JOURNAL OF BACTERIOLOGY, Dec. 2005, p. 8267–8277 Vol. 187, No. 24
0021-9193/05/$08.00+0 doi:10.1128/JB.187.24.8267–8277.2005

F-Like Type IV Secretion Systems Encode Proteins with Thioredoxin Folds That Are Putative DsbC Homologues

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Received 14 April 2005/Accepted 3 October 2005

F and R27 are conjugative plasmids of enteric bacteria belonging to the IncF and IncHI1 plasmid incompatibility groups, respectively. Based on sequence analysis, two genes of the F transfer region, *traF* and *trbB*, and three genes of the R27 transfer region, *trhF*, *dsbC*, and *htdT*, are predicted to encode periplasmic proteins containing a C-terminal thioredoxin fold. The C-X-X-C active-site motif of thioredoxins is present in all of these proteins except $TraF_F$. *Escherichia coli* carrying a *dsbA* mutation, which is deficient in disulfide bond formation, cannot synthesize pili and exhibits hypersensitivity to dithiothreitol (DTT) as monitored by mating ability. Overproduction of the *E. coli* disulfide bond isomerase DsbC, $TrbB_F$, $DsbC_{R27}$, or $HtdT_{R27}$, but not $TraF_F$ or $TrhF_{R27}$, reverses this hypersensitivity to DTT. Site-directed mutagenesis established that the C-X-X-C motif was necessary for this activity. Secretion into the periplasm of the C-terminal regions of $TrbB_F$ and $DsbC_{R27}$, containing putative thioredoxin folds, but not $TrhF_{R27}$, partially complemented the host *dsbA* mutation. A $trbB_F$ deletion mutant showed a 10-fold-lower mating efficiency in an *E. coli dsbC* null strain but had no phenotype in wild-type *E. coli*, suggesting redundancy in function between $TrbB_F$ and *E. coli* DsbC. Our results indicate that $TrbB_F$, $DsbC_{R27}$, and $HtdT_{R27}$ are putative disulfide bond isomerases for their respective transfer systems. $TraF_F$ is essential for conjugation but appears to have a function other than disulfide bond chemistry.

Bacterial conjugation is a fundamental mechanism for horizontal gene transfer that facilitates the transmission of genetic material, such as antibiotic resistance and other virulence factors, within and between bacterial species (67). It involves the transfer of single-stranded DNA from a donor cell to a recipient cell that has established close contact via mating pair formation. Although the F plasmid of *Escherichia coli* remains a paradigm for this process, conjugative plasmids have been reported in a variety of species, including many bacterial pathogens (42). More recently, conjugation has been considered to belong to the type IV secretion system (T4SS) family based on the similarities between the proteins involved in both processes (16).

Mating pair formation involves a complex apparatus spanning the donor cell envelope that assembles the conjugative pilus. This filamentous appendage interacts with the recipient cell and is thought to retract by depolymerization into the donor cell, thereby allowing intimate wall-to-wall contact during mating pair stabilization (1). The conjugative plasmid is transferred across both bacterial envelopes by a mechanism that remains poorly understood (17). All genes required for mating pair formation and DNA transfer are typically located in one or two clusters, the transfer regions, on conjugative plasmids.

Many components of the F plasmid transfer apparatus have homologues in other T4SS. For example, eight F transfer proteins are homologous to VirB proteins encoded by the T4SS of the Ti plasmid (42). However, the F plasmid encodes a unique group of proteins that are essential for pilus assembly and DNA transfer in F-like systems: TraF, -H, -N, -U, and -W and TrbC. All of these proteins reside in the periplasm, except the outer membrane protein TraN (24). Mutations in *traF*, *traG*, *traH*, *traW*, and *trbC* impair pilus assembly (5), whereas TraG, TraN, and TraU appear to be involved in mating pair stabilization and DNA transfer (38, 54).

Periplasmic and outer membrane proteins often contain disulfide bonds. Although disulfide bonds can form spontaneously in the oxidizing periplasmic environment (3), disulfide bond formation is facilitated in vivo by a number of specialized thiol-disulfide exchange enzymes. In *E. coli*, DsbA is a soluble, monomeric 21.1-kDa periplasmic protein (10) that randomly and rapidly oxidizes pairs of cysteine residues in secreted proteins through reduction of its own disulfide bond (68). The disulfide bond in DsbA is restored by the inner membrane protein DsbB, using the oxidizing power of the electron transport chain (8). *dsbA* mutants exhibit a pleiotropic phenotype including the rapid degradation of cell envelope proteins, a lack of motility due to incomplete assembly of the flagellar motor, hypersensitivity to metals and dithiothreitol (DTT), and resistance to M13 bacteriophage infection in F^+ cells because of the absence of F pili (35).

DsbC and DsbG are homodimeric disulfide isomerases that resolve incorrectly formed disulfide bonds (12, 50). They are maintained in a reduced state by DsbD, which is regenerated by the cytoplasmic thioredoxin reductase system (12, 61). DsbC stimulates proper folding and correct disulfide bond formation in a number of heterologous proteins (49). Although DsbC null mutants do not have an obvious phenotype, overexpressed *dsbC* can partially compensate for *dsbA* null mutants, possibly

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TABLE 1. Plasmids and *Escherichia coli* strains used in this study

Strain or plasmid	Relevant characteristics	Selective marker(s)[a]	Reference or source
Bacterial strains			
DH5α	*supE44* Δ*lacU169* (ϕ80d*lacZ*Δ*M15*) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Nal	Laboratory collection (29a)
DY330	W3110 Δ*lacU169 gal490* λ*c1857* Δ(*cro-bioA*)	Nal or Rif	69
MC4100	*araD139* Δ(*argF-lac*)*U169 rpsL150 relA1 flbB3501 deoC1 ptsF25 rbsR*	Sm	Manoil; laboratory collection
RI89	MC1000 *phoR* Δ*ara714 leu*$^+$	Sm	60a
RI90	RI89 *dsbA*::*kan1*	Km, Sm	60a
RI179	RI89 Δ*dsbC*::*cam*	Cm, Sm	60a
XK1200	F$^-$ *lac*Δ*U124* Δ(*nadA aroG gal att*λ *bio gyrA*)	Nal	K. Ippen-Ihler (54)
Plasmids			
PCR4Blunt-TOPO	4-Kb cloning vector with covalently linked topoisomerase	Amp, Km	Invitrogen
pBAD30	Cloning vector for controlled expression from $P_{ara\text{-}BAD}$	Amp	28
pOX38-Tc	pOX38::mini-Tn*10*	Tc	4
pOX38-Tc Δ*traF*::*kan*	pOX38-Tc with *kan* replacing codons 34 to 229 in *traF*	Tc, Km	This study
pOX38-Tc Δ*trbB*::*cat*	pOX38-Tc with *cat* replacing codons 14 to 160 in *trbB*	Tc, Cm	This study
pFTraF	F *traF* in pBAD30 (DPID)[b]	Amp	This study
pFTraF(CPYC)	F *traF* (D135C, I137Y, D138C)[c] in pBAD30 (CPYC)[b]	Amp	This study
pFTrbB	F *trbB* in pBAD30 (CPYC)[b]	Amp	This study
pFTrbB(Y63H)	F *trbB* (Y63H)[c] in pBAD30 (CPHC)[b]	Amp	This study
pFTrbB(SPYS)	F *trbB* (C61S, C64S)[c] in pBAD30 (SPYS)[b]	Amp	This study
pHTrhF	R27 *trhF* in pBAD30 (CQFC)[b]	Amp	This study
pHHtdT	R27 *htdT* in pBAD30 (CDGC)[b]	Amp	This study
pHDsbC	R27 *dsbC* in pBAD30 (CGFC)[b]	Amp	This study
pHDsbC(SGFS)	R27 *dsbC* (C107S, C110S)[c] in pBAD30 (SDGS)[b]	Amp	This study
pKDsbA	*E. coli* K-12 *dsbA* in pBAD30	Amp	This study
pKDsbA(NheI)	*E. coli* K-12 *dsbA* in pBAD30 with NheI site at codons 17 and 18	Amp	This study
pFBASS	*dsbA* signal sequence with A34 to K159 of F *trbB* in pBAD30	Amp	This study
pFBASS(Y63H)	pFBASS using F *trbB* (Y63H)[c]	Amp	This study
pHFASS	*dsbA* signal sequence with Q137 to R324 of R27 *trhF* in pBAD30	Amp	This study
pHCASS	*dsbA* signal sequence with A82 to K227 of R27 *dsbC* in pBAD30	Amp	This study

