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Thioredoxin-like proteins in F and other plasmid systems

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Abstract

Bacterial conjugation is the process by which a conjugative plasmid transfers from donor to recipient bacterium. During this process, single-stranded plasmid DNA is actively and specifically transported from the cytoplasm of the donor, through a large membrane-spanning assembly known as the pore complex, and into the cytoplasm of the recipient. In Gram negative bacteria, construction of the pore requires localization of a subset of structural and catalytically active proteins to the bacterial periplasm. Unlike the cytoplasm, the periplasm contains proteins that promote disulfide bond formation within or between cysteine-containing proteins. To ensure proper protein folding and assembly, bacteria employ periplasmic redox systems for thiol oxidation, disulfide bond/sulfenic acid reduction, and disulfide bond isomerization. Recent data suggest that plasmid-based proteins belonging to the disulfide bond formation family play an integral role in the conjugative process by serving as mediators in folding and/or assembly of pore complex proteins. Here we report the identification of 165 thioredoxin-like family members across 89 different plasmid systems. Using phylogenetic analysis, all but nine family members were categorized into thioredoxin-like subfamilies. In addition, we discuss the diversity, conservation, and putative roles of thioredoxin-like proteins in plasmid systems, which include homologs of DsbA, DsbB, DsbC, DsbD, DsbG, and CcmG from *Escherichia coli*, TlpA from *Bradyrhizobium japonicum*, Com1 from *Coxiella burnetii*, as well as TrbB and TraF from plasmid F, and the absolute conservation of a disulfide isomerase in plasmids containing homologs of the transfer proteins TraH, TraN, and TraU.

Keywords

plasmid; thioredoxin; oxidase; reductase; isomerase; disulfide

1. Introduction

1.1 The mechanism of bacterial conjugation

Self-transmissible conjugative plasmids encode nearly all of the machinery required to promote their horizontal transfer. The much-studied F plasmid is representative of conjugative plasmids. Like all conjugative plasmids, F encodes an array of proteins that combine to carry out DNA transfer. The transfer (*tra*) region of F plasmid encodes proteins

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that assemble into the pore complex, aid in the assembly of the pore complex, or play a direct role in the transfer of plasmid DNA (Firth 1996, Frost 1994).

For F and many other plasmids, bacterial conjugation initiates when a bacterium harboring a conjugative plasmid (donor) extends filamentous structures known as pili into the extracellular environment. When one of these pili contacts a non-plasmid-containing bacterium (recipient), the pilus retracts via disassembly, bringing the bacteria together (Curtiss 1969, Firth 1996, Helmuth 1978, Novotny 1968, Ou 1970). After retraction of the pilus, the donor and recipient bacterium form a stable mating pair, and at this point the plasmid DNA is transferred (Achtman 1975, Achtman 1977). Concurrent with pilus extension is the formation of a relaxase-ssDNA complex (Zechner 2000). After formation of the stable mating pair, the relaxase-ssDNA complex is delivered to the pore complex where it is translocated through the pore in an ATP-dependent manner (Gomis-Rüth 2002). After transport into the recipient bacterium, the relaxase ligates the linear, single-stranded copy of plasmid DNA (Dostál 2011, Sherman 1994). With the culmination of second strand synthesis, the recipient cell is considered F positive, as it can now act as a donor in subsequent rounds of bacterial conjugation. For additional information on these topics, the reader is directed to reviews from Firth (Firth 1996), Lawley (Lawley 2003), and Zechner (Zechner 2012).

1.2 Periplasmic transfer proteins

The *tra* operon of plasmid F encodes 35 different proteins. Ten of these *tra* genes encode proteins that either fully or partially reside in the bacterial periplasm, several of which contain multiple cysteine residues (Table 1). The *E. coli* periplasm is home to an array of enzymes that promotes the formation of inter- and intramolecular disulfide linkages. For proteins containing large numbers of cysteines, an enormous number of redox states are theoretically possible (Benham 1993, Cantor 1980, Kauzmann 1959, Sela 1959). We have calculated the theoretical numbers of redox states and cysteine connectivities for F-encoded periplasmic *tra* proteins that reside, at least partially, in the periplasm (Table 1). Although these calculations give insight into theoretical complexities, the constraints imposed by the conformations of the folded or folding protein effectively reduces the actual number of redox states a protein can adopt (Bardwell 1993, Martin 1993, Wunderlich 1993). Nevertheless, the conservation of periplasmic redox systems highlights the importance of thioredoxin-like proteins to both the folding and maintenance of disulfide bond-containing proteins in the periplasm.

1.3 Periplasmic redox systems

Gram negative bacteria can express a number of thioredoxin (trx)-like family members in their periplasm (Table 2). These proteins, collectively known as thiol-oxidoreductases, contain a thioredoxin-like fold and a C-X-X-C (C = Cys, X = any residue) redox active site that is responsible for performing specific redox chemistries on protein substrates. One subset of these proteins, the disulfide bond formation (Dsb) family, can be grouped into two primary functional groups (Figure 1). The first of these, the DsbB-dependent family, is responsible for the *de novo* synthesis of disulfide bonds through direct oxidation of free thiols (Darby 1995, Hiniker 2003, Nakamoto 2004). The second group, the DsbD-dependent family, is responsible for the shuffling of incorrect disulfide bonds in protein substrates (DsbC) or maintenance of single unpaired cysteines (DsbG) (Andersen 1997, Bessette 1999, Depuydt 2009, Missiakas 1994, Rietsch 1996, Shevchik 1994). The distinguishing characteristic of these families is their dependence on one of a pair of inner membrane proteins (DsbB or DsbD) for proper maintenance of their redox states (Bader 1999, Bessette 1999, Rietsch 1997).

1.3.1 De novo disulfide bond formation—As a protein containing an N-terminal signal peptide is secreted into the periplasm, DsbA, a 21 kDa monomeric thioredoxin-like family member that is active in its oxidized state (Figure 2), incorporates disulfide bonds between consecutive cysteine residues in the unfolded or partially folded protein substrate (Bardwell 1993, Martin 1993, Wunderlich 1993). In isolated cases, however, DsbA has been shown to promote proper folding of proteins containing nonconsecutive disulfide bonds, as exemplified in studies utilizing RNase I (Messens 2007). As DsbA oxidizes its substrate, the C-P-H-C (C = Cys, P = Pro, H = His) active site of DsbA is reduced, rendering it inactive. To reoxidize its active site, DsbA donates electrons to the inner membrane protein DsbB (Figure 1) (Bardwell 1993). DsbB is a 20 kDa monomeric protein with four membrane-spanning regions that each contain an essential cysteine (C41, C44, C104, and C130) positioned in the periplasmic space (Inaba 2006, Malojci 2008, Zhou 2008). DsbB accepts electrons from the DsbA active site and donates them to electron acceptors such as ubiquinone (aerobic conditions) or menaquinone (anaerobic conditions) in the inner membrane (Figure 1). Ubiquinone is then oxidized by cytochrome terminal oxidases within the electron transport chain while menaquinone transfers electrons to alternate final electron acceptors such as fumarate or nitrate (Bader 1999, Kobayashi 1997, Kobayashi 1999, Regeimbal 2002). After resolution of the DsbA-DsbB complex, the C-X-X-C active site of DsbA is oxidized, resulting in a recharged and enzymatically functional protein (Bardwell 1993).

1.3.2 Disulfide bond isomerization—There are estimated to be over 300 different *E. coli* proteins that localize to the periplasm (Dutton 2008, Hiniker 2004). Of these proteins, only a few, including RNase I, MepA, AppA, and End1, have been found to require a non-consecutive disulfide bond for proper folding and activity (Berkmen 2005, Hiniker 2004). DsbA-catalyzed disulfide bond formation can trap these proteins in non-native states. Additionally, random oxidation events and environmental stresses can also cause errant bond formation in cysteine-containing periplasmic proteins (Hiniker 2005). To resolve these incorrect bonds, bacteria utilize a secondary pathway composed of the periplasmic disulfide isomerase DsbC and the inner membrane protein DsbD to shuffle disulfide bonds (Berkmen 2005, Hiniker 2004, Rietsch 1996, Shevchik 1994, Zapun 1995).

DsbC, 23 kDa as a monomer, functions as a homodimer (Missiakas 1994, Shevchik 1994). Dimerization results in the formation of a V-shaped binding pocket lined with hydrophobic and uncharged residues (Figure 3) (Banaszak 2004, McCarthy 2000). DsbC is thought to recognize misfolded protein substrates through exposed hydrophobic patches on the protein surface (Darby 1998). Using this method of substrate recognition, DsbC can also promote protein folding as a molecular chaperone for non-cysteine containing proteins (Chen 1999). Each monomer of DsbC contains a C-G-Y-C (C = Cys, G = Gly, Y = Tyr) redox active site, in principle allowing the homodimer to catalyze multiple rounds of disulfide isomerization before becoming fully oxidized. In addition to disulfide isomerase and chaperone activity, dimeric DsbC was also shown to promote substrate reduction at levels similar to TrxA *in vitro* (Zhao 2003). Interestingly, experiments performed with a single amino acid substitution, DsbC G49R, which disrupts dimerization via electrostatic repulsion in the N-terminal dimerization domain (Bader 2001), or with a truncation mutant lacking a dimerization domain (Sun 2000) reveal that monomeric DsbC retains its disulfide reductase activity, but can no longer catalyze disulfide bond isomerization or function as a molecular chaperone.

