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The disulphide isomerase DsbC cooperates with the oxidase DsbA in a DsbD-independent manner

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Summary

In *Escherichia coli*, DsbA introduces disulphide bonds into secreted proteins. DsbA is recycled by DsbB, which generates disulphides from quinone reduction. DsbA is not known to have any proofreading activity and can form incorrect disulphides in proteins with multiple cysteines. These incorrect disulphides are thought to be corrected by a protein disulphide isomerase, DsbC, which is kept in the reduced and active configuration by DsbD. The DsbC/DsbD isomerization pathway is considered to be isolated from the DsbA/DsbB pathway. We show that the DsbC and DsbA pathways are more intimately connected than previously thought. *dsbA*$^-$*dsbC*$^-$ mutants have a number of phenotypes not exhibited by either *dsbA*$^-$, *dsbC*$^-$ or *dsbA*$^-$*dsbD*$^-$ mutations: they exhibit an increased permeability of the outer membrane, are resistant to the lambdoid phage Φ80, and are unable to assemble the maltoporin LamB. Using differential two-dimensional liquid chromatographic tandem mass spectrometry/mass spectrometry analysis, we estimated the abundance of about 130 secreted proteins in various *dsb*$^-$ strains. *dsbA*$^-$*dsbC*$^-$ mutants exhibit unique changes at the protein level that are not exhibited by *dsbA*$^-$*dsbD*$^-$ mutants. Our data indicate that DsbC can assist DsbA in a DsbD-independent manner to oxidatively fold envelope proteins. The view that DsbC's function is limited to the disulphide isomerization pathway should therefore be reinterpreted.

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Introduction

DsbA introduces disulphide bonds into secreted proteins in the *Escherichia coli* periplasm (Bardwell *et al*., 1991). DsbA has a CXXC catalytic site motif present within a thioredoxin fold. The cysteine residues of this motif are found oxidized *in vivo*. The disulphide bond of DsbA is very unstable and is rapidly transferred to secreted unfolded proteins. DsbA is then reoxidized by the inner membrane protein DsbB (Bardwell *et al*., 1993). DsbA forms a mixed disulphide complex with DsbB. Disulphide bond transfer occurs after conformational changes within the DsbB protein (Inaba *et al*., 2006). DsbB has two pairs of cysteine residues and generates disulphide bonds *de novo* from quinone reduction. Electrons are then successively transferred from quinone to cytochrome oxidases and finally to molecular oxygen (Bader *et al*., 1999).

DsbA is likely to be involved in the oxidative folding of many periplasmic and outer membrane proteins. A search in the sequence databases reveals that about 40% of the 700 secreted proteins in *E. coli* have at least two cysteine residues and are therefore potential DsbA substrates (D. Boyd, pers. comm.). It is therefore not surprising that *dsbA*$^-$ strains have a pleiotropic phenotype; they show an attenuated virulence, lack motility, form mucoidal colonies on minimal media and in the presence of some antibiotics such as tetracycline, and are more sensitive to dithiothreitol and cadmium (reviewed in Collet and Bardwell, 2002). About 15 DsbA substrates have been identified so far; these were obtained by trapping of mixed disulphides with DsbA mutants (Kadokura *et al*., 2004), from differential thiol trapping experiments (Leichert and Jakob, 2004), and by 2D-gel analysis, which showed decreased levels of expression of several cysteine-containing proteins (Hiniker and Bardwell, 2004).

Despite the significant number of proteins that are DsbA substrates, *dsbA*$^-$ strains are surprisingly healthy, particularly when grown on rich media. This in part may be due to the fact that small molecule oxidants, like cysteine,

are present in rich media (Bardwell *et al*., 1993). Consistent with this, *dsbA*$^-$ strains grow very poorly on minimal media lacking cysteine, particularly in some strain backgrounds. Although the rate of disulphide bond formation is about a 100-fold decreased in *dsbA*$^-$ strains (Bardwell *et al*., 1991), some slow residual disulphide bond formation does occur, and proteins that are stable in the absence of their disulphide accumulate in the oxidized form to near normal levels at steady state. This disulphide formation appears to be dependent on oxygen, as much lower levels of oxidized proteins are seen in *dsbA*$^-$ strains when they are grown anaerobically (Leichert and Jakob, 2004).

DsbA is a powerful oxidant that apparently lacks proofreading activity. DsbA oxidizes cysteine residues on secreted proteins as they emerge into the periplasm. If the native disulphide bond pattern involves cysteine residues that are consecutive in the amino acid sequence, DsbA can form disulphides correctly. However, when secreted proteins have disulphides that need to be formed between non-consecutive cysteines, DsbA can introduce non-native disulphides, leading to protein misfolding and degradation by proteases (Berkmen *et al*., 2005). Noteworthy, our recent work on RNase I, a periplasmic protein with one non-consecutive disulphide, showed that DsbA is more specific than generally assumed (Messens *et al*., 2007). The correction of non-native disulphides is thought to be the role of a disulphide isomerization system. This system is composed of two soluble periplasmic proteins, DsbC and DsbG, which are thought to function as disulphide isomerase proteins *in vivo* and *in vitro* (Zapun *et al*., 1995; Bessette *et al*., 1999). Like DsbA, DsbC and DsbG possess a thioredoxin fold and a CXXC catalytic site motif. In contrast to DsbA, whose CXXC active site motif is maintained in an oxidized form, the CXXC motif of DsbC and DsbG is kept reduced in the periplasm. This allows DsbC and DsbG to attack non-native disulphides, a necessary step in the isomerization reaction (reviewed in Messens and Collet, 2006). The protein that keeps DsbC and DsbG reduced is the inner membrane protein, DsbD. DsbD transfers reducing equivalents from the cytoplasmic thioredoxin system to the periplasm via a succession of disulphide exchange reactions (Rietsch *et al*., 1996; Katzen and Beckwith, 2000; Collet *et al*., 2002; Rozhkova *et al*., 2004). Residues that are important for this electron cascade have recently been identified (Cho and Beckwith, 2006; Hiniker *et al*., 2006), but the precise mechanism used by DsbD to transport electrons from one side of the membrane to the other is still obscure.

In contrast to the central role played by DsbA in oxidizing a large number of periplasmic proteins, DsbC is thought to be required for the expression of a limited subset of proteins that contain non-consecutive disulphides, including the penicillin insensitive endopeptidase MepA, the ribonuclease RNase I, and the acid phosphatase AppA (Hiniker and Bardwell, 2004; Berkmen *et al*., 2005). In agreement with the relatively small number of DsbC substrates, *dsbC*$^-$ strains have a milder phenotype than *dsbA*$^-$ mutants (reviewed in Collet and Bardwell, 2002). DsbC seems to be particularly important under some oxidative stress conditions. For instance, DsbC is required for growth in the presence of high concentrations of copper, a redox metal that catalyses the formation of non-native disulphide bonds (Hiniker *et al*., 2005).

The role of DsbG is less clear. DsbG was originally reported to be an essential oxidase (Andersen *et al*., 1997). However, subsequent work showed it to be non-essential for the growth of *E. coli* (Bessette *et al*., 1999). *DsbG* null mutants have no defect in the folding of heterologous proteins containing multiple disulphide bonds, and are unable to catalyse disulphide bond rearrangement using either hirudin or the bovine pancreatic trypsin inhibitor intermediate as substrates (Bessette *et al*., 1999; Hiniker *et al*., 2007). However, DsbG overexpression is able to restore the ability of *dsbC*$^-$ mutants to express some heterologous proteins containing multiple disulphide bonds (Bessette *et al*., 1999). It is also possible to select mutations in DsbG that complement DsbC, and these mutations show increased isomerase activity (Hiniker *et al*., 2007). These latter observations and DsbG's homology to DsbC (Heras *et al*., 2004) have led to the conclusion that DsbG is a disulphide isomerase with restricted substrate specificity.

