Proc. Natl. Acad. Sci. USA
Vol. 89, pp. 12058–12062, December 1992
Biochemistry

Translocation of a folded protein across the outer membrane in *Escherichia coli*

(protein secretion/pullulanase/disulfide bond)

ANTHONY P. PUGSLEY

Unité de Génétique Moléculaire, Centre National de la Recherche Scientifique URA1149, Institut Pasteur, 25, rue du Dr. Roux, Paris 75724 Cedex 15, France

Communicated by Werner Arber, September 11, 1992 (received for review June 1, 1992)

ABSTRACT **A mutation in the *Escherichia coli dsbA* gene (coding for a periplasmic disulfide oxidoreductase) reduces the rate of disulfide bond formation in the enzyme pullulanase and also reduces the rate at which the enzyme is secreted to the cell surface, as measured by protease accessibility. The enzyme did not become protease accessible when disulfide bond formation was completely prevented in the mutant strain by carboxymethylation. These results indicate that a disulfide bond may be required for, and certainly does not impede, the translocation of pullulanase across the outer membrane. Since it is unlikely that a disulfide bond could be formed and then reduced again in the periplasm, these results would appear to strengthen the argument that pullulanase polypeptides fold into or close to their final conformation before they are transported across the outer membrane. It is suggested that this might be a feature common to all proteins that are secreted by other Gram-negative bacteria by a pullulanase-like pathway.**

The first stage in the general secretory pathway (GSP) in *Escherichia coli*, the translocation of signal peptide-bearing presecretory proteins across the cytoplasmic membrane, is catalyzed by the products of six *sec* genes (1–3). Processing of the precursor by signal peptidase allows secretory proteins to be released into the periplasm, whence they may insert into or be transported across the outer membrane (1, 4). A special subfamily of secretory proteins, the lipoproteins, remain anchored to one or another of the membranes by fatty acids attached to their N-terminal cysteine residue (1).

E. coli is unusual among Gram-negative bacteria in that it does not naturally secrete extracellular enzymes by the GSP. To understand more about how other bacteria secrete extracellular proteins via the GSP, we cloned the *Klebsiella oxytoca* gene (*pulA*) for a secreted (cell surface anchored) amylolytic lipoprotein, pullulanase, together with 14 other genes (*pulS* and the *pulC-O* operon; refs. 4–7), which are required for its secretion in *E. coli*. Our studies show that pullulanase secretion is a two-step process involving the sequential action of the *sec* (8) and then the *pul* gene products (9). The existence of the normally short-lived secretion intermediate can be prolonged by uncoupling the two steps in the secretion pathway (9).

We have proposed that when the two secretion steps are uncoupled, pullulanase probably folds into or close to its final conformation before it is transported across the outer membrane, making it one of the very few examples of the transmembrane transport of folded proteins [other examples include nuclear protein import (1) and heat-labile enterotoxin or cholera toxin secretion by *Vibrio cholerae* (10)]. The aim of this study was to determine whether pullulanase does, or indeed must, fold prior to translocation across the outer membrane. To do this, we made use of the fact that pullulanase has six unmodified cysteine residues, at least two of which form an intramolecular disulfide bond (9, 11). The rate of formation of this disulfide bond was shown here to be lower in cells carrying a mutation in the *dsbA* (*ppfA*) gene that codes for a periplasmic disulfide oxidoreductase (12, 13). The effects of the *dsbA* mutation on the rate of pullulanase secretion were therefore examined.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

MATERIALS AND METHODS

Strains, Plasmids, and Culture Conditions. The *E. coli* strains used were JCB570 and its derivative JCB571 carrying the *dsbA*::*kan1* mutation (12). This mutation was introduced into strains PAP7172 (7) and pop3325 (14) by P1 phage transduction according to Miller (15) with selection for resistance to kanamycin (25 μg/ml). The presence of the *dsbA* mutation was tested by introducing a Tn*10*-tagged F′ *lac* plasmid and verifying that the cells were resistant to M13 phage when they carried the mutation [due to the absence of F pili, the M13 receptors (12)]. Plasmids were pCHAP231, a pBR322 derivative carrying the 15 *pul* genes required for pullulanase synthesis and secretion (4–6); pCHAP137, which is pBR322 carrying only the pullulanase structural gene (*pulA*) (8, 9); pCHAP656 (pBGS18 *lacZp–pulA*) (11); and pCHAP1051, a derivative of pCHAP656 in which *pulA* is fused to the first codons of *lacZ* with resulting elimination of the signal peptide (8). Cultures were incubated at 30°C in L broth (15) buffered to pH 7.0 with 10 mM phosphate, in M63 minimal salts medium (15), or in a low-potassium medium (8) containing 0.4% Casamino acids or a methionine-free amino acid mixture (8) as appropriate. Maltose (0.4%) was used to induce *pulA* and *pulC-O* expression, and *lacZp–pulA* fusions were induced with 1 mM isopropyl β-D-thiogalactoside. Plasmids were maintained by including ampicillin (200 μg/ml) in the medium.