After oxidation of its redox active site, DsbC interacts with and directly accepts electrons from the inner membrane protein DsbD (Figure 1) (Bessette 1999, Missiakas 1995, Rietsch 1997). DsbD is a 59 kDa monomeric inner membrane protein that consists of three domains: an N-terminal periplasmic α domain (DsbD α) (Haebel 2002), an inner membrane spanning

central β domain (DsbD β) that possesses 8 transmembrane regions (Cho 2007), and a C-terminal periplasmic γ domain (DsbD γ) (Rozhkova 2004). DsbD accepts electrons from cytoplasmic thioredoxin, and shuttles them sequentially through a well-defined cascade that includes cysteine pairs within each of its three domains. Electrons from cytoplasmic thioredoxin are initially passed to a cysteine pair in DsbD β , then to DsbD γ , and finally to DsbD α , where they are directly exchanged with DsbC after formation of a transient, but specific, DsbC/DsbD complex (Cho 2007, Katzen 2000, Katzen 2003, Stewart 1999). A recent bioinformatics analysis has revealed that the DsbD-like family can be further subdivided into three classes, which include DsbD-like proteins, CcdA-like proteins, and ScsB-like proteins (Cho 2012a, Cho 2012b). In comparison to the DsbD-like proteins described above, the N-terminal alpha domain of ScsB-like proteins is considerably different. As the N-terminal alpha domain facilitates substrate interactions, this difference suggests that although functionally similar, DsbD-like and ScsB-like families possess unique sets of substrates. Alternatively, CcdA-like proteins differ significantly from both their DsbD-like and ScsB-like relatives, lacking both the alpha and gamma periplasmic domains and possess only six transmembrane regions in comparison to the eight transmembrane regions found in the DsbD-like and ScsB-like families (Cho 2012b).

1.3.3 Maintenance of free thiols—DsbD is also responsible for the redox maintenance of the periplasmic thioredoxin-like family member DsbG. DsbG is a 26 kDa monomer and, like DsbC, exists as a functional V-shaped homodimer (Figure 3) (Heras 2004). DsbG has a C-P-Y-C (C = Cys, P = Pro, Y = Tyr) redox active site in each of its monomers, both of which are catalytically functional. DsbG was originally proposed to be a secondary disulfide isomerase in bacterial systems due to its ability to catalyze disulfide isomerization *in vitro*, albeit at lower rates than DsbC (Andersen 1997, Bessette 1999, Shao 2000). Structural comparisons of DsbC and DsbG revealed significant differences in their V-shaped binding pockets (Figure 3) (Heras 2004). While the binding pocket of DsbC is lined with uncharged and hydrophobic residues, the pocket of DsbG, which is wider than that of DsbC, contains several acidic residues, suggesting that DsbG is more likely to interact with folded protein substrates. Recent studies by Depuydt, *et al.*, (Depuydt 2009) revealed that DsbG preferentially interacts with the periplasmic proteins YbiS, ErfK, and YnhG, each of which are periplasmic L,D-transpeptidases containing a single unpaired cysteine in their native structure. *In vivo*, unpaired cysteines can be oxidized by radical oxidative species to form sulfenic acids (-SOH), while further oxidation leads to irreversible formation of sulfinic (-SO₂H) and/or sulfonic (-SO₃H) acids (Hamann 2002, Poole 2004). Depuydt, *et al.* were able to convincingly demonstrate that while DsbG can act as a disulfide bond isomerase, its primary responsibility is to maintain the reduced state of free thiols within the bacterial periplasm.

1.4 Cytochrome maturation proteins

A secondary set of thioredoxin-like proteins in the bacterial periplasm function in cytochrome maturation. Although cytochrome maturation and disulfide bond formation and maintenance are two distinct processes, both appear to rely on the same inner membrane proteins for redox state maintenance (Katzen 2000).

CcmG is a 21 kDa inner-membrane associated periplasmic thioredoxin-like protein that belongs to the cytochrome c maturation (Ccm) family within the cytochrome c biogenesis system I in *E. coli* (Figure 2) (Fabianek 1998). This system is responsible for the posttranslational attachment of heme to the C-X-X-C-H (C = Cys, X = any residue, H = His) motif of apocytochrome c in the bacterial periplasm (Stevens 2011). Although the specific details of the pathway for heme attachment are unclear, previous studies have demonstrated that CcmG: (1) exhibits specific reductase activity within the cytochrome c biogenesis

system (Fabianek 1998, Fabianek 1999), (2) is maintained in its reduced state by the periplasmic redox system protein DsbD (Katzen 2000, Stirnimann 2005), and (3) is able to associate with apocytochrome c in a cysteine-independent manner (Turkarslan 2008). In one model, CcmG directly reduces the C-X-X-C-H motif in apocytochrome c, effectively preparing it for heme attachment (Edeling 2002). In an alternative model, a second Ccm family member, CcmH, acts as an intermediate. In this model, CcmG reduces the C-X-X-C motif of CcmH, which then reduces apocytochrome c (Di Matteo 2007, Meyer 2005). Although CcmH contains a C-X-X-C motif, it lacks a thioredoxin-like fold, and therefore will not be discussed further in this review.

TlpA is a 23 kDa, monomeric, inner-membrane-anchored thioredoxin-like protein localized to the periplasm (Figure 2). TlpA was first discovered due to its role in the maturation of cytochrome aa3 in *Bradyrhizobium japonicum* (Loferer 1993, Loferer 1995b). More recently, TlpA has been associated with both the biogenesis of cytochrome c and the bacterial oxidative stress response in *Agrobacterium tumefaciens* (Tanboon 2009). Unlike CcmG, whose reductase activity is specific for the cytochrome maturation system, TlpA is able to directly reduce disulfide bonds in insulin *in vitro* (Loferer 1995a). Although the precise role and/or molecular targets of TlpA in cytochrome maturation are unknown, biophysical characterization has led to the hypothesis that like CcmG, TlpA is acting as a disulfide reductase (Loferer 1995).

1.5 The Com1-like family

Com1 of the rickettsial pathogen *Coxiella burnetii* is a 27 kDa, outer-membrane-associated thioredoxin-like family member (Hendrix 1993). Initially, Com1-like proteins were considered to be a prototype for a subgroup of the DsbA-like family, but this classification has recently been challenged (Jameson-Lee 2011). Our current knowledge of Com1-like proteins derives largely from studies on the Com1-like protein DsbA2 from *Legionella pneumophila*. Characterization of DsbA2 and the systems in which it is encoded reveals several important features. First, it was found that the DsbA2 lineage appears to be conserved in, but limited to bacteria that express a type IV secretion system. Moreover, bacteria encoding DsbA2 lack DsbC-like proteins. Finally, it was noted that a BLAST search utilizing DsbA2 as the query did not capture DsbA (Jameson-Lee 2011). This evidence suggests that Com1-like proteins may not be a DsbA-like subgroup. Interestingly, when we analyzed the Com1 protein sequence using the Conserved Domain Database (CDD) (Marchler-Bauer 2011) we found that Com1 appeared to contain a DsbA-like thioredoxin-like fold, but overall was more reminiscent of the DsbG-like family. To date there have been no published functional or structural studies of Com1-like proteins, leaving us with little insight to the functional role of the Com1-like family of proteins.

2. Previous studies on thioredoxin-like proteins in conjugative plasmids

The first thioredoxin-like protein identified in a conjugative system was the 181-residue periplasmic protein TrbB, found during the sequence analysis of genes in the F *tra* operon (Wu 1987). Subsequent experiments revealed no obvious difference in mating efficiency for a donor harboring a *trbB* plasmid, and the protein was deemed nonessential (Kathir 1991). Recently, however, Elton, *et al.* reported that in a *dsbC*⁻ background, a donor cell harboring a *trbB*⁻ plasmid exhibits a small but significant 10-fold decrease in mating efficiency that can be restored by *in trans* complementation with either DsbC or TrbB (Elton 2005). These authors also used a bioinformatics approach to identify TrbB-like proteins in several plasmids. Furthermore, they identified several plasmids that contain homologs of the putative disulfide isomerase DsbC_{R27}, a DsbC-like protein encoded in the IncH plasmid R27 (Sherburne 2000), and TraF, an essential periplasmic thioredoxin-like family member encoded in plasmid F that lacks a C-X-X-C redox motif (Frost 1994, Wu 1988).

Interestingly, TrbB-like and DsbC-like proteins were not found within the same conjugative systems, suggesting these two proteins may be functionally similar. TraF, which shares homology with TrbB (Elton 2005), was later found to associate with an outer membrane complex consisting of several *tra* proteins, including TraV and TraH, which may anchor TraF to the complex (Arutyunov 2010). Attempts to introduce a C-X-X-C active site into TraF based on homology studies with C-X-X-C-containing TraF-like family members resulted in a protein that was able to rescue mating in a *traF* plasmid, but unable to complement DTT hypersensitivity, suggesting it lacks DsbC-like activity (Elton 2005, Missiakas 1994).

Following this, we examined the activity of TrbB from plasmid F, showing that it can catalyze both disulfide bond isomerization and reduction in substrates not involved in conjugation, and demonstrating that it can partially rescue mating of plasmid R27 in a *dsbC⁻ / dsbC_{R27}⁻* background (Hemmis 2011). In addition, we found that, like DsbC, the TrbB redox active site (C-P-Y-C, where C = Cys, P = Pro, Y = Tyr) is maintained in the reduced state *in vivo* by DsbD. In the absence of the bacterially encoded DsbD, TrbB accumulates in an oxidized and inactive state.

