

The role of Dsb proteins of Gram-negative bacteria in the process of pathogenesis

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Introduction

Cysteine in *de novo* synthesized proteins is present in a reduced state. Formation of disulfide bonds in extracytoplasmic proteins takes place during their folding in cellular compartments that are separated from the cytosol. In Gram-negative bacteria, this process occurs in the periplasm and involves proteins for which the periplasm is the target cell compartment as well as proteins that are anchored in cell membranes or secreted outside the cell. Misfolded proteins initially accumulate in the reduced form (contain thiol SH groups) and are, for the great majority, subsequently degraded by periplasmic proteases.

Disulfide bond formation in extracytoplasmic proteins, best characterized for *Escherichia coli* cells, is catalyzed by the Dsb protein system composed of numerous proteins localized in the periplasm and inner membrane (Raina & Missiakas, 1997). Genetic and biochemical studies performed in *E. coli* so far permitted identification and characterization of seven proteins of the Dsb system. Five of these proteins are engaged in two independent metabolic pathways – the oxidation pathway (DsbA and DsbB) and the isomerization/reduction (DsbC, DsbD and DsbG) pathway.

Abstract

Tertiary and quaternary structures of extracytoplasmic proteins containing more than one cysteine residue often require introduction of disulfide bonds. This process takes place in an oxidative environment, such as the periplasm of Gram-negative bacteria, and is catalyzed by Dsb (disulfide bond formation) proteins. Mutations in *dsb* genes influence the conformation and stability of many extracytoplasmic proteins. Thus, many pathogens become partially or fully attenuated due to improper folding of proteins that act as virulence factors. This review summarizes the current knowledge on Dsb proteins and their effect on the pathogenicity of Gram-negative bacteria. The potential application of Dsb proteins in biotechnology is also discussed.

DsbA is the main protein catalyzing disulfide bond formation. It is reoxidized by the DsbB protein, which is an integral component of the cytoplasmic membrane (Missiakas *et al.*, 1993) (Fig. 1). DsbC, DsbG and DsbD participate in isomerization of nonnative disulfides. Specifically, DsbC and DsbG promote the rearrangement of incorrect disulfide bonds, whereas the DsbD inner membrane protein transports electrons necessary for catalyzing the isomerization reaction from the cytoplasmic thioredoxin (Missiakas *et al.*, 1995; Stewart *et al.*, 1999; Chung *et al.*, 2000) (Fig. 2). The next Dsb protein, DsbE/CcmG, is involved in the cytochrome maturation process (Fabianek *et al.*, 1999; Stirnemann *et al.*, 2005) and the role of the last Dsb protein, DsbF, which was identified by genetic screening, still needs further elucidation (Metheringham *et al.*, 1996).

Virulence factors of pathogenic microorganisms are mainly secretion proteins, which are transported from the periplasm or cytoplasm to the cell outer membrane, to the medium or directly to the eukaryotic cell. Many of the virulence factors are posttranslationally modified via disulfide bond formation. Some of them are part of large protein complexes, structured by intramolecular disulfide bonds. Inactivation of genes encoding for the Dsb system in