General Techniques. Procedures for labeling cells with ^{35}[S]methionine, immunoprecipitation, SDS/PAGE, and immunoblotting were as described (8, 9). Sample buffer for SDS/PAGE contained 0.1 M Tris·HCl (pH 8.0), 5% SDS, and 12.5% (vol/vol) glycerol. β-Galactosidase and pullulanase were assayed according to Miller (15) and Pugsley *et al.* (8), respectively. Specific modifications to these procedures are indicated in the text.

RESULTS

***dsbA*::*kan1* and Dithiothreitol (DTT) Reduce the Level of Pullulanase Production.** To test whether the *dsbA*::*kan1* mutation (12) had any effect on the steady-state levels of pullulanase production and secretion, plasmid pCHAP231, carrying *pulA* and all of the pullulanase-specific secretion genes (4, 5), was introduced into *E. coli* strains JCB570 (*dsbA*$^+$) and JCB571 (*dsbA*::*kan1*) (12). Pullulanase activity

Abbreviations: GSP, general secretory pathway; DTT, dithiothreitol; IA, iodoacetamide; NEM, *N*-ethylmaleimide.

in both strains was entirely exposed on the cell surface (Table 1), indicating that the enzyme was secreted in the absence of the DsbA protein. Total pullulanase activity was, however, reduced to only 20–30% of the wild-type level in the DsbA$^-$ mutant. Growth of the wild-type cells in the presence of DTT also caused a similar decrease in the level of pullulanase production (Table 1).

Quantitative immunoblotting experiments revealed that the pullulanase polypeptide was present in the mutant cells and in wild-type cells grown in medium containing 4 mM DTT at only 20–30% of the level in the control cells (data not shown; see below for examples). Before proceeding to investigate the possible effects of *dsbA*::*kan1* on pullulanase secretion, an attempt was made to determine the cause of its effect on pullulanase production. The *dsbA*::*kan1* mutation was transduced into strain PAP7172, which carries the pCHAP231-derived gene cluster in the chromosome (5, 7) and into a pCHAP231 transformant of strain pop3325, which carries a *malT*^{c} allele that renders pullulanase production independent of exogenous inducer (maltose) (14). In both cases, the *dsbA* mutation again lowered the level of pullulanase produced to about the same extent as in strain JCB571 (Table 1). Thus, the effect of *dsbA*::*kan1* on pullulanase production cannot be explained by changes in plasmid copy number or by lower levels of inducer uptake.

It will be reported elsewhere that *dsbA*::*kan1* and DTT treatment reduce the level of the major outer membrane porin in *E. coli* cells by reducing the level of *ompF* transcription (unpublished data). The possible adverse effects of *dsbA*::*kan1* or of 4 mM DTT on *pulA* transcription were therefore tested by using three previously constructed *pulA–lacZ* operon fusions in pCHAP231 (5). In no case was the level of fusion-encoded β-galactosidase activity lower in the mutant or DTT-treated cells than in wild-type cells (data not shown). Furthermore, the effects of *dsbA*::*kan1* and DTT treatment on pullulanase production were the same in cells carrying pCHAP656 [in which *pulA* is under *lacZ* promoter control (11)] as in cells carrying pCHAP137 (in which *pulA* is under the control of its own promoter) (Table 1). Thus, *pulA* transcription is not affected by *dsbA*::*kan1* or DTT. To rule out any possible effects of *dsbA*::*kan1* on expression of the *pulC-O* operon of secretion genes, expression of a *pulO–lacZ* operon fusion (16) was also tested in strains JCB570 and JCB571. Once again, the level of β-galactosidase activity was the same in the DsbA$^-$ mutant and wild-type cells.