Secondary structure comparisons between TrbB and DsbC reveal that TrbB lacks a conserved N-terminal dimerization domain (residues 1–61 in DsbC) and a helical subdomain (residues 123 to 166 in DsbC), both features of DsbC-like and DsbG-like families (Hemmis, manuscript in preparation; Heras 2004, McCarthy 2000). Analytical ultracentrifugation (Hemmis 2011) and size exclusion chromatography (Hemmis, unpublished data) each show that TrbB exists as a 17.7 kDa monomer, suggesting that unlike the dimeric DsbC and DsbG, TrbB may function as a monomer. Additional work has revealed a direct interaction between TrbB and the periplasmic α -domain of DsbD, consistent with a mechanism in which electrons are transferred directly between the bacterial and plasmid redox systems (Hemmis, manuscript in preparation). This discovery, along with structural characterization of the CcmG/DsbD α complex (Stirnemann 2005), reveals that dimerization is not necessary for facilitating a direct interaction with DsbD, in contrast to earlier models (Goldstone 2001, Haebel 2002, Inaba 2006).

A model for TrbB function was developed based upon the experimental evidence from these studies (Figure 4). In this model, a functionally active TrbB protein, in its reduced state, will form a disulfide linked enzyme-substrate complex with its substrate. For plasmid F, this substrate may be TraH, TraN, TraP or TraU, each of which contains at least six cysteine residues (Table 1). Depending on the origin of the secondary attacking cysteine, the disulfide bond will either be directly incorporated into the substrate protein (isomerization pathway), or the substrate will become completely reduced (reductase pathway). In the case of complete reduction, subsequent formation of the new disulfide bond requires a separate oxidation event that is facilitated by either DsbA or a reactive oxygen species. It is possible that a single disulfide isomerization event will fail to yield a properly folded substrate; therefore multiple rounds of disulfide reduction/oxidation may be required for the substrate to assume its native state. A similar mechanism has been previously described for the disulfide reductase TrxP from *Bacteriodes fragilis* (Shouldice 2010).

Plasmid F also encodes two periplasmic proteins, TraK and TraW, which contain only a single cysteine residue. A yeast-two-hybrid performed by Harris and Silverman (Harris 2004) identified a putative TraW/TrbB interaction. Based on this observation, we can speculate that in addition to disulfide isomerization, TrbB may also participate in maintaining these free thiols, much like DsbG in the bacterial redox system.

3. Identification of thioredoxin-like proteins in conjugative systems

Proteins annotated as disulfide isomerases, thiol-disulfide interchange proteins, and protein disulfide oxidoreductases were previously identified in enterobacteria and analyzed phylogenetically (Hemmis 2011). These studies revealed that TrbB-like proteins cluster independently and distinctly from all other *dsb* families. In addition, we found that each of the proteins clustered into the TrbB-like family are either encoded in a conjugative plasmid or within a genomic island on a bacterial chromosome corresponding to a plasmid transfer operon, commonly referred to as integrative and conjugative elements (ICEs). These results, combined with information derived from the biophysical characterization of TrbB, led us to conclude that TrbB is the prototype of a previously unrecognized family of plasmid-based disulfide bond isomerases.

No analogous examination of thioredoxin-like families within plasmid systems has been published. To address this issue, we report here our search for plasmid-encoded thioredoxin-like proteins. Annotated genes from plasmids were obtained from GenBank (<http://www.ncbi.nlm.nih.gov/GenBank>) and grouped into mobility (MOB) families as specified by the relaxase-based classification system proposed by de la Cruz and colleagues (Garcillán-Barcia 2009). Putative thioredoxin-like proteins were identified via a two-step selection process. Proteins were first selected based on the presence of a C-X-X-C motif in their protein sequence. Proteins meeting this criterion were then examined for conserved domains using the CDD (Marchler-Bauer 2011) using an E value cutoff of $1E-20$, which should reveal only highly significant matches within the database. Those proteins containing a C-X-X-C motif that were categorized as thioredoxin-like superfamily members were included in our final list (Table S1). This search identified 165 thioredoxin-family members that are encoded in plasmids from five of the seven MOB families. Of these proteins, 60 are encoded in MOB_F plasmids, 52 in MOB_H plasmids, 41 in MOB_P plasmids, 11 in MOB_Q plasmids, while only a single thioredoxin-like family member is encoded in a MOB_C plasmid (Table 3). Thioredoxin family members were not found in MOB_V or MOB_{HEN} plasmids. (The reasons for the absence of thioredoxin-like proteins in MOB_V or MOB_{HEN} plasmids are not obvious given the diversity of their hosts. While MOB_{HEN} plasmids have been isolated exclusively from Gram-negative bacteria, including many species potentially pathogenic to humans, MOB_V plasmids have been isolated from both Gram-negative and Gram-positive bacteria that occupy various niches.) Among the proteins identified are homologs of DsbA, DsbB, DsbC, DsbG and DsbD from the periplasmic redox system, CcmG and TlpA from cytochrome maturation systems, and TrbB and TraF from plasmid F. With the continuing inclusion of newly discovered plasmid systems, additional sequencing of plasmid genomes, and better annotation of open reading frames, the list of thioredoxin-like proteins in plasmid systems will grow.

3.1 Size distribution and mobility of *trx*-like containing plasmids

Proteins identified in this study were encoded across 89 different plasmid systems. The size of these plasmids ranges from 9 kbp (pLF5) to 1.68 Mbp (pSymB). A frequency distribution analysis shows a majority of these plasmids are between 90 kbp and 205 kbp in size, with the median size being 126 kbp (Figure 5). The size distribution of plasmids containing thioredoxin-like proteins is similar to that for conjugative plasmids as illustrated by Smillie, *et al.* (Smillie 2010). A closer inspection shows that 79 of the 89 plasmids (89%) containing thioredoxin-like proteins also encode proteins typical of a type IV secretion system/pore complex, suggesting they are conjugative in nature. To date, however, only 30 of the 89 plasmids (33%) have been shown to transfer experimentally (Ahmer 1999, Boyd 2004, Bröker 2008, Chatterjee 1972, Chen 2003, Chen 2007, Coetzee 1972, Falkow 1962, Furuichi 1984, Gniadkowski 1998, Hedges 1975, Jerke 2008, Johnson 2002, Johnson 2006, Kim 2008, Lederberg 1952, Lee 2008, Maher 1993, Paulsen 2003, Rees 1987, Romine

1999, Shintani 2006, Skyberg 2006, Terawaki 1967, van Kranenburg 2005, Wain 2003, Welch 2007, Zienkiewicz 2007). The association of thioredoxin-like proteins with conjugative plasmids suggests that these proteins confer some advantage on the plasmids, not surprising given the complexity of the periplasm-spanning pore complex found in conjugative plasmids.

The distribution of the 165 proteins within each plasmid system was also examined to determine how these proteins were spread across the 89 different plasmid systems. An overwhelming majority (78%) of the plasmids encoded only one or two thioredoxin-like proteins (Table 4). Of the remaining plasmids, pK29, R27, 1(c), 1(d), pUTI89, pSN254, and pSymB encoded three proteins, pHCM1, pMAK1, R478, Rts1, pAPEC-01-R, pAA1, pKMSM01, TC1, and pNF1 encoded four proteins, while pCAR1, pDSHI01, and pDSHI03 each encoded six (Table 4; also see Table S1). Of the 20 different plasmids that encode three or more proteins, ten belong to the MOB_H family, while five belong to MOB_P, four to MOB_F, and one to MOB_Q families.

4. Phylogenetic analysis of *trx*-like proteins

To determine which thioredoxin-like families are represented in conjugative plasmids, a two-part phylogenetic analysis was performed. First, protein sequence alignments were performed for each of the MOB groups using ClustalW with a pairwise alignment method (Larkin 2007) as implemented in the Mega 5 software package (Tamura 2011). After alignment, Maximum-likelihood phylogenetic trees of each group were built with Mega 5 using a bootstrap number of 100. Trees were built in presence of the prototypical *dsb* proteins DsbA, DsbB, DsbC, DsbD, and DsbG, the putative *dsb* protein Com1, the cytochrome maturation proteins CcmG and TlpA, and the F plasmid proteins TraF and TrbB to determine the classification for each of the plasmid-based thioredoxin-like proteins identified in this study. For the final analysis, any of the prototypical proteins listed above that did not group with any plasmid-based proteins were removed.

Sequences of plasmid-based thioredoxin family members were also analyzed by the protein sequence clustering program CD-HIT to identify putative protein “clusters” within each MOB group (Huang 2010). Specifically, analyses were performed using the CD-HIT algorithm with a sequence identity cut-off value of 0.4. Protein clusters identified by CD-HIT are labeled in each of the phylogenetic trees (Figures 6–8, hashed lines). Multiple sequence alignments of each cluster have been provided as supplemental material (Figures S1–S15).

4.1 MOB_F

The MOB_F family consists of 114 plasmid systems that are typically conjugative, as opposed to mobilizable, and large. We have identified 60 thioredoxin-like proteins that are encoded across 40 different MOB_F plasmids (Table 4; for additional information see Table S1). Phylogenetic analysis of these proteins reveals DsbA-, DsbC-, TrbB-, TraF-, Com1-, and TlpA-like proteins are encoded in at least one MOB_F plasmid (Table 3).

