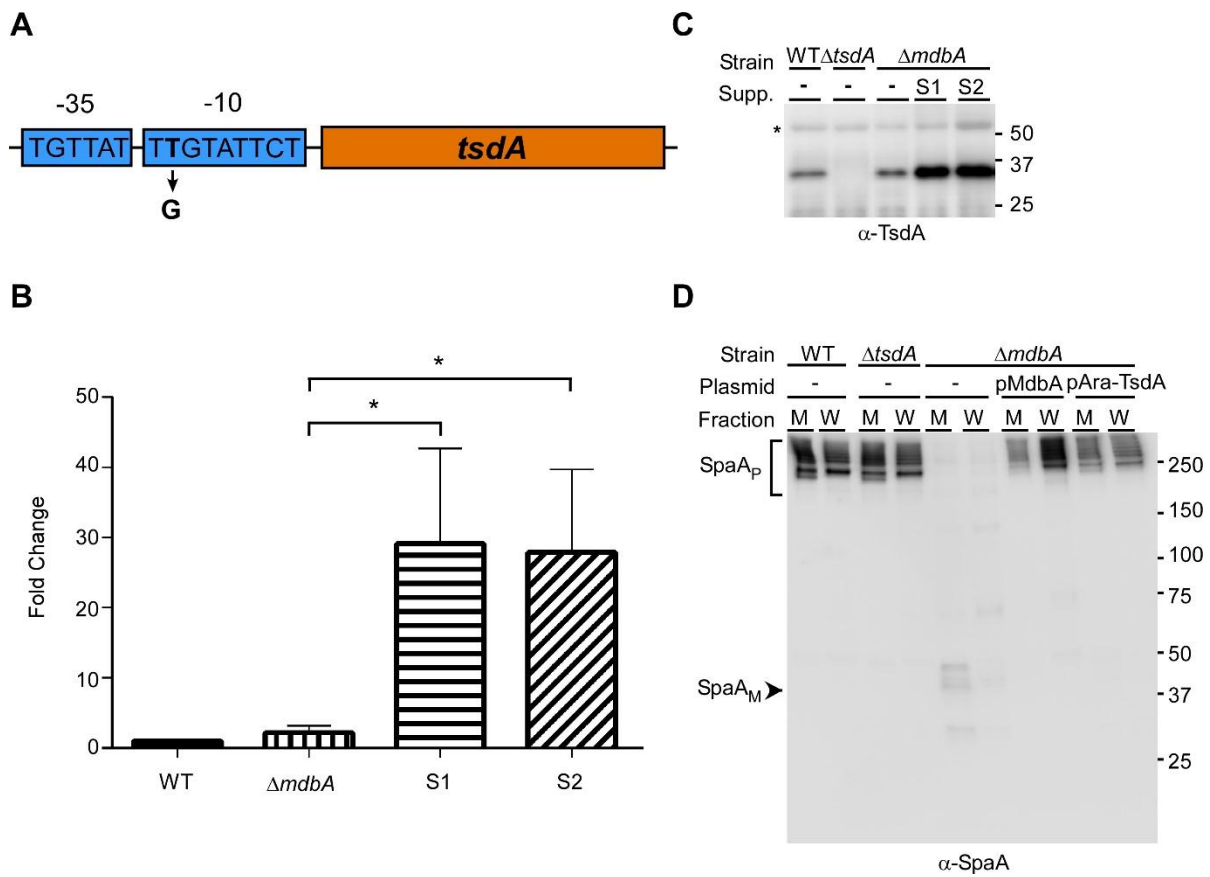


Figure 24. A point mutation within the *tsdA* promoter induces gene overexpression, which rescues disulfide bond formation in the *mdbA* mutant. (A) A representation of the point mutation identified in the *C. diphtheriae* $\Delta mdbA$ suppressor mutants S1, S2, and S3. A single T-to-G mutation was identified 29 bases upstream of the putative oxidoreductase *tsdA* near the predicted -10 box. **(B)** qRT-PCR was used to quantitate *tsdA* transcription. Expression levels were normalized to 16S rRNA levels, and the fold change values were calibrated against wild-type. An unpaired T-test was used to measure significance ($\Delta mdbA$ vs. S1 P-value = 0.0267; $\Delta mdbA$ vs. S2 P-value = 0.0196). **(C)** Membrane fractions were collected from *C. diphtheriae*, separated by SDS-PAGE, and immunoblotted with α -TsdA. A nonspecific band found at approximately 50 kDa was used as a loading control (*). **(D)**

Medium (M) and cell wall (W) fractions were isolated from *C. diphtheriae*, separated on a 4-20% Tris-Glycine gel, and immunoblotted with α -SpaA. Polymeric and monomeric forms of SpaA are indicated.