[a] Antibiotic resistances: Amp, ampicillin; Cm, chloramphenicol; Km, kanamycin; Nal, nalidixic acid; Rif, rifampin; Sm, streptomycin; Tc, tetracycline.
[b] Sequence of the four amino acids at the C-X-X-C motif indicated in Fig. 1 (in parentheses).
[c] The first letter indicates the wild-type amino acid; the second letter indicates the mutant amino acid; the number refers to the position of the amino acid within the mature protein.

due to a high level of redundancy among the disulfide isomerase proteins (53).

DsbA, DsbC, and other soluble Dsb proteins all contain a domain with homology to the thioredoxin fold. Thioredoxins form a large family of proteins that catalyze the formation and isomerization of disulfide bonds as well as other redox reactions (59). The thioredoxin fold consists of at least three α-helices and a four-stranded β-sheet (48). The active site consists of two cysteine residues in a C-X-X-C motif and is located in a loop connecting the first β-strand and the first α-helix within the thioredoxin fold. The cysteines are reversibly reduced and oxidized during the catalytic cycle. A conserved proline residue plays an important role in both structure and function of thioredoxins by forming van der Waals interactions between the loop in which it resides and the C-X-X-C disulfide bond (18).

Here we report that the F- and H-like (R27) conjugative plasmids encode proteins with a predicted C-terminal thioredoxin fold. Using assays developed for DsbC, we have determined that $TrbB_F$, $DsbC_{R27}$, and $HtdT_{R27}$ contain a functional C-X-X-C active site in their thioredoxin-like domains and that they are possibly disulfide bond isomerases. $TrhF_{R27}$, which contains a C-X-X-C motif, and $TraF_F$, which does not, apparently have a function not directly related to disulfide bond formation.

MATERIALS AND METHODS

Bacterial strains and plasmids. All strains and plasmids used in this study are listed in Table 1. Bacterial cultures were grown in Luria-Bertani medium with appropriate antibiotics at the following concentrations: ampicillin, 100 μg/ml; chloramphenicol, 20 μg/ml; kanamycin, 25 μg/ml; nalidixic acid, 40 μg/ml; rifampin, 20 μg/ml; streptomycin, 200 μg/ml; tetracycline, 10 μg/ml.

Recombinant DNA techniques, DNA sequencing, and PCR. Techniques for cloning restriction fragments or PCR fragments were as described previously (64). Blunt-end PCR products for cloning were purified from an agarose gel using the Qiaquick gel extraction kit (QIAGEN) to remove plasmid templates and were directly inserted into pCR4Blunt-TOPO (Invitrogen) according to the manufacturer's instructions. The sequences of PCR insertions into pCR4Blunt-TOPO (Invitrogen) were verified using the M13 Forward (5′ GTAAAACGA CGGCCAG) and M13 Reverse (5′ CAGGAAACAGCTATGAC) primers from Invitrogen. Restriction endonuclease digestion and ligation of DNA molecules were performed as described by Ausubel et al. (7). All enzymes were supplied by Roche Applied Science except Vent polymerase (New England Biolabs) and *Pfu* Turbo DNA polymerase (Stratagene). The method for automated sequencing has been described previously (38). Based on the method of Jonda et al. (34), the plasmid pKDsbA (NheI), which contains a single NheI restriction site at the end of the DsbA signal sequence without change to the amino acid sequence, was used in the construction of plasmids for periplasmic localization of thioredoxin fold variants. Thus the portion of $trbB_F$, $trhF_{R27}$, or $dsbC_{R27}$ encoding the thioredoxin fold could be amplified by PCR and cloned into pKDsbA (NheI).

TABLE 2. Primers used in this study[a]

Construct	Primer
pFTraF For	5′ GCTAAGGTACCCTCCGGTAATTATCTGG-3′
pFTraF Rev	5′ GGCATAAGCTTCCTGTCATTACGCTCAG-3′
pFTraF(CPYC) For	5′ CATGTTTTTTTACCGGGGGCAG**TG**CCCC**TACTG**CGGGCAACTGGCGCAGGTC-3′
pFTraF(CPYC)Rev	5′ GACCTGCGCCAGTTGCCCG**CAGTA**GGGG**CA**CTGCCCCCGGTAAAAAAACATG-3′
pFTrbB For	5′ GGTACCGAAGGGCAGCAGGAGGGC-3′
pFTrbB Rev	5′ GAAGCTTCCGGCAATGAGTAACAGCAC-3′
pFTrbB(Y63H) For	5′ CAGGGGCATTGCCCT**C**ACTGTCACCAGTTTGAC-3′
pFTrbB(Y63H) Rev	5′ GTCAAACTGGTGACAGT**G**AGGGCAATGCCCCTG-3′
pFTrbB(SPYS) For	5′ GTTTATGCAGGGGCATT**C**CCCTTACT**C**TCACCAGTTTGACCCGG-3′
pFTrbB(SPYS) Rev	5′ CCGGGTCAAACTGGTGA**G**AGTAAGGG**G**AATGCCCCTGCATAAAC-3′
pHTrhF For	5′ GGTACCATCATACCTCTATTCCATG-3′
pHTrhF Rev	5′ CAAGCTTCAAACGGCTAACGATAAAAG-3′
pHHtdT For	5′ GGTACCAGGCGAACCTAAAGAAAGTGAG-3′
pHHtdT Rev	5′ GAAGCTTGATGAACCAACAACCAGAGCGC-3′
pHDsbC For	5′ GGTACCTTCGCATGATAGTATTAACACTCC-3′
pHDsbC Rev	5′ GAAGCTTCATAAACACCTCTTGCAGTCG-3′
pHDsbC(SGFS) For	5′ GTTTACGGATATTACAT**C**CGGCTTTT**C**CCAGAAATTGCATCAAG-3′
pHDsbC(SGFS) Rev	5′ CTTGATGCAATTTCTGG**G**AAAAGCCG**G**ATGTAATATCCGTAAAC-3′
pKDsbA For	5′ GGTACCAGTTCTACAAGAACCCCCTTTGC-3′
pKDsbA Rev	5′ GAAGCTTCAGCGGCAGGATGCATTATCAG-3′
pKDsbA(NheI) For	5′ GTTTAGTTTTAGCGTTTAGCGC**TAGC**GCGGCGCAGTATGAAGATGG-3′
pKDsbA(NheI) Rev	5′ CCATCTTCATACTGCGCCGC**GCTA**GCGCTAAACGCTAAAACTAAAC-3′
pFBASS For	5′ GCTAGCGCGGCTCCCCGCTGGTTCCG-3′
pHFASS For	5′ GCTAGCGCGGCTCAGCAGTCGGTTATGAAAGATATTTTC-3′
pHCASS For	5′ GCTAGCGCCGCCACTAAAACAAAATCCATCG-3′

[a] Numbers present in the primer names indicate amino acid mutations in the active site. Restriction sites are underlined; KpnI or NheI were used for forward primers, whereas HindIII was used for reverse primers. Nucleotide changes for mutagenic primers are in boldface.