The situation in eukaryotic disulphide bond formation is more complex and controversial. Protein disulphide isomerase is thought to function as both an oxidase and an isomerase *in vivo.* However, there has been quite a bit of controversy regarding the relative importance of the oxidative and isomerase activities to the cell (Sevier and Kaiser, 2006). In addition to protein disulphide isomerase, yeast possesses several thioredoxin-like proteins that are localized to the endoplasmic reticulum. Those proteins are likely to play important roles in the isomerization and oxidation of proteins (reviewed in Gruber *et al*., 2006).

In *E. coli*, the current view is that the distinct disulphide catalytic pathways have well defined roles: the DsbA/DsbB system is important in oxidizing disulphide bonds, and the DsbC-G/DsbD system is important in isomerizing them. The results presented in this paper show that this conclusion needs to be reinterpreted. We show that the simultaneous absence of DsbA and DsbC has severe consequences on *E. coli*'s viability and outer membrane integrity. *dsbA*$^-$*dsbC*$^-$ mutants are resistant to the Φ80 bacteriophage and seem unable to fold the trimeric porin LamB. Using a two-dimensional liquid chromatographic

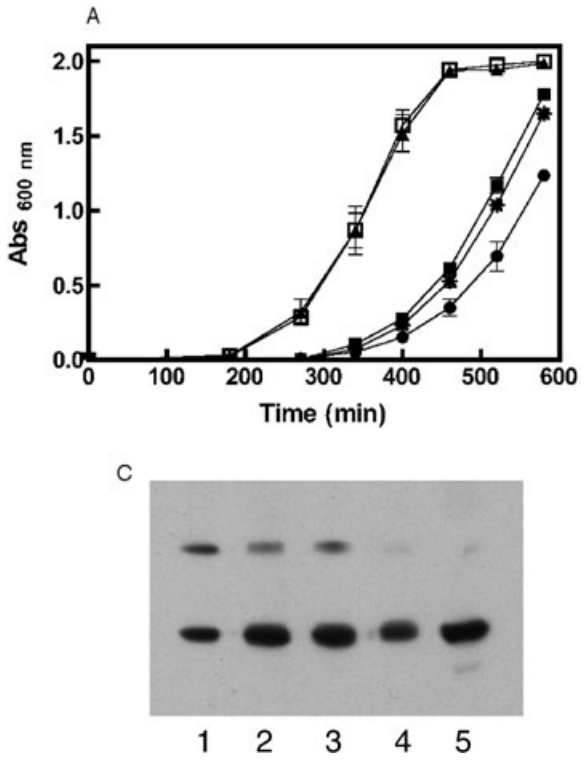

Fig. 1. The absence of *dsbA* and *dsbC* has phenotypical consequences.
A. Growth curves of wild-type (□), *dsbC*⁻ (▲), *dsbA*⁻ (★), *dsbA*⁻*dsbD*⁻ (■) and *dsbA*⁻*dsbC*⁻ (●) strains in M63 minimal media at 37°C. Growth was monitored at A_{600}.
B. SDS sensitivity of wild-type (lane 1), *dsbA*⁻ (lane 2), *dsbA*⁻*dsbC*⁻ (lane 3) and *dsbA*⁻*dsbD*⁻ (lane 4) strains. Strains were grown in LB at 37°C to an A_{600} of 0.5. The cultures were then serially diluted 10^7-fold in 10-fold increments. Ten microlitres of each dilution was then spotted on LB plates containing 2.5% SDS and grown overnight.
C. Western blot showing protein expression levels. The upper bands correspond to the LamB protein. Outer membrane proteins prepared from wild-type (lane 1), *dsbA*⁻ (lane 2), *dsbC*⁻ (lane 3), *dsbA*⁻*dsbC*⁻ (lane 4) and *LamB*⁻ (lane 5) strains. The lower bands correspond to an unknown protein recognized by the anti-LamB antibody, which was used as an internal standard.

mass spectrometry/mass spectrometry (2D-LC-MS/MS) proteomics approach, we show that the absence of DsbA and DsbC affects the global protein content of the periplasm and leads to a decreased abundance of several cysteine-containing proteins. Our data indicate that the function of DsbC goes beyond the correction of the non-native disulphides formed by DsbA. On the basis of our results, we propose a new model for the oxidative protein folding pathways in *E. coli*.

Results and discussion

dsbA⁻dsbC⁻ *double mutants have a more severe phenotype than* dsbA⁻ *mutants*

To test whether DsbC can assist DsbA outside the framework of the isomerization pathway, we made *dsbA*⁻*dsbC*⁻ double mutants and compared their phenotype with that of *dsbA* null strains. The logic behind these experiments is as follows: if DsbC's function is restricted to the correction of DsbA's mistakes, then *dsbA*⁻ and *dsbA*⁻*dsbC*⁻ mutants should have a similar phenotype. In contrast, if DsbC has functions in addition to the correction of DsbA's mistakes, then *dsbA*⁻*dsbC*⁻ double mutants should have a more severe phenotype than *dsbA*⁻ mutants. We observed that a *dsbA*⁻*dsbC*⁻ mutant has a more severe growth defect than a *dsbA*⁻ strain when cells are grown in minimal media (Fig. 1A and Table 1). Both the *dsbA*⁻ and *dsbA*⁻*dsbC*⁻ strains are more sensitive to antibiotics and detergents than wild-type strains. However, the sensitivity of the *dsbA*⁻*dsbC*⁻ double mutant is more severe than that of a *dsbA*⁻ mutant. The *dsbA*⁻*dsbC*⁻ mutant is more sensitive to SDS (Fig. 1B), and, unlike *dsbA*⁻ strains, is unable to grow in the presence of 4 μg ml^{-1} rifampin, a large hydrophobic antibiotic (Table 1). These phenotypes suggest that the permeability of the outer membrane is increased in the double mutant. A single *dsbC*⁻ mutant does not exhibit any growth defect or sensitivity towards antibiotics and detergents compared with isogenic wild-type strains.

dsbA⁻*dsbC*⁻ mutants form pink colonies on maltodextrin MacConkey agar, in contrast to wild-type, *dsbA*⁻ and *dsbC*⁻ colonies, which are red (Table 1). This pink phenotype is often indicative of a decreased abundance of LamB, the outer membrane component of the maltose transport system in *E. coli* (Duguay and Silhavy, 2002). LamB is a trimeric protein that possesses one disulphide bond per subunit. By Western blot analysis, we confirmed that the expression level of LamB is strongly decreased in a *dsbA*⁻*dsbC*⁻ mutant compared with wild-type, *dsbC*⁻ and *dsbA*⁻ strains (Fig. 1C). The *dsbA*⁻*dsbC*⁻ mutants are also significantly more resistant to the lambdoid Φ80 phage (Table 1). The *E. coli* receptor for this phage is the ferrichrome iron receptor protein FhuA. Interestingly, FhuA contains four cysteine residues that form two consecutive disulphide bonds. These data suggest that the function of DsbC is not restricted to the correct folding of proteins that have disulphides formed between non-consecutive cysteines. As the disulphides of LamB and

Table 1. Phenotypic characterization of *dsbA*⁻*dsbC*⁻ mutants.

