The EMBO Journal vol.14 no.14 pp.3415–3424, 1995

Identification and characterization of a new disulfide isomerase-like protein (DsbD) in *Escherichia coli*

Dominique Missiakas, Françoise Schwager and Satish Raina[1]

Département de Biochimie Médicale, Centre Médical Universitaire, 1 rue Michel-Servet, CH-1211 Genève 4, Switzerland

[1]Corresponding author

Previous studies have established that DsbA and DsbC, periplasmic proteins of *Escherichia coli*, are two key players involved in disulfide bond formation. A search for extragenic mutations able to compensate for the lack of *dsbA* function *in vivo* led us to the identification of a new gene, designated *dsbD*. Lack of DsbD protein leads to some, but not all, of the phenotypic defects observed with other *dsb* mutations, such as hypersensitivity to dithiothreitol and to benzylpenicillin. In addition, unlike the rest of the *dsb* genes, *dsbD* is essential for bacterial growth at temperatures above 42°C. Cloning of the wild-type gene and sequencing and overexpression of the protein show that *dsbD* is part of an operon and encodes an inner membrane protein. A 138 amino acid subdomain of the protein was purified and shown to possess an oxido-reductase activity *in vitro*. Expressing this subdomain in the periplasmic space helped restore the phenotypic defects associated with a *dsbD* null mutation. Interestingly, this domain shares 45% identity with the portion of the eukaryotic protein disulfide isomerase carrying the active site. We further show that in *dsbD* mutant bacteria the dithiol active sites of DsbA and DsbC proteins are mostly oxidized, as compared with wild-type bacteria. Our results argue that DsbD generates a reducing source in the periplasm, which is required for maintaining proper redox conditions. The finding that overexpression of DsbD leads to a Dsb$^-$ phenotype, very similar to that exhibited by *dsbA* null mutants, is in good agreement with such a model.

Key words: disulfide formation/folding/PDI-like domain

Introduction

How protein folding is mediated in the bacterial periplasmic space or in the outer membrane is of major interest, since little is known about the chaperone-like activities which may exist in these cell compartments. Recently a lot of progress has been achieved with the discovery of periplasmic catalysts of folding, such as peptidyl prolyl isomerase (PPI) (the *rotA* gene in *Escherichia coli*; Liu and Walsh, 1990) and protein disulfide isomerase (PDI) homologs (reviewed by Bardwell, 1994). Disulfide bonds are essential for the stability and activity of many extracellular proteins. Such a gain in stability is particularly relevant for the pathogenecity of bacteria, which secrete toxins, cellulases and pectate lyases which possess disulfide bonds in their native structure (Peek and Taylor, 1992; Yu *et al.*, 1992; Shevchik *et al.*, 1994). In eukaryotes the making or rearranging of disulfide bonds has been known for a long time to be a process catalyzed by PDI (reviewed by Freedman, 1989). In prokaryotes it is only recently that a similar family of proteins, called Dsbs, has been identified. Interestingly, and unlike the situation in eukaryotes, *E.coli* seems to possess many thiol:disulfide oxido-reductase activities (Bardwell *et al.*, 1991, 1993; Kamitani *et al.*, 1992; Dailey and Berg, 1993; Missiakas *et al.*, 1993, 1994). The first member of this family has been named *dsbA* for disulfide bond formation (Bardwell *et al.*, 1991) or *ppfA* (Kamitani *et al.*, 1992). DsbA was rapidly shown to be a powerful oxidant in the periplasmic space, even under acidic conditions (Wunderlich *et al.*, 1993; Zapun *et al.*, 1993), albeit with a weak isomerase activity (Zapun and Creighton, 1994). Using various genetic approaches we isolated mutant *E.coli* which lacked the ability to cope with changes in their redox environment. This led to the identification of different unlinked mutations assigned to the *trxA*, *trxB*, *dsbA* and *dsbB* genes (Missiakas *et al.*, 1993). In addition, we showed genetically that the lack of *dsbA* or *dsbB* could be compensated for by overexpression of the *dsbC* gene product (Missiakas *et al.*, 1994). To further investigate the catalytic activities of DsbA and DsbC we compared the disulfide-coupled refolding kinetics of bovine pancreatic trypsin inhibitor (BPTI) in their presence. DsbC was shown to mimic a PDI-like activity by catalyzing disulfide formation into a quasi-native intermediate species of BPTI (N*), a stage of refolding shown to be poorly, if at all, catalyzed by DsbA (Zapun and Creighton, 1994; Zapun *et al.*, 1995). The optimal refolding kinetics of BPTI were achieved by the dual and synergistic action of DsbA and DsbC, leading to the model in which oxidized DsbA forms cystine bonds in the first place and reduced DsbC 'proof-reads' their formation (Zapun *et al.*, 1995). So far, the inner membrane-embedded DsbB protein appears to be essential in recycling of the DsbA dithiol active site, but there is no direct evidence available regarding the intrinsic mechanism of such a process (Bardwell *et al.*, 1993; Missiakas *et al.*, 1994).

Here we describe an additional member of the Dsb family, DsbD. We isolated mutations in the *dsbD* gene as extragenic suppressors of *dsbA* mutant bacteria. Lack of DsbD function leads to global defects in disulfide bond formation and the gene is essential for bacterial growth >42°C. Our results suggest that DsbD acts as a reductase in the periplasmic space.

Table I. Phenotypic analyses of different *dsbD* mutants or plasmid-carrying strains

Strain genotypes	Growth on L-agar				Growth at 42°C	Motility	AP activity (U/mg protein)	Growth on M63 medium
	DTT (mM)		BP (μM)					
	7	10	20	30				
Wild-type CA8000	+	+	+	+	+	+	0.885	+
CA8000 *dsbD*::Tn*10*	+	–	–	–	-	+	0.712	+ (–)
CA8000 *dsbD*::ΩCm^r	+	–	–	–	-	+	0.730	+ (–)
CA8000 *dsbA*::Tn*10*	–	–	+	–	+	–	0.344	±
CA8000 *dsbB*::Tn*10*	–	–	+	–	+	–	0.350	±
CA8000 *dsbC*::Tn*10*	–	–	–	–	+	+	0.592	±
CA8000 *dsbA*::Tn*10 dsbD*::ΩCm^r	+	+	+	±	–	±	0.634	±
CA8000 *dsbB*::Tn*10 dsbD*::ΩCm^r	+	+	+	+	–	±	0.700	±
CA8000 *dsbC*::Tn*10 dsbD*::ΩCm^r	+	+	–	–	–	±	0.330	–
CA8000 *dsbD*::ΩCm^r/pDM1466 *dsbD*$^+$ (4)	+	+	+	+	+	+	0.690	+
CA8000 *dsbD*::ΩCm^r/pDM1467 *dsbD*$^+$ (>20)	–		–	–	–	–	0.353	–
CA8000 pDM1467 *dsbD*$^+$ (>20)	–	–	–	–	–	–	0.378	±
CA8000 pDM1663 PDI-like domain	+	+	+	+	±	+	0.740	+

Numbers in parentheses indicate the plasmid copy number.

Results

Mutations in the dsbD gene suppress some of the phenotypic defects associated with dsbA null mutant bacteria

In order to further study the roles of soluble periplasmic oxido-reductases and better understand disulfide bond formation *in vivo*, we used a variety of genetic approaches. The first one was to isolate spontaneous extragenic suppressors in bacteria carrying null mutations in the *dsbA* gene. To do this we took advantage of the fact that such mutant bacteria grow poorly in the presence of benzylpenicillin (BP). For example, they do not form colonies on L-agar supplemented with 30 μg/ml BP, whereas wild-type bacteria can grow normally at such a concentration. Such a sensitivity reflects the lack of folded penicillin binding protein 4, which contains two disulfide bonds in its active site (Mottl *et al.*, 1992). Spontaneous revertants capable of forming wild-type-like colonies occurred at a frequency of 10^{-6}. About 500 such revertants from five different independent cultures of *dsbA* mutant bacteria were isolated and characterized. To study whether any of these suppressors belonged to the *dsb* pathway, we checked to what extent they suppressed the other phenotypic defects associated with *dsbA* mutants, namely motility and DTT sensitivity. Altogether, 22 suppressor candidates were retained, since they were also revertants, at least partly, of these two phenotypes of *dsbA* mutant bacteria. Next the suppressor alleles were marked with Tn*10*–Kan or Tn*10*–Tet. This enabled us to quickly test whether they bred true and to assign them to different complementation groups. Among them, seven suppressors defined a unique complementation group which was characterized in more detail.