Gene deletion techniques. PCR amplification of the kanamycin resistance gene (including the native promoter) from the pUC4K plasmid (Amersham Pharmacia Biotech) was obtained using primers TEL-*traF-kan*-For (5′ GATGCAGGC TGGCAGTGGTATAACGAGAAAATAAATCCGAAAAGCCACGTTGTG TCTCAA) and TEL-*traF-kan*-Rev (5′ TCTTCAGAAACGTTCAGGAACTGT TTTGCCAGGTCGTCCTCGCTGAGGTCTGCCTCGTGA). The resulting *kan* linear DNA cassette had overhanging arms homologous to either nucleotides 15142 to 15182 or nucleotides 15767 to 15806 of *traF*. The PCR product was purified from an agarose gel using the Qiaquick gel extraction kit (QIAGEN) to remove the plasmid template and electroporated into *E. coli* strain DY330/F according to the procedure outlined by Yu et al. (69). The *E. coli* strain MC4100 was added as recipient cells and allowed to mate with DY330/F for 1 h. Recombinants were selected on kanamycin and streptomycin. To verify insertion of the *kan* cassette into F, clones were sequenced with TEL1 (5′ GGATCCAA AGA TGCAGGCTGGCAG) and TEL2 (5′ CAGAATTCCTCAGAAAAGAAATAA CCGG). PCR amplification of the Tn*9* chloramphenicol acetyltransferase gene (including the native promoter) from the pBAD33 plasmid (28) was obtained using primers TEL-*trbB-cat*-For (5′ CATGTCTCTCACTAAATCACTGCT GTTCACCCTGTTGCTGCTGTGACGGAAGATCACTTC) and TEL-*trbB-cat*-Rev (5′ CGTACATCTGCAAAACGGTATCCACCCGCGCCATAAAAC CTTATTCAGGCGTAGCACCAG). The resulting *cat* linear DNA cassette had overhanging arms homologous to nucleotides 16855 to 16894 and nucleotides 17333 to 17372 of *trbB*. The remainder of the protocol was completed as above. To verify insertion of the *cat* cassette into F, clones were sequenced with TEL26 (5′ GGTACCGAAGGGCAGCAGGAGGGC) and TEL27 (5′ GAAGCTTCC GGCAAT GAGTAACACCAC).

Site-directed mutagenesis. All mutagenesis was completed using QuikChange (Stratagene) site-directed mutagenesis according to the manufacturer's instructions. Briefly, 25 ng of pBAD30-*trbB* was used as a template for 125 ng of mutated nucleotide primers described in Table 2. PCR conditions were as follows: initial denaturing at 95°C for 2 min, followed by 18 cycles of denaturing at 95°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 7 min. After digestion with DpnI, 5 μl of PCR product was used to transform XL-1 Blue competent cells. Every mutant was sequenced to ensure that mutants were correct and in frame.

Mating assays. Mating assays have been described by Anthony et al. (4) and Klimke and Frost (38). To test transfer of pOX38-Tc, an F derivative, from RI89 (wild-type), RI90 (*dsbA*), or RI179 (*dsbC*) donor cells to XK1200 recipient cells, donor cells were grown to mid-log phase and mixed with various concentrations of DTT (0 mM to 7 mM) for 1 h prior to the mating assay. Two hundred microliters of mid-log-phase donor cells and recipient cells was incubated at 37°C for 45 min. The cultures were serially diluted in saline (between 10^{-2} and 10^{-7}), and 10-μl droplets were plated out on either tetracycline and streptomycin or tetracycline and nalidixic acid. If protein overproduction for vector constructs was required, then donor cells were induced with 0.1% arabinose 2 h prior to adding the recipient cells. When testing the DTT hypersensitivity of a *dsbA* null mutant, donor cells were grown to mid-log phase and mixed with 1 mM dithiothreitol for 1 h prior to the mating assay.

RESULTS

F- and H-like transfer regions encode putative thiol-disulfide oxidoreductases. The nonredundant protein sequence database was searched with the program PSIBLAST (2) to find homologues of $TraF_F$ encoded by the F plasmid (24, 27). PSIBLAST detected several TraF homologues encoded by the transfer regions of other F-like (E values = $4e^{-21}$ to e^{-136}) and H-like (E-values = $6e^{-4}$ to $3e^{-18}$) plasmids (Table 3). Using the position-sensitive scoring matrix (PSSM) based on this set of TraF homologues, a second cycle of PSIBLAST found a group of proteins that displayed homology to approximately 100 amino acids of the C-terminal region of $TraF_F$ (E values = $2e^{-6}$ to $4e^{-8}$) (Table 3). This group of proteins included $TrbB_F$ and its homologues encoded by R100 (5), pSLT (51), pED208 (45), and pYJ016 (19), as well as a few proteins annotated as thiol-disulfide isomerases. Subsequent cycles of PSIBLAST using a PSSM inclusion threshold of 0.001 revealed extensive homology to the thioredoxin/thiol-disulfide isomerase family (E values = $<8e^{-10}$) (Table 3). A search for conserved domains within homologues of $TraF_F$ using the program CD-Search (47) revealed thioredoxin-like regions (COG0526, TrxA) within TraF encoded by R391 (14) and SXT (11). Although $TraF_F$ lacks the C-X-X-C motif that forms the catalytic site of thioredoxins, closely related TraF orthologues encoded

TABLE 3. Homologies among TraF, TrhF, TrbB, DsbC, and HtdT proteins encoded by F-like, H-like, and I-like plasmids

Plasmid	Inc group	Organism	Plasmid reference(s) or accession no.	BLAST query			
				F TraF		R27 DsbC	
				Protein	E value	Protein	E value
F-like							
F	FI	*Escherichia coli*	24 and 27	TraF	e^{-136} (1)[a]		
				TrbB	$4e^{-07}$ (2)		
R100	FII	*Salmonella enterica* serovar Typhimurium	5	TraF	e^{-133} (1)		
				TrbB	$8e^{-08}$ (2)		
pSLT		*Salmonella enterica* serovar Typhimurium	51	TraF	e^{-107} (1)		
				TrbB	$2e^{-06}$ (2)		
pED208	FV	*Salmonella enterica* serovar Typhi	45	TraF	$1e^{-51}$ (1)		
				TrbB	$4e^{-08}$ (2)		
pYJ016		*Vibrio vulnificus*	19	TraF	$4e^{-21}$ (1)		
				TrbB	$1e^{-07}$ (2)		
H-like							
pNL1		*Novosphingomonas aromaticivorans*	63	TraF	$3e^{-18}$ (1)	Orf883	$2e^{-21}$ (2)
Rts1	T	*Proteus vulgaris*	55	Orf240	$8e^{-09}$ (1)	Orf212	$2e^{-16}$ (2)
R391	J	*Providencia rettgeri*	14	TrhF	$5e^{-13}$ (1)	DsbC	$3e^{-23}$ (2)
SXT		*Vibrio cholerae*	11	TrhF	$4e^{-13}$ (1)	S054	$3e^{-23}$ (2)
pCAR1		*Pseudomonas resinovorans*	46	TrhF	$1e^{-09}$ (1)	DsbC	$2e^{-14}$ (2)
pHCM1	HI1	*Salmonella enterica* serovar Typhi	57	TrhF	$7e^{-05}$ (1)	DsbC	e^{-140} (1)
						HtdT	$4e^{-04}$ (2)
R27	HI1	*Salmonella enterica* serovar Typhi	40, 41, and 65	TrhF	$7e^{-05}$ (1)	DsbC	e^{-140} (1)
						HtdT	$3e^{-04}$ (2)
R478	H2	*Serratia marcescens*	26	TrhF	$6e^{-04}$ (1)	DsbC	e^{-120} (1)
						HtdT	$1e^{-04}$ (2)
I-like							
R64	I1	*Salmonella enterica* serovar Typhimurium	25			TrbB	$5e^{-15}$ (3)
ColIb-P9	I1	*Shigella sonnei*	60			TrbB	$5e^{-15}$ (3)
pEL60		*Erwinia amylovora*	23			TrbB	$3e^{-07}$ (2)
pCTX-M3		*Citrobacter freundii*	NC_004464			TrbB	$4e^{-06}$ (3)
None	None	*Escherichia coli*		TrxA	$8e^{-10}$ (3)		
None	None	*Escherichia coli*				DsbC	$5e^{-32}$ (1)
None	None	*Escherichia coli*				DsbG	$5e^{-16}$ (2)