5.3 Discussion

Disulfide bonds are important for the stability and function of many secreted proteins. Within Gram-negative bacteria, these linkages are catalyzed in the extracellular periplasm by DsbA. Until recently, disulfide bond formation has been largely ignored in Gram-positive bacteria, which lack periplasmic compartments. Using the models *A. oris* and *C. diphtheriae*, we recently revealed that Gram-positive Actinobacteria rely on protein oxidation to help fold secreted proteins (Chapter 4). Disulfide bond formation is catalyzed in these organisms by the membrane-bound oxidoreductase MdbA. Similar to Gram-negative DsbA, MdbA is important for pathogenesis because it is required for the production of secreted virulence factors like adhesive pili and DT. However, unlike DsbA, MdbA is required for growth and division. We showed that *mdbA* mutants are associated with severe morphological division defects that may result from the failure to oxidize cell wall synthesis machinery like PBPs. Remarkably, stress induced by deleting *C. diphtheriae mdbA* selected for a suppressor mutation that caused the overexpression of another oxidoreductase called TsdA.

To study *A. oris mdbA in vivo*, we generated a depletion mutant by placing a plasmid-derived *mdbA* under control of an arabinose-inducible promoter. The depletion of *mdbA* was associated with cell chaining and bending (Fig. 19C) suggesting that MdbA is important for division. Eventually, we expected that depletion of *mdbA* would lead to death, but *A. oris* continued to divide. Western blotting revealed trace amounts of MdbA in the membrane even after prolonged incubation without arabinose (Fig. 19A). This suggested that the MdbA protein is stable, and/or the arabinose-controlled promoter is leaky. We reasoned that residual traces of MdbA may be enough to keep *A. oris* viable. In support of this, we failed to isolate colonies that had lost the plasmid carrying the only copy of *mdbA* after growing bacteria overnight without selective antibiotics (data not shown).

Since a *C. diphtheriae* *mdbA* deletion mutant was successfully generated, subsequent experiments focused on this bacterium. *C. diphtheriae* Δ *mdbA* exhibited a severe temperature-sensitive phenotype. The bacteria grew normally at 30°C, but became coccoid, chained, and eventually stopped dividing at 37°C (Fig. 20). It should be noted that the growth defect is not responsible for the pilus assembly phenotypes described in Chapter 4. Pilus assembly is dependent on peptidoglycan synthesis, so it is expected that non-growing *mdbA* mutants would not synthesize these factors at 37°C (Mandlik et al., 2008). However, all pilus assays were performed at the permissive temperature, so bacterial pathogenicity and growth were studied separately.

Peptidoglycan, a lattice of glycan and peptide chains, determines cell shape, and counters intracellular osmotic pressure to prevent lysis (Cava & de Pedro, 2014). The inhibition of cell wall synthesis or removal of existing peptidoglycan is known to convert rod-shaped *E. coli*, *C. glutamicum* and *Bacillus* into cocci (Botta & Buffa, 1981; Carballido-Lopez & Formstone, 2007; Tomasz, 1979; Valbuena et al., 2007). Based on this, we suspected that *C. diphtheriae* Δ *mdbA* morphological defects were caused by a compromise in cell wall synthesis. Using Van-FL staining, nascent cell wall was detected at apical sites of growth and division septa of wild-type cells, but found throughout the *mdbA* mutants (Fig. 21). Peptidoglycan is synthesized by a group of extracellular enzymes called PBPs, which are divided into two categories (Sobhanifar, King & Strynadka, 2013). High-molecular-weight (HMW) PBPs are transglycosylase and/or transpeptidase enzymes that polymerize the cell wall glycan backbone and cross-link peptide chains. LMW PBPs are typically D, D-carboxypeptidases that remove the terminal D-Ala residue from cross-linked peptide chains. A survey of the *C. diphtheriae* proteome revealed several PBPs, and most contained two or more Cys residues (Kanehisa & Goto, 2000) (Fig. 21B). We predicted that the *mdbA* mutant was defective in cell wall synthesis because the bacteria failed to form disulfide bonds

required for the stability and/or function of PBPs. Valbuena et al. (2007) investigated the role of HMW PBPs in *C. glutamicum* growth and division. Single deletions of most *pbp*-encoding genes produced no detectable defects, but phenotypes associated with double deletions were strikingly similar to *C. diphtheriae* $\Delta mdbA$. A double deletion of *C. diphtheriae* homologues *pbp1a* and *pbp1b*, or *pbp1a* and *pbp2a* induced cell rounding and inhibited septation. To test if HMW PBPs were compromised in the *mdbA* mutant, susceptibility to β -lactams ampicillin and penicillin, which specifically target PBPs, was assayed (Fig. 21C). $\Delta mdbA$ growth was inhibited at lower concentrations of antibiotics than wild-type. This result supports a role for MdbA in cell wall synthesis. Reduced levels of functional PBPs in the *mdbA* mutant could explain the temperature-sensitive phenotype. PBPs within the *C. diphtheriae* $\Delta mdbA$ may be sufficient to handle cell wall synthesis at slow-growth temperatures, but become overwhelmed by the high demand for peptidoglycan at 37°C.

Our findings reveal a major difference between Gram-negative and Gram-positive disulfide bond forming pathways. MdbA proteins are required for cell growth and division, while DsbA is not. Eukaryotic PDI is essential because of its dual function in oxidative protein folding; it forms new disulfide bonds, and reduces nonnative linkages (Laboissiere et al., 1995). Thus, it is possible that MdbA is more functionally similar to PDI than DsbA. The observation that MdbA is important for growth is not limited to *A. oris* and *C. diphtheriae*. An *M. tuberculosis* transposon library generated by Sasseti et al. (2003) revealed a low insertion frequency within *vkor* and an adjacent *dsbA*-like gene suggesting they are important for survival. In line with this, deletion of *M. tuberculosis* *vkor* confers a slow-growth phenotype (Dutton et al., 2008). Gram-negative DsbA is an attractive target for new antimicrobials because it is not required for growth (Heras et al., 2009). Inhibiting DsbA is proposed to lessen selective pressures that lead to mutation (Allen et al., 2014; Rasko & Sperandio,

2010). Although MdbA inhibitors would make powerful bactericides, we have shown that they would not be immune to bacterial resistance.