Strain	Genotype	MacConkey maltodextrin (1%)	Rifampin (4 μg ml^{-1})	Growth rate (h^{-1})[a]
JFC209	Wild-type	Mal+ (red)	+++	1.15
MD1	*dsbA*⁻	Mal+ (red)	++	0.79
JFC383	*dsbC*⁻	Mal+ (red)	+++	1.15
MD3	*dsbA*⁻*dsbC*⁻	Mal+/– (pink)	–	0.69
AH396	*dsbA*⁻*dsbD*⁻	Mal+ (red)	++	0.87

Strain	Genotype	Pfu with Φ80
JP114	Wild-type	5.5×10^{10}
JP220	*dsbA*⁻	1.0×10^{9}
JP539	*dsbC*⁻	5.1×10^{10}
JP557	*dsbA*⁻*dsbC*⁻	2.0×10^{4}
JP649	*dsbA*⁻*dsbD*⁻	7.0×10^{9}
C600	*fhua*⁻	0

a. Growth rate have been calculated using the following formula: $\mu = \ln(A_{600})/\Delta$time.

FhuA do not seem to be important for the function of these proteins (Ferenci and Stretton, 1989; Bos *et al*., 1998), it is tempting to speculate that in the absence of DsbA and DsbC intermolecular disulphides are formed, preventing these proteins from correctly folding in the outer membrane.

Our results clearly show that *dsbA*⁻*dsbC*⁻ mutants have a more severe phenotype than *dsbA*⁻ mutants and suggest that DsbC may be involved in the folding of LamB and FhuA, two proteins that have only consecutive disulphides. Our data thus support the hypothesis that DsbC's function is not restricted to the correction of non-native disulphides. DsbC also has chaperone activity that is independent of its active site cysteine residues (Liu and Wang, 2001). Thus we needed to consider the possibility that the defects observed in *dsbC*⁻ strains is due to the lack of DsbC's chaperone activity. We, however, observed that expression of a mutant of DsbC in which both catalytic site cysteines are replaced by serine failed to improve the growth rate of *dsbA*⁻*dsbC*⁻ strains, in contrast to expression of the wild-type DsbC protein. This mutant of DsbC is expected to lose its thiol-disulphide oxidoreductase activity, while retaining its chaperone activity (Liu and Wang, 2001). Thus the severe phenotype of the *dsbA*⁻*dsbC*⁻ mutant is unlikely to be due to a chaperone deficiency in *dsbC*⁻ strains, but rather a thiol-disulphide oxidoreductase defect.

DsbC is able to assist DsbA in a DsbD-independent manner

To test whether the activity of DsbC always depends on the presence of DsbD, we generated a *dsbA*⁻*dsbD*⁻ double mutant. This mutant is phenotypically similar to a *dsbA*⁻ strain in terms of detergent and antibiotics sensitivity. It is even slightly more resistant to detergent than a *dsbA*⁻ strain. As such, its phenotype is less severe than that of a *dsbA*⁻*dsbC*⁻ strain. DsbD functions to keep DsbC reduced in the periplasm so that DsbC can react with non-native disulphides to correct them. The lack of equivalence between a *dsbA*⁻*dsbC*⁻ mutant and a *dsbA*⁻*dsbD*⁻ mutant indicates that DsbC does not always require DsbD to function in the periplasm.

DsbC is reduced in a dsbA⁻dsbD⁻ *mutant*

DsbC is found reduced in wild-type cells, but is oxidized in strains lacking DsbD. We determined the *in vivo* redox state of DsbC in a *dsbA*⁻*dsbD*⁻ mutant using AMS trapping. DsbC is found reduced in this genetic background, even when cells are grown in Luria–Bertani (LB), a medium that contains small molecule oxidants (Fig. 2). Addition of diamide, a disulphide-generating compound, to growing *dsbA*⁻*dsbD*⁻ cells leads to immediate oxidation of DsbC (not shown). However, when this disulphide stress is over, DsbC goes back to its reduced state even though DsbD is absent. We propose that DsbC accumulates in the reduced state in the periplasm of *dsbA*⁻*dsbD*⁻ strains by donating its disulphide bond to folding proteins.

The deletion of DsbG has no effect

The *E. coli* periplasm contains another protein disulphide isomerase, DsbG. DsbG has been proposed to function

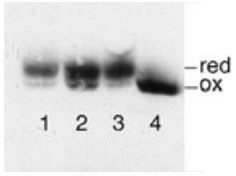

Fig. 2. *In vivo* redox state of DsbC. Exponentially growing cells (in LB) were TCA-precipitated, free cysteines were modified by AMS, and DsbC was detected by Western blot analysis. Lanes: 1, wild-type; 2, *dsbA*⁻; 3, *dsbA*⁻*dsbD*⁻; 4, *dsbD*⁻.

as an isomerase for essentially two reasons: first, like DsbC, DsbG is found reduced in the periplasm and second, DsbG can assist the folding of eukaryotic proteins with multiple cysteine residues when it is overexpressed (Bessette *et al*., 1999). However, no physiological substrate has been identified so far for DsbG, and the exact function of this protein remains unclear. To see whether DsbG is also able to function in the periplasm of *dsbA*$^-$ strains in a manner similar to DsbC, we constructed a *dsbA*$^-$*dsbG*$^-$ double mutant.

We found that deletion of DsbG does not affect the phenotype of a *dsbA*$^-$ mutant (data not shown). Similarly, a triple *dsbA*$^-$*dsbC*$^-$*dsbG*$^-$ mutant is phenotypically similar to a *dsbA*$^-$*dsbC*$^-$ double mutant. However, DsbG may be able to donate its disulphides in ways similar to DsbC as we found the protein to be mostly reduced in *dsbA*$^-$*dsbD*$^-$ double mutants.

2D-LC-MS/MS analysis of dsb$^-$ *strains*

The data presented above suggest that DsbC cooperates with DsbA in a DsbD-independent manner. These observations prompted us to characterize the periplasmic proteome of various *dsb*$^-$ strains by 2D-LC-MS/MS to see whether deletion of both *dsbA* and *dsbC* has specific consequences on the protein content of the periplasm. 2D-LC-MS/MS allows a global and semiquantitative analysis of protein expression ratios. The periplasmic proteomes of wild-type, *dsbA*$^-$, *dsbC*$^-$, *dsbA*$^-$*dsbC*$^-$ and *dsbA*$^-$*dsbD*$^-$ strains were compared.

Two proteins with multiple cysteine were not identified in the dsbC$^-$ *strains*

To examine the consequences of the absence of DsbC at the protein level, wild-type and *dsbC*$^-$ strains were grown in minimal media, and periplasmic extracts were prepared. Periplasmic proteins were then digested by trypsin, and the generated peptides were analysed by 2D-LC-MS/MS. The experiments were repeated three times for both strains. Each run allowed us to identify up to 175 secreted proteins, but only 115 proteins that could be reproducibly identified were kept for further analysis. To our knowledge, this is the first time that a proteomic approach allowed the identification of such a large number of secreted proteins, representing about 18% of all the proteins present in the cell envelope. A number of outer-membrane proteins, probably present in outer-membrane vesicles that did not pellet during the centrifugation, were reproducibly identified. As they also represent potential targets for DsbA and DsbC, they were kept for further analysis.

The same proteins were identified as being present in both the wild-type and *dsbC*$^-$ strains with the exception of three proteins that were absent in the latter strain. We discovered that these three proteins include DsbC itself and two proteins with multiple cysteine residues: a penicillin insensitive murine endopeptidase (MepA) and an endonuclease (End1). MepA had previously been found to depend on DsbC for expression (Hiniker and Bardwell, 2004), but End1 had not previously been reported to be a DsbC substrate. The structure of *Vibrio cholera* End1 shows four disulphide bonds, one of which is formed between non-consecutive cysteines (Altermark *et al*., 2006). The eight cysteines that form these four disulphide bonds are conserved in the *E. coli* End1 protein. The *E. coli* and *V. cholera* proteins are 66% identical at the amino acid sequence level; this suggests that they have similar structures and makes it almost certain that the *E. coli* protein shares the *V. cholera* disulphide bond pattern. As DsbC is required for the formation of non-consecutive disulphides, the folding of End1 is likely to require the presence of DsbC.