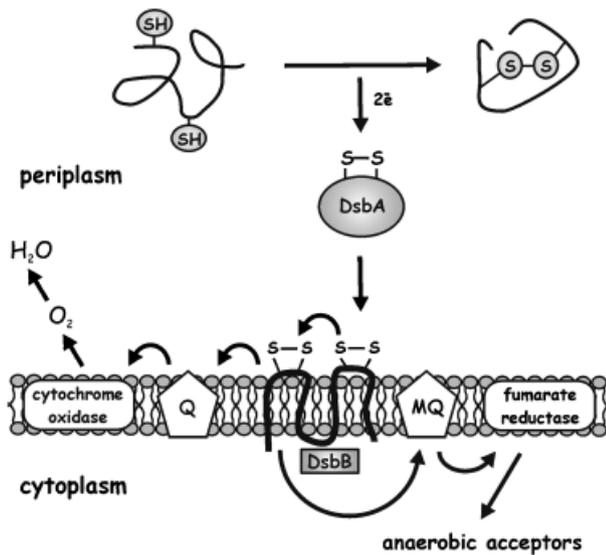


Fig. 1. The pathway of protein disulfide formation in *Escherichia coli*. The direction of electron flow is shown by black arrows. DsbA reacts with a newly translocated protein. Free thiols in this protein are oxidized to form a disulfide bond. After donating its disulfide bond to a target protein, DsbA is released in the reduced form. To start a new catalytic oxidation cycle, DsbA must be reoxidized. This reoxidation is accomplished by its partner enzyme DsbB, a quinone reductase. Electrons then flow from DsbB to ubiquinones and to terminal oxidases, such as cytochrome *bd* and *bo* oxidases. The terminal oxidases transfer the electrons to oxygen in reactions coupled to H^+ transfer and production of H_2O . Under anaerobic conditions, DsbB passes electrons from DsbA onto menaquinone (MQ), which is up-regulated upon oxygen depletion. Anaerobic oxidoreductases such as fumarate reductase serve to reoxidize menaquinone. This figure was reproduced from Messens & Collet (2006) with permission.

the cells of numerous pathogenic microbial species, e.g. *Vibrio cholerae*, *Pseudomonas aeruginosa*, *Salmonella enterica* or *Helicobacter pylori*, reduces their virulence (Peek & Taylor, 1992; Barbieri & Sun, 2004; Miki *et al.*, 2004; Godlewska *et al.*, 2006). Identification of direct substrate(s) of the Dsb system, which are responsible for the attenuation of the strain, is not easy. To recognize particular virulence factors, which are dependent on the activity of Dsb proteins, and to relate these data to a specific phenotype, a detailed analysis of the pathogen proteome for the presence of all Dsb system components and a wide knowledge of the pathogenesis process is needed. So far, global analysis of this kind was performed only for one pathogenic bacterium – *Salmonella enterica* sv. Typhimurium (Agudo *et al.*, 2004).

In recent years, several outstanding reviews have appeared which extensively describe the mechanism of the Dsb systems in *E. coli* (Collet & Bardwell, 2002; Kadokura, 2003; Nakamoto & Bardwell, 2004; Messens & Collet, 2006). In this study, we present a compendium of the current knowledge on the role of Dsb proteins in the virulence process of Gram-negative bacteria (Table 1).

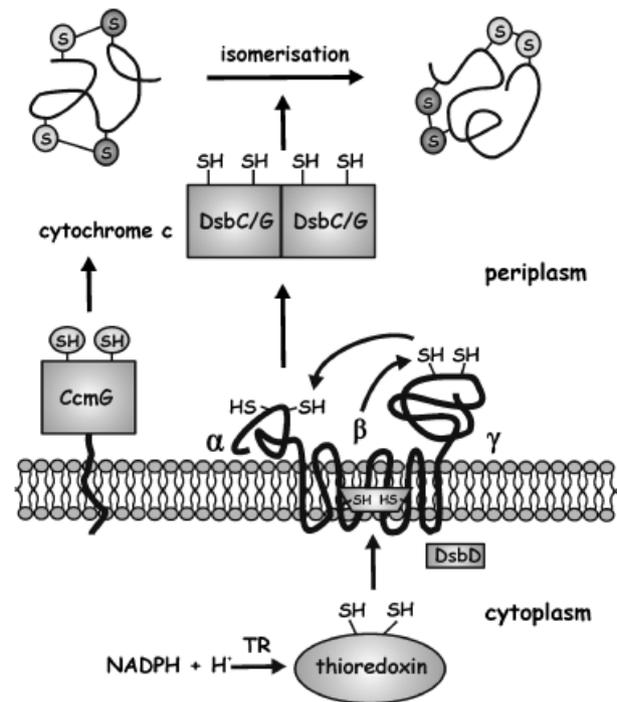


Fig. 2. The isomerization pathway in *Escherichia coli*. The direction of electron flow is indicated by the arrows. Disulfide-bond rearrangement is catalyzed by the thiol–disulfide oxidoreductases DsbC and DsbG, which are maintained in a reduced state by the membrane protein DsbD. DsbD is reduced by cytoplasmic thioredoxin, which is recycled by thioredoxin reductase (TR) in a NADPH-dependent manner. In DsbD, the electrons flow from the membranous (β domain) to the C-terminal domain (γ domain) and then to the N-terminal domain (α domain). This figure was reproduced from Messens & Collet (2006), with permission and modified by addition of CcmG protein, as described by Stirnimann *et al.* (2005).