[a] The numbers in parentheses indicate the number of cycles of PSI-BLAST.

by the H-like T4SS as well as all TrbB proteins contain this active-site motif (Fig. 1). Interestingly, TraF orthologues which lack the C-X-X-C motif are encoded only by plasmids that encode TrbB, whereas TrbB is not encoded by plasmids encoding a TraF homologue that contains a C-X-X-C motif. This suggests that TrbB may compensate for the lost redox function of TraF.

The IncHI1 R27-encoded transfer protein $DsbC_{R27}$ is annotated as a putative disulfide interchange protein (accession number, AAF69969) (40, 41, 65). A PSIBLAST search with this protein sequence revealed $DsbC_H$ homologues encoded by the transfer regions of H-like plasmids pHCM1 (57) and R478 (26) (E values = e^{-140} and e^{-120}, respectively) as well as the well-characterized *E. coli* chromosomally encoded DsbC (E value = $5e^{-32}$) (Table 3). The second cycle of PSIBLAST identified another group of $DsbC_H$ homologues from other H-like plasmids (E values = $2e^{-14}$ to $3e^{-23}$), establishing homology to H-like HtdT proteins (E value = $4e^{-4}$) and the chromosomally encoded *E. coli* DsbG (E value = $5e^{-16}$) (Table 3). Subsequent cycles of PSIBLAST using a PSSM inclusion threshold of 0.001 revealed homology to the TrbB proteins encoded by the IncI plasmids R64 (25), ColIb-P9 (60), pEL60 (23), and pCTX-M3 (accession number, NC_004464) (E values = $4e^{-6}$ to $5e^{-15}$). The naming of R64 TrbB and F TrbB was coincidental; discerning the relationship between these two proteins required many iterations of PSIBLAST, suggesting that they are, at best, distantly related. P-like T4SS (42), such as that of RP4 or the Vir region of the Ti plasmid of *Agrobacterium tumefaciens*, do not encode these homologues. Searches with CD-Search also revealed that all $DsbC_H$ and $HtdT_H$ homologues, with the exception of $HtdT_{R478}$, share homology with COG1651 (DsbG), showing E values between $7e^{-16}$ and 0.004. All these proteins contain the C-X-X-C active-site motif of thioredoxins (Fig. 1). The thioredoxin domain of *E. coli* DsbC contains an extra 43-residue α-helical subdomain (50). Cys^{141} and Cys^{163} within this subdomain form an intramolecular disulfide bond that is important for the stability of the molecule (44). Despite variations in length, all $DsbC_H$ and $HtdT_H$ proteins contain this extra domain, which includes the two conserved cysteine residues (Fig. 1).

Disulfide isomerization activity requires hydrophobic or aromatic amino acids in the third position of the C-X-X-C active site (13). All $DsbC_H$ and $TrbB_F$ proteins contain aromatic amino acids in this position. Most $TraF_F$ or $TrhF_H$ proteins with an active site have either aromatic or hydrophobic amino acids in this position, with the exception of $TraF_{pNL1}$, which has an alanine,