We then searched for proteins that, although present in both strains, vary substantially in their abundance. For quantification of abundance, we used the number of spectral counts (SC) reported for every protein. The number of SC for a protein is the total number of MS/MS spectra taken on peptides from this protein in a given 2D-LC-MS/MS analysis. This value is linearly correlated with the protein abundance over a dynamic range of two orders of magnitude (Liu *et al*., 2004). Protein ratios determined by spectral counting agree well with those determined from peak area intensity measurements and are consistent with independent measurements based on gel staining intensities (Old *et al*., 2005). To validate this quantification method, we added varying amounts (2–60 pmol) of two eukaryotic proteins, ovalbumin and carbonic anhydrase, to 300 μg of periplasmic proteins. Linear regression based on different sampling statistics was performed for each of the 2D-LC-MS/MS runs; the R^2 values obtained for the SC were 0.94 and 0.92 for ovalbumin and carbonic anhydrase respectively (Fig. 3). We concluded that this method is quantitative and that the ratio of SC reliably reflects changes in protein expression levels.

We selected proteins whose abundance was decreased or increased by at least twofold. To test the significance of the data, we used the unpaired Student's *t*-test and defined significance as a $P < 0.05$ (two-tail two-sample equal variance test). No protein was more abundant in the *dsbC*$^-$ strain (Table S1), and only two were decreased. YebF, a small protein with an unknown function, was the protein most decreased by the absence of DsbC (6 SC instead of 36), whereas Ivy, an inhibitor of lysozyme, was about twofold less abundant. Both of these proteins have two cysteine residues, and our results suggest that they may partially depend on DsbC for correct folding.

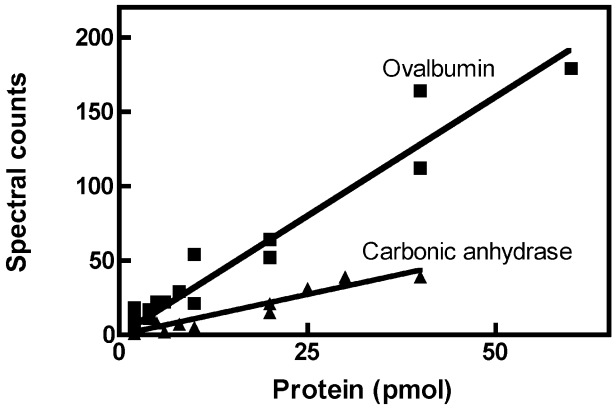

Fig. 3. The number of spectral counts (SC) correlates with the abundance of a protein. Varying amounts (2–60 pmol) of two eukaryotic proteins, ovalbumin and carbonic anhydrase, were added to 300 μg of periplasmic proteins. The SC values obtained for these two proteins in the various samples were then plotted against the corresponding protein amounts. After linear regression, we found that the R^2 values obtained for the SC were 0.94 and 0.92 for ovalbumin and carbonic anhydrase respectively. This indicates that the number of SC reliably reflects protein abundance in the sample.

Deletion of both dsbA *and* dsbC *affects the global protein content of the periplasm*

The results of the analysis of the periplasmic proteome of *dsbC*⁻ and wild-type strains by 2D-LC-MS/MS agree well with those obtained using 2D gels (Hiniker and Bardwell, 2004). This indicates that our 2D-LC-MS/MS method is reliable and should allow for the detection of changes in the periplasmic protein content of other *dsb*⁻ strains. Periplasmic extracts were prepared from *dsbA*⁻, *dsbA*⁻*dsbC*⁻ and *dsbA*⁻*dsbD*⁻ mutants, proteins were digested, and peptides were separated by high-performance liquid chromatography (HPLC) followed by LC-MS/MS analysis. The results from the MS/MS analysis were then compared with those obtained previously for the wild-type strain. First, the expression levels of several proteins were dramatically modified in all the strains that lack DsbA. These differences, which are described below, are consistent with the previously reported role of DsbA in oxidative protein folding. Second, we found that a *dsbA*⁻*dsbC*⁻ double mutant has a significantly altered periplasmic proteome when compared with *dsbA*⁻ and *dsbA*⁻*dsbD*⁻ strains. This is reflected by the graphs shown in Fig. 4. The SC values from the *dsbA*⁻ and *dsbA*⁻*dsbD*⁻ strains are linearly distributed, reflecting similar protein content. Comparison of the *dsbA*⁻ and *dsbA*⁻*dsbD*⁻ mutants shows indeed that there is only one protein (Spy) whose abundance is significantly different between these two strains (see Table S2). In contrast, when the SC values reported for proteins from the *dsbA*⁻*dsbC*⁻ mutant are plotted against those from the *dsbA*⁻ strain, the distribution is much more dispersed. This indicates a distinct overall protein content of the *dsbA*⁻*dsbC*⁻ mutant and confirms the deleterious effect of the simultaneous absence of DsbA and DsbC on the periplasm. One possibility is that the broader dispersion of the SC values observed in the *dsbA*⁻*dsbC*⁻ mutant reflects an increased sensitivity of this strain to the osmotic shock procedure. However, this would probably be reflected by an increased overall yield of proteins in periplasmic extracts, not decreased amounts of specific proteins in these extracts. The distinct protein content of *dsbA*⁻*dsbC*⁻ strains is a new finding and suggests that DsbC and DsbA cooperate in the folding of proteins. Specifically our finding suggests that DsbC can take over the function of DsbA. This is not consistent with the assigned roles of DsbA as an oxidase and DsbC as an isomerase. It suggests that a revision of the current model of disulphide bond formation in *E. coli* is called for. We note that a more

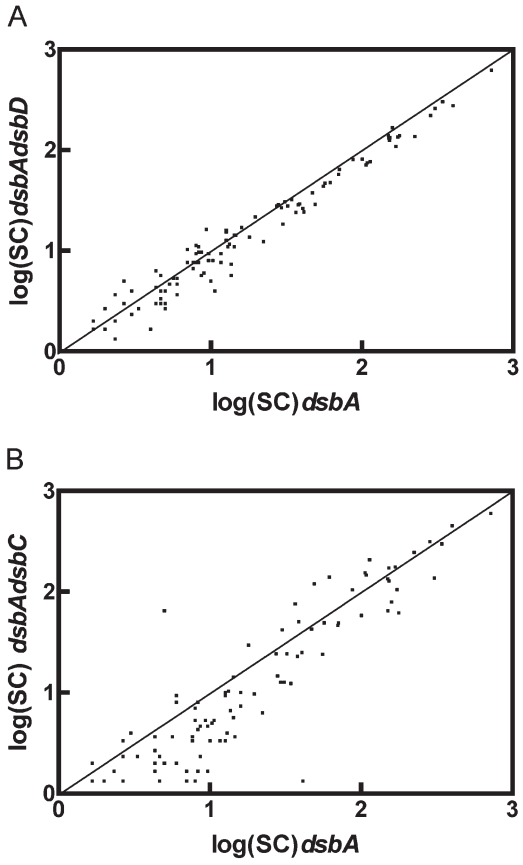

Fig. 4. The overall protein content of a *dsbA*⁻*dsbC*⁻ mutant is different compared with *dsbA*⁻ and *dsbA*⁻*dsbD*⁻ strains.
A. The logarithms of the SC values reported for the *dsbA*⁻ strain were plotted against those reported for the *dsbA*⁻*dsbD*⁻ mutant. Most of the SC values are similar in both strains, which is reflected by a quasi-linear distribution.
B. The logarithms of the SC values reported for the *dsbA*⁻ strain were plotted against those reported for the *dsbA*⁻*dsbC*⁻ mutant. The distribution is more dispersed, which indicates that the overall protein content of this double mutant is different.