To test if inhibiting disulfide bond formation in *C. diphtheriae* could lead to mutation, we screened for $\Delta mdbA$ suppressors. Three strains capable of growing at the nonpermissive temperature were isolated, and found to harbor a single point mutation within the predicted -10 box of the *tsdA* promoter (Fig. 24). This mutation was found to increase the expression of *tsdA*, which restores disulfide bond formation in the *mdbA* mutant. It is not yet clear how the mutation alters *tsdA* expression, but we suspect it extends the -10 box to increase its basal level of transcription. Extended -10 boxes are alternative σ^{70} -type promoter elements commonly found in Gram-positive bacteria (Burns & Minchin, 1994; Haenni, Moreillon & Lazarevic, 2007; Helmann, 1995; Sabelnikov, Greenberg & Lacks, 1995). The extended -10 box, which features a TRTGNTAATAAT consensus sequence, harbors a TG dinucleotide that lowers the thermal energy required to form an open RNA polymerase initiation complex (Haenni et al., 2007). The -10 box within the wild-type *tsdA* promoter (TTTIGTATTCT) is similar to the extended sequence, but it lacks the TG dinucleotide. The T-to-G substitution within the promoter creates this TG element (TTTIGGTATTCT), which may induce higher levels of *tsdA* transcription.

Multiple attempts to delete *tsdA* from $\Delta mdbA$ were unsuccessful suggesting its basal expression is required to keep the *mdbA* mutants viable at 30°C. To test if overexpression of *tsdA* was sufficient to rescue disulfide bond formation, the parental *mdbA* mutant was transformed with a copy of *tsdA* under control of an arabinose-inducible promoter. Remarkably, this construct restored SpaA polymerization, an event shown to require disulfide bond formation (Fig. 24D). Due to the presence of a CxxC motif, it is most likely that TsdA is an oxidoreductase, but its role in wild-type *C. diphtheriae* is not known. Based upon the residues making up its CPFC motif, we predict it is probably not a disulfide bond forming

enzyme (Fig. 8). The oxidoreductases PDI, DsbA, and MdbA contain a His residue in their CxxC consensus sequences. This positively charged amino acid contributes to the high redox potential of these enzymes by stabilizing a negative charge that forms in the active site after catalysis (Grauschopf et al., 1995; Guddat et al., 1997). Since TsdA lacks this residue, it is possible that it is a disulfide bond isomerase that corrects misoxidized proteins (Fig. 25). If this is the case, the T-to-G promoter mutation may simply increase its basal level of transcription to allow it to compensate for *mdbA*. In support of this, overexpression of *dsbC* restores disulfide bond formation in an *E. coli dsbA* mutant (Missiakas et al., 1994). It is also possible that TsdA is a specialized oxidoreductase that is activated in response to stress. In this case, the T-to-G mutation may help to relieve repression of *tsdA*. Future studies will focus on elucidating TsdA function, as well as its possible regulatory pathway.

In summary, we have revealed that oxidative folding pathways are essential in Gram-positive Actinobacteria. Using *A. oris* and *C. diphtheriae* as models, we showed that mutations targeting *mdbA* cause severe morphological and division defects. These phenotypes likely result because division components like PBPs require MdbA for proper folding. Remarkably, the deletion of *C. diphtheriae mdbA* selected for a suppressor mutation that upregulated *tsdA* expression. The isolation of suppressor mutants emphasizes the need to consider bacterial back-up plans when disulfide bond formation is inhibited. Our results provide valuable insight for combating future mechanisms of resistance against disulfide bond formation inhibitors.

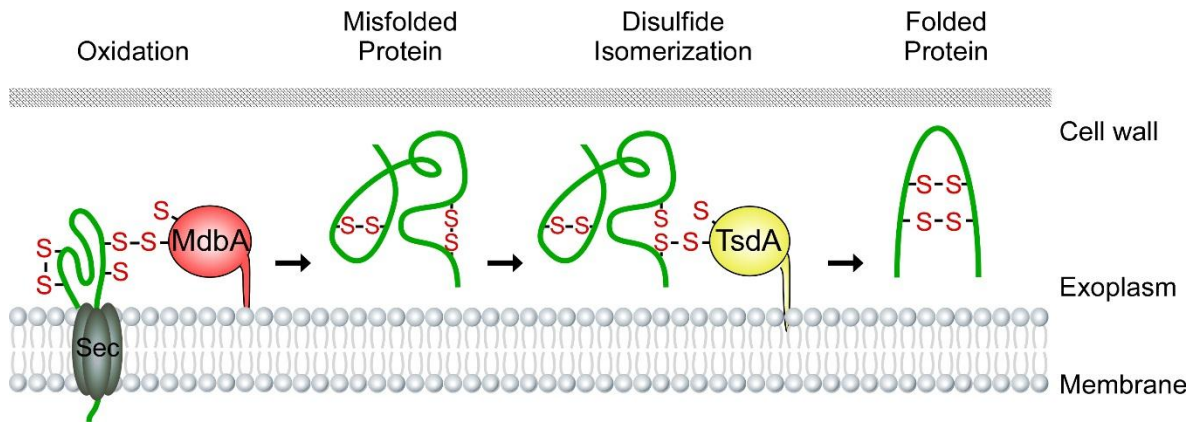


Figure 25. The role of TsdA in *C. diphtheriae*. TsdA is proposed to function as a disulfide bond isomerase that rearranges nonnative disulfide bonds formed by MdbA or the environment.

