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# Mutants in disulfide bond formation that disrupt flagellar assembly in *Escherichia coli*

**(motility/chemotaxis/protein folding)**

FRANK E. DAILEY AND HOWARD C. BERG

Department of Cellular and Developmental Biology, Harvard University, Cambridge, MA 02138

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**ABSTRACT** **We report the isolation and characterization of *Escherichia coli* mutants (*dsbB*) that fail to assemble functional flagella unless cystine is present. Flagellar basal bodies obtained from these mutants are missing the L and P rings. This defect in assembly appears to result from an inability to form a disulfide bond in the P-ring protein (FlgI). Cystine suppresses this defect in *dsbB* strains. We also show that *dsbA* strains [Bardwell, J. C. A., McGovern, K. & Beckwith, J. (1991) *Cell* 67, 581–589] fail to assemble P rings, apparently from a similar failure in disulfide bond formation. However, cystine does not completely suppress this defect in *dsbA* strains. Thus, disulfide bond formation in FlgI is essential for assembly. DsbA likely puts in that bond directly, whereas the DsbB product(s) play a role in oxidizing DsbA, so that it can be active.**

Cells of the bacterium *Escherichia coli* are motile and chemotactic. They are propelled by about six peritrichous flagella. The *fla* genes are genes that have been identified as required for flagellar assembly. The *fla* genes of *E. coli* (*flg*, *flh*, and *fli*) or *Salmonella typhimurium* (also *flj*) are clustered in three or four regions of the chromosome, respectively, in several operon hierarchies. The analysis of mutants in these genes has established, in broad outline, the order in which their products are synthesized. Roughly half of these products have been identified as structural components. A schematic drawing of the basal part of the flagellum is shown in Fig. 1. For an extensive review of the genetics and biogenesis of bacterial flagella, see ref. 1.

Undoubtedly, other gene products, the functions of which are important for flagellar assembly, remain to be identified. These proteins might include chaperones that ensure proper folding, proteins required for export of *fla* gene products (such as the L- and P-ring proteins, which have been shown to have a cleavable signal sequence; cf. refs. 2–4), or proteins that modify *fla* gene products. A component of the latter kind is described in this report—namely, a component required for disulfide bond formation.

We isolated a mutant defective in disulfide bond formation (*dsbB*) by an indirect route. Strains of *E. coli* K-12 that lack all cytoplasmic chemotaxis proteins except CheY swim smoothly under most conditions (5, 6), but they tumble in the presence of acetate (7). Although this behavior now appears to result from the phosphorylation of CheY by acetyl phosphate (ref. 8; unpublished work), we thought some other kinase might be involved. So we searched for this kinase by isolating strains that swam smoothly in the presence of acetate. One such strain proved motile only when grown in the presence of cystine or cystamine. The defect was traced to the assembly of L and P rings, resulting from the failure of disulfide bond formation in the P-ring protein (FlgI). Other strains known to be defective for disulfide bond formation in periplasmic and outer-membrane proteins (*dsbA*; ref. 9) also showed this assembly defect. However, the latter mutants were not cured by adding cystine. Also, our mapping data located *dsbB* to a different region of the *E. coli* chromosome.

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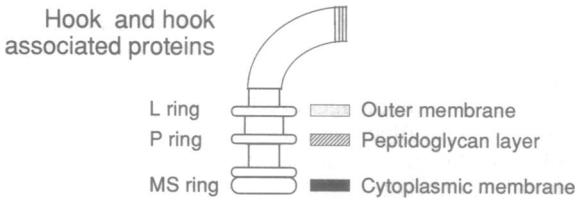


FIG. 1. Schematic of the wild-type hook–basal-body structure and its juxtaposition to components of the cell wall. The motor is assembled from the inside out: MS ring (FliF), rod (FliE, FlgB, -C, -F, -G, extending from the MS ring to the hook), P ring (FlgI), L ring (FlgH), hook (FlgE), and hook-associated proteins (FlgK, -L, FliD). The filament protein (FliC, not shown) is inserted between the two most distal hook-associated proteins. Proteins required at the earliest stages of assembly, including the switch components FliG, -M, -N, are not indicated. The latter are thought to reside at the cytoplasmic face of the MS ring. The operons controlling this synthesis are regulated in three hierarchies, in the following order: class I (*flhD*), class II (*flgA,-B, flhB, fliA,-E,-F,-L*), and class III (*flgK, fliC,-D*).