The TrbB-like family is the most populated within MOB_F plasmids, with a total of 26 different proteins that cluster into two distinct phylogenetic groups (Figure 6). The first group contains proteins homologous to the 19.7 kDa TrbB from plasmid F, while the second contains proteins homologous to the smaller 16.0 kDa TrbB from plasmids pS19501 and pS19502. In addition, we have found seven proteins that belong to the TraF-like family. Further examination of two of these proteins using the CDD reveals an E value of less than 1e-40 for the TrbB-like family, suggesting these two proteins, plp0027 from pLPL and plpp0035 from pLPP (Figure 6, asterisks), can be considered both TraF- and TrbB-like. A

closer inspection of the pLPL and pLPP plasmid genomes reveals that both encode a TraF-like protein that lacks a C-X-X-C motif, which is reminiscent of other TrbB-containing plasmids. For this reason, we propose that plp0027 and plpp0035 should be primarily considered to be TrbB-like proteins, although they cluster among the TraF-like family. We also identified 11 TlpA-like, nine Com1-like proteins, five DsbC-like, and two DsbA-like proteins in MOB_F plasmids (Table 3).

Only 13 of the 40 plasmids from this study encoded multiple thioredoxin-like proteins. Of these plasmids, plasmid pMT encodes TrbB- and Com1-like proteins, plasmid pSLT encodes both a TrbB- and a DsbA-like protein, while plasmids pAOVO01, pCAR3, pNL1, 1(a), and pSWIT01 encode both TraF- and DsbC-like proteins. Plasmids pKB1, pMKMS01, pAA1, and TC1 encode both TlpA- and Com1-like proteins, while pUTI89 encodes TrbB-, DsbA-, and TlpA-like proteins (Table 4; also see Table S1).

4.2 MOB_H

MOB_H is one of the newer classifications of plasmid systems, and currently includes 25 large, conjugative plasmids. We have identified 52 proteins encoded across 18 of these systems (Table 4; also see Table S1), including DsbA-like, DsbB-like, DsbC-like, DsbD-like, DsbG-like, TraF-like, Com1-like, and TlpA-like family members (Table 3).

Of the 52 thioredoxin-like proteins in MOB_H plasmids, 39 are encoded within only eight systems: the plasmids pCAR1, Rts1, pAPEC-01-R, R478, pHCM1, pMAK1, pK29 and R27, and the ICE pAGI2 (Table 4). Each of these plasmids encodes at least one protein belonging to each of the DsbC-, Com1-, and TraF-like families. In some cases, however, the plasmid encodes multiple proteins within the same family. For instance, pCAR1 encodes three different Com1-like proteins, while Rts1 encodes two. Plasmids pAPEC-01-R, R478, pHCM1, and pMAK1 each encode two DsbC-like proteins that cluster into two distinct phylogenetic groups. Closer inspection reveals that the proteins in the first group, which consists of protein clusters H4 and H5 along with DsbC (pCAR1) and Rts1-211 (Rts1), are more distantly related to DsbC than the proteins in the second group, which consists entirely of proteins within cluster H3 (Figure 7). Phyre analysis (Kelley 2009) suggests that the first group (clusters H4 and H5, Rts_211 (Rts1), and DsbC (pCAR1)) possess an elongated N-terminus in comparison to DsbC. Structural predictions using the Phyre algorithm suggest this extension is composed of an additional beta strand-loop-helix within the N-terminal dimerization domain when compared to DsbC from *E. coli* (data not shown). In addition, the genomic island pAGI2 encodes one protein each within the DsbG-, DsbD-, TlpA-, and Com1-like families. Interestingly, orf-c8 from pAGI2 is the lone DsbG homolog identified in this study.

Overall, in MOB_H plasmids we have identified 18 DsbC-like proteins, 18 Com1-like proteins, 10 TraF-like proteins, two DsbD-like proteins, one DsbA-like protein, one DsbB-like protein, one DsbG-like protein, and one TlpA-like protein (Table 3).

4.3 MOB_P

MOB_P is an interesting plasmid family in that it contains two subgroups, MOB_Q and MOB_{HEN} (Garcillán-Barcia 2009). For these studies, MOB_P, MOB_Q, and MOB_{HEN} were treated as three distinct phylogenetic groups. Within the 180 plasmids that are strictly considered MOB_P, we found 37 thioredoxin-like proteins belonging to the Com1-like, CcmG-like, DsbB-like, and DsbD-like families (Table 3).

Like MOB_H, there are several MOB_P plasmid systems that encode more than one thioredoxin-like protein, although the trends are much less apparent (Table 4). Of special note, however, are the plasmids pDSHI01 and pDSHI03, which each encode six

thioredoxin-like proteins; two Com1-like proteins, two DsbB-like proteins, one CcmG-like protein, and one DsbD-like protein. Phylogenetic analysis (Figure 8) revealed that the two Com1-like proteins and the two DsbBs cluster differently from one another, which may suggest the existence of two specific Com1/DsbB systems that are responsible for unique functions *in vivo*. Finally, we identified four proteins, EF-B0054 and EF-B0055 from pTEF2, pFL5-05 from pFL5, and pNF1 1020 from pNF1 that, according to the CDD, do not have homology to any specific thioredoxin-like subfamily, bringing the total number of thioredoxin like proteins in MOB_P plasmids to 41.

4.4 MOB_Q

As mentioned above, MOB_Q is a subgroup within the MOB_P family. MOB_Q consists of 21 plasmid systems in which we identified 11 different thioredoxin-like proteins (Table 3). Included in these proteins are the TlpA-like proteins Nham_4643 from plasmid 3 and SMb20213 from pSymB, the TrbB-like pLDTEXKL-p22 from pLD-TEX-KL, and three proteins, pRL11-0200 from pRL11, Smed-5325 from pSMED02, orf28 from pWCFS103, that do not group well with any thioredoxin-like families (data not shown). According to the CDD, however, Smed_5325 seems to have small regions of homology to both TlpA- and DsbD-like families. In addition there are four proteins that have homology to protein families not discussed in this review. Two of these, nuoE2 from pSymA and Smed-3619 from pSMED01 are predicted to be members of the NADH:ubiquinone oxidoreductase family (Nuo)/ Thioredoxin-like [2Fe-2S] Ferredoxin family of proteins. The Thioredoxin-like [2Fe-2S] ferredoxin family is characterized by a four cysteine cluster organized in an approximate C-X₁₀-C-X₂₉-C-X₃-C motif that functions in binding of an iron-sulfur cluster (Meyer 2011). Further inspection of the nuoE2 and Smed-3619 sequences, which are 91% identical, reveals that the identified C-X-X-C motif is due to the presence of a fifth cysteine within this conserved four cysteine cluster, resulting in a C-X₄-[C-X₂-C]-X₃₂-C-X₃-C motif in both nuoE2 and Smed-3619. How this extra cysteine affects binding of the iron-sulfur cluster has not been experimentally determined, however, the positioning of the C-X-X-C motif would suggest that neither nuoE2 nor Smed-3619 possess functional redox active sites. The remaining two proteins, Caul-5154 from pCAUL01 and SMb20194 from pSymB are predicted to be members of the Peroxiredoxin-like1 subfamily (cd02969). Peroxiredoxins are typically characterized by a catalytic triad, T-X-X-C-X(x82)-R/S (T = Thr, C = Cys, R = Arg, S = Ser), responsible for its enzymatic activity. The Prx1 subfamily, however, lacks the triad, and only contains only a single conserved cysteine residue. Coincidentally, the conserved cysteine is different than the perioxodatic cysteine seen in the catalytic triad (Fomenko 2003).

4.5 MOB_C

MOB_C is a small plasmid family that was organized by the clustering of 54 different relaxase proteins encoded in conjugative plasmids, mobilizable plasmids, and ICEs. In our studies, we found only a single thioredoxin-like protein in the 22 different MOB_C plasmids identified by Garcillan-Barcia, *et al.* (Garcillán-Barcia 2009) (Table 3). This protein, EFA0039 from plasmid pTEF1, does not cluster well with any of the thioredoxin-like families mentioned in this study (data not shown). A search of the CDD shows homology to a small family of oxidases found only in photosynthetic organisms. This finding is surprising, as pTEF1 was isolated from the commensal gut bacterium *Enterococcus faecalis* (Paulsen 2003).

5. Trends and Perspectives

5.1 TrbB-containing plasmids

Through phylogenetic analysis, we have found that of the 29 identified TrbB-like family members, 28 are found in MOB_F plasmids. Continuing a trend observed earlier, none of these TrbB-like proteins were found on a plasmid encoding a DsbC-like protein. Instead, we found that 28 of the 29 TrbB-containing plasmids also encode TraF-like family members, but these TraF-like proteins lack C-X-X-C motifs in their mature form, and for this reason were not part of our analysis. Conversely, in 14 of the 15 plasmids encoding TraF-like proteins that contain a C-X-X-C motif, we also find DsbC-like family members. Although there is yet no experimental data that allows a comparison of DsbC, TrbB, and TraF functions, the trends revealed in this work raise important questions about the relationship between these proteins.