For this purpose the mutations were transduced into an otherwise wild-type genetic background, with the help of the linked Tn*10* insertion, and their phenotypes were examined. Surprisingly, such mutations conferred a highly pleiotropic phenotype, such as DTT and BP sensitivities. Sensitivity to BP was even more severe than that exhibited by *dsbA* null mutant bacteria (Table I). When we examined other reporter systems to monitor loss of disulfide bond formation in native proteins, such as flagellar proteins or alkaline phosphatase (AP), we did not detect any significant differences from wild-type bacteria (Table I). However, all of them conferred a severe temperature-sensitive growth defect (Ts$^-$) at temperatures >42°C.

On the basis of this finding we re-examined our collection of previously isolated DTT-sensitive candidates from mini-Tn*10* transposon insertions (Missiakas *et al.*, 1993). We found that eight such mutants exhibited the Ts$^-$ phenotype and were linked to the Tn*10* transposons used to mark the suppressors of *dsbA* mutant bacteria. Therefore, we designated this locus *dsbD*.

Mapping and cloning of the dsbD gene

Starting with a previously described *E.coli* chromosomal DNA library constructed in a low copy number cosmid vector (Koomey *et al.*, 1982), a clone (pDM1440) was obtained which restored the ability of all eight *dsbD*::Tn*10* mutant bacteria to grow on 20 μg/ml BP and 10 mM DTT agar media. This cosmid candidate was further shown to complement the Ts$^-$ phenotype of all *dsbD* mutants and to carry the corresponding *dsbD*$^+$ wild-type gene, as opposed to a multicopy suppressor gene. In addition, all seven putative *dsbD* point mutations identified were found to be recessive.

Mapping of *dsbD*$^+$ cosmid clones by hybridization to the ordered *E.coli* DNA library (Kohara *et al.*, 1987) placed the *dsbD* gene in the 4390–4410 kb region of the *E.coli* physical map, corresponding to bacteriophage λ clones 647 (21H1), 648 (5G7), 649 (9B1) and 650 (1D4). These results were confirmed by an observed 50% co-transduction of *dsbD*::Tn*10* mutations with *groES*30 zid-1::Tn*10* and ~90–95% with *htrI*::Tn*5*, two known markers mapping in this region. The *htrI*::Tn*5* insertion was identified in a previous independent screen aimed at isolating *htr* (high temperature requirement) mutations in genes whose products are required for bacterial viability at high temperatures (Raina, 1987).

Using standard DNA manipulation techniques, further subcloning experiments were performed with DNA prepared from cosmid pDM1440, by always selecting for the ability to suppress sensitivity to DTT and BP at 42°C, in the *dsbD*::Tn*10* mutant background. The results are

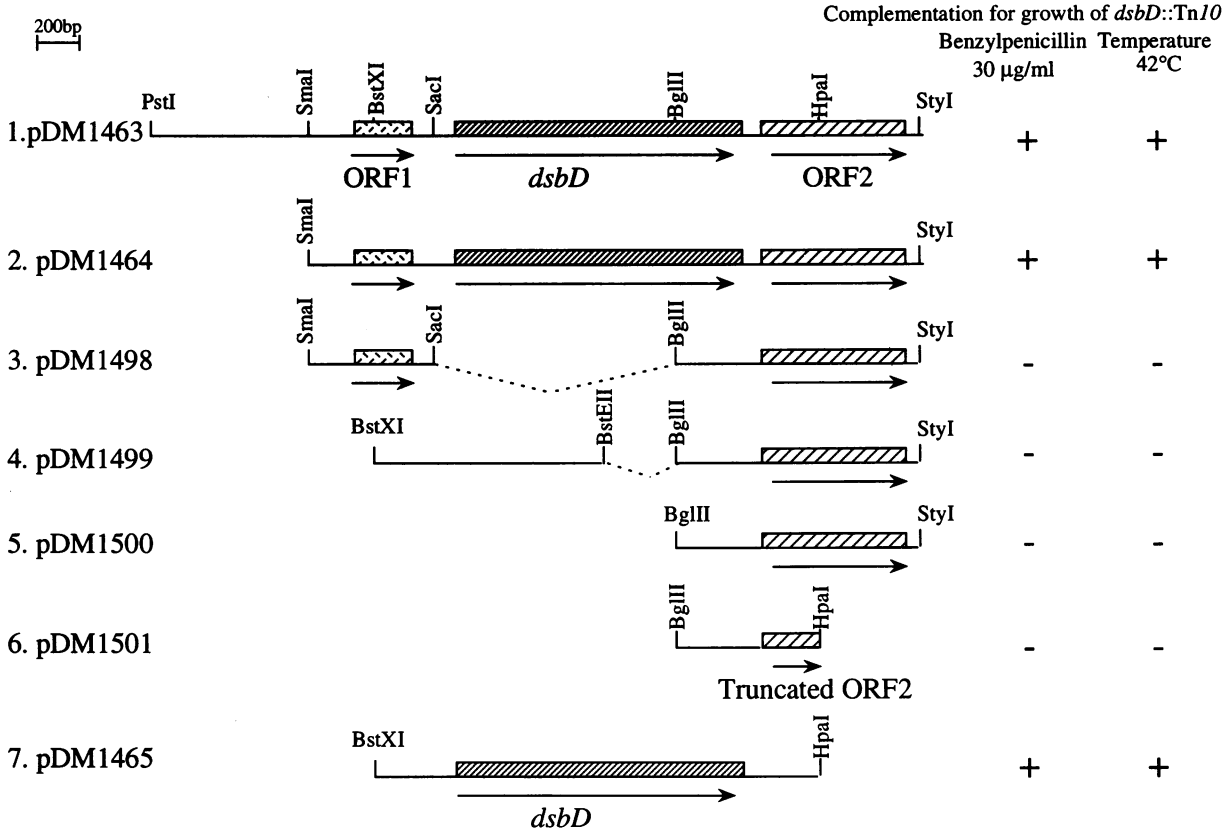

Fig. 1. Restriction map of the *dsbD* gene and surrounding DNA sequences. Columns at the right side indicate the ability of the different clones to complement *dsbD*::Tn*10* mutant bacteria.

presented in Figure 1. Since complementation by the minimal *dsbD*$^+$ clone was seen only with low copy number vectors, further subcloning experiments were performed using vectors pWSK29/30 (Wang and Kushner, 1991). However, cloning of the *dsbD* gene in a high copy number vector was not lethal for the cells (pDM1468), it simply did not reverse the Dsb$^-$ phenotype of the chromosomal mutation (Table I). Clones carrying either the 2.8 kbp *Sma*I–*Sty*I DNA fragment (pDM1464) or the minimal 2 kbp *Bst*XI–*Hpa*I fragment (pDM1465) were found to be sufficient to complement all of the phenotypes exhibited by *dsbD* null mutations, as well as recombine the *dsbD*::Tn*10* insertions.