Table 2. Proteins more than twofold less abundant in a *dsbA*⁻*dsbC*⁻ mutant than in *dsbA*⁻ and *dsbA*⁻*dsbD*⁻ strains ($P < 0.05$).[a]

Protein	# Cysteines	Spectral counts			RNA[b] (*dsbA*⁻*dsbC*⁻ versus *dsbA*⁻)
		dsbA⁻	*dsbA*⁻*dsbC*⁻	*dsbA*⁻*dsbD*⁻	
Proteins with at least two cysteine residues					
Cn16	2	2	0	3	NSD
DsbC	4	12	0	8	↓ (110)
GltI	2	5	0	6	NSD
OppA[c]	2	305	137	259	NSD
YggN	2	2	0	2	NSD
TreA[c]	2	8	1	11	nd
YhjJ	2	10	2	9	NSD
Proteins with one or no cysteine residue					
FepB	0	5	0	3	NSD
FliY	0	151	65	132	NSD
SubI	0	9	0	6	NSD
YcfS	1	2	0	2	NSD
GgT	0	8	3	8	NSD
MalE	0	41	1	24	nd
MppA	0	14	3	15	NSD
PhnD	0	159	79	168	NSD
Slp	1	5	0	3	↓ (2.9)
YliB	1	10	4	11	NSD
YjhT	0	4	0	2	NSD
YnjE	1	14	0	6	NSD
YncE	0	15	4	14	NSD

The levels of expression of the genes coding for these proteins in the *dsbA*⁻*dsbC*⁻ double mutant relative to the *dsbA*⁻ mutant are shown in the last column.
A complete list with all the identified proteins is available in Table S2. Microarray data for the genes corresponding to the identified proteins are available in Table S3.
a. All proteins that were identified in the *dsbA*⁻ and *dsbA*⁻*dsbD*⁻ strains but were absent in all three independent analysis of the *dsbA*⁻*dsbC*⁻ were also considered as significantly less abundant in the latter strain.
b. Genes whose expression ratios were > twofold different ($P < 0.05$) in the *dsbA*⁻*dsbC*⁻ double mutant relative to the *dsbA*⁻ mutant were considered as induced (↑) or repressed (↓). All other genes were considered as not significantly different (NSD). The ratio indicated next to the arrows is the fold increase or decrease in transcript level in the *dsbA*⁻*dsbC*⁻ strain compared with the *dsbA*⁻ strain. 'nd' means that no data were obtained for the corresponding gene.
c. $P = 0.05$ when SC values from the *dsbA*⁻*dsbC*⁻ are compared with those from the *dsbA*⁻*dsbD*⁻.

likely interpretation is that DsbC acts to compensate for the absence of DsbA, and DsbA and DsbC cooperate in helping to fold proteins.

Several cysteine-containing proteins are less abundant in a dsbA⁻dsbC⁻ *strain*

We searched for proteins with a significantly decreased abundance in the *dsbA*⁻*dsbC*⁻ strain, compared with the *dsbA*⁻ and *dsbA*⁻*dsbD*⁻ strains. Ten proteins were not detected in the double *dsbA*⁻*dsbC*⁻ mutant (Cn16, DsbC, FepB, GltI, Slp, SubI, YcfS, YggN, YjhT and YnjE), and 10 were at least twofold less abundant in the *dsbA*⁻*dsbC*⁻ than in the *dsbA*⁻ and the *dsbA*⁻*dsbD*⁻ mutants ($P < 0.05$) (Table 2). Interestingly, six of these proteins (Cn16, GltI, YggN, OppA, TreA and YhjJ) have two cysteine residues. Noteworthy, GltI, YggN and OppA are known DsbA substrates (Hiniker and Bardwell, 2004; Kadokura *et al.*, 2004). Two other cysteine-containing DsbA substrates, PhoA and DppA, were also more than twofold less abundant in the *dsbA*⁻*dsbC*⁻ mutant than in the *dsbA*⁻ or the *dsbA*⁻*dsbD*⁻ mutant. However, the decrease was not statistically significant when compared with the *dsbA*⁻*dsbD*⁻ mutant (see Table S2) and these proteins were not included in Table 2. Altogether, our data show that the simultaneous absence of both DsbA and DsbC decreases the level of several proteins, including several that contain cysteine residues. In contrast, the absence of DsbD has no effect on *dsbA*⁻ strains. Determination of RNA expression levels (Table 2) showed that, for most of the decreased proteins, their lower abundance is not due to a decreased transcription and is therefore likely to represent a direct consequence of the absence of DsbC. Altogether, our results further support the hypothesis that DsbC can assist DsbA in a DsbD-independent manner.

Proteins are decreased in all strains impaired in disulphide bond formation

In all strains lacking DsbA, the relative abundance of several proteins is dramatically modified compared with wild type (Table 3). In 125 proteins that were identified, we observed that the abundance of about 50 proteins was modified by at least twofold.

Table 3. Proteins whose abundance is significantly changed in *dsbA*⁻ strains.

Proteins > twofold less abundant in *dsbA*⁻ strains ($P < 0.05$)[a]				Proteins > twofold more abundant in *dsbA*⁻ strains ($P < 0.05$)			
	Spectral counts				Spectral counts		
Protein	Wild type	*dsbA*⁻	RNA[b] *dsbA*⁻ versus wild-type	Protein	Wild-type	*dsbA*⁻	RNA[b] (*dsbA*⁻ versus wild-type)
ArgT (2 cys)[c]	54	9	NSD	DegP (2 cys)	1	27	↑ (2.0)
ArtJ (2 cys)[c]	301	150	↑ (3.5)	FkpA	24	62	NSD
DppA (4 cys)	99	34	NSD	GgT	0	8	↑ (2.3)
GltI (2 cys)	49	5	NSD	IvY (2 cys)	26	224	↑ (7.9)
HisJ (2 cys)	170	84	↓ (2.6)	OmpA (2 cys)	131	401	NSD[d]
PhoA (4 cys)	212	29	↑ (19.4)	OmpX	27	152	NSD[d]
ProX (2 cys)[c]	46	8	NSD	OpgG	8	32	NSD
YfhM (2 cys)	1	0	NSD	OsmE	16	39	NSD
YebF (2 cys)[c]	36	1	↑ (3.3)	OsmY	104	285	↑ (6.0)
YggN (2 cys)	5	2	NSD	PotF (2 cys)	9	31	↑ (9.6)
YtfQ (2 cys)[c]	8	0	↑ (27.7)	RseB	0	3	nd
CirA (2 cys)[c]	8	0	NSD	Spy	0	10	↑ (5.3)
CreA (2 cys)[c]	2	0	NSD	TreA (2 cys)	1	8	nd
End1 (8 cys)[c]	1	0	nd	WzA	0	8	↑ (9.3)
MepA (6 cys)	4	0	NSD	YbiS	8	30	↑ (4.5)
RNase I(8 cys)	4	0	NSD	YdeI	11	108	↑ (9.7)
YebY (2 cys)[c]	8	0	NSD	YehZ	7	41	↑ (2.8)
DsbA	131	0	↓ (3.4)	YggG (3 cys)	1	14	↑ (3.7)
FlgH	5	0	NSD	YgiW	6	20	NSD)
FliC	219	0	↑ (92.5)	YhjJ (2 cys)	3	10	NSD
OmpF	136	13	(10.7)	YjbG	0	6	↑ (52.0)
PhoE	86	9	↑ (3.9)	YjbH (3 cys)	0	5	↑ (5.9)
FlgG	5	0	↓ ([e])	YjbF	0	2	nd
OmpN	2	0	NSD	YpfG (6 cys)	0	3	↑ (4.5)
				YraP	1	3	NSD

The levels of expression of the genes coding for these proteins in the *dsbA*⁻ mutant relative to the wild type are shown in the fourth and the last columns.
DsbA substrates are underlined.
A complete list with all the identified proteins is available in Table S1. Microarray data for the genes corresponding to the identified proteins are available in Table S4.
a. All proteins that were identified in the wild-type strain but were absent in all three independent analyses of the *dsbA*⁻ were also considered as significantly less abundant in the latter strain.
b. Genes whose expression ratios were >twofold different ($P < 0.05$) in the *dsbA*⁻ mutant relative to the wild type were considered as induced (↑) or repressed (↓). All other genes were considered as not significantly different (NSD). The ratio indicated next to the arrows is the fold increase or decrease in transcript level in the *dsbA*⁻ strain compared with the wild-type strain. 'nd' means that no data were obtained for the corresponding gene.
c. Newly identified DsbA substrates.
d. *osmE* and *opgG* transcription rates were increased by 1.82 ($P = 0.026$) and 1.7 ($P = 0.0039$) respectively.
e. *flgG* transcripts were not detected in the *dsbA*⁻.