5.2 Com1-like proteins

Com1-like proteins are arguably the least understood of all the thioredoxin-like families, as their *in vivo* function, structure, and regulation have not been thoroughly characterized. Our analysis has found that although the C-X-X-C active site of Com1-like proteins are embedded within a DsbA-like thioredoxin fold as judged by the CDD, these proteins seem to have both DsbA-like and DsbG-like characteristics. In all, Com1-like proteins were found in 39 different plasmids within the MOB_F, MOB_H and MOB_P families. Six of these 39 plasmid systems encode multiple Com1-like proteins. Specifically, pCAR1 and 1(d) encode three, while Rts1, pMKMS01, pDSH01, and pDSH03 each encode two (Table 4).

A previous analysis of bacteria encoding homologs of the Com1-like protein DsbA2 from *Legionella pneumophila* revealed an absence of DsbC-like proteins (Jameson-Lee 2011), however, this trend is not upheld in plasmid systems. Of the 24 Com1-like containing plasmids that encode more than one thioredoxin-like protein, 12 possess DsbC-like proteins. Additionally, 8 of these 12 also include a C-X-X-C-containing TraF-like protein.

Although the redox active site of DsbA2 is maintained in a DsbA-like ratio *in vivo* (60% oxidized: 40% reduced) (Jameson-Lee 2011), its putative dependence on the inner membrane protein DsbB for redox maintenance has not been directly examined. In this study, we identified three plasmids that encode both Com1-like and DsbB-like proteins (pCAR1, pDSHI01, and pDSHI03). Most interesting are plasmids pDSHI01 and pDSHI03, which encode two Com1-like proteins and two DsbB-like proteins, suggesting they may possess two independent Com1/DsbB systems. In each of these plasmids, one of the DsbA/B pairs is encoded sequentially within the plasmid genome, further supporting this hypothesis. There is, however, little evidence for either the *in vivo* function or substrates of Com1 or DsbA2, hampering speculation on the role of the Com1-like family in conjugative systems.

5.3 Cytochrome biogenesis proteins

Thioredoxin-like proteins belonging to cytochrome biogenesis families have been found in 17 different plasmids. Eight of these contain homologs of CcmG, while nine contain homologs of TlpA. To further understand the presence of these proteins, we searched these 17 plasmid systems for additional cytochrome biogenesis proteins. This search revealed that 16 of the 17 plasmids containing CcmG- or TlpA-like proteins also encoded a cytochrome c biogenesis island or additional cytochrome c maturation proteins (data not shown). In addition, we found that 23 of the 24 proteins within these 16 plasmids resided directly within the biogenesis island, or next to other cytochrome maturation proteins.

The conservation of cytochrome biogenesis proteins within plasmid systems has not yet been investigated. However, the conservation of both CcmG-like and TlpA-like proteins proximal to cytochrome c maturation proteins further supports the role of TlpA-like proteins in the biogenesis of cytochrome c that was first revealed in *A. tumefaciens*.

5.4 Redox state maintenance of thioredoxin-like proteins in plasmids

As thioredoxin-like proteins rely on inner membrane proteins for redox state maintenance *in vivo*, the presence of proteins such as DsbB and DsbD are essential for the regulation of plasmid-based thioredoxin family members.

Phylogenetic analysis of thioredoxin-like proteins in MOB_F plasmids has revealed an overall dearth of DsbB or DsbD-like proteins, which may reflect a dependence on host-encoded proteins for each of these systems, much like the dependence of the F plasmid-based disulfide isomerase TrbB on the *E. coli* encoded DsbD. In fact, only six of the 89 plasmid systems discussed in this study encode a DsbB or DsbD homolog. One of these, the MOB_H plasmid pBVIE02, encodes a DsbD homolog, yet does not possess another thioredoxin-like protein, which raises questions about its primary function. Alternatively, the MOB_P plasmids pDSHI01 and pDSHI03 appear to encode a self-contained system, including two possible Com1/DsbB pairs and one CcmG/DsbD pair. The MOB_H plasmid pCAR1 encodes a DsbB homolog that may be responsible for the maintenance of its three different Com1-like proteins. The MOB_P plasmid pFL5 encodes two sequential thioredoxin-like proteins; the DsbB homolog pFL5-p05 and pFL5-p06, the latter of which does not appear to have homology to any of the prototypical thioredoxin-like families. The sequential positioning of pFL5-p05 and pFL5-p06, however, may suggest that pFL5-p06 is maintained in the oxidized form by pFL5-p05 *in vivo*. Finally, the genomic island pAGI2 encodes a DsbD homolog along with Com1-, DsbG-, and TlpA- like family members. One possible explanation for the lack of DsbB and DsbD homologs in plasmids is that bacterially-encoded DsbB and DsbD function as a general oxidase and reductase, respectively, thereby enabling interactions with and subsequent redox state maintenance of proteins encoded within these plasmid systems.

5.5 Potential substrates of trx-like proteins

Of obvious importance to the mechanistic understanding of thioredoxin-like proteins in these systems are the identification of their plasmid encoded substrates. Using a bioinformatics approach, putative substrates within trx-like containing plasmids were selected using two criteria: (1) the presence of two or more cysteine residues and (2) the presence of an N-terminal signal peptide as predicted by the SignalP 4.0 server (Peterson 2011). This approach led to the identification of more than 1000 potential trx-like substrates, including 375 within MOB_F plasmids, 241 within MOB_H plasmids, 129 within MOB_P plasmids, and 298 within MOB_Q plasmids. As this number of proteins is quite difficult to analyze, this search was refined to include only those proteins containing 5 or more cysteine residues in their mature state. This additional criterion reduced the overall number of potential substrates to 193. Of these, 114 were encoded in MOB_F plasmids, 36 in MOB_H plasmids, 17 in MOB_P plasmids, and 22 in MOB_Q plasmids. We decided to focus further efforts on those proteins encoded by MOB_F plasmids, which are summarized in Table 5. Those proteins found within MOB_H, MOB_P, and MOB_Q families have been provided as supplemental information (Table S2). The set of MOB_F proteins containing five or more cysteines originate from 36 of the 40 trx-like containing MOB_F plasmids discussed in this review. Within the MOB_F family, only plasmids A, pAA1, pKB1, and pLEW279b contain no proteins with five or more cysteines.

Only seven of the 40 MOB_F plasmids identified in this review lack a disulfide bond isomerase. These include plasmids A, pAA1, pENTE01, pKB1, pLEW279b, pMKMS01,

and TC1. Of the plasmids encoding disulfide bond isomerases, 28 contain TrbB homologs, while five possess DsbC homologs. Our current model proposes that these disulfide bond isomerases aid in the folding and redox state maintenance of a subset of plasmid-encoded disulfide-containing proteins. As would be expected from this hypothesis, our analysis reveals that proteins containing five or more cysteines are predominantly encoded in plasmids containing either TrbB or DsbC (Table 5). In fact, only seven of the 118 proteins identified in MOB_F plasmids were encoded in plasmids lacking a disulfide bond isomerase. Of special note is plasmid pENTE01, which encodes five of those seven proteins. A closer inspection of the pENTE01 genome reveals the presence of a TrbB homolog with a serine at C-terminal redox active site position (C-X-X-S). Although it is not a canonical redox active site, this motif has been previously identified in a number of redox enzymes (Fomenko 2002, Fomenko 2003), suggesting that TrbB from pENTE01 may be catalytically active. If this is the case, we then find only two proteins with five or more cysteines, Mkms_5650 from plasmid pMKMS01 and Aaur_pCT10011 from TC1 (Table 5), encoded in a plasmid lacking a disulfide bond isomerase.

Another striking observation is the conservation TraH, TraN, and TraU homologs in these systems. Of the 118 proteins identified here, 32 are TraH homologs, 32 are TraN homologs, and 33 are TraU homologs, which together comprise 82% of the identified sequences (Table 5). Moreover, if pENTE01 is to be considered a disulfide bond isomerase containing plasmid, we observe a perfect correlation between the encoding of TraH/TraN/TraU-like proteins and a disulfide bond isomerase, at least within MOB_F plasmids. In other words, the 34 plasmids encoding a disulfide bond isomerase are the only plasmids that encode TraH, TraN, or TraU-like proteins. Interestingly, 29 of these 34 plasmids encode all three of the *tra* proteins. The remaining five each encode two of the three: ColMB encodes TraN and TraU, pAPEC-01-ColV encodes TraH and TraN, pPBR1 encodes TraN and TraU, pSF0157 encodes TraH and TraU, and R100 encodes TraH and TraU (Table 5). Further investigation of these plasmid genomes reveals that three of these plasmids, pAPEC-01-ColV, pSF0157, and R100 encode the third, but the homolog from that respective system is not predicted to have an N-terminal signal peptide. Therefore, only two plasmids, ColBM and pPBR1, which both appear to lack a TraH homolog, do not encode all three. To further establish this correlation, we have found that disulfide bond isomerases are encoded in all but one plasmid containing TraH, TraN, or TraU across all MOB families, with the exception being the MOB_H plasmid 1(b) (Figure S2).

Together, these trends suggest that plasmid-based disulfide bond isomerases are indeed responsible for the folding and maintenance of a select subset of plasmid-encoded proteins, and that the core of this subset consists of the transfer proteins TraH, TraN, and TraU. These results also highlight the importance of further examination of the remaining potential substrates we have identified within the MOB_P, MOB_H, and MOB_Q families, for which trends are much less apparent.