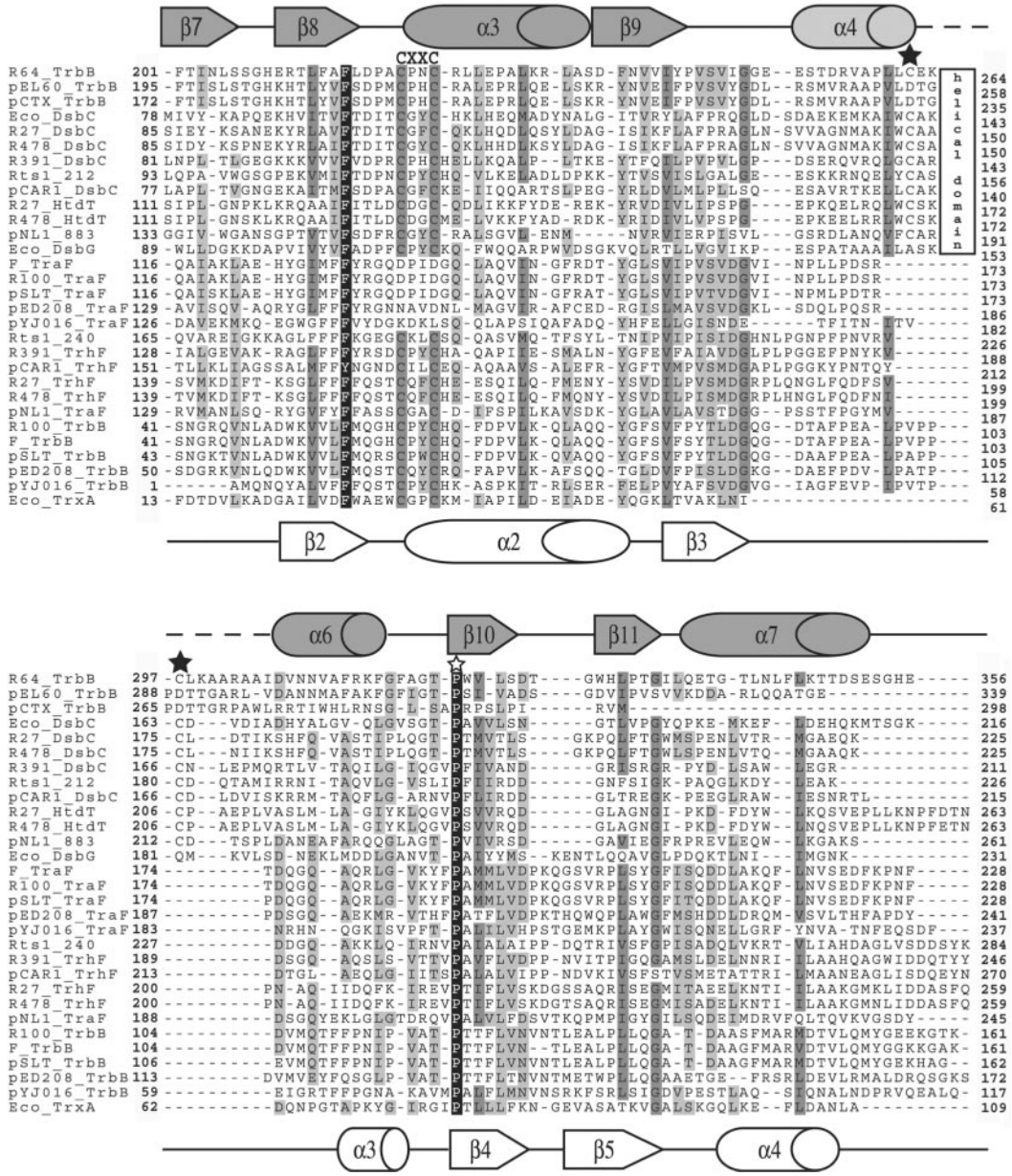


FIG. 1. Multiple sequence alignment of TraF, TrhF, TrbB, HtdT, and DsbC encoded by the F-like, H-like, and I-like transfer regions with the TrxA, DsbC, and DsbG proteins from *E. coli*. Each protein in the alignment is preceded by the plasmid name or a three-letter abbreviation for the organism if the gene is carried on the chromosome (Eco = *Escherichia coli*). The following information regarding these proteins is arranged as plasmid name (if necessary), protein name, GenBank accession number, and host organism: R64, TrbB, BAB91646, *S. enterica* serovar Typhimurium; pEL60, TrbB, AAQ97939, *Erwinia amylovora*; pCTX-M3, TrbB, AAN87722, *Citrobacter freundii*; DsbC, AAC75931, *E. coli*; R27, DsbC, AAF69969, *S. enterica* serovar Typhi; R478, DsbC, CAE51723, *Serratia marcescens*; R391, DsbC, AAM08034, *Providencia rettgeri*; Rts1, Orf212, BAB93774, *Phaseolus vulgaris*; pCAR1, DsbC, BAC41659, *Pseudomonas resinovorans*; R27, HtdT, AAF69865, *S. enterica* serovar Typhi; R478, HtdT, CAE51541, *S. marcescens*; pNL1, Orf883, AAD03960, *Novosphingobium aromaticivorans*; DsbG AAC73705, *E. coli*; F, TraF, BAA97961, *E. coli*; R100, TraF, BAA78873, *E. coli*; pSLT, TraF, AAL23500, *S. enterica* serovar Typhimurium; pED208, TraF, AAM90720, *S. enterica* serovar Typhi; pYJ016, TraF, BAC97739, *Vibrio vulnificus*; Rts1, Orf240, BAB93802, *P. vulgaris*; R391, TraF, AAM08018, *P. rettgeri*; pCAR1, TraF, BAC41668, *P. resinovorans*; R27, TrhF, AAF69964, *S. enterica* serovar Typhi; R478, TrhF, CAE51730, *S. marcescens*; pNL1, TraF, AAD03949, *N. aromaticivorans*; R100, TrbB, BAA78876, *E. coli*; F, TrbB, BAA97965, *E. coli*; pSLT, TrbB, AAL23502, *S. enterica* serovar Typhimurium; pED208, TrbB, AAM90721, *S. enterica* serovar Typhi; pJY016, TrbB, BAC97741, *V. vulnificus*; TrxA, AAA24694, *E. coli*. Proteins from other plasmid systems with sequences identical to a protein listed above were not included in Fig. 1, but their accession numbers are as follows: ColIb-P9, TrbB, BAA75142, *Shigella sonnei*; pHCM1, DsbC, CAD09838, *S. enterica* serovar Typhi; SXT, S054, AAL59715, *V. cholerae*; pHCM1, HtdT, CAD09683, *S. enterica* serovar Typhi; SXT, TraF, AAL59678, *V. cholerae*; pHCM1, TrhF, CAD09843, *S. enterica* serovar Typhi. The secondary structures of DsbC (top) and thioredoxin (bottom) are as determined by X-ray structure and nuclear magnetic resonance analysis (33, 50). The active sites are labeled as CXXC. The black stars indicate cysteines known or proposed to be important in forming a disulfide bond. The white star indicates a conserved proline residue that is found in close proximity to the active sites of DsbA, DsbC, and thioredoxin from *E. coli*. The box marked "helical domain" indicates the presence of an extra helical domain in some DsbC-like proteins. Residues in black indicate 100% conservation, residues in dark gray indicate more than 80% conservation, and residues in light gray indicate at least 50% conservation.

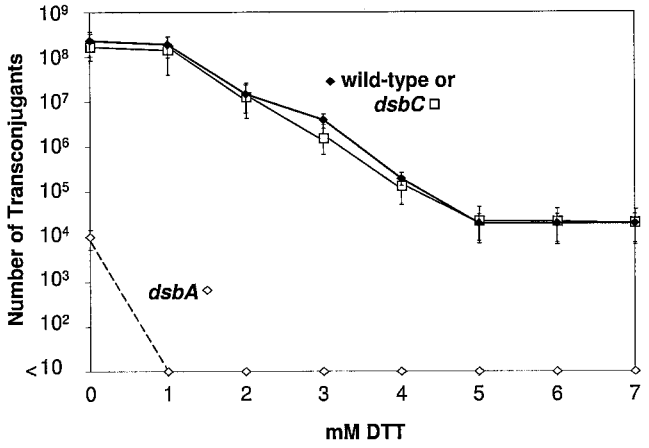


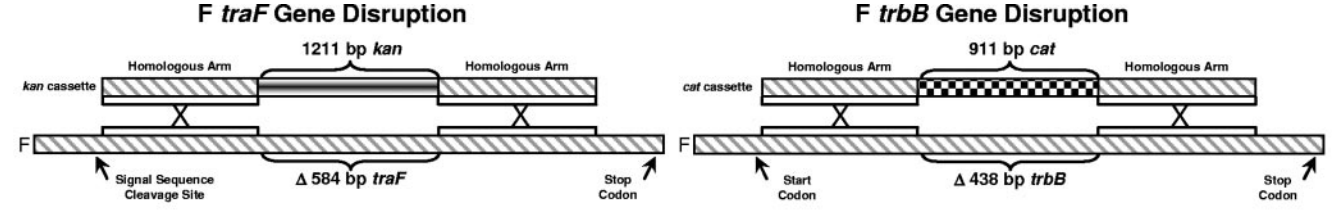
FIG. 2. Mating efficiency of pOX38-Tc is greatly decreased by the reducing agent DTT. The mating efficiencies of *E. coli dsbA* and *dsbC* mutants containing pOX38-Tc were plotted against increasing concentrations of DTT.

and the $HtdT_H$ proteins, which have glycine. A proline in close structural proximity to the active site is required for maintaining an active conformation in *E. coli* thiol-disulfide oxidoreductases, (36) and is conserved in all $TraF_F$, $TrhF_H$, $TrbB_F$, $HtdT_H$, and $DsbC_H$ proteins discussed here (Fig. 1).

$TraF_F$, $TrhF_H$, $TrbB_F$, $DsbC_H$, and $HtdT_H$ are predicted to be soluble periplasmic proteins. $TraF_F$ and $TrbB_F$ are known to contain leader peptides (24), and SignalP predictions (56) detected leader peptides in all but one of the $TrhF_H$, $HtdT_H$, and $DsbC_H$ proteins. The exception is $HtdT_{R27}$ (accession number, AAF69865), for which a leader sequence could not be found; however, when the sequence of $htdT_{R27}$ was compared to the sequence of the nearly identical $htdT_{pHCM1}$ (accession number, CAD09683), it became apparent that $htdT_{R27}$ should begin 35 codons upstream from its current predicted start, at the rare TTG start codon. Analysis of this longer sequence revealed a 22-residue leader peptide in this protein. No other putative transmembrane regions were predicted in the mature proteins. Full-length $TraF_F$ and $TrbB_F$ can be expressed as soluble proteins (6; L. S. Frost, unpublished data), suggesting that $TrhF_{R27}$, $DsbC_{R27}$, and $HtdT_{R27}$ are also soluble periplasmic proteins.