## MATERIALS AND METHODS

**Bacteria.** Bacteria, plasmids, and phage are listed in Table 1. P1 transductions and transformations were done per Silhavy *et al.* (15). Strain FD597 was isolated as a tetramycin-sensitive ($Tet^S$) derivative of FD596 by using fusaric acid plates (19). FD600 was constructed by using λgt4 to isolate temperature-sensitive lysogens (15).

**Media.** LB medium (20) was used for routine growth of cells and for transformation and transduction experiments. Tryptone broth (TB) was used to assess motility or chemotactic ability in rich medium. M63 minimal medium (15) was supplemented with sodium citrate (10 mM). Glucose or glycerol (0.4% wt/vol) was added as a carbon source and, when required, amino acids were added at the concentrations specified by Davis *et al.* (21). To determine the amino acid requirement of *dsbB* strains for motility, auxanography was done, as described in ref. 21, except that only amino acids were added. Ampicillin (Amp), kanamycin (Kan), and tetracycline (Tet) were added to minimal or rich medium, as required (21). Agar (Difco) was added at a concentration of 1.5% for standard procedures or at 0.3% for swarm plates (to determine chemotactic behavior or motility).

**Isolation of *dsbB* Strains.** Strain FD572 was mutagenized with λNK1098, as described in Way *et al.* (16). Approximately $10^5$ independent tetramycin-resistant ($Tet^R$) derivatives were isolated and then grown at 30°C in TB/0.4% glycerol in the presence of 25 mM L-arabinose (to induce CheY expression) until the cells were fully motile ($\approx 10^9$ cells per ml). Aliquots (0.1 ml) were layered on top of glycerol gradients, as in ref. 22, except that sodium acetate was added

at a uniform concentration of 25 mM, and attractants were deleted. We used 10-cm columns of liquid in clear plastic tubes 1.5 cm in diameter. The tubes were incubated at 30°C for 4 hr, and the bottom 1 ml was collected and plated out on LB/tetracycline plates to isolate individual colonies.

**Electrophoresis.** SDS/PAGE (23) was done at a polyacrylamide concentration of 10%. One volume of sample was mixed with an equal volume of sample buffer [4% SDS/20% (vol/vol) glycerol/0.12 M Tris, pH 6.8]. 2-Mercaptoethanol was added to the samples at a final concentration of 5%, except where otherwise indicated.

**Immunodetection.** Cells were grown at 30°C in glycerol minimal medium with or without L-cystine (100 μg/ml) to ≈1 × $10^9$ cells per ml, concentrated quickly, and put in sample buffer. Immunodetection of flagellin and the hook protein was done by using Western immunoblots (24), with rabbit polyclonal serum to either protein as the primary antibody, followed by anti-rabbit IgG alkaline phosphatase conjugate (Sigma). Western Blue reagent (Promega) was used to detect the alkaline phosphatase conjugate. A semi-dry blotter (Owl Scientific, Cambridge, MA) was used to transfer proteins from the acrylamide gel to the nitrocellulose membrane (Bio-Rad).

**Enzyme Assays.** β-Galactosidase assays (20) were done on cells grown in glycerol minimal medium at 30°C with or without cystine (100 μg/ml) to ≈1 × $10^9$ cells per ml. Alkaline phosphatase assays were done as in ref. 25, except cells were grown at 37°C in bis-Tris medium (26) with a 1 mM citrate supplement and 0.4% glucose as the carbon source. The concentration of phosphate was either 0.067 mM (derepressed) or 1 mM (repressed).