Dissemination of Dsb proteins of oxidation pathway in proteomes of pathogenic bacteria

Most Gram-negative pathogenic bacteria have complete oxidation pathways, analogous to those in *E. coli*. *In silico* analysis of numerous microbial genomes conducted by Tinsley *et al.* showed that most species possess only one *dsbA* gene. Yet, genomes that encode several paralogous proteins (DsbA and SrgA of *S. enterica* sv. Typhimurium or DsbA 1, 2 and 3 of *Neisseria meningitidis*) (Bouwman *et al.*, 2003; Tinsley *et al.*, 2004) as well as genomes which lack the components of the oxidation pathway (*Helicobacter hepaticus*) (Godlewska *et al.*, 2006) were also identified. For *Campylobacter jejuni*, genome sequencing of several strains carried out in recent years revealed the presence of two genes encoding DsbA proteins. These observations raised a question of why some Gram-negative bacteria demand the activity of one, whereas others need the activity of several *dsbA* genes to ensure proper functioning of the cell (the

Table 1. Examples of the processes involved in pathogenesis affected by Dsb protein activity

Process/bacterial species	Target of Dsb system	Dsb protein involved*	Reference
Motility			
<i>Salmonella enterica</i>	FliC and FliB	DsbA	Agudo <i>et al.</i> (2004)
<i>Escherichia coli</i>	FlgI and FliC	DsbA	Hiniker & Bardwell (2004)
<i>Campylobacter jejuni</i>	Unknown	Dsbl and DsbB	Raczko <i>et al.</i> (2005)
Type III secretion system			
<i>Salmonella enterica</i>	SpiA, InvH?	DsbA	Miki <i>et al.</i> (2004), Altmeyer <i>et al.</i> (1993)
<i>Shigella flexneri</i>	Spa32	DsbA	Watarai <i>et al.</i> (1995), Yu <i>et al.</i> (2000)
<i>Yersinia pestis</i>	YscC	DsbA	Jackson & Plano (1999)
<i>Pseudomonas aeruginosa</i>	ExsA	DsbA	Ha <i>et al.</i> (2003)
Type IV secretion system			
<i>Bordetella pertussis</i>	One of the Ptl proteins	DsbC	Stenson & Weiss (2002)
Adhesion			
<i>Neisseria meningitidis</i>	Protein/s involved in pili biogenesis	DsbAs	Tinsley <i>et al.</i> (2004)
<i>Salmonella enterica</i>	PefA	SrgA and DsbA	Bouwman <i>et al.</i> (2003)
<i>Escherichia coli</i>	Bfp	DsbA	Zhang & Donnenberg (1996)
Toxins			
<i>Bordetella pertussis</i>	Subunit A and B of PTX toxin	DsbA	Stenson & Weiss (2002)
<i>Vibrio cholerae</i>	Subunit B of the CT toxin	DsbA	Yu <i>et al.</i> (1992)
<i>Escherichia coli</i>	Subunit A and B of the LT toxin	DsbA	Okamoto <i>et al.</i> (1998), Hardy & Hedges (1996)
Extracellular enzymes			
<i>Pseudomonas aeruginosa</i>	Elastase, lipase	DsbA	Braun <i>et al.</i> (2001), Urban <i>et al.</i> (2001)
<i>Erwinia chrysanthemi</i>	Pectinase, cellulase	DsbA	Shevchik <i>et al.</i> (1995)

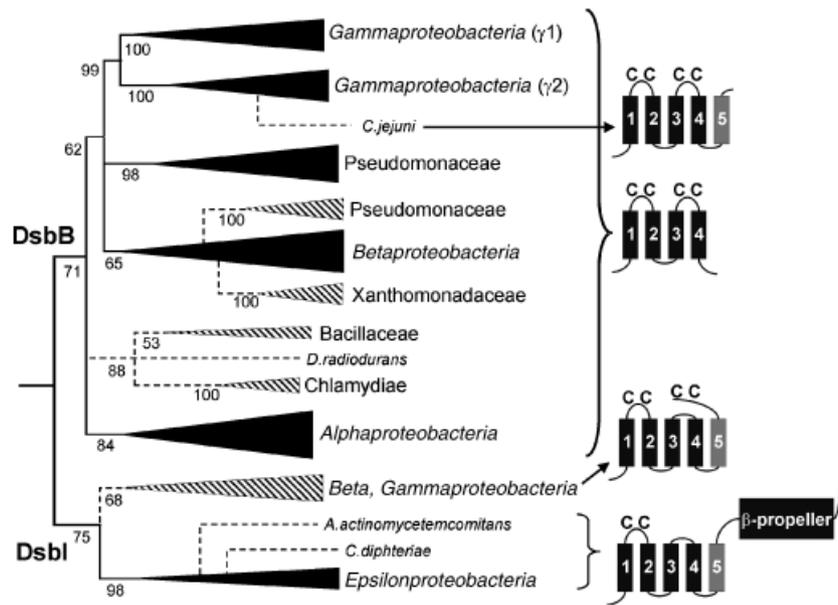
*The majority of experiments were performed on *dsbA*⁻ mutated cells. DsbC influence has been exclusively proven on the *Bordetella pertussis* PTX secretion system. One can speculate that the other type IV secretion systems, which play an important role in virulence of many bacterial species, are also affected by DsbC.