5.6 Membrane association via lipid modification

Further analysis of the N-terminal signal peptides of plasmid-encoded thioredoxin like proteins was performed using the LipoP server (Juncker 2003), which analyzes the N-terminal sequence for the presence of a lipobox. A lipobox is a conserved [LVI][ASTVI][GAS]C amino acid sequence within the C-terminal region of a protein's N-terminal signal peptide (Babu 2006). This motif results in the covalent attachment of a diacylglycerol moiety onto the free thiol of the conserved cysteine by lipoprotein diacylglycerol transferase (Babu 2006). After attachment of the diglycerol moiety, the signal peptide is cleaved N-terminal to the modified cysteine by lipoprotein signal peptidase (Tokunga 1982). In Gram-negative bacteria (and some Gram-positive bacteria), a second round of modification occurs after cleavage, whereby an acyl group will be attached to the free amine of the now N-

terminal cysteine by lipoprotein N-acyl transferase (Gupta 1993). Addition of these lipid moieties facilitates association of the modified proteins with the periplasmic face of either the inner or outer membrane (Narita 2004).

Of the 165 proteins discussed in this review, only 14 were predicted to possess a lipobox. Of these, six belong to the TlpA-like family, six to the CcmG-like family, while one each is a member of the TrbB-like and DsbG-like families. In this review we identified only 14 TlpA-like and 10 CcmG-like proteins, which translates to 43% of TlpA-like and 60% of CcmG-like proteins identified in plasmid systems possessing lipoboxes, thus suggesting a critical importance of membrane-association to thioredoxin-like cytochrome maturation family proteins. This trend is not surprising, as previous work has shown both TlpA and CcmG-like proteins to be membrane anchored (see Section 1.4). Alternatively, only 2 of the remaining 141 thioredoxin-like family proteins (1.4%) are predicted to possess lipoboxes, indicating an overall lack of membrane associated thioredoxin-like proteins outside of the cytochrome maturation system.

5.7 Concluding Remarks and Perspectives

Through our examination of thioredoxin-like proteins in mobile plasmids, we have identified 89 different plasmid systems that encode at least one protein within the thioredoxin-like superfamily. Together these plasmid systems encode more than 160 different thioredoxin-like proteins that belong to at least 10 different thioredoxin-like families (DsbA, DsbB, DsbC, DsbD, DsbG, Com1, CcmG, TlpA, TraF, and TrbB).

Based on our analysis, we can draw several conclusions. First, nearly all of the thioredoxin proteins identified are encoded on large, apparently conjugative plasmids. This observation suggests that thioredoxin-like proteins may play an important role in the construction or regulation of the type IV pore complex. In addition, we have found a significant number of cytochrome maturation proteins in these plasmids, suggesting that there is a selective advantage for conjugative plasmids encoding at least part of these systems.

Despite the relative frequency of thioredoxin-like proteins in conjugative plasmids, these proteins are observed in less than one quarter of the conjugative plasmids examined. Clearly these proteins are not generally required for maintenance or transfer of a conjugative plasmid. There are, however, some intriguing trends. We observe no thioredoxin-like proteins in 105 MOB_V plasmids and a single thioredoxin-like protein in 22 MOB_C plasmids, while 18 of 33 MOB_H plasmids encode one to as many as six such proteins. Are the periplasmic proteins encoded by MOB_H plasmids more prone to misfolding or oxidation, allowing thioredoxin-like proteins a comparatively greater selective advantage? Do MOB_H plasmid hosts frequently inhabit niches that favor plasmids bearing these proteins?

In another example, in MOB_F plasmids we frequently observe both a TrbB-like and a TraF-like protein encoded on the same plasmid, however, the TraF-like proteins in these cases lack a C-X-X-C motif. Alternatively, MOB_F plasmids encoding TraH, TraN, and TraU homologs that lack TrbB were found, in all cases, to encode both a DsbC-like protein and a TraF-like protein that possesses a complete C-X-X-C redox active site motif. We have yet to observe TrbB-like and DsbC-like proteins encoded together in MOB_F plasmids. While the roles of thioredoxin-like protein families have been studied within bacterial systems, to date only three different plasmid-based family members have been investigated experimentally: TrbB and TraF from plasmid F, and DsbC from plasmid R27. Although TrbB and DsbC each exhibit disulfide bond isomerase activity, we have not yet experimentally determined their specific protein targets, nor do we understand the extent of their roles within assembly of the pore complex and/or DNA translocation. In addition, studies have shown that TraF, although lacking a C-X-X-C motif, is an essential protein for pilus extension in the F

plasmid system. The *in vivo* function and protein targets of TraF have not been determined, however it is currently thought to act as a molecular chaperone. In addition to homologs of the canonical *dsb* family, we have also identified plasmid proteins belonging to the CcmG-like, TlpA-like, and ComI-like families, for which there exists little experimental data.

Although thioredoxin-like proteins appear to play an important part in the conjugative process, there is an overall lack of experimental data addressing the unique and apparently numerous roles of these proteins. For this reason, many important questions remain unanswered. One of the most important is how each of these proteins contributes to the process of bacterial conjugation. Although we speculate that plasmid-based *dsb* family members are responsible for the correct folding and maintenance of plasmid-encoded proteins, there is no empirical evidence to support this. It has also been hypothesized that plasmid-based *dsb* proteins could aid in the maintenance of certain host-encoded proteins, or that they are specifically required when bacteria are under particular environmental stresses. Alternatively, these proteins may only be required when the plasmid is in a host that does not possess a complete disulfide bond formation system. We have also identified a large number of proteins belonging to the ComI-like family, whose function is unknown even within bacterial systems. Lastly, we must address why certain plasmids encode cytochrome *c* biogenesis systems. Are these proteins essential for plasmid transfer or persistence? Are they functionally redundant to proteins within the host-encoded system? For the first time, we have at our disposal a cumulative list of thioredoxin-like proteins conserved within plasmid systems. With continued investigations of the functions of these proteins, great strides can be made into further understanding the intricacies of the conjugative process.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Mechanisms of DsbB- and DsbD-dependent proteins within the *E. coli* periplasmic redox system. (A) The periplasmic thiol-oxidase DsbA incorporates a disulfide linkage into a substrate protein, becoming reduced in the process. After reduction, DsbA donates electrons from its active site to the inner membrane protein DsbB in order to reoxidize its active site to reassume its catalytically active state. DsbB will then pass these electrons to ubiquinone (UQ) or menaquinone (MQ) and enter the electron transport chain where they will eventually be passed to terminal electron acceptors. (B) The disulfide bond isomerase DsbC and the disulfide bond isomerase/sulfenate (–SOH) reductase DsbG are both active in their reduced states. Upon isomerization, as shown for DsbC, or sulfenate reduction of substrates, as shown for DsbG, the redox active sites can become oxidized. To reassume their active states, DsbC and DsbG accept electrons from the periplasmic α -domain of the inner membrane protein DsbD. DsbD, in turn, will re-reduce itself by accepting electrons from the cytoplasmic protein thioredoxin A (TrxA), and shuttling them from the cytoplasm, through the inner membrane, and finally into the periplasm.

Figure 2.

High-resolution structures of prototypical thioredoxin-like family proteins. Shown are cartoon model representations of the structures of thioredoxin-like proteins (clockwise from top-left) DsbA (PDB 1DSB), DsbB (PDB 2K73), and CcmG (PDB 1KNG) from *E. coli*, TlpA (PDB 1JFU) from *B. japonicum*, and lastly, DsbG (PDB 1V57) and DsbC (PDB 1EEJ) from *E. coli*. Shown in red spheres are the redox active cysteines within each protein. Absent are ComI from *C. burnetii*, TraF and TrbB from plasmid F, and DsbD from *E. coli*, for which high resolution structures are not available.

Figure 3. Comparison of reduced forms of DsbC and DsbG homodimers. Space filling models of DsbC (A, pdb 1TJD) and DsbG (B, pdb 1V58) structures displaying hydrophobic and non-charged residues (green), acidic residues (red), basic residues (blue), and the C-X-X-C active sites (yellow). Note the elongated V-shaped binding cleft of DsbG (43.3Å internal, 100.3Å external) in comparison to DsbC (27.6Å internal, 76.5Å external). Additionally, note the presence of acidic residues within the binding cleft of DsbG (B, bottom, red residues), while the DsbC binding cleft (A, bottom) contains only hydrophobic and uncharged residues.

Figure 4.

Proposed mechanism of TrbB function on a substrate containing three cysteines. Reduced TrbB recognizes a substrate and forms a transient enzyme:substrate complex by means of a direct attack from the N-terminal active site cysteine (C81) of TrbB. Upon formation of the complex, TrbB is thought to catalyze isomerization by one of two pathways, depending on the origin of the resolving cysteine. (1) When the C-terminal cysteine in the TrbB active site (C84) acts as the resolving cysteine, the substrate becomes fully reduced and TrbB becomes fully oxidized. This mechanism then requires a separate oxidation event to re-oxidize the substrate. This event most likely occurs as a second interaction with DsbA or a reactive oxygen species. Oxidized TrbB will then interact with and accept electrons from the inner

membrane protein DsbD, effectively recharging it for additional rounds of catalysis. (2) Alternatively, when the resolving cysteine originates from the substrate protein, a new disulfide linkage is directly introduced, and TrbB is released in its reduced state. In this mechanism, TrbB remains active and can immediately catalyze additional rounds of disulfide isomerization. As the folding of the substrate is not directed by the disulfide isomerase, it is possible that a non-native disulfide bond may be reintroduced, making additional rounds of disulfide isomerization necessary.