Sequencing of the dsbD gene and identification of its product

Nucleotide sequence determination of the 3.8 kbp *Pst*I–*Sty*I DNA fragment (pDM1463) revealed three open reading frames (ORFs). The first ORF, ORF1, spans 336 nucleotides (nt) and potentially encodes a 12 kDa polypeptide. The second ORF (DsbD) is located 206 nt after ORF1 and is predicted to encode a 53 kDa polypeptide of 489 amino acids. The predicted sequence of the DsbD polypeptide showed at least six to eight potential transmembrane domains in the N-terminal 37 kDa part of the protein, indicating a cytoplasmic membrane location. The most striking features of the DsbD protein are carried on the last 16 kDa C-terminal domain. This domain is predicted to be highly hydrophilic and to face the periplasmic space. From its deduced amino acid sequence it is related to the thioredoxin superfamily of proteins,

```
DsbD    : ALVEAKGKPVMLDLYADWCVACKEFEKYTFSDPQVQKALADTVLLQANVTAND
          A+V    K V+++ YA WC CK+ E    S + K   D V+ + + TAND
PDI (1): AIVMDPKKDVLIEFYAPWCGHCKQLEPIYTSLGKKYKGQKDLVIAKMDATAND

DsbD    : ANVTANDAQDVALLKHLNVLGLPTILFFDGQGQ
          A V AN+ + L    +V G PTI F  G+
PDI (2): AKVDANEEHNKDLASENDVKGFPTIKIFRNGGK
```

Fig. 2. Alignment of the amino acid sequences of the DsbD protein and PDI around the active site and the conserved C-terminal region which form part of the thioredoxin fold. PDI (1) and PDI (2) are proteins ER72 and a plant PDI (*Medicago sativa*) with accession numbers P08003 and P29828 respectively. The one letter amino acid code is used. Conserved residues are indicated by +.

including eukaryotic PDI and prokaryotic Dsbs. These proteins possess a conserved dithiol active site cys-X-Y-cys. Such a motif is also present in the DsbD C-terminal domain, including the unique Pro residue in *cis*, a very conserved residue in the thioredoxin fold (summarized by Creighton and Freedman, 1993). A global sequence search showed that this C-terminal part is most closely related to eukaryotic PDI (Figure 2). A score of ~40–45% sequence identity with a range of eukaryotic PDIs was obtained. This score is the highest recorded so far among all identified Dsb proteins.

The third ORF, designated ORF2 (Figure 1), was predicted to encode a 199 amino acid long polypeptide. The organization of the three ORFs suggested that DsbD

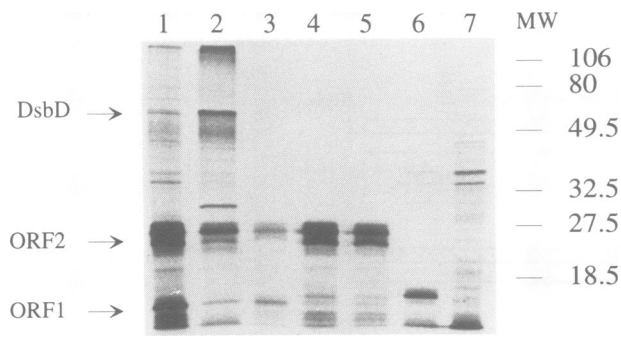

Fig. 3. Identification of the DsbD protein. Cultures of strains carrying vector alone or the different plasmids with ORF1, *dsbD* and ORF2 cloned under the T7 RNA polymerase promoter were grown at 37°C in M9 minimal medium and labeled with [^{35}S]methionine (50 μCi/ml) for 5 min as described in Materials and methods. The proteins were resolved by 12.5% SDS–PAGE; an autoradiogram of the dried gel is shown. Lanes 1–6 correspond to cell extracts carrying respectively plasmids 1–6 described in Figure 1; lane 7 corresponds to a cell extract carrying the vector alone. Arrows indicate the protein species synthesized. Numbers to the right indicate the positions and sizes in kDa of pre-stained protein molecular size standards (BioRad).

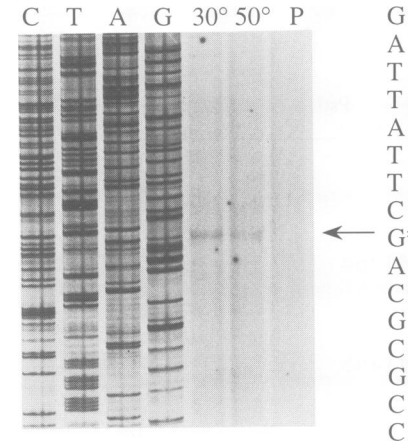

Fig. 4. Mapping of the 5′ termini of *dsbD* transcripts. Primer extension reactions of total cellular RNA hybridized to a ^{32}P-end-labeled DNA oligonucleotide probe, complementary to the sequence from nt 13–33 of the *dsbD* coding region. RNA was extracted from *dsbD*$^+$ bacteria (MC4100) grown at 30 and 50°C. Lanes labeled C, T, A and G correspond to the dideoxy sequencing reactions carried out, using the same oligonucleotide as primer. The lane labeled P was loaded with the radiolabeled primer alone as a control. The asterisk and the arrow point towards the transcriptional start site.

and ORF2 form an operon and all three genes are transcribed counter-clockwise to the origin of replication of the *E.coli* chromosome.

Identification of the *dsbD* gene product and the other two ORFs was carried out by labeling BL21(DE3) cells carrying one of plasmids 1–6 depicted in Figure 1 with [^{35}S]methionine. The results presented in Figure 3 further verified the assignment of the two adjacent ORFs. Introduction of plasmid pDM1463, as well as plasmid pDM1464, induced the synthesis of three proteins corresponding to the full-length products of the three predicted ORFs (Figures 1 and 3). However, plasmid pDM1498, which lacks the N-terminal region of the DsbD ORF (Figures 1 and 3, lane 3) made only ORF1 and ORF2. As can be seen in Figure 3 (lanes 4 and 5), plasmids pDM1499 and pDM1500 encode only the 24 kDa protein corresponding to ORF2. This was further confirmed by using plasmid pDM1501, which carries a truncated ORF2-encoding gene, leading to the synthesis of a truncated ORF2 (Figures 1 and 3, lane 6). Since the molecular weight of the observed DsbD protein is close to 53 kDa, as judged from its migration profile on SDS–PAGE, it further supported the conclusion that the predicted initiation codon (GTG) is the one used *in vivo*.

The positions of three of the eight *dsbD*::Tn*10* insertions able to recombine with the *dsbD*$^+$ plasmid were determined by sequencing the DNA junctions between Tn*10* and the *dsbD* gene. The sites of the mini-Kan::Tn*10* insertion (SR2447, SR2459 and SR2502) were found to be within the deduced *dsbD* gene at nt positions 45, 49 and 101 respectively after the initiation codon. In addition, we also sequenced the Tn*5* insertion, which defined the *htrI* gene. Interestingly, this insertion, which exhibited a comparatively weaker Ts$^-$ phenotype, was located towards the end of the *dsbD* gene at the corresponding codon position 462.

The proof that it is the larger ORF which encodes DsbD is based on the following observations: (i) a clone where ORF1 has been disrupted (*Bst*XI–*Sty*I, pDM1467) was still able to complement in low copy number a *dsbD* null mutation; (ii) removal of ORF2 from the plasmid construct (*Bst*XI–*Hpa*I, pDM1465), did not interfere with the ability of such a construct to fully complement a *dsbD* null mutation; (iii) clones carrying any of the Tn*10*–Kan insertions within the 53 kDa ORF did not complement a *dsbD* null mutation and neither did pDM1529, a derivative plasmid construct of pDM1463 (Figure 1) carrying a Cm cassette inserted at the unique *Bgl*II site; (iv) the presence of the conserved thioredoxin fold could be predicted from the amino acid sequence of the DsbD ORF, which was consistent with its role in disulfide bond formation.

Transcriptional regulation of the dsbD gene

The transcriptional organization of the *dsbD* gene was examined by mapping the 5′ termini of *dsbD*-specific transcripts using primer extension. It was found that *dsbD* transcription is initiated from a unique site, located 162 nt upstream of the putative GTG initiation codon (Figure 4). The sequences of the –10 (TGCCGG) and –35 (TGC-TAC) regions do not resemble canonical Eσ^{70}-transcribed promoters. Since *dsbD* mutants are temperature sensitive for growth, we examined the putative regulatory effect of temperature on *dsbD* transcription. Transcription of the gene was found to be decreased at 50°C, but not completely turned off (Figure 4), as otherwise observed for housekeeping genes. Also, these promoter mapping results clearly showed that the *dsbD* gene is transcribed from its own promoter and that ORF1 is not part of the DsbD–ORF2 operon.