Table 1. *E. coli* strains, plasmids, and phage

| Name | Relevant genotype | Ref. |
|---|---|---|
| Strain | | |
| RP437 | Wild type for chemotaxis | 10 |
| HCB721 | Δ(*cheA–cheY*)1590::XhoI Tn*5* Δ(*tsr*)7021 *trg*::Tn*10* | 6 |
| FD571 | pJH120/HCB721 | * |
| FD572 | $Tet^S$ of FD571 | * |
| FD577 | *dsbB*::mini-Tet of FD572 | * |
| FD596 | *dsbB*::mini-Tet of RP437 | * |
| FD597 | $Tet^S$ of FD596 | * |
| FD600 | FD597 λ$cI^{ts}$ | * |
| FD619 | YK3421 *fliC*::Mu*dI* (*lac*, Ap) | 11 |
| FD620 | YK4337 *fliA*::Mu*dI* (*lac*, Ap) | 11 |
| FD621 | *dsbB*::mini-Tet of FD619 | * |
| FD622 | *dsbB*::mini-Tet of FD620 | * |
| MS912 | *fliC*(am) | 12 |
| JTG10 | *gshA*::Tn*10*-kan | 13 |
| JCB572 | *dsbA*::kan1 | 9 |
| FD695 | *dsbA*::kan1 of RP437 | * |
| PB111 | *minB1 zcf-117*::Tn*10* | 14 |
| PB103$T_{mk}$17 | *minB*::$T_{mk}$17 | 14 |
| KF31 | *minB*::$T_{mk}$17 of RP437 | † |
| KF32 | *minB1 zcf-117*::Tn*10* of RP437 | † |
| FD697 | pOH20/KF32, *flgHI*$^+$ on plasmid | * |
| FD699 | *minB1 zcf-117*::Tn*10* of FD597 | * |
| FD700 | pOH20/FD699, *flgHI*$^+$ on plasmid | * |
| Plasmid | | |
| pJH120 | para*cheY* | ‡ |
| pOH20 | *flgHI*$^+$ | 3 |
| pPB102 | *minB*$^+$ | 14 |
| Phage | | |
| λgt4 | λ$cI^{ts}$ | 15 |
| λNK1098 | element 8, mini-Tet | 16 |
| λPB37 | *minB*$^+$ | 14 |
| λ11G8 | | 17 |
| λ2A3 | | 17 |
| λ7C10 | | 17 |

mk, Mini::Tn*10*-kan; $Tet^S$, tetracycline sensitive; ts, temperature sensitive. Flagellar nomenclature is as defined in ref. 18.
*This work.
†K. Fahrner, Harvard University, Cambridge, MA.
‡F. W. Dahlquist, University of Oregon, Eugene, OR.

**Detection of Plasmid-Encoded Gene Products.** Cells were grown at 34°C to a density of ≈2 × $10^9$ cells per ml in minimal medium supplemented with all 20 amino acids (except asparagine, cysteine, and glutamine) at concentrations specified in ref. 21. Minicells were isolated as described in Homma *et al.* (27). Cells were labeled in the same growth medium, except methionine was omitted. Two microliters of L-[$^{35}$S]methionine [>10 mCi/ml (New England Nuclear; 1 Ci = 37 GBq)] was added to 100 μl of purified minicells ($OD_{660}$ = 1.0) for 30 min at 34°C. Where noted, L-cystine (100 μg/ml) was added to cells when they were grown and/or when they were labeled.

**Electron Microscopy.** Cells were grown in glycerol minimal medium at 30°C to ≈1 × $10^9$ cells per ml with or without L-cystine (100 μg/ml). Hook–basal body structures were isolated (28) and negatively stained with 2% phosphotungstate.