Since DTT and *dsbA*::*kan1* also caused a decrease in the level of pullulanase in strains carrying pCHAP656 or pCHAP137 (Table 1), both of which carry *pulA* but not the cognate secretion genes, their effects are not restricted to pullulanase that is transported to the cell surface. In contrast to pullulanase produced by cells carrying these plasmids, that produced by cells bearing pCHAP1051 is retained in the cytoplasm due to the absence of a signal peptide (8). The level of pullulanase activity detected in these cells was not affected by *dsbA*::*kan1* (Table 1) or by DTT treatment (data not shown).

These results indicate a posttranscriptional (and probably posttranslational) effect on pullulanase production that is only observed when the enzyme is translocated across the cytoplasmic membrane. The most likely explanation is increased proteolysis of pullulanase by periplasmic proteases. However, repeated pulse-labeling and immunoblotting experiments failed to provide any evidence for increased proteolysis of pullulanase in cells carrying the *dsbA*::*kan1* mutation (data not shown; see below for examples), although products resulting from rapid degradation during translocation across the cytoplasmic membrane might not have been immunodetected.

***dsbA* Is Not Essential for Cell-Surface Exposition of Pullulanase.** The results presented above rule out the possibility that only a proportion of the total amount of pullulanase made by the DsbA$^-$ mutant is in an active conformation and validate the pullulanase assay as an indicator of secretion efficiency. Therefore, the results in Table 1 show that growth in DTT or the presence of the *dsbA*::*kan1* mutation do not markedly affect pullulanase secretion. Accessibility to proteinase K was also used as an alternative method to determine whether pullulanase was efficiently secreted by the DsbA$^-$ mutant. Proteinase K reduces the size of native pullulanase polypeptide by ≈8 kDa (9). Pullulanase was almost totally clipped when proteinase K was added to whole cells carrying pCHAP231, irrespective of the presence of *dsbA*::*kan1* (Fig. 1). Pullulanase produced by strains carrying pCHAP137, which carries *pulA* but not the cognate secretion genes, was only clipped by proteinase K when the cells were lysed by detergent (octyl polyoxyethylene) or by sonication (data not shown; see ref. 9, for example).

These results are reminiscent of those obtained previously by Pronase treatment of pullulanase-producing *Klebsiella planticola* cells, which causes an active fragment of pullulanase lacking its N-terminal fatty acylated cysteine residue to be released from the outer membrane (17). In the present study, however, the large pullulanase fragment produced by proteinase K remained cell associated, retained the fatty

Table 1. Pullulanase activity and iodoacetamide (IA) sensitivity in strains of *E. coli* carrying wild-type or mutant *dsbA* alleles

Strain	Relevant chromosomal alleles	Plasmid	Induction	DTT	Pullulanase activity		
					Whole cells	Lysed cells	+IA
JCB570	*dsbA*$^+$	pCHAP231	Maltose	–	1415	1460	1360
JCB570	*dsbA*$^+$	pCHAP231	Maltose	+	210	205	25
JCB571	*dsbA*::*kan1*	pCHAP231	Maltose	–	370	410	45
pop3325	*dsbA*$^+$ *malT*^{c}	pCHAP231	—	–	930	870	870
pop3325*dsbA*	*dsbA*::*kan1 malT*^{c}	pCHAP231	—	–	210	200	25
PAP7172 F′ *lacI*^{q1}	*dsbA*$^+$ *pulA pulS pulCO*	—	Maltose	–	315	295	270
PAP7172 F′ *lacI*^{q1} *dsbA*	*dsbA*::*kan1 pulA pulS pulCO*	—	Maltose	–	80	90	10
JCB570	*dsbA*$^+$	pCHAP137	Maltose	–		630	570
JCB571	*dsbA*::*kan1*	pCHAP137	Maltose	–		130	35
JCB570 F′ *lacI*^{q1}	*dsbA*$^+$	pCHAP656	IPTG	–		2690	2570
		pCHAP1051	IPTG	–		1490	35
JCB571 F′ *lacI*^{q1}	*dsbA*::*kan1*	pCHAP656	IPTG	–		410	40
		pCHAP1051	IPTG	–		1695	55

Cells were grown at 30°C under the indicated inducing conditions for at least five generations in L broth containing 10 mM phosphate and, where indicated, 4 mM DTT. Cells were harvested and then washed and resuspended in 25 mM Tris·HCl buffer (pH 7.5) and incubated at 37°C for 90 min. Pullulanase activity is expressed as nmol of reducing sugar released from pullulan per min per mg of total cell protein at 37°C. Where appropriate, cells were lysed with 0.4% octyl polyoxyethylene. IA was added to the washed cell suspension at 25 mM. No further reduction in activity was observed after >90 min of preincubation with IA. IPTG, isopropyl β-D-thiogalactoside.