Phenotypic analyses of the dsbD null mutation

In order to characterize the *dsbD* gene in more detail, we analyzed the phenotypes of the *dsbD* null mutation alone, as well as in combination with other *dsb* mutations. In order to obtain multiple combinations of *dsb* alleles, such as *dsbA*/*dsbD*, *dsbB*/*dsbD* or *dsbC*/*dsbD*, we constructed a new null allele using an ΩCm cassette (Fellay *et al.*, 1987) as the resistance marker, inserted at the *Bgl*II

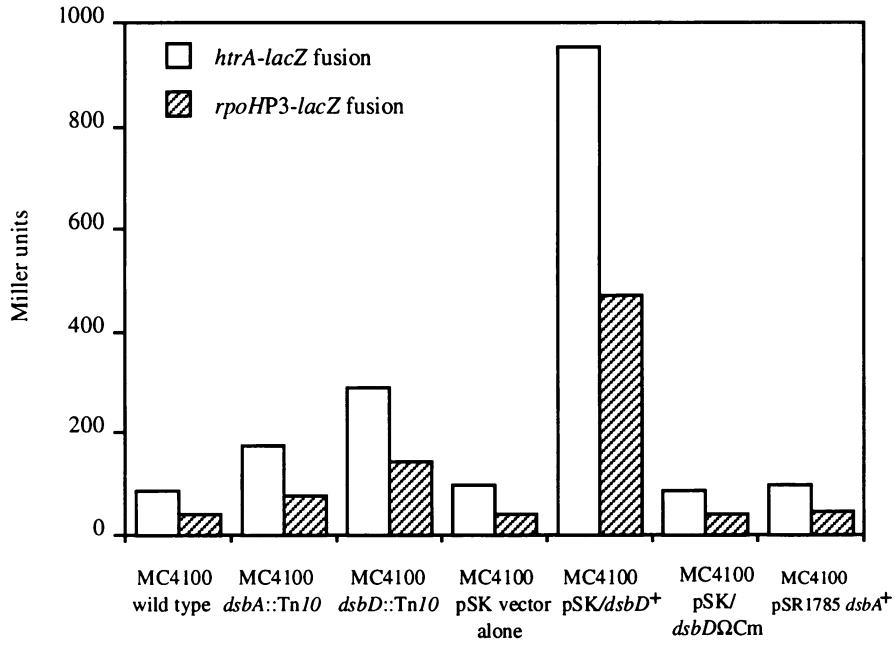

Fig. 5. Absence or excess of *dsbD* gene product induces a σ^E-dependent stress response. β-Galactosidase activities were determined as described by Miller (1992). The bacterial cultures were grown overnight in LB at 30°C, diluted 1:100 and allowed to reach an $OD_{595\ nm}$ of 0.2. SDS (0.1%) and chloroform were used to disrupt the cell membrane. Samples were assayed in duplicate and the data presented here are the average of four independent experiments. Miller units were used to depict β-galactosidase activity.

restriction site within the *dsbD* gene. Bacteria carrying either a Tn*10* insertion or the ΩCm cassette in the *dsbD* gene grew poorly on DTT-containing media, albeit not as poorly as *dsbA*, *dsbB* or *dsbC* null mutant bacteria (Table I). Mutations in these three last genes interfered with the ability of *E.coli* to cope with small changes in the redox potential of the medium. The efficiency of plating of such mutants was <0.1% when incubated in the presence of 7 mM DTT, whereas *dsbD* null mutants showed a comparable phenotype only in the presence of 10 mM DTT (Table I). However, a null mutation in *dsbD* conferred hypersensitivity to BP, since bacterial growth was inhibited at concentrations as low as 20 μg/ml in L-agar medium (Table I).

Most of the *dsb* mutants grow poorly on minimal media and are slightly mucoid (*dsbA* or *dsbC* mutants), presumably because such media (M9 and M63) do not contain any small oxidizing molecules or amino acids such as cystine. Growth of the *dsbD* null mutant in M9 or M63 was only affected in certain wild-type strains such as MC4100, but not in the CA8000 background, for example. To understand how a *dsbD* null mutation affected disulfide bond formation, we constructed double null mutations with *dsbA*::Tn*10*–Tet, *dsbB*::Tn*10*–Tet or *dsbC*::Tn*10*–Kan. We have previously shown that *dsbC* mutations are phenotypically additive to *dsbA* mutations (Missiakas *et al.*, 1994). In contrast to these results, the *dsbA/dsbD* double mutant was able to grow on DTT- or benzylpenicillin-containing media, whereas the *dsbA* null mutant did not grow in the presence of 7 mM DTT or 30 μg/ml BP. This is in agreement with the finding that *dsbA* extragenic suppressors mapped in the *dsbD* gene. This suppression effect was tested for other phenotypes exhibited by *dsbA* null mutants, such as lack of motility or inability to use bromochloroindolyl phosphate (XP) as a substrate for the periplasmic alkaline phosphatase containing two disulfide bonds. The double *dsbA/dsbD* null mutant was motile and able to use XP, albeit not to the same extent as the isogenic wild-type strain (Table I). Hence, inactivation of both the *dsbA* and *dsbD* genes does not lead to full recovery of the wild-type redox potential in the periplasm, but to an intermediate level, such that some oxidation reactions are properly catalyzed.

The best suppression was obtained when a *dsbD* null mutation was introduced into a *dsbB* background. Motility and growth in the presence of DTT or BP were all restored (Table I). The *dsbC/dsbD* double mutant behaved quite differently from *dsbA/dsbD* or *dsbB/dsbD* double mutants. While some *dsbC* phenotypes were restored (e.g. sensitivity to DTT), others turned out to be additive (Table I). For example, motility was not affected in either a *dsbC* or *dsbD* single mutant as compared with a wild-type bacterium, but the double *dsbC/dsbD* mutant was not as motile. This double mutant was also more sensitive to BP than either of the single mutants alone (Table I).

We have previously shown that mutations in the *dsbA* or *dsbC* genes induce the heat shock response dependent on the σ^E transcription factor. This regulon is specifically induced by misfolding of proteins in the periplasmic space and the outer membrane and in *dsb* mutants folding of such secreted proteins is affected (Mecsas *et al.*, 1993; Raina *et al.*, 1995). The σ^E-dependent response in *dsb* mutants was monitored using two promoter fusions known to be transcribed by $E\sigma^E$, namely *htrA–lacZ* and *rpoH*P3–*lacZ*. We carried out similar experiments by transducing the *dsbD*::ΩCm allele into strains carrying these *lacZ* fusions (Figure 5). Consistent with our expectations that DsbD plays an active role in the folding of disulfide bond-containing proteins, a *dsbD* null mutation led to a 3-fold induction of the σ^E regulon, which is even more than that observed with *dsbA* or *dsbC* single mutations (Figure 5).