Chapter VI:

Discussion and future directions

6.1 Summary

Disulfide bonds result when neighboring Cys residues in a protein are oxidized. The formation of these covalent linkages, which is often required for the stability of secreted proteins, is an active process that occurs in extracellular compartments. In eukaryotic cells, disulfide bond formation is catalyzed in the ER by the chaperone PDI. In Gram-negative bacteria, Cys-oxidation is performed by DsbA in the periplasm. Unlike PDI, DsbA is not required for growth, but it is important for pathogenesis (Heras et al., 2009; Laboissiere et al., 1995; Vertommen et al., 2008). Bacteria that lack functional Dsb enzymes are often attenuated in virulence because factors required for host colonization, immunomodulation, and/or death become misfolded (Heras et al., 2009).

Oxidative protein folding in Gram-positive bacteria is less understood. The cell wall envelopes of these bacteria are comprised of a single membrane that is surrounded by peptidoglycan. Because peptidoglycan is porous, these organisms are not thought to possess enclosed compartments to fold secreted proteins. This presents a potential problem for the translocation of Cys-containing proteins. If the exoplasm is exposed to the extracellular milieu, proteins could suffer oxidative damage. Proteomic analyses have revealed that the majority of proteins secreted by Gram-positive Firmicutes contain one or no Cys residues suggesting that they do not rely on disulfide bond formation (Daniels et al., 2010; Dutton et al., 2008). Gram-positive Actinobacteria, however, appear to be exceptions. Unlike Firmicutes, proteins secreted by these bacteria are abundant in Cys residues, which strongly indicate they contain intramolecular disulfide bonds (Daniels et al., 2010; Dutton et al., 2008). However, to date, protein oxidation pathways have not been explored in these organisms.

This thesis elucidates disulfide bond forming pathways in Actinobacteria using the model substrates FimA and SpaA secreted by *A. oris* and *C. diphtheriae*, respectively. FimA and SpaA are major components of adhesive pili, which mediate adherence to host tissues,

induce inflammation, and promote biofilm formation (Mandlik et al., 2008). Pilin precursors are translocated to the exoplasm where they are folded, and then processed by sortase enzymes to form pili (Hendrickx et al., 2011). Recently, the crystal structures for FimA and SpaA were solved, and predicted that they form disulfide bonds (Kang et al., 2009; Mishra et al., 2011). Using an alkylation assay, we demonstrated that these linkages form *in vivo* (Chapter 3). Mutational analysis showed that FimA and SpaA are not polymerized when Cys residues are mutated to Ala. Instead, degradation products of FimA and SpaA were secreted into the culture media. Together, the data demonstrated that disulfide bonds are an important folding feature for these pilin proteins.

We proposed to use FimA and SpaA as models to study disulfide bond formation in Actinobacteria. Using a combination of bioinformatics and transposon mutagenesis, we identified *A. oris* MdbA and VKOR and *C. diphtheriae* MdbA. Protein disulfide bond formation is catalyzed in both bacteria by the membrane-bound MdbA (Chapter 4). MdbA oxidizes proteins by donating a disulfide bond found in a CxxC motif. In turn, it is reduced and requires re-oxidation by VKOR in *A. oris* and an unidentified protein (MdbB) in *C. diphtheriae*.

Since the majority of signal-peptide-containing proteins in *A. oris* (74%) and *C. diphtheriae* (60%) harbor two or more Cys residues, we predicted that MdbA targets multiple virulence factors (Chen, 2005; Kanehisa & Goto, 2000). DT, a secreted protein with two disulfide bonds, was chosen as an additional substrate to test this idea. In the *C. diphtheriae* *mdbA* mutant, we found that Cys residues within diphtheria toxin were reduced, and that the protein was heavily degraded. In line with this, the same deletion strain was attenuated in virulence in an animal model.

Unexpectedly, this work revealed that Actinobacterial MdbA is also required for growth (Chapter 5). The depletion of *A. oris* *mdbA* and deletion of *C. diphtheriae* *mdbA* was associated with abnormal morphological and division phenotypes. Due to the increased

susceptibility to β -lactam antibiotics and atypical Van-FL staining, it was suspected that *mdbA* deletion phenotypes were caused by a defect in peptidoglycan synthesis. The high number of Cys residues within PBPs hinted that cell wall machinery may require disulfide bonds. In the absence of *mdbA*, we predict that these factors misfold and, therefore, malfunction.