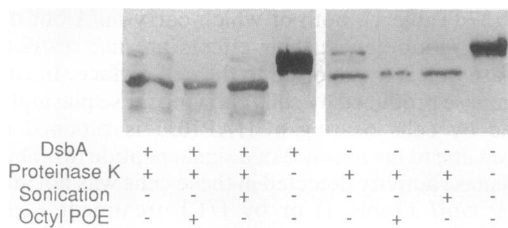

FIG. 1. Cleavage of cell-surface exposed pullulanase in JCB570 and JCB571 (*dsbA::kan1*) carrying pCHAP231. Cells were harvested during exponential growth, and resuspended in 25 mM Tris·HCl (pH 7.4). Samples of the cell suspension were then lysed with 0.4% octyl polyoxyethylene (POE) or by sonication, and the lysed or whole cells were incubated at 30°C for 5 min with proteinase K (100 μg/ml). Proteolysis was arrested by adding 2 mM phenylmethylsulfonate. The samples were immediately chilled on ice, precipitated with 12.5% trichloroacetic acid, washed with 95% ethanol, resuspended in sample buffer containing 5% 2-mercaptoethanol, and heated to 100°C for 5 min. SDS/PAGE was followed by electrotransfer and blotting with antipullulanase antiserum.

acylated N terminus (data not shown), and was at least 80% as active as that in the control (untreated) cells. Proteinase K therefore removes a small C-terminal fragment from pullulanase without dramatically affecting its catalytic activity. These results indicate that at least the C-terminal region of pullulanase reaches the cell surface irrespective of the presence of the *dsbA* mutation.

Pullulanase Produced by the DsbA⁻ Mutant Has a Disulfide Bond but Is Sensitive to Carboxymethylation. Failure of the DsbA⁻ mutant to form a disulfide bond in pullulanase should be revealed by changes in its electrophoretic mobility on SDS/PAGE under nonreducing conditions (9). However, pullulanase produced by cells carrying the *dsbA* mutation or by cells grown in the presence of DTT comigrated with the enzyme produced by control cells, indicating the presence of at least one disulfide bond (Fig. 2; data not shown).

Pullulanase produced by wild-type strains carrying pCHAP231 or pCHAP137 is insensitive to IA, which carboxymethylates free sulfhydryl groups (9), but the enzyme produced by the *dsbA::kan1* mutant carrying the same plasmids was found to be IA sensitive (Table 1). Unlike pullulanase variants that remain cytoplasmic because of mutations affecting the signal peptide (e.g., pCHAP1051; ref. 9), the enzyme produced by the DsbA⁻ mutant was only slowly inhibited (<50% inhibition after 5 min of IA treatment relative to >90% in the case of the pCHAP1051-encoded enzyme), presumably due to poor accessibility of the IA-reactive group.

Thus, pullulanase produced by the DsbA⁻ mutant is IA sensitive despite having one or more disulfide bonds (as

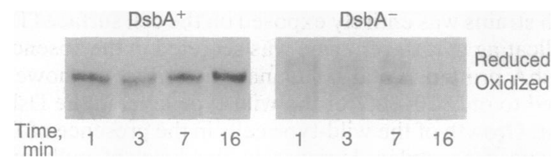

FIG. 3. Reduced rate of disulfide bond formation in pullulanase in the absence of DsbA. Cells of strains JCB570 (pCHAP231) or JCB571 (pCHAP231) were pulse-labeled with ^{35}[S]methionine (60 μCi/ml; 1 Ci = 37 GBq) for 1 min and then chased by adding unlabeled methionine (0.2 μg/ml). SDS (final, 0.9%) and IA (final, 25 mM) were added to samples removed at time intervals, which were immediately heated to 100°C for 5 min. Pullulanase was immunoprecipitated (8) and examined by SDS/PAGE and autoradiography. Samples from equal volumes of the cell suspension were loaded onto the gels.

shown by the gel-shift experiment). Similar results were obtained with cells treated with *N*-ethylmaleimide (NEM), another sulfhydryl group alkylating agent, but once again the pullulanase from the NEM-treated cells could not be electrophoretically distinguished from pullulanase in control cells (data not shown). Furthermore, pullulanase in DsbA⁻ cells carrying pCHAP137 exhibited IA and NEM sensitivity and electrophoretic mobility identical to that produced by the pCHAP231-bearing DsbA⁻ cells. Therefore, the IA/NEM sensitivity of pullulanase in the DsbA⁻ mutants is not related to translocation across the outer membrane. Possible explanations for this phenomenon are presented in the *Discussion*.