record holder is *Shewanella oneidensis*, containing four *dsbA* genes in the genome).

Multiplication of the DsbA protein does not increase the number of DsbB paralogs, as was observed for *Bacillus subtilis* cells, where every DsbA homolog is oxidized by a cognate DsbB protein (protein pairs BdbA-B and BdbD-C) (Bouwman *et al.*, 2003; Tinsley *et al.*, 2004; Moller & Hederstedt, 2006). DsbA paralogs usually demonstrate different substrate specificity; yet, in some cases they can complement each other or participate in the same cellular process. A fine example is the virulence plasmid-encoded SrgA protein of *S. enterica* sv. Typhimurium. The substrate for SrgA is PefA, the main protein subunit of the adhesion fimbriae. Mutation in the *srgA* gene is partially complemented by the product of the chromosome-localized *dsbA* gene (Bouwman *et al.*, 2003). Analysis of the virulence of the mutant strain *in vivo* showed that *srgA* inactivation does not cause attenuation of the strain; yet, this phenotype, characteristic for the chromosomal *dsbA* gene mutant of *Salmonella*, is significantly intensified in the double *srgA*⁻*dsbA*⁻ mutant. Further experiments led to the identification of a second substrate, common for both SrgA and DsbA, the SpiA protein (SsaC) (Miki *et al.*, 2004). The *spiA* gene is confined to the second pathogenicity island (SPI-2; *Salmonella* pathogenicity island 2) which encodes for activities responsible for pathogen survival in macrophages and for induction of the systemic mice infection (Ochman *et al.*,

1996; Hensel *et al.*, 1998). The SpiA protein is localized in the cell outer membrane, contains one sulfur bridge, and is a structural component of the type III secretion system (TTSS). Inactivation of the *spiA* gene and loss of the correct conformation of its product due to the absence of DsbA/SrgA decrease the TTSS effector protein transport efficiency and impair the SCV (*Salmonella* containing vacuole) biogenesis, which in effect lead to attenuation of the strain (Knodler & Steele-Mortimer, 2003). Reduction of virulence of *Salmonella* strains, which do not produce the DsbA protein, can also be due to the impairment of the second *Salmonella* TTSS system encoded by genes localized in the SPI-1 pathogenicity island (Miki *et al.*, 2004). SPI-1 genes control the internalization of pathogen into the epithelium cells of the gut (Galan, 2001). The *dsbA*⁻ mutants exhibit a reduced secretion level of TTSS effector SPI-1 proteins and lack the ability to induce changes in the actin cytoskeleton of eukaryotic cells, which significantly lowers the invasion capacity of the pathogen. So far, the component of the type III SPI-1 secretion machinery that at the same time could be the substrate for DsbA remains unidentified. One of the candidates is the *invH* gene product, containing one disulfide bridge. By interacting with InvG (SpiA homolog; does not contain cysteine residues), it facilitates pore formation in the outer membrane, through which effector proteins are transmitted to the cytosol of the host cell. Possibly, to obtain its native conformation, InvH demands additionally the

Fig. 3. Phylogenetic tree of the DsbB/DsbI family. Triangles indicate 'compressed' branches, with the names of taxons indicated. Hatched triangles and broken lines indicate subfamilies (or single genes) that underwent horizontal gene transfer. Numbers at the nodes indicate the bootstrap support in percentile values. Nodes with bootstrap support < 50% are regarded as unresolved and were grouped together with their sister lineages. The right panel illustrates the topology of members of each lineage, including the number of TM segments and position of periplasmic Cys residues. The periplasmic regions are shown at the top of the helical bundle, and the cytoplasmic regions are at the bottom. This figure was reproduced from Raczko *et al.* (2005).