This was a surprising finding since disulfide bond forming enzymes are nonessential in Gram-negative bacteria (Bessette et al., 2001; Vertommen et al., 2008). *E. coli* DsbA is considered a valuable drug target because it is not required for viability, but is important for the folding of secreted virulence factors. Due to this characteristic, DsbA inhibitors are proposed to allow human hosts to clear infections without providing a selective pressure to mutate (Cascioferro et al., 2014; Cegelski et al., 2008). Efforts are ongoing to develop drugs that target disulfide bond forming pathways in the Gram-negative periplasm (Adams et al., 2014; Halili et al., 2015). This thesis presents evidence that anti-Dsb drugs may not be resistance-proof for all bacteria. To test if inhibiting protein oxidation in Actinobacteria could lead to resistance, we isolated *C. diphtheriae* $\Delta mdbA$ suppressor mutants (Chapter 5). Whole genome sequencing revealed that the suppressors harbored a T-to-G mutation within the promoter of a gene encoding an oxidoreductase called TsdA. This single nucleotide change resulted in TsdA overexpression, which restored disulfide bond formation in *C. diphtheriae* $\Delta mdbA$.

Due to the importance of MdbA for virulence and growth, we predicted that it serves a housekeeping role in disulfide bond formation. Therefore, TsdA is probably an accessory oxidoreductase. The enzyme could function as a disulfide isomerase that re-folds proteins with non-native Cys residues (Fig. 24). Similar to our results, the over-expression of *E. coli* DsbC, a known disulfide isomerase, was shown to restore oxidative protein folding in a *dsbA* mutant (Missiakas et al., 1995). Under normal conditions, we hypothesize that TsdA is repressed, or expressed at low levels. The consequences of a single point mutation within *C.*

diphtheriae $\Delta mdbA$ provide valuable insight for the development of antibacterial agents. Although inhibitors of Cys oxidation would make powerful bactericides, they would not be immune to natural selection. Similar to *C. diphtheriae*, other bacteria could activate alternative disulfide bond forming pathways to circumvent drugs targeting MdbA or other DsbA-like enzymes. To prevent or delay drug resistance, it is important to consider developing drugs that will target more than one disulfide bond forming factor. Given that *C. diphtheriae* can survive with MdbA or TsdA, this organism is an excellent model to study broad-spectrum disulfide bond inhibitors.

6.2 Disulfide bond formation in the Gram-positive exoplasm.

Due to the lack of an outer membrane, it is proposed that Gram-positive bacteria do not possess periplasmic compartments. Potentially, this makes the secretion of Cys-containing proteins hazardous due to the risk of aberrant oxidation. However, this thesis has revealed that disulfide bonds are important folding features for proteins secreted by Actinobacteria like *A. oris* and *C. diphtheriae*. How do these Gram-positive models catalyze oxidative folding without enclosed extracellular compartments?

The argument that Gram-positives do not possess periplasmic spaces may not be valid. Corynebacteria, including *C. diphtheriae* and *M. tuberculosis*, exhibit unique cell envelope architectures. Their peptidoglycan is cross-linked to arabinogalactan, which is esterified by mycolic acid. Mycolic acid, a type of long chain fatty acid, forms a hydrophobic surface layer that is visible by thin section EM (Bayan et al., 2003). Interestingly, this layer contributes to the high impermeability of Corynebacteria, and forms liposomes when cells are treated with detergent (Bayan et al., 2003; Puech et al., 2001). These fatty acids are proposed to form a so-called mycomembrane, which may be analogous to the Gram-negative

outer membrane. Therefore, it is possible that some Gram-positives contain protected compartments to regulate protein oxidation (Fig. 25A).

Although *A. oris* does not produce mycolic acid, it must have evolved to successfully oxidize proteins since 74% of its predicted secretome contains two or more Cys residues (Chen et al., 2005). It is possible that *A. oris* possesses an outer lipid layer that has not been characterized. Its environment may also contribute to disulfide bond formation in the exoplasm. *A. oris*, a pioneer colonizer of the oral cavity, comprises the anaerobic layers of mature biofilm (Kolenbrander, 2000; Kolenbrander et al., 2006). The lack of oxygen within its niche may help avoid environmentally-induced protein oxidation (Fig. 25B). However, this does not explain how *A. oris* survives during the early stages of biofilm development when exposure to oxygen is high. To alleviate oxidative stress, *A. oris* secretes catalase, and benefits from other antioxidants released by oral co-colonizers. For example, co-aggregation between *A. oris* and *S. gordonii* induces the oral cocci to synthesize arginine (Jakubovics et al., 2008). Together, these compounds may provide some protection against oxidative damage until an anaerobic environment within the biofilm is established.

Finally, Actinobacteria could avoid random oxidation by coordinating translocation with extracellular folding. Specialized zones of secretion have been identified in multiple Gram-positive bacteria including *S. pyogenes*, *Enterococci faecalis*, and *C. diphtheriae* (Guttilla et al., 2009; Kline et al., 2009; Rosch & Caparon, 2005). For example, the Sec machinery was shown to co-localize with sortase and pilus subunits (Guttilla et al., 2009; Kline et al., 2009). In *C. diphtheriae*, pilus subunits devoid of their C-terminal membrane anchor were found to be incorporated into pilus structures (Chang et al., 2011). Since the membrane anchor is required to prevent the release of pilins into the culture medium, it was expected that these mutants would not be polymerized by sortase. However, since they were polymerized, this strongly suggested that sortase processes its substrates during or immediately following

translocation (Chang et al., 2011). Thus, *C. diphtheriae* secretion and pilus assembly may be coupled.