## RESULTS

**Isolation of *dsbB* Strains.** Strain FD572 (see Table 1), a $Tet^S$ derivative of HCB721 in which *cheY* is expressed, is deleted for *cheA*, *-W*, *-R*, *-B*, and *-Z*. This strain has a smooth-swimming phenotype, unless acetate is present. After mutagenesis with the transposon mini-Tet (16), we selected for strains that swam smoothly in the presence of acetate. Such cells swim to the bottom of a glycerol gradient containing acetate (see *Materials and Methods*), presumably because the cell body sediments more rapidly than the flagellar bundle, and the cells are unable to reorientate their swimming direction; nonmotile cells or cells that tumble stay at the top. One such strain, FD577, was used as a donor for P1 transduction to move the mutation causing the smooth-swimming behavior into the wild-type chemotactic strain RP437 by selecting for $Tet^R$. When one of these transductants, FD596, was tested in soft-agar plates containing T broth, the chemotactic rings for serine and aspartate were normal. However, the strain failed to respond to mannose or proline in soft agar containing minimal medium. Upon microscopic examination, we found that FD596 was not motile ($Mot^-$) in glycerol, mannose, or proline minimal medium. Thus, surprisingly, strain FD596 is $Mot^-$ when grown in minimal medium but $Mot^+$ when grown in a rich medium, such as TB. The difference did not appear to be due to growth defects because FD596 and RP437 grew at identical rates in glycerol minimal medium.

**Requirement for L-Cystine for Motility in *dsbB* Strains.** Addition of vitamin-free Casamino acids (Difco) to glycerol minimal medium at a concentration of 0.25% or higher partially restored chemotaxis of *dsbB* strains in soft-agar plates. Tests of individual amino acids showed that only L-cystine was required (Table 2), although the cells had to grow for several generations in its presence. Concentrations of ≈25 μg/ml or higher completely restored wild-type motility and chemotaxis, whereas concentrations below ≈5 μg/ml had no apparent effect. D-Cystine also restored motility. On the other hand, freshly prepared solutions of L-cysteine were ineffective. Supplementation with 10 mM $MgSO_4$ or L-methionine or cystathionine also had no effect, suggesting that cystine does not work because of some nonspecific defect in sulfur metabolism. *dsbB* strains did not appear to be $Mot^-$ as a result of a defect in glutathione synthesis, because not only did solutions of oxidized or reduced glutathione fail to restore motility, but strain JTG10

Table 2. Compounds tested for ability to restore motility in *dsbB* strains

| Compound | Fully motile, μg/ml | Partially motile, μg/ml | Max. conc. tested, μg/ml |
|---|---|---|---|
| L-Cystine | 25 | 5 | 100 |
| L-Cysteine | — | | 200 |
| D-Cystine | 100 | 25 | 200 |
| Cystamine | 100 | 50 | 100 |
| Cysteamine | — | | 100 |
| Cystathionine | — | | 100 |
| Glutathione (ox) | — | | 200 |
| Glutathione (red) | — | | 200 |
| L-Methionine | — | | 100 |

All compounds (Sigma) were tested in glycerol minimal medium in liquid or in swarm plates at 30°C. Max. conc., maximum concentration; ox, oxidized form; red, reduced form; —, no concentration tested stimulated motility.

(defective in glutathione synthesis) was motile in minimal medium. Furthermore, although cysteamine (a compound found in the lumen of the endoplasmic reticulum) did not stimulate motility in *dsbB* strains, the oxidized form, cystamine, did. None of the compounds tested interfered with the motility of strain RP437.