***dsbA::kan1* Reduces the Rate of Disulfide Bond Formation and Delays Secretion.** The results presented so far show that *dsbA::kan1* does not dramatically affect either disulfide bond formation in pullulanase or pullulanase secretion. To measure kinetic effects, the pulse–chase analysis was combined with assays for proteinase K accessibility. pCHAP231-encoded pullulanase produced by DsbA⁺ cells was found to be entirely in the oxidized form within 1 min of ^{35}[S]methionine pulse labeling (Fig. 3) and was completely clipped by proteinase K within 5 min (Fig. 4). These results show that both disulfide bond formation and secretion occur rapidly, making it difficult to determine which happens first. However, previous studies in which the two secretion steps were uncoupled showed that disulfide bond formation can occur before transport across the outer membrane (9).

Both oxidized and reduced forms were detected in the DsbA⁻ mutant after 1 min of pulse labeling, with the initially predominant reduced form being chased into the oxidized form (Fig. 3). Only one band (the reduced form) was observed when the samples were reduced with 2-mercaptoethanol before being applied to the gel (data not shown), confirming that the two bands shown in Fig. 3 correspond to reduced and oxidized forms of pullulanase and not to precursor and

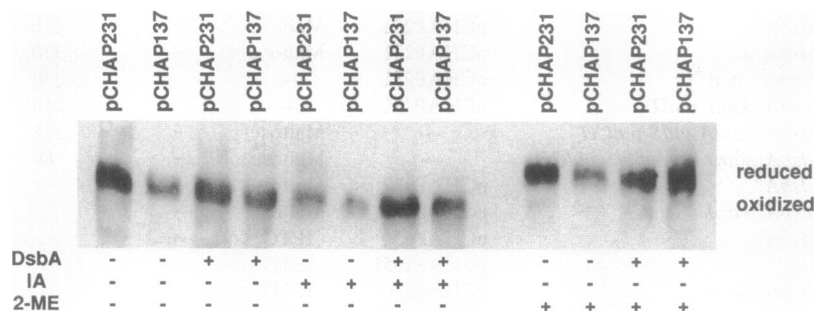

FIG. 2. Oxidized and reduced forms of pullulanase in cells of strains JCB570 and JCB571 (*dsbA::kan1*) carrying pCHAP231 or pCHAP137. Cells were harvested during exponential growth and resuspended in sample buffer with or without 25 mM IA or 5% 2-mercaptoethanol (2-ME), heated at 100°C for 5 min, and then subjected to SDS/PAGE, electrotransfer, and blotting with antipullulanase antiserum. Prior incubation of cells for 2 h in Tris·HCl buffer (25 mM; pH 7.5 or pH 8.0) containing 50 mM IA at 37°C did not affect the electrophoresis profile. Samples were adjusted to contain approximately the same amount of immunoreactive material.

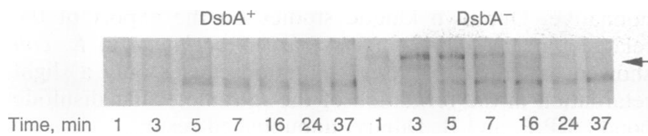

FIG. 4. Pullulanase produced by DsbA− cells is not immediately accessible to proteinase K. Proteinase K (100 μg/ml) was added at t_0 of the chase to cells of strains JCB570 (pCHAP231) or JCB571 (pCHAP231) labeled and chased as described in Fig. 3. Samples removed at time intervals were treated as described in Fig. 1 and resuspended in 0.9% SDS. Pullulanase was immunoprecipitated (8) and examined as described in Fig. 3. Arrow, position of an intermediate cleavage product detected in the cells carrying the *dsbA* mutation.

mature forms (8). Furthermore, clipping of the pulse-labeled pullulanase by proteinase K was also delayed in the DsbA− strain (Fig. 4). Control experiments showed that this was due to initial inaccessibility of pullulanase to the protease rather than to a reduced rate of proteolysis. In some experiments, minor amounts of another, intermediate-sized clipped pullulanase product were also detected in the DsbA− mutant (Fig. 4, arrow).