Ideally, sortase would not process FimA and SpaA until they are folded. Since disulfide bonds are required for the stability of these proteins (Chapter 3), we predict that they are processed by MdbA prior to interacting with sortase. Therefore, if sortase is co-localized with the SecYEG translocon, MdbA must be also. The coupling of secretion with protein folding could be an adaptation for secreting proteins into unfavorable environments (Fig. 25C). The ability of MdbA to oxidize substrates as they emerge from the cytoplasm may increase the likelihood that disulfide bonds are catalyzed by cellular machinery, rather than the extracellular milieu.

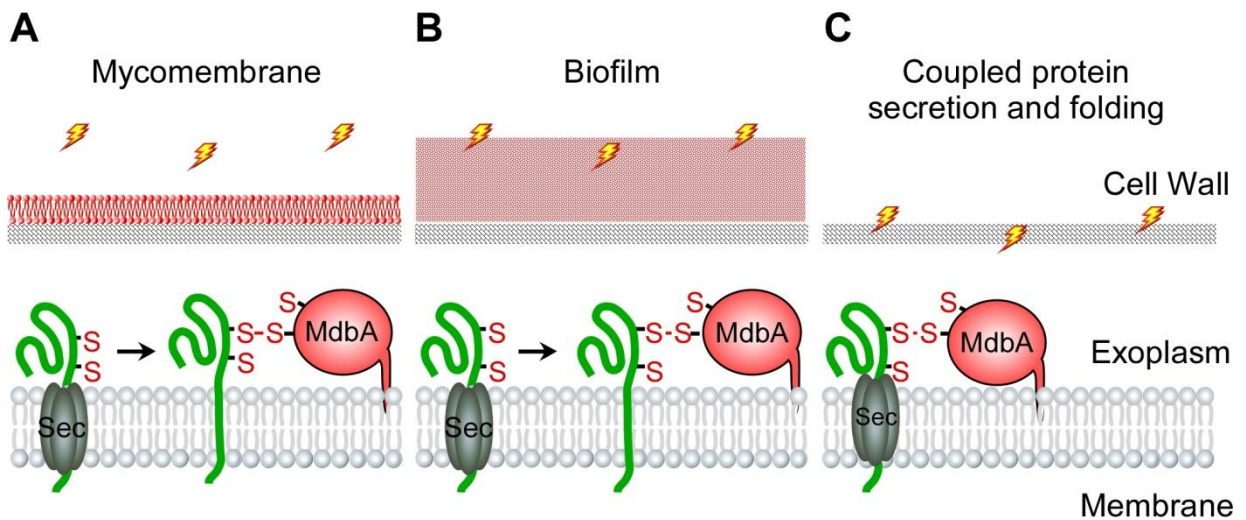


Figure 26. Disulfide bond formation in the Gram-positive exoplasm. Three mechanisms for catalyzing disulfide bond formation in the Gram-positive exoplasm are proposed **(A)** Similar to Gram-negative bacteria, Gram-positives may possess an enclosed extracellular compartment to regulate the folding and maturation of secreted proteins. Actinobacteria including *C. diphtheriae* and *M. tuberculosis* synthesize mycolic acid, which is incorporated into the cell envelope. This lipid layer may form a barrier between the exoplasm and extracellular milieu (symbolized by lightning bolts). **(B)** Bacterial co-colonizers and the extracellular matrix that comprise the oral biofilm may protect proteins secreted by *A. oris* from oxidative stress. **(C)** Actinobacteria may couple secretion and folding events to protect Cys-containing proteins from aberrant oxidation within an exoplasm that is exposed to the extracellular milieu.

6.3 The importance of disulfide bond forming pathways for Actinobacteria and Gram-negative bacteria.

This work revealed a significant difference between disulfide bond forming enzymes expressed by Gram-negative bacteria and Actinobacteria. *E. coli* Dsb proteins are nonessential, while MdbA is required for growth and division (Chapter 5). When grown at the nonpermissive temperature, *C. diphtheriae* *mdbA* mutants became spherical, formed chains, and eventually stopped dividing. These phenotypes indicated a defect in cell wall synthesis (Valbuena et al., 2007). PBPs secreted by *C. diphtheriae* harbor multiple Cys residues, which suggest they contain disulfide bonds. We hypothesized that the failure of the *mdbA* mutant to catalyze these linkages in growth factors caused the observed defects.

Since *E. coli* PBPs are secreted proteins, it was possible that they contained disulfide bonds. Why, then, are *E. coli* *dsbA* mutants not associated with growth defects? A survey of 12 known PBPs expressed by *E. coli* revealed that half harbored one or no Cys residues (Zhou & Rudd, 2013). This indicated that the majority of these factors may not require oxidative protein folding. Exceptions included PBP1a and PBP1b, which are vital for the insertion of new peptidoglycan during cell growth. A double deletion of *E. coli* *pbp1a* and *pbp1b* is lethal (Denome et al., 1999). Since disulfide bonds are important for proper protein folding, it was logical to assume that both PBP1a and PBP1b would misfold in the *dsbA* mutant leading to cell death. However, Chalut et al. (1999) found that disrupting disulfide bond formation within PBP1b did not affect its activity. This implies that (at least) one essential PBP would be functional in the absence of *dsbA*. Therefore, *dsbA* mutant phenotypes would not mimic the conditionally lethal *pbp1a pbp1b* deletion. Unlike Actinobacteria, the data suggests that protein oxidation and cell wall synthesis are not linked in Gram-negative bacteria like *E. coli*.