**Characterization of the Motility Defect in *dsbB* Strains.** To determine at what step flagellar assembly was blocked (see Fig. 1 legend) we measured the production of flagellar filament (FliC) and hook protein (FlgE) by immunoblots. Little FliC or FliE protein was present in strain FD596 (*dsbB*::mini-Tet) as compared with the parental strain RP437, unless cells were grown in the presence of cystine (data not shown). We next determined whether this might be the result of differences in the transcription of the genes by monitoring the synthesis of β-galactosidase in strains carrying *lacZ* fusions to the transcriptional promoter of *fliA* or *fliC*. *fliA* encodes for the flagellum-specific σ factor and is a middle gene in the hierarchy. The level of its expression was the same in strains FD620 (*fliA–lacZ*) and FD622 (*fliA–lacZ dsbB*::mini-Tet), grown in the presence or absence of cystine. *fliC* encodes for the flagellar filament and is a late gene in the operon hierarchy. The level of its expression was the same in strain FD619 (*fliC–lacZ*) grown in the presence or absence of cystine as in strain FD621 (*fliC–lacZ dsbB*::mini-Tet) grown in its presence. However, the level of expression of *fliC* was depressed ≈2-fold when strain FD621 was grown without cystine. These results suggest that transcription of genes in the early or middle part of the hierarchy, which includes *flgE*, is normal, and that transcription of late genes does occur, although at a reduced level. Therefore, the much lower levels of hook and filament protein found in *dsbB* strains do not appear due to a transcriptional defect of *flgE* and *fliC* but might reflect some assembly defect affecting the translation or stability of the proteins.

**Analysis of Flagellar Basal Bodies in *dsbB* Strains.** Electron micrographic images of negatively stained basal-body preparations are shown in Fig. 2. The control (Fig. 2*a*), prepared from filament-minus (*fliC*) strain MS912, shows the normal complement of rings; compare with Fig. 1. When strain FD596 (*dsbB*) was grown in minimal medium without cystine, the isolated structures lacked discernible L and P rings. Usually, the hook and filament also were missing (Fig. 2*b*). Occasionally, they were present (Fig. 2*c*). Structures of these kinds have been seen previously in *flgA* or *flgI* strains (29). However, when strain FD596 was grown with cystine, the isolated structures appeared normal, as expected (Fig. 2*d*).

**Analysis of L- and P-Ring Proteins in Minicells.** Because the above results suggested that *dsbB* mutants are Mot⁻ because of a defect in L-ring or P-ring assembly, we analyzed the products of the *flgH* and *flgI* genes in minicells to see whether we could detect differences in their expression level. Because the *E. coli flgH* and *flgI* genes have not been well characterized, we used for these analyses plasmid pOH20, which expresses *S. typhimurium flgH* and *flgI* genes, as well as β-lactamase (*bla*). These genes are functionally homologous to the *E. coli* genes: they complement *flgH* or *flgI* mutants in *E. coli* (27). Furthermore, minicells carrying the *dsbB*::mini-Tn*10* mutation exhibited the same motility defect as strain FD596—i.e., they were Mot⁻ in the absence of cystine, even when wild-type *flgH* or *flgI* genes were present on a high-copy-number plasmid.

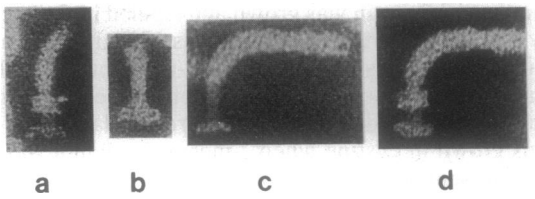


FIG. 2. Wild-type and *dsbB* hook–basal-body structures. (*a*) Structure from *fliC* strain MS912 grown without cystine; this hook–basal body is wild type. (*b* and *c*) Structures from *dsbB* strain FD596 grown without cystine; all structures were missing L and P rings, but some had hooks and filaments or filament stubs (*c*). (*d*) Structure from FD596 grown with cystine. (Scale: the MS ring is ≈2.6 nm in diameter.)

We analyzed the proteins specified by this plasmid for their mobility in SDS gels in the presence or absence of 2-mercaptoethanol (Fig. 3). We did this because it had been shown previously that the mobility of FlgI (2, 30) and β-lactamase (32) decreases when a reducing agent is added. This difference probably results from the presence of a disulfide bond: the reduced form of a protein often has decreased mobility in SDS/polyacrylamide gels (33). Therefore, we also wished to examine whether the *dsbB* mutation affected disulfide bond formation. This mutation did not affect the synthesis of FlgH, FlgI, and β-lactamase or their mobilities in samples containing 2-mercaptoethanol (Fig. 3*a*, lanes 1 and 2). Therefore, *dsbB* does not adversely affect processing of the N-terminal signal sequences of FlgH and FlgI. (In other gels, the pre-FlgI and FlgI bands were of comparable intensity with or without 2-mercaptoethanol.) However, *dsbB* does affect the formation of a disulfide bond in the FlgI protein and in β-lactamase.