In addition to DsbA, eight proteins with at least two cysteine residues were missing in *dsbA*⁻ strains. Two of these proteins (MepA and End1) were also missing in the *dsbC*⁻ mutant, which suggests they require the presence of both DsbA and DsbC. The other cysteine-containing proteins are a periplasmic ribonuclease (RNase I), the outer membrane colicin 1 receptor protein (CirA), a protein that is exported to the periplasm according to Psort (CreA), and three proteins with an unknown function (YebY, YtfQ and YfhM). We also found nine proteins with two or more cysteine residues whose abundance was decreased by at least twofold ($P < 0.05$) (Table 3). HisJ, GltI, DppA PhoA and YggN are known DsbA substrates (Hiniker and Bardwell, 2004; Kadokura *et al.*, 2004), but the other proteins (ProX, ArgT, ArtJ and YebF) had not previously been identified to depend on DsbA for correct folding. Sequence analysis revealed that the cysteine residues of these proteins are conserved in homologous sequences, which suggests that they are structurally important and probably form disulphide bonds. Our results allow us to add these proteins to the list of the potential DsbA substrates. The formation of a disulphide in YtfQ, YebF and ArtJ was confirmed by differential thiol trapping (see below).

The absence of DsbA also leads to decreased levels of several proteins that do not contain cysteine residues (Table 3). This can either be a direct consequence of a misfolding problem caused by the absence of DsbA or can result from a decreased transcription of their genes. To discriminate between these two possibilities, we

determined the RNA expression levels of the genes coding for these proteins (Table 3). Our data show that the absence of DsbA has no consequence on the transcription of most of these genes and that some genes, such as *artJ*, *phoA* and *yebF* are even induced. This indicates that the decreased protein abundance is due to an impaired folding of these proteins. Because DsbA does not have a chaperone activity, we propose that envelope perturbations in *dsbA*$^-$ strains prevent these proteins to correctly fold in the periplasm. In contrast, we found that the RNA expression levels of *ompF* and *flgG* were significantly decreased, which indicates that the diminution in the corresponding protein abundance is due to a decreased transcription of their genes. Regarding the outer membrane protein OmpF, our data agree with previous results (Pugsley, 1993). The other protein, FlgG, is a protein of the bacterial flagellum. Previous reports have shown that in a *dsbA*$^-$ strain, the flagellar P-ring protein FlgI is not properly folded and is degraded (Dailey and Berg, 1993). The degradation of FlgI prevents the assembly of a functional flagellum, which leads to the repression of the transcription of other flagellum genes (Chilcott and Hughes, 2000). The misfolding of FlgI is therefore the reason why the transcription of *flgG* is repressed and the corresponding protein is not identified in the *dsbA*$^-$ strain. Noteworthy, we found that the transcription of *flgH*, the gene coding for the other flagellum protein that was decreased in our proteomics analysis, was also diminished, but by less than twofold.

Several stress-related proteins are more abundant in strains lacking DsbA

The absence of DsbA leads to increased levels of 25 proteins, including nine proteins that were not identified in the wild type. Similar data were obtained for the *dsbA*$^-$*dsbC*$^-$ and *dsbA*$^-$*dsbD*$^-$ mutants (see Tables S1–4). Determination of the RNA expression levels allowed us to show that the transcription rates of 14 of the genes coding for these proteins are increased (Table 3).

Seven of these 25 proteins are part of the SigmaE regulon. SigmaE is a transcriptional activator that controls the expression of a variety of genes involved in maintaining the integrity of the cell envelope (reviewed in Ruiz and Silhavy, 2005). SigmaE is induced under conditions of stress in the cell envelope, including accumulation of misfolded outer membrane proteins in the periplasm, aberrant lipopolysaccharides, and lack of periplasmic folding agent. Gross and coworkers already showed that the absence of DsbA leads to an increased transcription of the gene coding for SigmaE (Mecsas *et al*., 1993), but this is the first time that the induction of SigmaE was confirmed at the protein level. The SigmaE regulon members whose abundance is increased in the *dsbA*$^-$ strain include the outer membrane proteins OmpA and OmpX, the periplasmic chaperone FkbA, the periplasmic proteases DegP and YhjJ, a protein involved in the biosynthesis of osmoregulated glycans (OpgG) and a negative regulator of SigmaE activity (RseB). The induction of these proteins further indicates that lack of disulphide bond formation leads to a global stress in the cell envelope.

In addition to the induction of SigmaE-regulated proteins, we observed the induction of proteins that are known to be induced under high osmotic pressure: OsmE, an osmotically inducible lipoprotein (Bordes *et al*., 2002), OsmY, a small protein of unknown function that has been proposed to interact with phospholipids on both sides of the periplasm (Lange *et al*., 1993), and the periplasmic trehalase TreA (Repoila and Gutierrez, 1991). Determination of the expression levels of the genes coding for these proteins allowed us to confirm that the increased protein abundance of OsmE and OsmY can be attributed to an increased RNA synthesis. Similar changes in the abundance of these three proteins, as well as a decreased transcription of *ompF* (see above), have been observed in strains grown under high osmotic pressure, suggesting that *dsbA*$^-$ strains mimic the effects of increased osmotic pressure. The induction of Spy, a protein that is specifically induced in spheroplasts (Hagenmaier *et al*., 1997), suggests that the induction of these osmo-related proteins in *dsbA*$^-$ strains may be the consequence of an altered peptidoglycan layer.

Determination of the in vivo *redox state of periplasmic proteins by differential thiol trapping*

To confirm the presence of disulphide bonds in the newly identified DsbA substrates, we adapted the differential thiol trapping technique developed by Leichert and Jakob (2004) to determine the redox state of the cysteine residues present in the periplasm of *dsbA*$^-$ and wild-type strains.

As expected, the majority of the cysteine residues identified in peptides from the wild-type strain were oxidized (Table 4). In contrast, more reduced cysteine residues were found in proteins from the *dsbA*$^-$ strain. In particular, cysteine residues from known DsbA substrates including OmpA, PhoA, DppA and GltI were found oxidized in the wild-type and, when detected, reduced in the *dsbA*$^-$ strain. The differential thiol trapping technique allowed us to confirm the formation of a disulphide bond in three newly identified DsbA substrates, ArtJ, YebF and YtfQ.

We also determined the redox state of the cysteine residues in the periplasm of the *dsbA*$^-$*dsbC*$^-$ and *dsbA*$^-$*dsbD*$^-$ double mutants and we found that they are similar to those observed in the *dsbA*$^-$ strain.

Table 4. Redox state of cysteine-containing peptides.