E. coli LptD is another essential protein that contains disulfide bonds, but does not require Dsb proteins. Together with the accessory protein LptE, LptD forms a channel that transports newly synthesized lipopolysaccharide (LPS) to the outer leaflet of the outer membrane (Whitfield & Trent, 2014). LptD harbors two nonconsecutive disulfide bonds (C31-C724 and C173-C725) that position its N- and C-termini in the periplasm (Ruiz et al., 2010). LptD function is dependent of the formation of only one of these linkages, which suggests they are redundant (Ruiz et al., 2010). This may serve as a failsafe for LptD folding when Dsb pathways are inhibited. Random oxidation could be sufficient to induce the formation of at least one disulfide bond to keep *E. coli* viable. However, this may not be the case because LptD folding appears to be independent of Dsb machinery. Chng et al. (2012) showed that DsbA targets LptD, but catalyzes nonnative Cys linkages. Normally, this is corrected by DsbC, but LptD function is not defective in a *dsbC* mutant. It turns out that the Cys linkages within the translocon become rearranged when it binds to LptE, a protein with no known oxidoreductase activity. Importantly, the DsbA-catalyzed nonnative disulfide bonds may not represent a folding intermediate since reduced LptD still interacts with LptE (Ruiz et al., 2010). Because *dsbA* and *dsbC* do not appear to be required for LptD oxidation, it is possible that its interaction with LptE is sufficient to induce disulfide bond formation.

Both Actinobacteria and Gram-negative organisms use disulfide bond formation to help fold secreted proteins. However, only Actinobacteria require oxidative folding for proper growth and division. It is remarkable that multiple virulence factors misfold in an *E. coli dsbA* mutant, but essential proteins do not (Chalut, Remy & Masson, 1999; Chng et al., 2012b; Heras et al., 2009). Gram-negatives are proposed to be descendants of a Gram-positive ancestor (Koch, 2003). It is possible that disulfide bond formation was once essential in this ancestor, and remained so in Actinobacterial descendants. However, over the course of

evolution, Gram-negatives may have fine-tuned disulfide bond forming pathways to be important for competitive fitness (i.e. pathogenesis), but not essential for growth and division.

6.4 Disulfide bond forming pathways in Actinobacteria and Firmicutes.

Bioinformatics analysis revealed a trend in which the majority of proteins secreted by Gram-positive Actinobacteria contained multiple Cys residues, while Firmicutes tended to exclude the residue altogether (Daniels et al., 2010; Dutton et al., 2008). This suggested that disulfide bond formation is an important protein folding pathway for the first, but not the latter. The thesis demonstrates that Actinobacteria rely on thiol oxidation to help fold secreted proteins *in vivo* (Chapter 4). Why do Firmicutes seem to lack similar pathways?

It is not that Firmicutes cannot use thiol oxidation to help fold proteins. Although proteins secreted with disulfide bonds are relatively rare, instances have been identified in *B. subtilis* and *S. aureus*. *B. subtilis* encodes four known oxidoreductases (*bdbA-D*) that are arranged in operons with known targets. *bdbA* and *bdbB* cluster with a gene encoding the lantibiotic sublancin, which contains two disulfide bonds (Dorenbos et al., 2002). The additional oxidoreductases *bdbC* and *bdbD* are located in a DNA competence operon. These enzymes are required for the formation of the ComCG pseudopilus, which also has disulfide bonds (Meima et al., 2002). In *S. aureus*, a single DsbA-like enzyme (SaDsbA) has been identified, but its only known substrate is also the ComCG pseudopilus (van der Kooi-Pol et al., 2012). Curiously, a DsbB-like protein for the re-oxidation of SaDsbA has not been identified (Dutton et al., 2008).

B. subtilis and *S. aureus* mutants lacking oxidoreductase enzymes are still viable (Kouwen et al., 2007; van der Kooi-Pol et al., 2012). Unlike Actinobacteria, this indicates that their Dsb-like proteins do not play a housekeeping role in protein folding. Since Dsb-like enzymes are arranged in gene operons, they probably specialize in oxidizing specific

substrates. However, *B. subtilis* and *S. aureus* oxidoreductases may not be strictly limited as they have been shown to target exogenous substrates. *E. coli* PhoA, which contains two disulfide bonds, is properly oxidized when it is secreted from *B. subtilis* (Kouwen et al., 2007). Furthermore, SaDsbA expressed in *B. subtilis* devoid of *bdbA-D* is sufficient to oxidize PhoA, sublancin, and the ComCG pseudopilus (Kouwen et al., 2007).