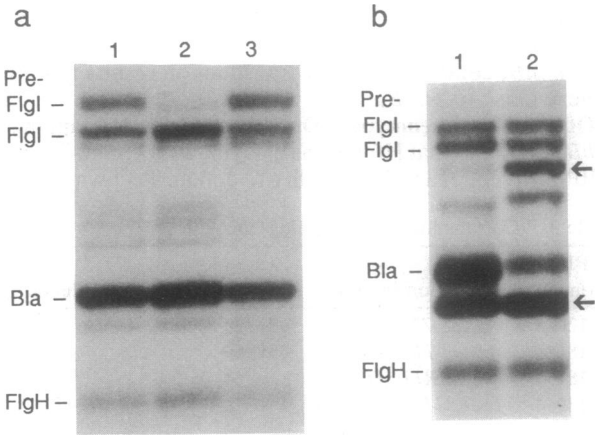


FIG. 3. Production of P-ring protein (FlgI) in minicells. (*a*) With 2-mercaptoethanol: wild-type strain FD697 (lane 1); *dsbB* strain FD700 grown without cystine (lane 2); strain FD700 grown with cystine (lane 3). (*b*) Without 2-mercaptoethanol: *dsbB* strain FD700 grown without cystine (lane 1); strain FD700 grown with cystine (lane 2). Pre-FlgI, FlgI before removal of signal sequence; Bla, β-lactamase; FlgH, L-ring protein. Arrows indicate the oxidized forms of FlgI and Bla.

When the *dsbB* strain was grown and labeled in the presence of cystine, more of the oxidized forms of FlgI and β-lactamase appeared in gels not containing 2-mercaptoethanol (Fig. 3*b*, lanes 1 and 2, arrows). Patterns of synthesis of FlgI and β-lactamase in wild-type strains were similar to the *dsbB* strains grown in cystine when 2-mercaptoethanol was omitted in the samples (data not shown).

**Comparison with *dsbA* Strains.** The gene *dsbA* encodes a protein, DsbA, which appears to play an essential role in catalyzing disulfide bond formation in several periplasmic and outer-membrane proteins, such as alkaline phosphatase and OmpA (9). Strains lacking DsbA were nonmotile when grown in the absence of cystine. When grown with cystine (100 μg/ml), as many as 1% of the cells in the population appeared partially motile. We isolated basal bodies from strain FD695 (*dsbA*::kan1) grown in the presence of cystine (100 μg/ml). Most structures examined had no L or P rings, as found earlier for *dsbB* strains. Occasionally, wild-type structures were seen (data not shown). Analysis of the synthesis of FlgI and FlgH in *dsbA* strains also suggested that these strains fail to assemble the P ring because of a defect in disulfide bond formation in FlgI: the mobility of this protein was not affected by 2-mercaptoethanol (data not shown).