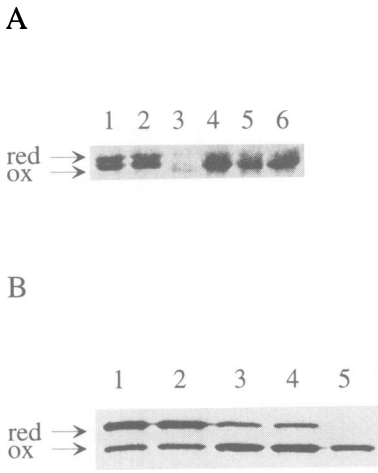

Fig. 6. The *in vivo* equilibrium between oxidized and reduced states of DsbA and DsbC is affected in *dsbD*::Tn*10* mutant bacteria. Bacterial cultures were grown in M9 medium and labeled with [^{35}S]methionine for 2 min followed by chases with excess cold methionine for various time periods. Cells were lysed and immunoprecipitated with antisera to either DsbA (A) or DsbC (B). Further non-specific oxidation reactions were blocked as described in Materials and methods. Autoradiograms of dried gels which were electrophoresed under non-reducing conditions are shown. The positions of oxidized (ox) and reduced (red) forms are indicated by arrows. (**A**) Lanes 1 and 2, CA8000 (*dsbD*$^+$), 0 and 5 min of chase with cold methionine; lane 3, control SR1790 (CA8000 *dsbA*::Tn*10*); lanes 4 and 5, SR2447 (CA8000 *dsbD*::Tn*10*), 0 and 5 min of chase; lane 6, CA8000 (*dsbD*$^+$) treated with oxidized DTT after labeling. (**B**) Lanes 1 and 2, CA8000 (*dsbD*$^+$), 0 and 5 min of chase; lanes 3 and 4, SR2447 (CA8000 *dsbD*::Tn*10*), 0 and 5 min of chase; lane 5, CA8000 (*dsbD*$^+$) treated with oxidized DTT after labeling.

Effect of dsbD mutants on the redox state of DsbA and DsbC proteins

As described above, single *dsbD* null mutants exhibited some phenotypes observed with *dsbA* or *dsbC* mutants and these defects were partially corrected in the double *dsbA/dsbD* mutant and to some extent in a double *dsbC/dsbD* mutant. These observations led us to the hypothesis that the *dsbD* mutation could directly alter the redox state of the DsbA and DsbC proteins. To test this, each protein was immunoprecipitated from extracts of *dsbD* null mutant or wild-type bacteria. The results, shown in Figure 6, indicate that both oxidized and reduced states of DsbA and DsbC proteins could be immunoprecipitated from wild-type bacteria grown in minimal medium, as well as from *dsbD* mutant bacteria. However, the ratio between the two forms was reversed in the *dsbD* mutant background as compared with wild-type bacteria, meaning that the equilibrium between oxidized and reduced DsbA and DsbC proteins was shifted towards their oxidized state. As a control, one sample in each case was treated with oxidized DTT to convert all the proteins to the oxidized form (Figure 6A, lane 6, and B, lane 5).

Overexpression of DsbD protein confers a Dsb$^-$ phenotype

The above experiments suggested that the *dsbD* gene product could play a role in counter-balancing the oxidizing effect of the major disulfide oxidases in the periplasm, such as DsbA. To confirm this, we cloned the minimal *dsbD* coding gene under the control of the p*tac* promoter with *lacI*^{q} on the plasmid to allow its controlled expression. Induction of *dsbD* led to the loss of alkaline phosphatase activity and motility in an otherwise wild-type bacterium (Table I). These phenotypes had previously been attributed to loss of proper oxidase activity. Thus it appears that overexpressing DsbD leads to the reduction/misfolding of disulfide bond-containing periplasmic proteins. This is probably the reason why complementation of the *dsbD* null mutations by the corresponding wild-type gene was achieved only when the *dsbD* gene was cloned into low copy number vectors.

In addition, the presence of *dsbD* on a high copy number plasmid turned on the σ^E-dependent response, a phenotype that can be attributed to partial loss of DsbA or DsbC functions, i.e. limitation of oxidation processes in the periplasm (Figure 5). In fact, we earlier found in independent experiments that certain plasmids carrying genes from the 94–95 min region induced the σ^E-dependent response (Raina *et al.*, 1995). We were able to verify that these clones overexpress the *dsbD* gene and can recombine the *dsbD*::Tn*10* insertions. Therefore, in agreement with the results described in the above section, DsbD is probably a reducing factor acting in the periplasmic space.

The PDI-like domain of DsbD exhibits an oxido-reductase activity

As stated above, the DsbD protein contains at its C-terminus a 16 kDa domain which shares 40–45% identity with eukaryotic PDI. This domain is predicted to be very hydrophilic and presumably protrudes towards the periplasmic space. The finding that lack of DsbD protein directly affected the redox state of periplasmic DsbA and DsbC proteins was in good agreement with such a predicted location. To unambiguously attribute the catalytic properties of the DsbD protein to this particular domain, the DNA fragments encoding for either the last 153 amino acids of the protein (17 kDa domain) or a smaller subdomain of only 138 amino acids (15.4 kDa) were cloned independently, in frame with the *pelB* signal sequence using the T7 expression vector pET-22b (Novagen). Since the 17 kDa domain was not completely processed, we used the minimal 15.4 kDa subdomain, which was efficiently translocated into the periplasmic space. First we tested its ability to complement a *dsbD* null mutation. We found that despite the fact that the Ts$^-$ phenotype was only partially suppressed, the DTT- and BP-sensitive phenotypes were almost fully reversed (Table I). However, just as for the full-length DsbD protein, overexpression of this subdomain led to a Dsb$^-$ phenotype, i.e. an excess of reducing processes in the periplasmic space.

To further substantiate the catalytic activity of this 15.4 kDa domain as a potential thiol:disulfide oxido-reductase, this truncated protein was purified from an osmotic shock fluid using anion exchange chromatography (Figure 7A). Its ability to catalyze reduction of the two chains of insulin in the presence of DTT (1 mM) is shown in Figure 7B. It has previously been reported that thioredoxin will actively reduce insulin upon addition of DTT, leading to aggregation of the B-chain (Holmgren, 1979). Both purified DsbC and DsbA have been shown to be active in this assay (Bardwell *et al.*, 1991; Missiakas *et al.*, 1994).

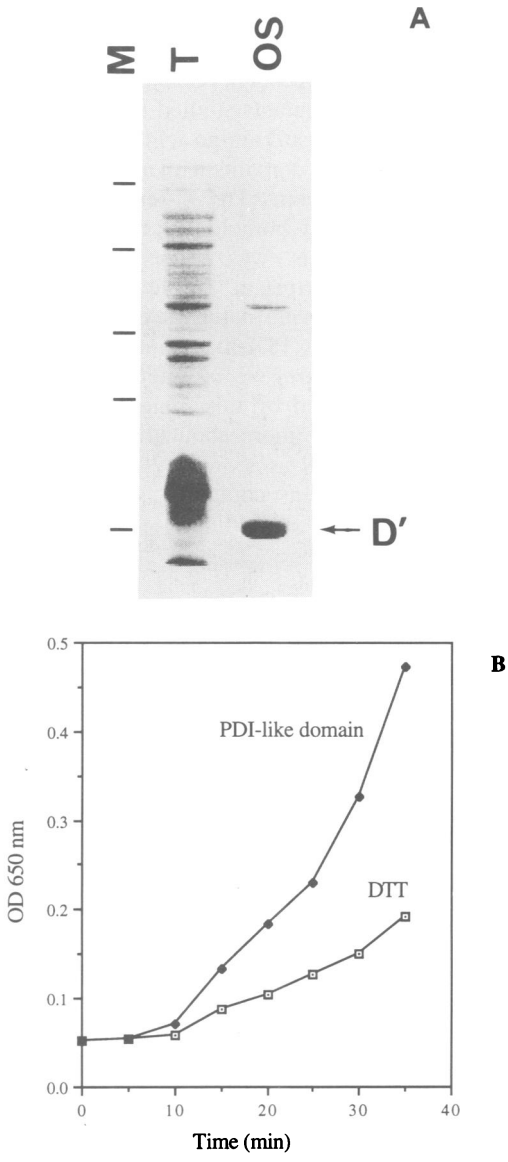

Fig. 7. The purified PDI-like domain of DsbD is able to reduce insulin in the presence of DTT. (**A**) Purification of the domain. Cultures of strains carrying clone pDM1663 were induced for 2 h with 5 mM IPTG, harvested and subjected to an osmotic shock followed by Q-Sepharose chromatography. Proteins were electrophoresed by 12.5% SDS–PAGE and the gel was stained with Coomassie brilliant blue. Lane M, lines indicate the position of pre-stained protein molecular weight standards (BioRad) of respectively 80, 49.5, 32.5, 27.5 and 18.5 kDa, from top to bottom; lane T, total cell extract carrying plasmid pDM1663; lane OS, purified domain from osmotic shock fluid from cells carrying plasmid pDM1663. (**B**) Assay of the reductase activity. The reaction was performed as described by Holmgren (1979) in 0.1 M potassium phosphate buffer, pH 7, containing 2 mM EDTA, 0.3 mM DTT and 131 μM insulin. The reaction was initiated by adding purified DsbD C-terminal domain (5 μM final concentration).