Altogether, these findings suggest that Firmicutes are capable of catalyzing general oxidative folding, but do not normally do so. Simply, this may be because they lack the available substrates. Oxidative folding pathways in *E. coli* appear to be nonessential because Dsb enzymes are not required for disulfide bond formation in essential proteins (i.e. PBPs and LptD). Over the course of evolution, Firmicutes may have excluded Cys residues from secreted proteins to avoid the consequences of random protein oxidation. Therefore, if they are not used for other purposes, it makes sense to cluster *bdbA-D* with their substrates. A lack of substrates could also explain why SaDsbA does not appear to possess a DsbB-like redox partner. Disulfide bond forming enzymes like *E. coli* DsbA exhibit high redox potentials to ensure that disulfide bonds are transferred from the enzyme to substrates (Grauschopf et al., 1995). Following catalysis, DsbA is converted to a reduced state that is extremely stable, so it requires re-oxidation by DsbB (Bardwell et al., 1993; Missiakas et al., 1993). *In vitro* studies have revealed that SaDsbA's redox potential is low enough to allow it to self-re-oxidize (Heras et al., 2008). If disulfide bond forming pathways are not vital for *S. aureus*, it may not be necessary for a DsbB-like protein to maintain SaDsbA in an active state. Over the course of evolution, we speculate that Actinobacteria adapted to the challenges of extracellular protein oxidation, while Firmicutes have largely eliminated the need for it.

6.5 Future directions

6.5.1 Elucidate a complete disulfide bond forming pathway in *C. diphtheriae*.

This thesis has revealed that Actinobacteria *A. oris* and *C. diphtheriae* use disulfide bond formation to help fold secreted proteins. In both organisms, Cys residues are oxidized by MdbA. Following catalysis, *A. oris* is re-oxidized by VKOR, which probably shuttles acquired electrons to the electron transport chain (Kadokura & Beckwith, 2010). The redox partner for *C. diphtheriae* MdbA has not been identified. Several oxidoreductase candidates were identified in *C. diphtheriae* (Chapter 3). We expected that the deletion of *mdbA* and its redox partner would result in identical pilus assembly phenotypes. However, only the deletion of *mdbA* produced a detectable defect (Chapter 4). We suspected that MdbA may have multiple redox partners, but double deletions constructed thus far have also failed to reveal any phenotype (data not shown). Since MdbA is important for growth and division, it is possible that its active state is maintained by multiple oxidoreductases. This could be explored by generating more extensive combinations of deletions. Since homology between redox-active enzymes is generally low, it is also possible that our search parameters were not sufficient to identify this factor by BLAST analysis. We could expand this search by including other redox-active enzymes including known Dsb-like proteins from other bacteria, peroxidases, and glutathione reductases.

Finally, *E. coli* DsbA has been engineered to trap reaction intermediates with both DsbB and its substrates (Inaba et al., 2009; Kadokura et al., 2004). Similar mutations could be generated in *C. diphtheriae* to trap MdbA's redox partner. Since MdbA is predicted to target multiple substrates, one problem with this approach is that it might be difficult to enrich MdbA-redox partner complexes. Assuming that MdbA and this factor are co-localized, a cross-linker may be more effective in identifying this factor.

6.5.2 Identify essential substrates of *C. diphtheriae* MdbA.

The *C. diphtheriae* *mdbA* mutant exhibits a severe growth defect at the nonpermissive temperature (Chapter 5). To elucidate the importance of MdbA for cell viability, we must identify its essential substrate(s). It is hypothesized that secreted PBPs require MdbA for disulfide bond formation. In the absence of *mdbA*, PBPs may malfunction, which would lead to the observed defects. First, disulfide bond formation within PBPs will need to be confirmed using an alkylation assay. Next, since the levels of detectable SpaA and DT are reduced in the *mdbA* mutant, the same would be expected for PBPs. PBP levels in the parental and mutant can be compared using Boc-FL, a fluorescent derivative of penicillin (Valbuena et al., 2007).

6.5.3 Determine the function of TsdA in wild-type conditions.

The overexpression of *tsdA* in the *C. diphtheriae* *mdbA* mutant rescues disulfide bond formation. What is the function of this factor under normal conditions? The deletion of *tsdA* produced no pilus assembly defect. Therefore, it is probably an accessory oxidoreductase that is normally expressed at low levels, or activated under stress conditions. Measuring its redox potential *in vitro* could provide some functional insight (Grauschopf et al., 1995). If TsdA is an isomerase, we would expect its redox potential to be lower than disulfide bond forming proteins like *E. coli* DsbA or MdbA. Finally, to test if TsdA is required to reverse aberrant Cys oxidation, the ability of a deletion mutant to grow in the presence of oxidative stress-inducing compounds like hydrogen peroxide or copper can be tested (Hiniker et al., 2005).

6.6 Overall Conclusions.

This thesis has revealed disulfide bond forming machinery in the Actinobacteria *A. oris* and *C. diphtheriae*. To our knowledge, we are the first to elucidate general oxidative folding

pathways in Gram-positive bacteria. In both organisms, MdbA is required for disulfide bond formation in secreted proteins important for virulence and growth. Due to its apparent housekeeping function in protein folding, MdbA inhibitors would make powerful bactericides against important Actinobacterial pathogens. *C. diphtheriae*, in particular, is an excellent model to study potential inhibitors *in vivo*, as well as mechanisms of resistance.

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Vita

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