Transfer efficiency of pOX38-Tc is affected by DTT or *dsbA* null mutations. DsbA null mutants have been reported to affect assembly of the flagellar motor; pilus assembly, as determined by monitoring by M13 infection; and hypersensitivity to DTT (10, 21). We therefore determined the mating efficiencies of wild-type and *dsbA* and *dsbC* mutant donor cells containing the F derivative pOX38-Tc. The mating assays were subsequently repeated following incubation of the donor cells with various concentrations of DTT for 1 hour. DTT is a small reducing agent that can diffuse into the periplasm and maintain sulfhydryl groups in the reduced state in vivo. Wild-type bacteria have been shown to tolerate DTT concentrations up to 7 mM (52). The mating efficiency of wild-type and *dsbC* donor cells was not affected by 1 mM DTT, but higher concentrations reduced mating efficiency up to 4 orders of magnitude in a dose-dependent manner before a plateau was reached at 5 mM DTT (Fig. 2). Donor cells with a chromosomal *dsbA* null mutation had transfer efficiencies 4 logs lower than that of wild-type cells; addition of 1 mM DTT to these cells completely abolished DNA transfer, indicating a DTT-hypersensitive phenotype for this strain (Fig. 2).

Construction of *traF* and *trbB* null mutants of pOX38-Tc. The essential role of $TraF_F$ in pilus formation was initially determined using a *traF* insertion mutant that could produce a C-terminally truncated TraF protein (24). To rule out any effect of the N-terminal region of TraF, we used the λ Red recombination system to replace codons 34 to 229 with a *kan* cassette, thus creating pOX38-Tc Δ*traF*::*kan* (Fig. 3; Table 1). This mutation completely abolished DNA transfer and phage f1 infection, an indication that no F pili were assembled. The



Construct	Mating efficiency (no. of transconjugants)	f1 phage sensitivity
pOX38-Tc RI89 (wildtype)	1.2×10^8	S
pOX38-TcΔ*traF*::*kan* RI89	<10	R
+ pTEF uninduced	8.1×10^7	S
+ pTEF induced	1.1×10^8	S

Construct	Mating efficiency (no. of transconjugants)	f1 phage sensitivity
pOX38-Tc RI89 (wildtype)	1.2×10^8	S
pOX38-Tc Δ*trbB*::*cat* RI89	1.2×10^8	S

FIG. 3. Gene disruption of F *traF* and F *trbB*. Above are schematic diagrams of a *kan* and *cat* cassette insertion into F *traF* and F *trbB*, respectively, through homologous recombination. The native *kan* and *cat* promoters are present in both gene disruptions. Below the diagram are the mating efficiencies and f1 phage sensitivities of the *traF* and *trbB* null mutants.

TABLE 4. Effect of *dsbC* and *trbB* null mutations on pOX38-Tc transfer

Construct	No. of transconjugants	Mating efficiency (±10) (no. of transconjugants/100 donor cells)
pOX38-Tc/RI89 (wild type)	1.2×10^8	80
pOX38-Tc/RI179 (*dsbC*)	1.1×10^8	73
pOX38-TcΔ*trbB*::*cat*/RI89 (wild type)	1.0×10^8	67
pOX38-TcΔ*trbB*::*cat*/RI179 (*dsbC*)	1.1×10^7	7
pOX38-TcΔ*trbB*::*cat*/RI179 (*dsbC*) + pFTrbB	1.0×10^8	67
pOX38-TcΔ*trbB*::*cat*/RI179 (*dsbC*) + pBAD30	1.0×10^7	7

TABLE 5. Effect of overproduction of F- and H-like transfer proteins in a *dsbA* mutant[a]

E. coli strain	DTT concn (mM)	Plasmid construct	No. of transconjugants
pOX38-Tc/RI89 (wild type)			1.2×10^8
pOX38-Tc/RI90 (*dsbA*)			1.1×10^4
pOX38-Tc/RI90 (*dsbA*)	1		<10
pOX38-Tc/RI90 (*dsbA*)	1	pBAD30	<10
pOX38-Tc/RI90 (*dsbA*)	1	pFTraF[b]	<10
pOX38-Tc/RI90 (*dsbA*)	1	pFTraF(CPYC)[b]	<10
pOX38-Tc/RI90 (*dsbA*)	1	pFTrbB[b]	9.0×10^3
pOX38-Tc/RI90 (*dsbA*)	1	pFTrbB(Y63H)[b]	3.0×10^3
pOX38-Tc/RI90 (*dsbA*)	1	pFTrbB(SPYS)[b]	<10
pOX38-Tc/RI90 (*dsbA*)	1	pHTrhF[c]	<10
pOX38-Tc/RI90 (*dsbA*)	1	pHHtdT[a]	8.0×10^3
pOX38-Tc/RI90 (*dsbA*)	1	pHDsbC[c]	1.0×10^4
pOX38-Tc/RI90 (*dsbA*)	1	pHDsbC(SGFS)[c]	<10

[a] All vector constructs were induced with the addition of 0.1% arabinose 2 hours prior to adding the recipient cells for the mating assay.
[b] F-like.
[c] H-like.

effects could be fully complemented by *traF* supplied in *trans* on pFTraF (Fig. 3).

Similarly, the previous insertion of a *kan* cassette between codons 66 and 67 of *trbB* might express a truncated version of TrbB with a functional thioredoxin motif (37). To ensure against this, codons 14 to 160 of *trbB* were replaced with the chloramphenicol acetyltransferase gene (*cat*) from Tn*9* to give pOX38-Tc Δ*trbB*::*cat* (Fig. 3; Table 1). This null mutation affected neither plasmid transfer nor f1 phage infection (Fig. 3).

Mating efficiency is reduced in an *E. coli dsbC trbB$_F$* double null mutant. The *trbB$_F$* null mutant has no obvious phenotype, similar to strains encoding several *E. coli* periplasmic thiol-redox proteins (35). Thus, significant functional redundancy could exist among this group of enzymes (62). To investigate whether a *trbB dsbC* double mutation affects F conjugation, pOX38-Tc or pOX38-Tc Δ*trbB*::Cm was introduced into RI189 (wild-type) or RI179 (*dsbC*) cells. Mating assays revealed that transfer efficiency decreased 10-fold in the *dsbC* strain containing pOX38-Tc Δ*trbB*::Cm compared to donor/plasmid combinations that contained either *trbB* or *dsbC* single mutations or completely wild-type donor cells (Table 4). Furthermore, complementation of RI179/pOX38-Tc Δ*trbB*::Cm with TrbB supplied in *trans* from pFTrbB resulted in the restoration of wild-type mating efficiency (Table 4).

Overproduced TrbB$_F$, DsbC$_{R27}$, or HtdT$_{R27}$ complements the DTT hypersensitivity of a *dsbA* null mutant. The overexpression of *E. coli dsbC* from a multicopy plasmid has been shown to alleviate the general hypersensitivity to DTT exhibited by *dsbA* null mutants (53, 66). Although pFTrbB (Table 1) could not complement the effect of the *dsbA* mutation on the mating ability of RI190/pOX38-Tc (*dsbA*) cells (data not shown), it could overcome the hypersensitivity to DTT as demonstrated by a mating assay (Table 5). This complementation required that pFTrbB be first induced with 0.1% arabinose, as uninduced samples showed no effect (data not shown). Similarly, overexpression of *dsbC$_{R27}$* or *htdT$_{R27}$* (pHDsbC or pHHtdT, respectively; Table 1) yielded comparable results (Table 5).