Discussion

The bacterial periplasm is presumed to have an oxidizing environment due to the presence of strong oxidants such as DsbA and DsbC. Such an environment is needed to facilitate disulfide bond formation of a variety of proteins. Isolation and characterization of extragenic suppressors of *dsbA* null mutants led us to the identification of a previously unreported reducing activity in the periplasmic space. The gene encoding for it was designated *dsbD*. Further analyses of a defined null mutation in *dsbD* showed that the *dsbD* null mutant could also correct the phenotypic defects exhibited by *dsbA*$^-$, such as sensitivity to DTT and BP. Interestingly, bacteria carrying mutations in the *dsbD* gene alone were also found to have a Dsb$^-$ phenotype. Quite surprisingly, *dsbD* null mutant bacteria were Ts$^-$ for growth at temperatures >42°C. This phenotype is unique so far to *dsbD* mutants, since none of the mutations in the other known *dsb* genes (*dsbA*, *dsbB* or *dsbC*) exhibited such a temperature-sensitive growth defect. This suggested an absolute requirement for DsbD function, particularly under such stress conditions, and a multiplicity of roles in the physiology of *E.coli*. Consistent with *in vivo* involvement of the *dsbD* gene product in disulfide bond formation, null mutations in *dsbD* were also isolated from our previously described collection of DTT-sensitive mutants which had led to identification of the *dsbB* and *dsbC* genes (Missiakas *et al.*, 1993, 1994).

Double *dsbA*/*dsbD* or *dsbB*/*dsbD* combinations behaved almost like isogenic wild-type bacteria. These observations confirmed the current reported model assuming that DsbA and DsbB belong to the same pathway (Bardwell *et al.*, 1993; Missiakas *et al.*, 1994). The picture is somewhat different with the *dsbC* mutation. Although the double combination *dsbC*/*dsbD* mutations restored the ability of cells to grow on DTT-containing medium, an additivity of the phenotypic defects was otherwise observed for certain phenotypes. As an example, bacteria carrying both *dsbC* and *dsbD* mutations were less motile than bacteria carrying any of the two mutations alone. These findings suggest that the functions of DsbA and DsbC *in vivo* do not completely overlap.

Overall, the redox properties of the thioredoxin-like proteins are intricately bound up with their stability. Whereas disulfide bonds stabilize the folded conformation of proteins, including that of thioredoxin (Kelley *et al.*, 1987), reduced DsbA was found to be more stable than oxidized DsbA (Wunderlich *et al.*, 1993; Zapun *et al.*, 1993). The active dithiol of DsbC has quite similar properties, hence reduced DsbC is also a more stable protein than oxidized DsbC (Zapun *et al.*, 1995). Our data clearly show that *in vivo* these ratios between oxidized and reduced forms are modulated by the presence of active DsbD. Absence of DsbD led to an excess of oxidized DsbA and DsbC in the cell, meaning that wild-type DsbD probably acts as a reductase. As a consequence, the oxidation potential is increased in the periplasm, leading to more thiol:disulfide exchange reactions. Non-specific mixed disulfide species are generated between DsbA or DsbC and thiol groups of substrate proteins, i.e. the introduction of incorrect disulfides is favored under more oxidizing conditions. Addition of DTT does not restore the wild-type situation; although DTT will reduce disulfide bonds (including incorrect ones), it won't substitute for the folding catalysts required for oxidation of the correct disulfides.

Since not all DsbA molecules are in the oxidized form in *dsbD* mutant bacteria and, moreover, DsbC functions are also altered in such mutants, this explains how the phenotypic defects exhibited by *dsbD* are not exactly the same as those exhibited by either *dsbA* or *dsbC* single mutants. On the other hand, an excess of DsbD confers a

similar phenotype to a *dsbA*::Tn*10* mutation. If DsbD acts as a reductase, its overexpression probably leads to random reduction of disulfide bond-containing proteins and as a consequence to the accumulation of misfolded proteins in the periplasm. This model is supported by the fact that a σ^E-dependent response was observed. This response comprises a regulon which is specifically induced by misfolding of extracytoplasmic proteins. In fact, we previously reported that a clone carrying a gene around the 94–95 min region on the *E.coli* chromosome could induce a σ^E-dependent response when present on a high copy number plasmid (Raina *et al.*, 1995). We have shown during this study that this gene is *dsbD*.

Absence of both the *dsbA* and *dsbD* gene products is an 'artificial' situation, where both a major oxidant and a major reductase have been removed from the system. Probably a new equilibrium between the remaining Dsb proteins, namely, DsbB, DsbC and DsbE (D.Missiakas and S.Raina, in preparation), is established, which is able to cope with the new redox conditions. Absence of both *dsbD* and *dsbC* is more critical for proper protein folding. This is not surprising according to our knowledge of DsbC functions. This protein can act not only as an oxidant, but also as an isomerase. Reduced DsbC in catalytic amounts was able to accelerate the rate limiting step of BPTI refolding and the isomerization between various intermediates of folding containing two native disulfide bonds and the native protein with all three disulfides (N) (Zapun *et al.*, 1995). Recent data have revealed that in a *dsbD* mutant background, folding of BPTI is blocked at the N* stage (a refolding intermediate containing two native disulfide bonds) and the native conformation (N) is almost never obtained (G.Georgiou, personal communication). This suggests that DsbD is involved either in activating reduced DsbC or acts itself as an isomerase, or both. Whether the two proteins belong to a same pathway for this particular function or have some cross-specificities towards some substrates remains to be elucidated. Nevertheless, both these possibilities fit with the observation that the double *dsbC*/*dsbD* null mutant exhibited combined protein folding defects.

After the completion of this work and during a recent homology search we came across recently submitted sequences with accession numbers X77707, L35347 and U14003 (this last sequence was released by the *E.coli* Genome Sequencing Project). The X77707 locus has been cloned on the basis of its requirement for cytochrome biogenesis (J.Cole, personal communication; Sambongi and Ferguson, 1994; Crooke and Cole, 1995). This sequence was identical to the sequence we submitted (P36655) and carried the *dsbD* gene. The L35347 locus was cloned because of its presumed involvement in metal tolerance (J.Camakaris, personal communication; Fong *et al.*, 1995). A defect in cytochrome biogenesis is in good agreement with our finding that DsbD provides a reducing source in the periplasm. It is likely that one of the functions of DsbD is to maintain the cys residues of the apocytochrome in a reduced state to allow proper covalent linkage with the haem. However, DsbD function is not restricted to cytochrome biogenesis, since mutations in genes other than *dsbD* which also lead to lack of c-type cytochromes in the periplasm (D.Missiakas and S.Raina, unpublished results) do not exhibit the Dsb$^-$ phenotype produced by *dsbD* mutations. Such cytochrome-deficient mutants exhibit wild-type-like DTT or BP resistances and do not induce the σ^E-dependent response, which is greatly altered in all the *dsb* mutants studied up to now.

Examination of the DsbD amino acid sequence predicted that the protein has two domains with a cys-X-Y-cys motif present in each of them. The C-terminal hydrophilic domain, which is highly homologous to PDI, thioredoxin and other Dsb proteins, was expressed and produced independently in the periplasm. This led to the suppression of most of the phenotypic defects exhibited by a *dsbD* null mutant. Since this PDI-like domain also exhibited reductase activity *in vitro*, we assume that the active site of the protein is carried by this moiety. It would be of major interest to know more about the redox properties of this domain, as well as its ability to directly interact with periplasmic proteins containing disulfide bonds or with the other catalysts of folding.