**Chromosomal Location of *dsbB*.** We mapped *dsbB* by Hfr linkage using an Hfr transposon collection (34) with strain FD597 as the recipient. Exconjugates were selected on LB streptomycin plates plus kanamycin or tetracycline, depending upon the Hfr used. According to this analysis, *dsbB* was located between 22 and 35 min on the *E. coli* K-12 chromosome (data not shown). A more precise location was determined by P1 cotransduction. We tested for cotransduction with Tn insertions in that region (34). The results, given in Table 3, suggest that *dsbB* maps near *fadR*, at ≈26 min on the *E. coli* chromosome (35). This position is not near any *fla* operon. When we tried to construct a *minB* (cell-division locus) *dsbB* strain for analysis of L- and P-ring proteins described earlier, we found that *dsbB*::mini-Tet and *minB*::$T_{mk}$17 cotransduced at a frequency of 100%. However *dsbB* is not part of the *minB* complex, because λPB37, a phage carrying genes from the *minB* region, and plasmid pPB102, a plasmid carrying just genes of the *minB* complex (14), failed to complement strain FD600 (described in *Materials and Methods*). We also tested λ transducing phages from the *minB* region from the bank of Kohara *et al.* (17): λ2A3 and λ1168 did complement our *dsbB* strain for motility, but λ7C10 did not. The two λ transducing phages that do complement *dsbB* overlap in the *umuCD* region of the chromosome (17) near *minB*, confirming the P1 transduction results that *dsbB* is very close to *minB*.

**Other *dsbB* Phenotypes.** During preliminary mapping of *dsbB*, we found that Hfr strains carrying *dsbB*::mini-Tet were ≈$10^3$-fold reduced in their ability to donate $Tet^R$ when grown in the absence of cystine (data not shown). This result is probably due to their inability to express the F pilus, as has been shown for *dsbA* strains (9). *dsbB* strains also produced mucoid colonies when grown on minimal plates, as do *dsbA* strains (9). Cystine completely suppresses this phenotype in *dsbB* but not in *dsbA* strains (data not shown). *dsbA* also has been shown to affect the activity of alkaline phosphatase (9, 36). When grown in minimal medium at low concentrations of phosphate (0.067 mM), *dsbB* strains showed ≈20-fold lower activities of alkaline phosphatase than did wild-type cells; however, the levels were normal when the cells were grown in the presence of cystine (data not shown). Thus, the *dsbB* mutation causes the same phenotypes as does *dsbA*, except that in *dsbB* strains, they are all completely suppressible by cystine (at 100 μg/ml).

Table 3. Cotransductional mapping of *dsbB*

| P1 donor | Insertion in donor | Colonies scored | Cotransduction, % |
|---|---|---|---|
| CG12078 | *zce-726*::Tn*10* | 23 | <5 |
| CG18463 | *zcf-117*::Tn*10* | 59 | 41 |
| CG18497 | *fadR13*::Tn*10* | 64 | 78 |
| CG18544 | *fadR3115*::Tn*10*-kan | 79 | 47 |
| CG12016 | *zcg-3060*::Tn*10* | 73 | 6 |
| CG12106 | *zcg-3116*::Tn*10*-kan | 90 | <2 |
| CG18445 | *trp*-B83::Tn*10* | 68 | <2 |

The recipient was FD597. Transductants were isolated by selection for tetracycline resistance (when the insertion of the donor was Tn*10*) or for kanamycin resistance (when the insertion was Tn*10*-kan). Cotransduction frequencies to $dsbB^+$ were scored by testing recipients for motility on glycerol minimal swarm plates. For additional information on the P1 donors, see ref. 34.

## DISCUSSION

*dsbB* strains represent a specific class of flagellar-assembly mutants in which assembly of the P ring (and thus the L ring) depends upon the presence of cystine or cystamine during growth (Fig. 2). Previous work by others (29) has shown that *flgA* or *flgI* strains also fail to assemble the P ring (and the L ring) to the rod. FlgA does not appear to be part of the final flagellar complex; FlgI is the P-ring structural protein. The *dsbB* mutation was found in a search for motile cells containing CheY that fail to tumble when exposed to acetate; however, the reasons for the smooth-swimming phenotype are not known.