Protein	Cysteine-containing peptides found	Wild type (% red)	*dsbA*⁻ (% red)	*dsbA*⁻*dsbD*⁻ (% red)	*dsbA*⁻*dsbC*⁻ (% red)
PhoA	ATYHGNIDKPAVTCTPNPQR	2	45	66	66
	CYGPSATSEK	0	–	–	
	QDHAANPCGQIGETVDLDEAVQR	0	50	33	50
OmpA	AALIDCLAPDRR	0	47	32	52
	GMGESNPVTGNTCDNVK	2	85	83	90
ArtJ	QMQAECTFTNHAFDSLIPSLK	11	100	100	80
YebF	SADIHYQVSVDCK	15	–	–	–
	CEDLDAAGIAASVK	0	–	–	–
DppA	NECQVMPYPNPADIAR	0	–	–	–
GltI	PQSQEAYGCMLR	0	–	–	–
MepA	TPPPLPPSCQALLDEHVI	0	–	–	–
RNase I	AVKLTCQGNPAYLTEIQISIK	0	–	–	
YneA	VLTWDSDTKPECR	0	–	–	–
YtfQ	KPCNVVELQGTVGASVAIDR	0	–	–	–
EcoT	VSSPVSTMMACPDGK	–	100	100	100
	VELLIGQTLEVDCNLHR		100	100	100
YedD	VDRPTAECAAALDK	–	75	100	60
YggG	TLSDQACQEMDSK	–	66	66	50

A revised model for the oxidative protein folding pathways in E. coli

In conclusion, our results show that the simultaneous absence of DsbA and DsbC leads to a decreased integrity of the cell envelope and affects the global protein content of the periplasm. In contrast, strains lacking both DsbA and DsbD, the protein that is responsible for keeping DsbC active as an isomerase, do not share these characteristics. Our results suggest therefore that DsbC cooperates with DsbA in a DsbD-independent manner to ensure the correct folding of *E. coli* envelope proteins.

Kinetic, structural and genetic data showed that DsbB is unable to oxidize DsbC at physiological rates, unless the dimerization domain is removed and DsbC is expressed as a monomeric protein (Bader *et al*., 2001). Similarly, DsbD is unable to reduce DsbA (Rozhkova *et al*., 2004). This led to the assumption that the DsbA/DsbB oxidation pathway was isolated from the DsbC/DsbD isomerization pathway. Our results show that one can open a door in the barrier separating the oxidative and isomerization pathways. Our results indicate that, in contrast to the current view, DsbC can function independently of DsbD and is therefore able to function in both the oxidation and isomerization pathways. When DsbC gets oxidized upon reduction of a non-native disulphide, it is either reduced by DsbD or by transferring its disulphide to a reduced protein. DsbC may possibly be acting as a stand-alone protein folding catalyst that is able to cycle from the reduced to the oxidized state upon substrate oxidation and substrate reduction respectively. This activity of DsbC seems important to maintain the integrity of the cell envelope and is not restricted to the correction of non-consecutive disulphides. Our results extent those from Bader and coworkers who showed that monomeric mutants of DsbC are substrates for DsbB and can catalyse disulphide bond formation (Bader *et al*., 2001). On the basis of our results, we have adapted the model of disulphide bond formation in the *E. coli* periplasm (Fig. 5).

Experimental procedures

Bacterial strains and growth conditions

The bacterial strains used in this study are described in Table 5. Strains JP114, JP220, JP539, JP557, JP649 and C600 were used for the titration experiment with phage Φ80. All the other experiments were performed with strains AH50, JFC383, AH396, MD1 and MD3 in the MC1000 background. Strains JFC383, MD1 and MD3 were constructed by P1 transduction. Cells were grown aerobically in either LB or M63 minimal media, at 37°C. Unless otherwise indicated, M63 minimal medium was supplemented with 0.2% glucose, vitamins (Thiamine 10 μg ml^{-1}, Biotin 1 μg ml^{-1}, Riboflavin 10 μg ml^{-1} and Nicotinamide 10 μg ml^{-1}), 1 mM $MgSO_4$, leucine (20 μg ml^{-1}) and isoleucine (20 μg ml^{-1}). Sensitivity to antibiotics was assayed by streaking the strains on LB plates containing 4 μg ml^{-1} rifampin. To test the sensitivity to SDS, strains were grown in LB at 37°C to an A_{600} of 0.5. The cultures were then serially diluted 10^7-fold in 10-fold increments. Ten microlitres of each dilution was then spotted on LB plates containing 2.5% SDS and grown overnight. To study the ability of *dsb*⁻ strains to assemble a functional LamB protein, strains were streaked on MacConkey agar indicator plates containing 1% maltodextrin.

Φ80 phage titration

Cells were grown overnight to late logarithmic phase at 30°C. One hundred microlitres of cells was used to inoculate 3 ml of LB top agar (0.7% LB agar). The suspension was vortexed and plated onto a pre-warmed LB plate. Serial dilutions of Φ80 stock (> 10^{11} pfu ml^{-1}) were made at 10^{-3}, 10^{-6} and 10^{-9}.

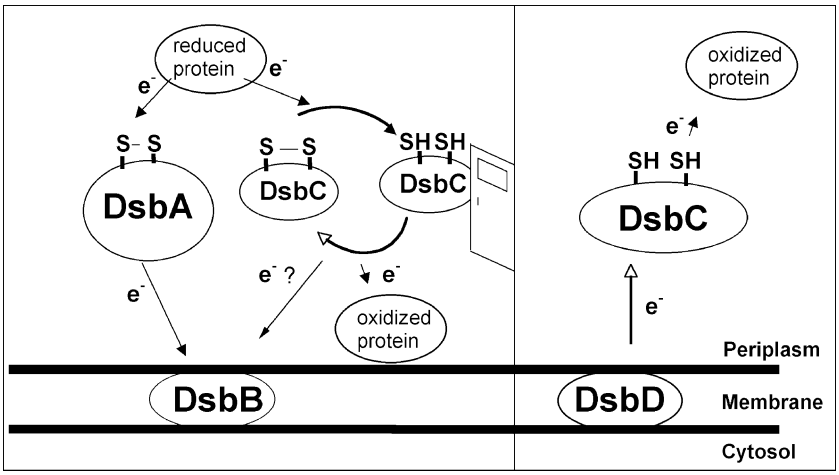

Fig. 5. A revised model for the formation of disulphide bonds in the *E. coli* periplasm. Disulphide bonds are introduced by the DsbA/DsbB pathway. Non-native disulphides are corrected by DsbC, which is recycled by DsbD. Both pathways are kinetically isolated. Our results indicate that DsbC is also able to function on the other side of the barrier where it assists DsbA in a DsbD-independent manner. DsbC may be acting as a stand-alone protein folding catalyst that cycles from the reduced to the oxidized state upon substrate oxidation and substrate reduction respectively. Although kinetics data showed that DsbC is not a good substrate for DsbB, we cannot exclude that a slow oxidation of DsbC by DsbB may play a more significant role in the absence of DsbA. The Western blot data presented in Fig. 2 also suggest that in the absence of DsbD, DsbA may be responsible for the oxidation of DsbC. The redox potentials of DsbA and DsbC are –125 mV and –130 mV respectively.

Five microlitres of these serial dilutions was spotted onto the LB agar plate containing cells, and allowed to incubate at 30°C overnight (16–18 h). The number of plaques and plaque sizes were tabulated.

Expression of a DsbC SXXS mutant

The catalytic site cysteine residues of DsbC were replaced by serine residues using the QuickChange Mutagenesis Protocol (Stratagene). Both the wild-type and mutated DNA sequence were then inserted in the pBAD33 expression plasmid. The plasmids were transferred into the MD3 strain and DsbC expression was induced by adding L-arabinose (0.2%).

Periplasmic extracts preparation

Cells (100 ml) were grown aerobically at 37°C in M63 minimal media to an A_{600} of 0.8, and periplasmic extracts were prepared as in Hiniker and Bardwell (2004). Protein concentration was determined using the Bradford assay.