Disulfide bond formation in pullulanase was completely and indefinitely prevented in the DsbA− cells by adding IA during the chase (Fig. 5). Under these conditions, the protein was stable for up to 20 min but did not become accessible to proteinase K, suggesting that secretion was prevented by blocking disulfide bond formation (Fig. 5). However, pullulanase that accumulated in the IA-treated DsbA− cells was completely degraded (not just clipped) by the protease when the cells were lysed by sonication (data not shown), and was apparently spontaneously degraded, presumably by periplasmic proteases, after prolonged incubation (Fig. 5*B*). Pullulanase produced by IA-treated cells of JCB571 (pCHAP137) was likewise spontaneously degraded after 20–40 min. IA treatment of JCB570 (pCHAP231) under identical conditions did not affect the accessibility of pullulanase to proteinase K (data not shown). This indicates that the components of the pullulanase secretion machinery are not affected by IA treatment. Disulfide bond formation is therefore required both for correct folding of pullulanase and possibly for its secretion.

DISCUSSION

The number of exported proteins whose activity or stability is adversely affected by the absence of disulfide oxidoreductase has not been determined but appears to be small (refs. 12 and 13; unpublished data). Instead, studies presented here and by others (12, 13) indicate that *dsbA* mutations retard but do not prevent disulfide bond formation. Presumably, the cysteine residues in pullulanase and in other proteins affected by DsbA are positioned such that, when these proteins fold in the periplasm, they must inevitably form a disulfide bond. Pullulanase appears to be perfectly stable in the absence of the DsbA enzyme, although it may be slowly degraded when disulfide bond formation is completely prevented by carboxymethylation.

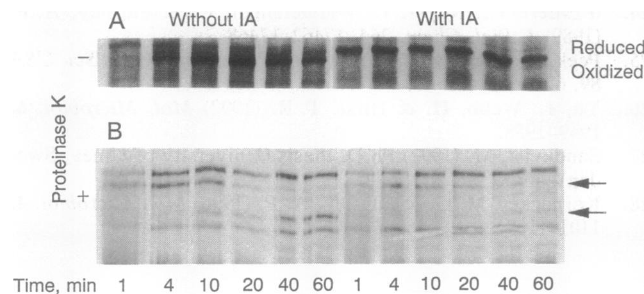

FIG. 5. Effects of IA treatment on accessibility of pullulanase to proteinase K in strain JCB571 (*dsbA*::*kanl*) (pCHAP231). Cells were labeled and chased as described in Fig. 3 except that proteinase K (100 μg/ml) and/or IA (10 mM) were added as indicated during the chase. Cells were then treated as described in Fig. 3 (*A*; for visualization of reduced and oxidized forms of pullulanase) or Fig. 4 (*B*; for visualization of proteinase K-clipped product). Note that samples in *B* were not immunoprecipitated. Upper and lower arrows indicate positions of full-length and clipped pullulanase, respectively.

The aim of this study was to determine whether disulfide bonds prevent or assist the translocation of pullulanase across the outer membrane. The fact that the reduced form of pullulanase cannot be detected in DsbA+ cells implies that the disulfide bond(s) forms rapidly, possibly during translocation across the cytoplasmic membrane and prior to translocation to the cell surface. Thus, the disulfide bond does not impede the second step of the GSP, in line with our observations that disulfide-bonded pullulanase can be stockpiled on the periplasmic face of the cytoplasmic membrane (9) or in the periplasm (18) before its transport across the outer membrane. Furthermore, proteolysis of pullulanase by external proteinase K during translocation produces exactly the same clipped product as when the protease is added after translocation. This implies that the pullulanase polypeptide does not emerge at the cell surface in an incompletely folded state, which, like the reduced, carboxymethylated form produced by the IA-treated DsbA− cells, would be degraded by the protease.