FlgI (and the L-ring protein FlgH) were produced at the same levels in *dsbB* strains as in wild-type strains (Fig. 3). The N-terminal signal sequence of FlgI appeared to be cleaved, indicating that the protein is probably exported. However, shifts in the positions of FlgI bands on SDS/polyacrylamide gels in the presence or absence of reducing agents did show that *dsbB* strains are defective in FlgI disulfide bond formation. An effect of reducing agents on the mobility of FlgI has been shown (2, 30): the mobility of FlgI is slightly higher in the absence of 2-mercaptoethanol. This result suggests the presence of an intramolecular disulfide bond (33) between the only two cysteines present in the mature form of FlgI, at positions 254 and 338 (4). When we looked at the mobility of FlgI (and β-lactamase) in *dsbB* strains, we found that FlgI (and β-lactamase) migrated predominantly at the slower rate, even under nonreducing conditions (Fig. 3). Because cystine suppresses both this defect and the P-ring assembly defect, the failure to put in the disulfide bond in FlgI is probably a direct cause of the P-ring (and L-ring) assembly defect.

Our work does not determine why a failure to put the disulfide bond in FlgI leads to an assembly defect. A failure in disulfide bond formation might affect the stability of the protein, although our results to date (e.g., Fig. 3) suggest that it does not. Alternatively, this failure might affect the possible interaction of FlgI and FlgA. Recently, the number of FlgI molecules that make up one P ring has been determined by two different approaches to be ≈26 (37, 38). The lack of a disulfide bond might affect the folding and aggregation of FlgI around the rod to form the P ring. The failure of L-ring assembly in the wake of a failure in P-ring assembly, also seen in *flgA* and *flgI* mutants (29), is not surprising, given that the two rings appear physically attached (39, 40).

Recently, two groups have isolated strains of *E. coli* with defects in disulfide bond formation in periplasmic proteins, such as alkaline phosphatase. These defects have been shown to result from a mutation in the same gene, *dsbA* (9) or *ppfA* (36), which maps in a different region of the chromosome as *dsbB*. The gene product of *dsbA* (*ppfA*) has a region of local similarity to the active sites of the bacterial oxidoreductase thioredoxin and to disulfide-isomerase found in the lumen of

the endoplasmic reticulum of eukaryotes. To determine the relationship between the *dsbA* and *dsbB* gene products, we tested whether *dsbA* affected disulfide bond formation in FlgI and β-lactamase. Both FlgI and β-lactamase lacked disulfide bonds when synthesized in *dsbA* strains (as in *dsbB* strains), even in the presence of cystine (100 μg/ml). *dsbB* also affected the activity of alkaline phosphatase, presumably because the disulfide bond required for catalysis or stability was not formed (as in *dsbA* strains, ref. 9).

Thus, both DsbA and DsbB are required for disulfide bond formation in several proteins in *E. coli*. Although our results do not show the exact relationship between these two gene products, a simple model can be proposed, based on the data presented here and in Bardwell *et al.* (9) and Kamitani *et al.* (36). Because the active site of DsbA is similar to the active site of other oxidoreductases and partially purified DsbA can reduce insulin (9), it is likely that DsbA directly catalyzes disulfide bond formation. However, once the disulfide bond is formed, DsbA would become reduced at the vicinal cysteines at its active site and no longer be able to oxidize another protein. *dsbB* strains are probably defective in regenerating this active site. The complete suppression of all known defects in *dsbB* strains by cystine or cystamine suggests that these compounds are effective oxidants of DsbA. Thus, the role of DsbB might be to produce such an oxidant in the periplasm.

Other properties of DsbB are discussed in an accompanying paper by Bardwell *et al.* (41), who isolated *dsbB* mutants by a different route. Their *dsbB*$^+$ plasmid p73-1 complements our *dsb* strains for motility on glycerol minimal medium; therefore, the two loci appear the same.

Although neither *dsbA* (9, 36) nor *dsbB* appears to be an essential gene in *E. coli*—null mutants grow well in minimal medium—there are probably certain conditions in the natural environment in which Dsb$^-$ strains would be at a severe disadvantage—for example, when chemotaxis is useful or phosphate is limiting. Homologues of *dsbA* have been found in *Vibrio cholera* that are essential for the expression of virulence factors (31, 42). Thus, homologues of *dsbB* are likely to be found in other bacteria, also.

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