The C-X-X-C motif of TrbB$_F$ and DsbC$_{R27}$ is required to complement DTT-hypersensitive *dsbA* mutants. To determine whether the C-X-X-C motifs of TrbB$_F$ and DsbC$_{R27}$ were important in the rescue of *dsbA* donor cells in the presence of DTT, site-directed mutagenesis (QuikChange; Stratagene) was used to convert the putative active-site cysteines to serines to give pFTrbB(SPYS) and pHDsbC(SGFS), respectively. Mating was undetectable using RI190/pOX38-Tc (*dsbA*) donor cells containing either pFTrbB(SPYS) or pHDsbC(SGFS), even after induction with 0.1% arabinose. Thus, the C-X-X-C motif appears to be essential for plasmid-encoded TrbB and DsbC function, and it acts as an active redox site in these proteins. Because TraF$_F$ lacks the putative active-site cysteines, site-directed mutagenesis was used to restore a C-P-Y-C motif at amino acids 135 to 138 of the mature protein. This mutant plasmid, pFTraF(CPYC), when supplied in *trans*, encoded a protein that behaved like wild-type TraF and complemented the transfer deficiency of RI89/pOX38-Tc Δ*traF*::*kan* donor cells (data not shown) without induction with arabinose, suggesting that basal expression of TraF was sufficient for complementation. However, mating was undetectable when either the wild-type (pFTraF) or mutated [pFTraF(CPYC)] TraF$_F$ was provided in *trans* in RI90 (*dsbA*) donor cells treated with 1 mM DTT (Table 5). Wild-type TrhF$_{R27}$ (pHTrhF) was also unable to complement DTT hypersensitivity when provided in *trans* even though TrhF$_{R27}$ contains a C-X-X-C motif (Table 5). Thus TraF and TrhF do not appear to have DsbC-like activity and the presence or absence of the C-X-X-C motif does not affect their function in pilus formation or conjugation.

Periplasmic secretion of the C-terminal regions containing thioredoxin folds of TrbB$_F$ and DsbC$_{R27}$, but not TrhF$_{R27}$, partially complements a *dsbA* mutation. *E. coli* thioredoxin, a disulfide oxidoreductase, can be secreted to the periplasm via the DsbA signal sequence. Although periplasmic wild-type thioredoxin cannot replace DsbA, thioredoxin variants containing the Grx-type (C-P-Y-C) or DsbA-type (C-P-H-C) active-site sequences could complement a DsbA deficiency by approximately 40 to 60% as measured by motility on a swarm plate (34). Furthermore, a DsbC variant with a 76-amino-acid deletion in its dimerization domain is able to complement the effect of a *dsbA* null mutation by 44% as measured by alkaline phos-

TABLE 6. Effect of periplasmic secretion of C-terminal putative thioredoxin folds of F- and H-like transfer proteins in a *dsbA* mutant[a]

E. coli strain	Plasmid construct	No. of transconjugants
pOX38-Tc/RI89 (wild type)		1.2×10^8
pOX38-Tc/RI90 (*dsbA*)		1.1×10^4
pOX38-Tc/RI90 (*dsbA*)	pBAD30	1.0×10^4
pOX38-Tc/RI90 (*dsbA*)	pKDsbA uninduced	8.0×10^7
pOX38-Tc/RI90 (*dsbA*)	pKDsbA induced	5.5×10^4
pOX38-Tc/RI90 (*dsbA*)	pFBASS uninduced	1.2×10^5
pOX38-Tc/RI90 (*dsbA*)	pFBASS induced	1.3×10^3
pOX38-Tc/RI90 (*dsbA*)	pFBASS(Y63H) uninduced	1.6×10^5
pOX38-Tc/RI90 (*dsbA*)	pFBASS(Y63H) induced	1.1×10^3
pOX38-Tc/RI90 (*dsbA*)	pHFASS uninduced	3.0×10^4
pOX38-Tc/RI90 (*dsbA*)	pHFASS induced	2.1×10^4
pOX38-Tc/RI90 (*dsbA*)	pHCASS uninduced	2.0×10^5
pOX38-Tc/RI90 (*dsbA*)	pHCASS induced	1.1×10^3

[a] Induced refers to the addition of 1% arabinose 2 hours prior to adding the recipient cells for the mating assay; uninduced refers to the continued growth for 2 hours without the addition of arabinose.

phatase activity (9). To determine whether the C-terminal regions containing putative thioredoxin folds of $TrbB_F$, $DsbC_{R27}$, or $TrhF_{R27}$ could complement a DsbA deficiency during conjugation, we constructed fusions between the DsbA signal sequence and our truncated proteins using the method of Jonda et al. (34) (Table 1). Mating was assayed using RI190/pOX38-Tc (*dsbA*) donor cells in the absence of DTT. As a control, the expression of *E. coli dsbA* from uninduced pKDsbA was shown to nearly fully complement a *dsbA* mutation as measured by mating efficiency (Table 6). The expression of genes encoding the C-terminal putative thioredoxin domains of $TrbB_F$ and $DsbC_{R27}$ (pFBASS and pHCASS, respectively) was also shown to increase mating efficiency by 11- to 20-fold in the *dsbA* mutant (Table 6). To test whether having a DsbA-type (C-P-H-C) active site rather than the wild-type (C-P-Y-C) active site of truncated $TrbB_F$ would be more effective in complementing the *dsbA* mutation, we constructed pFBASS(Y63H) (Table 1), which gave results similar to pFBASS and pHCASS (Table 6), implying that DsbA has attributes other than the thioredoxin domain that contribute to its activity. In contrast, the mating efficiency of pOX38-Tc in RI90 (*dsbA*) did not increase when the putative thioredoxin domain of $TrhF_{R27}$ was expressed from pHFASS despite the presence of a C-X-X-C motif (Table 6).

Interestingly, induction of these constructs (pKDsbA, pFBASS, and pHCASS) with 1% arabinose gave a decrease in mating efficiency. Perhaps this overexpression caused an excessive increase of oxidizing potential in the periplasm that interferes with the disulfide bond isomerization. For instance, overproduction of DsbB, but not DsbA, results in a defect in periplasmic disulfide bond formation, supporting the idea that isomerization has an optimal upper limit (32).

DISCUSSION

Sequence homology searches detected a putative thioredoxin fold within the C-terminal regions of $TraF_F$ and $TrbB_F$ encoded by F-like T4SS and $TrhF_H$, $DsbC_H$, and $HtdT_H$ within or near regions encoded by the T4SS in IncH plasmids. $TrbB_F$ and homologues encoded by the three H plasmids contain the thioredoxin C-X-X-C active-site motif, as well as the essential proline residue that is adjacent to the active-site loop. They also contain a hydrophobic or aromatic residue at the third position, which is consistent with a possible role in disulfide bond isomerization (13). The second position can vary and affects interactions with the substrate (43), suggesting substrate specificity in each system. $DsbC_H$ and $HtdT_H$ also conserve the extra helical domain that is present in *E. coli* DsbC, including the disulfide bond between residues Cys^{141} and Cys^{163}. This disulfide bond is required for the chaperone activity of *E. coli* DsbC (44), further suggesting that $DsbC_H$ and $HtdT_H$ are homologues of DsbC.