Figure 5.
Size distribution of plasmids containing thioredoxin-like proteins. Sizes of the 89 plasmids that encode thioredoxin-like family members were obtained from GenBank and grouped using bin sizes of 25kb.

Figure 6.

Maximum-likelihood tree of thioredoxin-like proteins within MOB_F plasmids. Included in this analysis are 60 plasmid-based thioredoxin-like proteins in addition to the thioredoxin-like prototype proteins TrbB, TraF, TlpA, DsbC, DsbA, and Com1. Proteins cluster into groups consisting of TrbB-like, TraF-like, TlpA-like, DsbC-like, DsbA-like, and Com1-like families (solid lines). Clusters identified by CD-HIT (F1–F9) are shown as dashed lines. Within the TraF-like cluster are two proteins plpl0027 and plpp0035 (denoted by asterisks), which have been proposed to be members of the TrbB-like family.

Figure 7.

Maximum-likelihood tree of thioredoxin-like proteins within MOB_H plasmids. Included in this analysis are 52 plasmid-based thioredoxin-like proteins in addition to the thioredoxin-like prototype proteins DsbC, DsbG, DsbB, DsbD, Com1, and TlpA. Proteins cluster into groups consisting of DsbC-like, DsbG-like, TraF-like, DsbA-like, DsbB-like, DsbD-like, Com1-like, and TlpA-like families (solid lines). Clusters identified by CD-HIT (H1–H6) are shown as dashed lines.

Figure 8.

Maximum-likelihood phylogenetic analysis of thioredoxin-like proteins within MOBp plasmids. Included in this analysis are 40 plasmid-based thioredoxin-like proteins in addition to the thioredoxin-like prototype proteins Com1, CcmG, DsbB, and DsbD. Proteins cluster into groups consisting of Com1-like, CcmG-like, DsbB-like, DsbD-like families (solid lines), while 4 proteins do not share homology with any prototypical subfamilies. Clusters identified by CD-HIT (P1–P9) are shown as dashed lines.

Table 1Periplasmic F plasmid *tra* proteins containing one or more cysteine residues

Protein	Localization	No. Cys	theoretical redox states	cysteine connectivity
TraK	P/OM	1	1	-
TraW	P	1	1	-
TraB	P/IM	2	2	1
TraF	P	2	2	1
TrbB	P	2	2	1
TrbC	P	2	2	1
TraP	C/IM/P	6	76	15
TraH	P/OM	7	232	15
TraU	P	10	9496	945
TraN	P/OM	22	6.20×10^{11}	1.37×10^{10}

Table 2

Thioredoxin-like families overview

Family	Prototype Host	Localization	Function
DsbA	<i>E. coli</i>	P	thiol-oxidase; <i>de novo</i> disulfide bond formation
DsbB	<i>E. coli</i>	P/IM	redox maintenance of periplasmic thiol-oxidases
DsbC	<i>E. coli</i>	P	disulfide bond isomerase, molecular chaperone
DsbD	<i>E. coli</i>	P/IM	redox maintenance of periplasmic disulfide bond isomerases and disulfide reductases
DsbG	<i>E. coli</i>	P	sulfenate reductase, disulfide bond isomerase, molecular chaperone
CcmG	<i>E. coli</i>	P/IM	disulfide reductase, cytochrome c maturation
TlpA	<i>B. japonicum</i>	P/IM	unknown, putative disulfide reductase, cytochrome aa3/c maturation
ComI	<i>C. burnetii</i>	P	unknown, putative thiol-oxidase or disulfide bond isomerase
TrbB	Plasmid F (<i>E. coli</i>)	P	disulfide bond isomerase
TraF	Plasmid F (<i>E. coli</i>)	P	putative molecular chaperone, lacks C-X-X-C active site

Table 3

Thioredoxin-like proteins within MOB families

Protein Family	MOBF	MOBH	MOBP	MOBQ	MOBC	MOBV	MOBHEN
DsbA	2	1	0	0	0	0	0
DsbB	0	1	5	0	0	0	0
DsbC	5	18	0	0	0	0	0
DsbD	0	2	2	0	0	0	0
DsbG	0	1	0	0	0	0	0
CemG	0	0	10	0	0	0	0
TlpA	11	1	0	2	0	0	0
ComI	9	18	20	0	0	0	0
TrbB	28	0	0	1	0	0	0
TraF	5	10	0	0	0	0	0
Other	0	0	4	8	1	0	0
TOTAL	60	52	41	11	1	0	0

Table 4

Thioredoxin-like proteins by plasmid systems

Plasmid	MOB	DsbA	DsbB	DsbC	DsbD	DsbG	CcmG	TlpA	ComI	TrbB	TraF	Other	TOTAL
pTEF1	C	-	-	-	-	-	-	-	-	-	-	1	1
I(a)	F	-	-	1	-	-	-	-	-	-	1	-	2
A	F	-	-	-	-	-	-	-	1	-	-	-	1
ColBM	F	-	-	-	-	-	-	-	-	1	-	-	1
F	F	-	-	-	-	-	-	-	-	1	-	-	1
MT	F	-	-	-	-	-	-	-	1	1	-	-	2
p1658/97	F	-	-	-	-	-	-	-	-	1	-	-	1
pAA1	F	-	-	-	-	-	-	3	1	-	-	-	4
pAOVO01	F	-	-	1	-	-	-	-	-	-	1	-	2
pAPEC-02-ColV	F	-	-	-	-	-	-	-	-	1	-	-	1
pAPEC-02-R	F	-	-	-	-	-	-	-	-	1	-	-	1
pAsa5	F	-	-	-	-	-	-	-	-	1	-	-	1
pC15-1a	F	-	-	-	-	-	-	-	-	1	-	-	1
pC4602-1	F	-	-	-	-	-	-	-	-	1	-	-	1
pCAR3	F	-	-	1	-	-	-	-	-	-	1	-	2
pED208	F	-	-	-	-	-	-	-	-	1	-	-	1
pENTE01	F	-	-	-	-	-	-	-	1	-	-	-	1
pG8786	F	-	-	-	-	-	-	-	1	1	-	-	2
pKB1	F	-	-	-	-	-	-	1	1	-	-	-	2
pKPN3	F	-	-	-	-	-	-	-	-	1	-	-	1
pKPN4	F	-	-	-	-	-	-	-	-	1	-	-	1
pLEW279b	F	-	-	-	-	-	-	1	-	-	-	-	1
pLPL	F	-	-	-	-	-	-	-	-	1	-	-	1
pLPP	F	-	-	-	-	-	-	-	-	1	-	-	1
pMAR7	F	-	-	-	-	-	-	-	-	1	-	-	1
pMKMS01	F	-	-	-	-	-	-	2	2	-	-	-	4
pNL1	F	-	-	1	-	-	-	-	-	-	1	-	2
pNR1	F	-	-	-	-	-	-	-	-	1	-	-	1
pO86A1	F	-	-	-	-	-	-	-	-	1	-	-	1

Plasmid	MOB	DsbA	DsbB	DsbC	DsbD	DsbG	CcmG	TlpA	ComI	TrbB	TraF	Other	TOTAL
pOU1113	F	-	-	-	-	-	-	-	-	1	-	-	1
pPBPR1	F	-	-	-	-	-	-	-	-	1	-	-	1
pS18501	F	-	-	-	-	-	-	-	-	1	-	-	1
pS19501	F	-	-	-	-	-	-	-	-	1	-	-	1
pS19502	F	-	-	-	-	-	-	-	-	1	-	-	1
pSF0157	F	-	-	-	-	-	-	-	-	1	-	-	1
pSLT	F	1	-	-	-	-	-	-	-	1	-	-	2
pSWIT01	F	-	-	1	-	-	-	-	-	-	1	-	2
pUTI89	F	-	-	-	-	-	-	1	1	1	-	-	3
pYJ016	F	-	-	-	-	-	-	-	-	1	-	-	1
R100	F	-	-	-	-	-	-	-	-	1	-	-	1
TC1	F	-	-	-	-	-	-	3	1	-	-	-	4
l(b)	H	1	-	-	-	-	-	-	-	-	1	-	2
pAGI2	H	-	-	-	1	1	-	1	1	-	-	-	4
pAPEC-01-R	H	-	-	2	-	-	-	-	1	-	1	-	4
pASA4	H	-	-	-	-	-	-	-	1	-	-	-	1
pBVIE02	H	-	-	-	1	-	-	-	-	-	-	-	1
pCAR1	H	-	1	1	-	-	-	-	3	-	1	-	6
pHCM1	H	-	-	2	-	-	-	-	1	-	1	-	4
pIP1202	H	-	-	1	-	-	-	-	1	-	-	-	2
pK29	H	-	-	1	-	-	-	-	1	-	1	-	3
pMAK1	H	-	-	2	-	-	-	-	1	-	1	-	4
pMAQU02	H	-	-	-	-	-	-	-	1	-	-	-	1
pP91278	H	-	-	1	-	-	-	-	1	-	-	-	2
pSN254	H	-	-	2	-	-	-	-	1	-	-	-	3
pYR1	H	-	-	1	-	-	-	-	1	-	-	-	2
R27	H	-	-	1	-	-	-	-	1	-	1	-	3
R391	H	-	-	1	-	-	-	-	-	-	1	-	2
R478	H	-	-	2	-	-	-	-	1	-	1	-	4
Rts1	H	-	-	1	-	-	-	-	2	-	1	-	4
2	P	-	-	-	-	-	1	-	-	-	-	-	1