Both translocation to the cell surface and disulfide bond formation are delayed by the *dsbA*::*kanl* mutation and by DTT treatment. This correlation suggests that a disulfide bond must be formed in pullulanase before it can be translocated across the outer membrane. Furthermore, secretion was completely prevented by carboxymethylating pullulanase produced by the DsbA− mutant, which again implies that a disulfide bond is required for secretion. Can these effects of DsbA (or DTT) and IA be explained by alteration of components of the GSP? Although several of the pullulanase secretion factors have one or more cysteine residues, almost all of them are buried in putative membrane-embedded segments. Secretion factors with large periplasmic domains (PulS, PulG, PulH, PulI, PulJ) and the integral outer membrane protein PulD do not have cysteine residues (see ref. 6 for review). PulE has five cysteine residues, but this polypeptide is believed to be entirely cytoplasmic (7). Furthermore, IA treatment blocked pullulanase secretion only in the DsbA− mutant, indicating that the observed effect is not due to carboxymethylation of preformed components of the pullulanase secretion machinery. Since simultaneous production of components of this pathway and of pullulanase is not required for pullulanase secretion (9), this implies that the observed combined effects of the *dsbA*::*kanl* mutation and IA are indeed due to inhibition of disulfide bond formation in pullulanase.

The idea that proteins can be transported through membranes in a folded state is a relatively new concept that deserves further investigation. Although translocation through the *E. coli* cytoplasmic membrane may not require the complete absence of secondary or tertiary structure (19–21), the formation of complex structures incompatible with translocation must be prevented by cytoplasmic molecular chaperones (22). Studies presented here and by others show that such constraints do not apply to transport across the outer membrane. We have previously drawn parallels between pullulanase secretion and the assembly and release of filamentous bacteriophages by *E. coli* and heat-labile enterotoxin and cholera toxin secretion by *V. cholerae* (4). The former requires an outer membrane protein (gpIV) (23)

with substantial similarity to the sole integral outer membrane protein required for pullulanase secretion (PulD; ref. 24), and the two events may be mechanistically similar. The latter is an excellent example of the type of phenomenon presented here since it involves the outer membrane translocation of a preassembled hexameric toxin complex from the periplasm (10). Toxin secretion seems to be adversely affected by a mutation in the *V. cholerae* gene that is equivalent to *dsbA* in *E. coli* (25, 26). It was recently shown that the terminal branch of the GSP by which cholera toxin is secreted is probably very similar to that used for pullulanase secretion (27), and it may transpire that translocation of prefolded proteins across the outer membrane is a feature common to other bacteria that secrete proteins by this pathway.

In view of the large size of cholera toxin and filamentous phages, it is perhaps not surprising that the pullulanase-specific terminal branch of the GSP can accommodate a folded polypeptide with at least one disulfide bond. This has important implications regarding the identity and location of the hypothetical signal that allows pullulanase to be specifically selected for transport across the outer membrane, since it is likely to be exposed only in the completely folded polypeptide; i.e., disulfide bond formation may be required to construct the three-dimensional secretion patch signal. Nevertheless, the tertiary structure of pullulanase can be substantially altered by replacing the C-terminal end of the enzyme by the entire mature segment of the normally periplasmic enzyme β-lactamase without dramatically affecting secretion (28). It may be significant that all six cysteine residues in pullulanase that could form disulfide bonds were retained in this hybrid.

PulD and related proteins in other bacteria that secrete extracellular proteins (4) may form large channels through which folded proteins are translocated across the outer membrane. The dimensions of these channels must be close to those of nuclear pores (1), but they must also be tightly gated to prevent the leakage of periplasmic proteins and the entry of toxic compounds such as lysozyme, proteases, antibiotics, and detergents. Some of the many secretion factors involved in the second step of the GSP (4, 6) may be required to open and close the channel.

The reason for the IA sensitivity of pullulanase produced by the DsbA$^-$ mutant remains to be determined. One explanation could be that pullulanase normally has more than one intramolecular disulfide bond, only one of which forms in the DsbA$^-$ mutant. This implies that we cannot resolve pullulanase with only one disulfide bond from the normal, fully disulfide-bonded form by SDS/PAGE. Alternatively, the full complement of disulfide bonds may be formed by the DsbA$^-$ mutant but, because they form more slowly, the enzyme folds into a nonnative but active conformation that allows IA to penetrate and carboxymethylate a normally inaccessible amino acid. Somewhat similar results were recently reported by Peek and Taylor (25), who found that type IV pilins produced by *V. cholerae* carrying a mutation in the *dsbA* homologue *tcpG* are secreted and assembled apparently normally but are unable to mediate bacterial adherence to epithelial cells, presumably because their conformation is nonnative. Our own kinetic studies on the export of the related type IV pilin of *Neisseria gonorrhoeae* in *E. coli* showed that the *dsbA*::*kan1* mutation causes only a slight retardation in the formation of the intramolecular disulfide bond (A.P.P. and B. Dupuy, unpublished result).