Proteins that promote disulfide bond formation have previously been shown to be essential for the proper assembly of a number of periplasmic protein complexes, including the *E. coli* flagellar apparatus, the enteropathogenic *E. coli* bundle-forming pilus, the *E. coli* pap pili, the *Klebsiella oxytoca* type II secreton, and *Salmonella enterica* serovar Typhimurium plasmid-encoded fimbriae (15, 58). In addition to previous studies on the effect of a *dsbA* mutation on F piliation as measured by filamentous phage sensitivity and electron microscopy (10), we observed that the mating efficiency of the F plasmid is also affected by the presence of a *dsbA* mutation in the donor cell and that the addition of DTT further decreases mating ability to undetectable levels. Interestingly, the F-like T4SS-specific proteins TraN, -U, -H, and -W and TrbC have 22, 11, 6, 1, and 2 conserved cysteines, respectively. The F-like universal T4SS

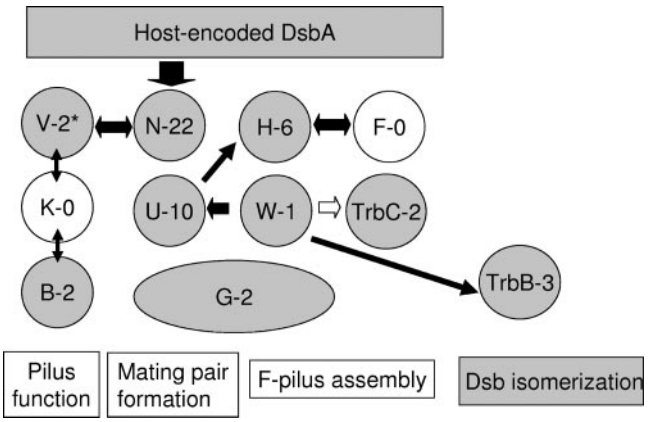


FIG. 4. A representation of possible interactions in the F pilus assembly and mating pair formation system. The proteins for F pilus assembly and mating pair formation are divided into three groups: those that are highly conserved among T4SS and are involved in pilus assembly and function (TraV, -K, and -B), F-T4SS proteins involved in mating pair stabilization (TraN, -U, and -G), and F-T4SS proteins involved in pilus assembly (TraF,-G [N-terminal region], -H, and -W and TrbC). DsbA is known to affect pilus assembly (10) and TraN stability (39). Host-encoded DsbC and -G may be involved in disulfide bond isomerization (not shown) as might be F TrbB. For the R27 plasmid, DsbC and HtdT might also be involved (not shown). The number of cysteines is shown after each protein (single letter for Tra proteins), indicating a potential for disulfide bond formation. Proteins with no cysteines are emphasized in white. The possible interactions between the proteins are summarized according to Harris and Silverman (31). White arrows indicate inferred interactions from sequence data for TrbC and TraW, which are fused into one protein in R27 (42).

envelope proteins TraB, -V, and -G also have 2, 3, and 2 cysteines, respectively, suggesting there are many candidates for disulfide bond formation and isomerization (Fig. 4). Nonreducing gel electrophoresis has demonstrated that wild-type TraV and a $TraV_{C18S}$ mutant can form mixed disulfides with many cell envelope proteins (30). Similar experiments based on protein mobility under nonreducing conditions have determined the presence of intra- and intermolecular disulfide bonds in TraN (39). In addition, the T4SS of the Ti plasmid, which has no apparent thioredoxin-like homologues, relies on disulfide bond formation for the stability of its transfer protein complex. In this case, VirB7 stabilizes VirB9 by a disulfide cross-link and the B7-B9 dimer in turn stabilizes other VirB proteins (16). Since the *dsbA* mutation affects F piliation, TraB, -G, -H, -V, and -W and TrbC, which are involved in pilus assembly, are the best candidates. The effect of a *dsbA* mutation on other proteins that are specific to F-like T4SS, which are involved in mating pair stabilization and possibly pilus retraction, cannot be readily assessed in the absence of the pilus.

In addition to $TraF_H$, the H-like T4SS also encode homologues ($DsbC_H$ and $HtdT_H$) that closely resemble the host DsbC protein, which is known to be involved in disulfide bond isomerization (49). $DsbC_{R27}$ and $HtdT_{R27}$ along with $TrbB_F$ could act as disulfide bond isomerases, rather than disulfide bond-forming proteins, because (i) $TrbB_F$ cannot complement a host *dsbA* mutant; (ii) $TrbB_F$, $DsbC_{R27}$, and $HtdT_{R27}$ can alleviate the DTT hypersensitivity of a *dsbA* null mutant, similar to the *E. coli* DsbC disulfide bond isomerase (53, 66); (iii) truncated $TrbB_F$ and $DsbC_{R27}$ proteins, containing the predicted thioredoxin fold, can partially complement a *dsbA* null mutation; (iv) single *trbB* and *E. coli dsbC* null mutants have no phenotype whereas a *trbB dsbC* double mutant has a 10-fold decrease in mating efficiency; and (v) $DsbC_{R27}$ and $HtdT_{R27}$ both conserve the extra helical domain of *E. coli* DsbC, including the important disulfide bond between Cys^{141} and Cys^{163}, which determines specificity for chaperone activity.

$TraF_F$ is essential for pilus assembly even though it does not contain a C-X-X-C active-site motif or any other cysteines. When a C-P-Y-C motif was introduced by mutagenesis, $TraF_F$ remained able to complement a *traF* mutation in *trans* but could not alleviate the DTT hypersensitivity of a *dsbA* donor cell. Similarly, $TrhF_{R27}$, which has the conserved C-X-X-C motif, is unable to counteract the hypersensitivity of a *dsbA* mutant to DTT. Furthermore, the cloned C-terminal domain of $TrhF_{R27}$, containing the thioredoxin fold element, was unable to complement the *dsbA* mutation. $TrhF_{R27}$ might have substrate specificity or redox potential requirements that do not allow it to affect general periplasmic disulfide bond formation. Liu and Wang (44) have shown that the active-site cysteine residues of *E. coli* DsbC are necessary for enzyme activity but are not required for substrate binding and chaperone function. Therefore, $TraF_F$ and $TrhF_{R27}$ may act as chaperones during pilus assembly. Yeast two-hybrid studies have indicated that $TraF_F$ interacts with $TraH_F$ (six cysteines) (31), which has a C-terminal coiled-coil domain (42), making it an excellent candidate for chaperone-assisted assembly in the periplasm.

It should be noted that thioredoxin has been found to play a role in processes unrelated to disulfide bond chemistry. It has a role in processivity of the T7 DNA polymerase (29) and in filamentous phage assembly (22), suggesting that TraF and TrhF might also have an unexpected role in pilus biogenesis.

There are more proteins with disulfide bonds than there are disulfide bond isomerases, suggesting there may be overlapping specificities that can explain the lack of a phenotype in *trbB* and *E. coli dsbC* null mutants (35). Interestingly, many large F- and H-like conjugative plasmids encode at least one Dsb homologue containing the C-X-X-C motif. H-like transfer regions such as those of pNL1 (63), SXT (11), R391 (14), Rts1 (55), and pCAR1 (46) encode either $DsbC_H$ or $HtdT_H$ but not both, whereas R27 (40, 41, 65), R478 (26), and pHCM1 (57) have two transfer regions, each encoding either $HtdT_H$ or $DsbC_H$. Both F- and H-like transfer regions encode TraF or TrhF; however, *trbB* is found only in plasmids encoding TraF lacking the C-X-X-C motif. The strict conservation of at least one functional Dsb homologue in each plasmid suggests a need for assisted disulfide bond formation. This conserved presence of plasmid-derived Dsb homologues, in spite of the chromosomally expressed DsbC, might be due to the relatively low isomerase activity of *E. coli* DsbC compared to eukaryotic protein disulfide isomerase, for instance (20). Since very few periplasmic and outer membrane proteins require more than two disulfide bonds per subunit, a high isomerase activity might not be necessary (20) whereas a resident plasmid producing several cell envelope proteins containing multiple disulfide bonds might need auxiliary isomerase activities. Unfortunately, we cannot determine definitively at this time whether these plasmid-encoded proteins are disulfide bond isomerases for their respective DNA transfer systems since there are no assays for their activity beyond pilus formation and conjugation. We are currently examining which of the many candidate proteins encoded by the F- and H-like transfer regions besides TraN (39) require DsbA for pilus assembly and transfer.

ACKNOWLEDGMENTS

This work was supported by grants from NSERC and CIHR to L.S.F. and the Alberta Heritage Foundation for Medical Research to B.H.

We thank Diane Taylor and members of her laboratory for useful discussions and providing plasmid R27.

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