Materials and methods

Bacterial strains and plasmids

The bacterial strains and plasmids used in this study are listed in Table II.

Media and chemicals

Luria-Bertani (LB) broth and M9 and M63 minimal media were prepared as described by Miller (1992). When necessary the media were supplemented with ampicillin (Amp, 100 μg/ml), tetracycline (Tet, 15 μg/ml), kanamycin (Kan, 50 μg/ml) or chloramphenicol (Cm, 20 μg/ml). The indicator dye 5-bromo-4-chloro-3-indolylphosphate (XP) was added in M63 medium to assay active alkaline phosphatase present in the different bacteria.

Genetic selections and methods

Chromosomal mutations which render *E.coli* sensitive to DTT and/or BP were isolated as described earlier (Missiakas *et al.*, 1993, 1994) in wild-type *E.coli* strain CA8000. The suppressors of *dsbA* null mutants were isolated using five different Tn*10* insertion mutants of *dsbA*. About 10^8 cells of each culture were plated on L-agar medium supplemented with 30 μg/ml BP. Resistant colonies were obtained at a frequency of 10^{-6}. Five hundred such independent colonies were retained and spot tested on L-agar medium containing 10 mM DTT, as well as stabbed onto 0.3% agar plates to test their motility. The suppressor candidates which were positive for all these tests were marked with either Tn*10*–Kan^r or Tn*10*–Tet^r by infecting the exponentially growing cultures with λ1105 and λ1098 (Way *et al.*, 1984) respectively. The marked mutations were freshly transduced in various backgrounds using bacteriophage P1. The assignment of mutations to the *dsbD* locus was done using *groES*30 zid-1::Tn*10* (Fayet *et al.*, 1989) and *htrI*::Tn*5* (Raina, 1987). In the course of this study it became desirable to construct a new null allele of the *dsbD* gene with a different drug marker. An ΩCm cassette (Fellay *et al.*, 1987) was inserted at the unique *Bgl*II site within the *dsbD* gene by *in vitro* DNA manipulation of plasmid pDM1463 (Figure 1). Transfer of this null allele onto the chromosome was performed as described previously (Raina *et al.*, 1995).

Cloning of the wild-type gene was achieved by transforming the mutant bacteria with a cosmid library, scoring for the regain of resistance to 20 μg/ml BP in the medium at 42°C. To confirm that the wild-type *dsbD* gene was being cloned, we verified that the corresponding cosmid could recombine the chromosomal mutation.

Mapping of the *dsbD* gene was achieved by synthesizing ^{32}P-radiolabeled probes using random primers (Sambrook *et al.*, 1989) and the derivative cosmid pDM1440 (*dsbD*$^+$) as the template DNA. The *E.coli* DNA library in bacteriophage λ (Kohara *et al.*, 1987) was hybridized using probes described previously (Missiakas *et al.*, 1993).

Transduction and linkage analyses were performed using bacteriophage P1 as described by Miller (1992). β-Galactosidase activities were determined according to Miller's procedure (1992). Alkaline phosphatase activity was monitored as described earlier (Missiakas *et al.*, 1994).

Table II. Bacterial strains and plasmids

	Relevant characteristics	Reference or source
Strains		
CA8000	HfrH *thi*	Our collection
MC4100	F⁻ *araD*139 Δ(*argF-lac*) U169	Our collection
SR38	CA8000 *htrI*1386::Tn*5*Kan^r=*dsbD*1386::Tn*5*Kan^r	S.Raina
SR2609	CA8000 *dsbD*::ΩCm^r	This study
SR2447	CA8000 *dsbD*45::Tn*10*Kan^r	This study
SR2459	CA8000 *dsbD*49:Tn*10*Kan^r	This study
SR2502	CA8000 *dsbD*101::Tn*10*Kan^r	This study
SR1790	CA8000 *dsbA*101::Tn*10*Tet^r	Missiakas *et al.*, 1993
SR1753	CA8000 *dsbB*65::Tn*10* Tet^r	Missiakas *et al.*, 1993
SR1798	CA8000 *dsbC*58::Tn*10*Kan^r	Missiakas *et al.*, 1994
DM1586	CA8000 *dsbA*101::Tn*10*Tet^r*dsbD*::ΩCm^r	This study
DM1594	CA8000 *dsbB*65::Tn*10*Tet^r*dsbD*::ΩCm^r	This study
DM1596	CA8000 *dsbC*58::Tn*10*Kan^r*dsbD*::ΩCm^r	This study
DM1579	MC4100 *htrA-lacZ dsbD*::ΩCm^r	This study
DM1581	MC4100 P3*rpoH-lacZ dsbD*::ΩCm^r	This study
CG712	W3110 *galE relA groES*30 zid-1::Tn*10*	Fayet *et al.*, 1989
Plasmids		
pDM1463	pWSK30 (Wang and Kushner, 1991), a pSC101-based vector carrying the 3.8 kbp *Pst*I–*Sty*I DNA region including the *dsbD*$^+$ gene and ORF1 and ORF2 in orientation with the T7 promoter	This study
pDM1464	pWSK30 carrying the 2.8 kbp *Sma*I–*Sty*I DNA region including the *dsbD*$^+$ gene and ORF1 and ORF2	This study
pDM1498	pWSK30 carrying the 1.9 kbp DNA region *Sma*I–*Sty*I DNA region without the *Sac*I–*Bgl*II DNA segment (encoding ORF1 and ORF2)	This study
pDM1499	pWSK30 carrying the 2.1 kbp *Bst*XI–*Sty*I DNA region without the *Bst*EII–*Bgl*II DNA segment (encoding ORF2 only)	This study
pDM1500	pWSK30 carrying the 850 bp *Bgl*II–*Sty*I DNA region encoding only ORF2	This study
pDM1501	pWSK30 carrying the truncated ORF2 *Bgl*II–*Hpa*I DNA region	This study
pDM1467	pWSK30 carrying the complete operon (DsbD and ORF2) corresponding to the *Bst*XI–*Sty*I DNA region	This study
pDM1468	As pDM1467 but in high copy vector pKS (Stratagene)	This study
pDM1662	pET-22b (Novagen) carrying the 153 amino acid PDI-like domain fused to the *pelB* signal sequence and transcribed from the T7 promoter	This study
pDM1663	pET-22b carrying the 138 amino acid PDI-like subdomain fused to the *pelB* signal sequence	This study

Subcloning experiments, DNA sequence analysis and mapping of 5′ termini of the dsbD gene

DNA manipulations were performed as described by Sambrook *et al.* (1989). The *dsbD* gene was subcloned from the derivative cosmid clone pDM1440, which was able to recombine the *dsbD*::Tn*10* insertions and complement the phenotypes of such mutant bacteria. A 3.8 kbp *Pst*I–*Sty*I DNA fragment was first cloned (pDM1463) and shown to confer DTT resistance when carried on a low copy number vector (pWSK29/30 Amp^r, containing the T7 RNA polymerase-transcribed promoter; Wang and Kushner, 1991). Further subcloning resulted in construction of the plasmids described in Figure 1 and Table II. The 3.8 kbp *Pst*I–*Sty*I DNA fragment of pDM1463 and the recombinant plasmids carrying three of the *dsbD*::Tn*10* mutations and one *htrI*::Tn*5* mutation were sequenced using the US Biochemicals Sequenase kit, as described previously (Missiakas *et al.*, 1993).