I thank Jim Bardwell for strains, and Jon Beckwith, Isabelle Poquet, Odile Possot, and Maxime Schwartz for helpful discussions.

1. Pugsley, A. P. (1989) *Protein Targeting* (Academic, San Diego).
2. Schatz, P. & Beckwith, J. (1990) *Annu. Rev. Genet.* **24**, 215–248.
3. Wickner, W., Driessen, A. J. J. & Hartl, F.-U. (1991) *Annu. Rev. Biochem.* **60**, 101–124.
4. Pugsley, A. P. (1992) in *Prokaryotic Structure and Function: A New Perspective*, eds. Mohan, S. B., Dow, C. & Cole, J. A. (Soc. Gen. Microbiol. Symp. 47, Cambridge Univ. Press, Cambridge, U.K.), pp. 223–248.
5. d'Enfert, C., Ryter, A. & Pugsley, A. P. (1987) *EMBO J.* **6**, 3531–3538.
6. Pugsley, A. P., d'Enfert, C., Reyss, I. & Kornacker, M. G. (1990) *Annu. Rev. Genet.* **24**, 67–90.
7. Possot, O., d'Enfert, C., Reyss, I. & Pugsley, A. P. (1992) *Mol. Microbiol.* **6**, 95–105.
8. Pugsley, A. P., Kornacker, M. G. & Poquet, I. (1991) *Mol. Microbiol.* **5**, 343–352.
9. Pugsley, A. P., Poquet, I. & Kornacker, M. G. (1991) *Mol. Microbiol.* **5**, 865–873.
10. Hirst, T. R. & Holmgren, J. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 7418–7422.
11. Kornacker, M. G. & Pugsley, A. P. (1990) *Mol. Microbiol.* **4**, 73–85.
12. Bardwell, J. C. A., McGovern, K. & Beckwith, J. (1991) *Cell* **65**, 581–589.
13. Kamitani, S., Akiyama, Y. & Ito, K. (1992) *EMBO J.* **11**, 57–62.
14. Débarbouillé, M., Shuman, H., Silhavy, T. J. & Schwartz, M. (1978) *J. Mol. Biol.* **124**, 359–371.
15. Miller, J. H. (1972) *Experiments in Molecular Genetics* (Cold Spring Harbor Lab., Cold Spring Harbor, NY).
16. Pugsley, A. P. & Reyss, I. (1990) *Mol. Microbiol.* **4**, 3565–3579.
17. Pugsley, A. P., Kornacker, M. G. & Ryter, A. (1990) *Mol. Microbiol.* **4**, 59–72.
18. Poquet, I., Faucher, D. & Pugsley, A. P. (1993) *EMBO J.*, in press.
19. Lecker, S. H., Driessen, A. J. M. & Wickner, W. (1990) *EMBO J.* **9**, 2309–2314.
20. Tani, K., Tokuda, H. & Mizushima, S. (1990) *J. Biol. Chem.* **265**, 17341–17347.
21. Reed, K. E. & Cronan, J. E., Jr. (1991) *J. Biol. Chem.* **266**, 11425–11428.
22. Kumamoto, C. A. (1991) *Mol. Microbiol.* **5**, 19–22.
23. Brisette, J. L. & Russel, M. (1990) *J. Mol. Biol.* **211**, 565–580.
24. d'Enfert, C., Reyss, I., Wandersman, C. & Pugsley, A. P. (1989) *J. Biol. Chem.* **264**, 17462–17468.
25. Peek, J. A. & Taylor, R. K. (1992) *Proc. Natl. Acad. Sci. USA* **89**, 6210–6214.
26. Yu, J., Webb, H. & Hirst, T. R. (1992) *Mol. Microbiol.* **6**, 1949–1958.
27. Sandkvist, M. (1992) Ph.D. thesis (University of Umea, Sweden).
28. Kornacker, M. G. & Pugsley, A. P. (1990) *Mol. Microbiol.* **4**, 1101–1109.