Differential thiol trapping and digestion

Three hundred micrograms of periplasmic proteins was precipitated by adding trichloroacetic acid (TCA) to a final concentration of 10% w/v, followed by incubation on ice for 30 min. Samples were then centrifuged at 14 000 r.p.m. for 20 min and the resulting pellets washed with 5% ice cold TCA. The pellets were then resuspended in 100 µl denaturing buffer (6 M urea, 200 mM Tris-HCl pH 8.5, 10 mM EDTA) supplemented with 100 mM iodoacetamide. At this stage, various amounts ranging from 2 to 60 pmol of carbonic anhydrase and ovalbumin were added to the samples as internal standards. After a 20 min incubation at 25°C, the reaction was stopped by adding 10 µl of ice cold 100% TCA and left on ice for 20 min. The alkylated proteins were centrifuged and the pellet washed as described above. The proteins were

Table 5. Strains used in this study and their relevant genotypes.

Strain	Relevant genotype	Source
AH50	MC1000 *phoR* Δ*ara714leu*$^{+}$ *phoA68*	Hiniker *et al.* (2005)
JFC383	AH50 *dsbC::kan*	This study
AH396	AH50 *dsbD::cm*, *dsbA::kan1*	Hiniker *et al.* (2005)
MD1	AH50 *dsbA::kan1*	This study
MD3	AH50 *dsbC::cm*, *dsbA::kan*	This study
JP114	ER1821	New England Biolabs
JP220	JP114 Δ*dsbA::kan*	This study
JP539	JP114 Δ*dsbC::cm*	This study
JP557	JP220 Δ*dsbC::cm*	This study
JP649	JP114 Δ*dsbA::kan dsbD*$^{-}$*::cm*	This study
C600	JP114 *fhuA::kan*	This study

then dissolved in 100 µl of 10 mM DTT in denaturing buffer. After a 1 h incubation at 25°C, 100 µl of denaturing buffer supplemented with 100 mM N-ethylmaleimide was added to titrate out the remaining DTT and alkylate all newly reduced cysteines. The reaction was stopped by addition of 10% TCA and the proteins collected by centrifugation. The resulting pellet was successively washed with TCA and ice cold acetone, dried in a Speedvac, resuspendend in 0.1 M NH_4HCO_3 pH 8.0 with 3 µg sequencing grade trypsin, and digested overnight at 30°C. Peptide samples were then acidified to pH 3.0 with formic acid and stored at –20°C.

Differential analysis of periplasmic proteins by label-free 2D-LC-MS/MS

Peptides were loaded onto a strong cation exchange column GROM-SIL 100 SCX (100 × 2 mm, GROM, Rottenburg, Germany) equilibrated with solvent A (5% acetonitrile v/v, 0.05% v/v formic acid pH 2.5 in water) and connected to an Agilent 1100 HPLC system. Peptides were separated using a 50 min elution gradient that consisted of 0–50% solvent B (5% acetonitrile v/v, 1 M ammonium formate adjusted to pH 3.0 with formic acid in water) at a flow rate of 200 µl min^{-1}. Fractions were collected at 2 min intervals (20 in total) and dried using a Speedvac. Peptides were resuspended in 10 µl of solvent C (5% acetonitrile v/v, 0.01% v/v TFA in water) and analysed by LC-MS/MS as described below.

The LC-MS/MS system consisted of an LCQ DECA XP Plus ion trap mass spectrometer (ThermoFinnigan, San José, CA, USA) equipped with a microflow electrospray ionization source and interfaced to an LCPackings Ultimate Plus Dual gradient pump, Switchos column switching device, and Famos Autosampler (Dionex, Amsterdam, Netherlands). Two reverse phase peptide traps C18 Pepmap 100 Dionex (300 µm × 5 mm) were used in parallel with two analytical BioBasic-C18 columns from ThermoElectron (0.18 mm × 150 mm). Samples were injected and desalted on the peptide trap equilibrated with solvent C at a flow rate of 30 µl min^{-1}. After valve switching, peptides were eluted in backflush mode from the trap onto the analytical column equilibrated in solvent D (5% acetonitrile v/v, 0.05% v/v formic acid in water) and separated using a 100 min gradient from 0% to 70% solvent E (80% acetonitrile v/v, 0.05% formic acid in water) at a flow rate of 1.5 µl min^{-1}.

The mass spectrometer was set up to acquire one full MS scan in the mass range of 400–2000 m/z, followed by three MS/MS spectra of the three most intense peaks in the mass range 400–1500 m/z. The dynamic exclusion feature was enabled to obtain MS/MS spectra on coeluting peptides, and the exclusion time was set at 2 min.

Protein identification

Raw data collection of approximately 54 000 MS/MS spectra per 2D-LC-MS/MS experiment was followed by protein identification using the TurboSequest algorithm in the Bioworks 3.2 software package (ThermoFinnigan) against an *E. coli* protein database (SWISSPROT) using the following constraints: only tryptic peptides up to one missed cleavage site were allowed; tolerances for MS and MS/MS fragment ions were set to 1.2 Da and 1.0 Da respectively; and methionine oxidation (+16.0 Da), carboxamidomethyl cysteine or N-ethylmaleimide cysteine (+57.0 Da or +125.0 Da respectively) were specified as variable modifications. The identified peptides were further evaluated using charge state versus cross correlation number (Xcorr). The criteria for positive identification of peptides were Xcorr > 1.5 for singly charged ions, Xcorr > 2.0 for doubly charged ions, and Xcorr > 2.5 for triply charged ions. Protein scores (Su, Xcorr), peak areas and SC were calculated within BioWorks 3.2. The data were converted into Microsoft Excel spreadsheets by the export function contained in BioWorks and the output files were compared and processed by an in-house software program. Relative quantification of protein abundance was estimated by calculating the ratio of SC determined within the BioWorks software package. This parameter was shown to follow a linear relationship over two orders of magnitude, as determined from spiked internal standard proteins, within a dynamic range of at least 10^3.

Preparation of outer membrane proteins

Outer membrane proteins were prepared from strains grown in LB. Cultures were grown to an A_{600} of 0.8; cells were harvested by centrifugation at 6000 r.p.m. for 10 min then resuspended in 25 mM Tris pH 8.0, 0.5 M sucrose, 1 mM EDTA and 0.25 mg ml^{-1} lysozyme. After 15 min at room temperature, 20 mM $MgCl_2$ was added. The extracts were then centrifuged for 5 min at 12 000 r.p.m. The pellets were discarded and supernatants were centrifuged at 45 000 r.p.m. for 1 h at 4°C. The pellets were resuspended in Laemli buffer and loaded on SDS-PAGE.

In vivo *redox state of DsbC*

Cells were grown in LB at 37°C to an A_{600} of 0.8, and 1 ml samples were taken. Proteins were precipitated with 5% ice cold TCA and centrifuged at 16 000 *g* for 15 min. The pellets were washed with acetone, dried and resuspended in 50 mM Tris-HCl, pH 7.5, 0.1% SDS, 10 mM EDTA and 10 mM AMS. AMS is a reagent that covalently reacts with free thiol groups, adding a 490 Da group. This leads to a major mobility shift respective of modified protein in SDS-PAGE gels. Samples were analysed by SDS-PAGE under non-reducing conditions.

Antibodies

Antibodies against LamB were kindly provided by Natividad Ruiz and Tom Silhavy (Princeton), and antibodies against DsbC were provided by Jon Beckwith (Harvard).

Microarray analysis

RNA from WT, *dsbA*$^-$ and *dsbA*$^-$*dsbC*$^-$ strains was extracted using the Tripure reagent and the RNeasy purification kit (Qiagen). Microarray analysis was performed in triplicates by using 'GeneChip® *E. coli* Genome 2.0 Array' and the protocol provided by Affymetrix for prokaryotic expression analysis.

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Supplementary material

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