Subcloning of the 17 and 15.4 kDa subdomains at the C-terminal end of the protein was achieved using vector pET-22b (Novagene, Amp^r), taking advantage of the presence of the PelB protein signal sequence, as well as the T7 promoter expression system. DNA fragments were amplified by the polymerase chain reaction method (Innis *et al.*, 1990) using the primers 5′-AACGCGGCTCCATGGGTATTGTGC-3′ and 5′-GCAAGTGTCGAATTCGGTTGGCGA-3′ or 5′-CATTGGTTACCATGGGCCCACTTC-3′ and 5′-GCAAGTGTCGAATTCGGTTGGCGA-3′ respectively for the 17 and 15.4 kDa domains.

Total cellular RNA was isolated using the hot SDS–phenol extraction procedure (Sambrook *et al.*, 1989). To define the transcriptional start site(s), ~10 ng of an oligonucleotide probe 5′-ATCTTCATGCCAGACGCCTTG-3′, which is complementary to nt positions 13–33 of the *dsbD* coding region, were annealed to 10 μg total cellular RNA. The annealed primer was extended by AMV reverse transcriptase, essentially as described (Raina *et al.*, 1995).

Protein overexpression, labeling and immunoprecipitation

Bacterial cultures were first grown in M9 minimal medium (Sambrook *et al.*, 1989) supplemented with 20 μg/ml each amino acid (except methionine) and 0.4% glucose. The *dsbD* gene and surrounding area were cloned under the T7 RNA polymerase promoter (Studier and Moffat, 1986) in vector pWSK29 or pET-22b (15.4 and 17 kDa domains) and protein expression was induced by the addition of 5 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Following a 30 min incubation, cells were treated with rifampicin (200 μg/ml) for another 20 min and then labeled with [^{35}S]methionine (50 μCi/ml) for 5 or 10 min.

The oxido-reduction states of DsbA and DsbC in *dsbD* null and isogenic wild-type bacteria were assessed by immunoprecipitation, using anti-DsbA and anti-DsbC antisera. Cultures of wild-type or *dsbD* mutant bacteria grown in M9 minimal medium as above were pulse-labeled with 100 μCi/ml [^{35}S]methionine for 2 min and chased with a 1000-fold excess of cold methionine. Further oxidation processes were quenched by dropping the pH and blocking the cysteinyl groups, as described by Pollitt and Zalkin (1983).

Purification and biochemical assays

The thioredoxin-like subdomain of DsbD was purified from bacterial cultures carrying plasmid pDM1663. Cultures were induced with 5 mM IPTG at an $OD_{595\ nm}$ of 0.2 for 2 h, collected and submitted to an osmotic shock (Neu and Heppel, 1965). The extract was loaded on Q-Sepharose using 0.05 M Tris–HCl, pH 7.2, 1 mM EDTA, 0.05 M KCl. The turbidimetric assay for the reduction of insulin was performed as described by Holmgren (1979). The concentration of DsbD subdomain was estimated by using a molar extinction coefficient at 278 nm of 14100/M/cm, as well as using a standard BioRad assay.

The Swiss-Prot accession number of DsbD is P36655.

Acknowledgements

We are grateful to T.Creighton and P.Model for helpful discussions, C.Georgopoulos for initial support for this work and careful reading of the manuscript and J.Cole and G.Georgiou for communicating results before publication. This work was supported by grant FN31-42429-94 from the Fond National Scientifique Suisse.

References

Bardwell,J.C.A. (1994) *Mol. Microbiol.*, **14**, 199–205.

Bardwell,J.C.A., McGovern,K. and Beckwith,J. (1991) *Cell*, **67**, 581–589.

Bardwell,J.C.A., Lee,J.-O., Jander,G., Martin,N., Belin,D. and Beckwith,J. (1993) *Proc. Natl Acad. Sci. USA*, **90**, 1038–1042.

Creighton,T.E. and Freedman,R.B. (1993) *Curr. Biol.*, **3**, 790–793.

Crooke,H. and Cole,J. (1995) *Mol. Microbiol.*, **15**, 1139–1150.

Dailey,F.E. and Berg,H.C. (1993) *Proc. Natl Acad. Sci. USA*, **90**, 1043–1047.

Fayet,O., Ziegelhoffer,T. and Georgopoulos,C. (1989) *J. Bacteriol.*, **171**, 1379–1385.

Fellay,R., Frey,J. and Krisch,H. (1987) *Gene*, **52**, 147–154.

Fong,S.-T., Camakaris,J. and Lee,B.T.O. (1995) *Mol. Microbiol.*, **15**, 1127–1137.

Freedman,R.B. (1989) *Cell*, **57**, 1069–1072.

Holmgren,A. (1979) *J. Biol. Chem.*, **254**, 9627–9632.

Innis,M.A., Gelfand,D.H., Sninsky,J.J. and White,T.J. (1990) *PCR Protocols: A Guide to Methods and Applications*. Academic Press, New York, NY.

Kamitani,S., Akiyama,Y. and Ito,K. (1992) *EMBO J.*, **11**, 57–62.

Kelley,R. F., Shalongo,W., Jagannadham,M.V. and Stellwagen,E. (1987) *Biochemistry*, **26**, 1406–1411.

Kohara,Y., Akiyama,K. and Isono,K. (1987) *Cell*, **50**, 495–508.

Koomey,J.M., Gill,R.E. and Falkow,S. (1982) *Proc. Natl Acad. Sci. USA*, **79**, 7881–7885.

Liu,J. and Walsh,C.T. (1990) *Proc. Natl Acad. Sci. USA*, **87**, 4028–4032.

Mecsas,J., Rouvière,P.E., Erickson,J.W., Donohue,T.J. and Gross,C.A. (1993) *Genes Dev.*, **7**, 2618–2628.

Miller,J. (1992) *A Short Course in Bacterial Genetics: A Laboratory Manual and Handbook for Escherichia coli and Related Bacteria*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Missiakas,D., Georgopoulos,C. and Raina,S. (1993) *Proc. Natl Acad. Sci. USA*, **90**, 7084–7088.

Missiakas,D., Georgopoulos,C. and Raina,S. (1994) *EMBO J.*, **13**, 2013–2020.

Mottl,H., Nieland,P., de Kort,G., Wierenga,J.J. and Keck,W. (1992) *J. Bacteriol.*, **174**, 3261–3269.

Neu,H.C. and Heppel,L.A. (1965) *J. Biol. Chem.*, **240**, 3685–3692.

Peek,J.A. and Taylor,R.K. (1992) *Proc. Natl Acad. Sci. USA*, **89**, 6210–6214.

Pollitt,S. and Zalkin,H. (1983) *J. Bacteriol.*, **153**, 27–32.

Raina,S. (1987) PhD Thesis, IARI, New Delhi, India.

Raina,S., Missiakas,D. and Georgopoulos,C. (1995) *EMBO J.*, **14**, 1043–1055.

Sambongi,Y. and Ferguson,S.J. (1994) *FEBS Lett.*, **353**, 235–238.

Sambrook,J., Fritsch,E.F. and Maniatis,T. (1989) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Shevchik,V.E., Condemine,G. and Robert-Baudouy,J. (1994) *EMBO J.*, **13**, 2007–2012.

Studier,F.W. and Moffatt,B.A. (1986) *J. Mol. Biol.*, **189**, 113–130.

Wang,R.F. and Kushner,S.R. (1991) *Gene*, **100**, 195–199.

Way,J.C., Davis,M.A., Morisato,D., Roberts,D.E. and Kleckner,N. (1984) *Gene*, **32**, 369–379.

Wunderlich,M., Otto,A., Seckler,R. and Glockshuber,R. (1993) *Biochemistry*, **32**, 12251–12256.

Yu,J., Webb,H. and Hirst,T.R. (1992) *Mol. Microbiol.*, **6**, 1949–1958.

Zapun,A. and Creighton,T.E. (1994) *Biochemistry*, **33**, 5202–5211.

Zapun,A., Bardwell,J.C.A. and Creighton,T.E. (1993) *Biochemistry*, **32**, 5083–5092.

Zapun,A., Missiakas,D., Raina,S. and Creighton,T.E. (1995) *Biochemistry*, **34**, 5075–5089.

Received on March 22, 1995; revised on April 28, 1995