Plasmid	MOB	DsbA	DsbB	DsbC	DsbD	DsbG	CcmG	TlpA	ComI	TrbB	TraF	Other	TOTAL
1(c)	P	-	-	-	-	-	2	-	1	-	-	-	3
1(d)	P	-	-	-	-	-	-	-	3	-	-	-	3
3(b)	P	-	-	-	-	-	1	-	-	-	-	-	1
C	P	-	-	-	-	-	-	-	1	-	-	-	1
Collb-p9	P	-	-	-	-	-	-	-	1	-	-	-	1
pBVE04	P	-	-	-	-	-	-	-	1	-	-	-	1
pCTX-M3	P	-	-	-	-	-	-	-	1	-	-	-	1
pDC3000A	P	-	-	-	-	-	-	-	1	-	-	-	1
pDC3000B	P	-	-	-	-	-	-	-	1	-	-	-	1
pDSH01	P	-	2	-	1	-	1	-	2	-	-	-	6
pDSH03	P	-	2	-	1	-	1	-	2	-	-	-	6
pEL60	P	-	-	-	-	-	-	-	1	-	-	-	1
pFL5	P	-	1	-	-	-	-	-	-	-	-	1	2
pNF1	P	-	-	-	-	-	2	-	1	-	-	1	4
pSC138	P	-	-	-	-	-	-	-	1	-	-	-	1
pSERB1	P	-	-	-	-	-	-	-	1	-	-	-	1
pSI-1	P	-	-	-	-	-	1	-	1	-	-	-	2
pTEF2	P	-	-	-	-	-	-	-	-	-	-	2	2
R64	P	-	-	-	-	-	-	-	1	-	-	-	1
TC2	P	-	-	-	-	-	1	-	-	-	-	-	1
3	Q	-	-	-	-	-	-	1	-	-	-	-	1
pCAUL01	Q	-	-	-	-	-	-	-	-	-	-	1	1
pLD-TEX-KL	Q	-	-	-	-	-	-	-	-	1	-	-	1
pSMED01	Q	-	-	-	-	-	-	-	-	-	-	1	1
pRL11	Q	-	-	-	-	-	-	-	-	-	-	1	1
pSMED02	Q	-	-	-	-	-	-	-	-	-	-	1	1
pSymA	Q	-	-	-	-	-	-	-	-	-	-	1	1
pSymB	Q	-	-	-	-	-	-	1	-	-	-	2	3
pWCFS103	Q	-	-	-	-	-	-	-	-	-	-	1	1
TOTAL		2	6	23	4	1	10	14	48	29	15	13	165

Table 5Periplasmic proteins from MOB_F plasmids containing five or more cysteine residues

Protein	Plasmid	Accession #	Cys #	Plasmid dsb isomerase
Bcen2424_6808	l(a)	116687184	13	DsbC
Bcen2424_6872	l(a)	116687248	8	DsbC
Bcen2424_6920	l(a)	116687295	6	DsbC
TraH	l(a)	116687190	6	DsbC
TraN	l(a)	116687185	22	DsbC
TraU	l(a)	116687182	16	DsbC
CoBM53	ColBM	157418136	12	TrbB
TraN	ColBM	157418127	22	TrbB
TraU	ColBM	157418125	11	TrbB
TraH	F	9507811	6	TrbB
TraN	F	9507802	22	TrbB
TraU	F	9507800	11	TrbB
TraH	MT	9507811	6	TrbB
TraN	MT	9507802	22	TrbB
TraU	MT	9507800	11	TrbB
TraH	p1658/97	32460003	6	TrbB
TraN	p1658/97	32469994	22	TrbB
TraU	p1658/97	32469992	11	TrbB
TraH	pAOVO01	121582472	6	DsbC
TraN	pAOVO01	121582475	22	DsbC
TraU	pAOVO01	121582478	16	DsbC
TraH	pAPEC-02-ColV	84060759	6	TrbB
TraN	pAPEC-02-ColV	84060766	22	TrbB
TraH	pAPEC-02-R	58000387	6	TrbB
TraN	pAPEC-02-R	58000380	22	TrbB
TraU	pAPEC-02-R	58000378	11	TrbB
TraH	pAsa5	1453014122	6	TrbB
TraN	pAsa5	145301419	23	TrbB
TraU	pAsa5	145301417	11	TrbB
TraH	pC15-1a	41056006	6	TrbB
TraN	pC15-1a	41056998	24	TrbB
TraU	pC15-1a	41056995	11	TrbB
TraH	pC4602-1	153971630	6	TrbB
TraN	pC4602-1	153971627	34	TrbB
TraU	pC4602-1	153971624	11	TrbB
TraH	pCAR3	113473815	6	DsbC
TraN	pCAR3	113473812	36	DsbC
TraU	pCAR3	113473810	14	DsbC
TraH	pED208	22539465	6	TrbB

Protein	Plasmid	Accession #	Cys #	Plasmid dsb isomerase
TraN	pED208	22539458	23	TrbB
TraU	pED208	22539455	11	TrbB
Ent638_4232	pENTE01	146284558	5	TrbB *
Ent638_4233	pENTE01	146284559	6	TrbB *
TraH	pENTE01	146284629	6	TrbB *
TraN	pENTE01	146284624	24	TrbB *
TraU	pENTE01	146284622	9	TrbB *
pG8786_073	pG8786	52788125	6	TrbB
TraH	pG8786	52788156	6	TrbB
TraN	pG8786	52788152	24	TrbB
TraU	pG8786	52788150	11	TrbB
TraH	pKPN3	229269588	6	TrbB
TraN	pKPN3	229269580	26	TrbB
TraU	pKPN3	229269513	11	TrbB
TraH	pKPN4	152973722	6	TrbB
TraN	pKPN4	152973714	26	TrbB
TraU	pKPN4	152973712	11	TrbB
plpl0040	pLPL	54292946	10	TrbB
TraH	pLPL	54292934	6	TrbB
TraN	pLPL	54292931	28	TrbB
TraU	pLPL	54292929	11	TrbB
TraH	pLPP	54295879	6	TrbB
TraN	pLPP	54295876	28	TrbB
TraU	pLPP	54295874	13	TrbB
TraH	pMAR7	190014912	6	TrbB
TraN	pMAR7	190014921	22	TrbB
TraU	pMAR7	190014923	11	TrbB
Mkms_5650	pMKMS01	119855041	6	-
TraH	pNL1	10956932	6	DsbC
TraN	pNL1	10956935	36	DsbC
TraU	pNL1	10956937	15	DsbC
TraH	pNR1	133756539	6	TrbB
TraN	pNR1	133756531	24	TrbB
TraU	pNR1	133756528	11	TrbB
TraH	pO86A1	116006902	6	TrbB
TraN	pO86A1	116006894	24	TrbB
TraU	pO86A1	116006891	11	TrbB
pOU1113_32	pOU1113	71559029	7	TrbB
TraH	pOU1113	71559045	6	TrbB
TraN	pOU1113	71559050	23	TrbB
TraU	pOU1113	71559053	11	TrbB

Protein	Plasmid	Accession #	Cys #	Plasmid dsb isomerase
TraN	pPBR1	47104038	30	TrbB
TraU	pPBR1	47104036	11	TrbB
DNaseI	pS18501	152998512	6	TrbB
Shew_185_4421	pS18501	152998518	5	TrbB
TraH	pS18501	152998530	6	TrbB
TraN	pS18501	152998534	28	TrbB
TraU	pS18501	152998536	10	TrbB
DNaseI	pS19501	160872986	6	TrbB
TraH	pS19501	160872937	6	TrbB
TraN	pS19501	160872940	28	TrbB
TraU	pS19501	160872942	10	TrbB
DNaseI	pS19502	160873009	6	TrbB
Sbal195_4604	pS19502	160873014	5	TrbB
TraH	pS19502	160873029	6	TrbB
TraN	pS19502	160873032	28	TrbB
TraU	pS19502	160873034	10	TrbB
TraH	pSF0157	149930800	6	TrbB
TraU	pSF0157	149930802	11	TrbB
w0012	pSF0157	149930757	6	TrbB
w0020	pSF0157	149930775	6	TrbB
PSLT014	pSLT	17233479	6	TrbB
TraH	pSLT	17233464	6	TrbB
TraN	pSLT	17233459	24	TrbB
TraU	pSLT	17233456	11	TrbB
Swit_5220	pSWIT01	148550657	6	DsbC
TraH	pSWIT01	148550818	6	DsbC
TraN	pSWIT01	148550815	30	DsbC
TraU	pSWIT01	148550813	15	DsbC
TraH	pUTI89	91206377	6	TrbB
TraN	pUTI89	91206368	24	TrbB
TraU	pUTI89	91206365	11	TrbB
TraH	pY016	37595840	6	TrbB
TraN	pY016	37595836	36	TrbB
TraU	pY016	37595833	11	TrbB
VVP49	pY016	37595870	6	TrbB
TraH	R100	9507644	6	TrbB
TraU	R100	9507632	11	TrbB
Aaur_pCT10011	TC1	119952502	10	-

* protein contains a C-X-X-S redox active site