DsbA or/and SrgA activity (Altmeyer *et al.*, 1993; Miki *et al.*, 2004).

DsbA paralogs can differ not only in substrate specificity, but also in cell localization. In *N. meningitidis*, genes encoding three DsbA proteins were identified. One of the products is similar to DsbA of *E. coli* and localizes in the periplasm, the two remaining proteins are lipoproteins anchored in the inner membrane. Products of the three *N. meningitidis* *dsbA* genes complement each other. Inactivation of one of them is compensated for by the activity of the other two and only inactivation of all three genes leads to the inhibition of cell growth at 37 °C. Yet, mutation of the two genes coding for the DsbA membrane lipoproteins increases the strain sensitivity towards reductive agents and results in the loss of functional adhesion structures – pili (Tinsley *et al.*, 2004). Elucidation of the mutual interactions between the three DsbA proteins and their substrates as well as reoxidation of their active forms by the same DsbB membrane protein demands further studies. One possible explanation is that not all DsbA types are reoxidized by DsbB, as the *dsbB*⁻ mutant phenotype does not correspond to the phenotype of the triple *N. meningitidis* *dsbA*⁻ 1, 2, 3 mutant (Tinsley *et al.*, 2004).

A database search performed in our laboratory led to the identification of over 100 proteins belonging to the DsbB/DsbI family. A great majority of the analyzed genomes were found to possess only one *dsbB* gene homolog; yet, some genomes contained several paralogs (*S. enterica* sv. Typhi, *C. jejuni* or *Pseudomonas putida*) (Kimball *et al.*, 2003; Raczko *et al.*, 2005). Figure 3 presents the phylogenetic tree of the DsbB/DsbI protein family made by multiple amino acid

sequence comparison and a scheme of potential tertiary structures of particular DsbB types. Primary and secondary structure comparison allowed seven DsbB subfamilies to be distinguished, which differ in the number of transmembrane helices and active disulfide bridges or in localization in the proteome. Apart from the classical DsbB proteins, recent studies allowed identification and initial characterization of another type of membrane-located disulfide oxidoreductase. Proteins from this group are encoded by the *dsbI* genes and are classified as DsbB paralogs (proteins that arose by duplication). The N-terminal domain of the DsbI protein contains five transmembrane helices and an active center containing a CXXC motif characteristic for all Dsb proteins. The DsbI C-terminal domain is most likely localized in the periplasm and adapts a β-propeller structure, which is absent in DsbB proteins (Raczko *et al.*, 2005). The *dsbI* gene was identified in the genomes of 13 microbial species (*C. jejuni*, *Campylobacter coli*, *Campylobacter lari*, *Campylobacter upsaliensis*, *Campylobacter curvus*, *Campylobacter concisus*, *Campylobacter fetus*, *H. pylori*, *Helicobacter acinonychis*, *Clostridium diphtheriae*, *Actinobacillus actinomycetemcomitans*, *Shewanella* sp. and *Thiomicrospira denitrificans*), nine of which represent *Epsilonproteobacteria*. All microbial species listed above, which contain the *dsbI* gene, except *Shewanella* sp. and *T. denitrificans*, are classified as human pathogens. Genes encoding 'shorter' versions of the DsbI protein were also identified. Representatives of this group of proteins, e.g. present in the proteomes of *Salmonella enterica* sv. Typhi and *Ralstonia solanacearum*, also contain five transmembrane helices, but do not possess the C-terminal β-propeller. These proteins, instead of the WD amino acid

'tail', carry a region characteristic for typical DsbB proteins which is absent in 'longer' DsbI, containing a second pair of cysteine residues after the fifth transmembrane helix (Raczko *et al.*, 2005).

Currently, genomic sequences of 35 strains from the *Epsilonproteobacteria* class, including 18 *Campylobacter* strains, are known or remain in progress. Exploration for DsbA, DsbB and DsbI orthologs in proteomes of these bacteria has shown a correlation in occurrence of DsbA and DsbB (Raczko *et al.*, 2005). The only exception is the absence of a DsbB ortholog in *C. lari* cells. Interestingly, the proteome of this bacteria contains an additional copy ('in-paralog') of the DsbI protein. Moreover, most genomes of *Campylobacter* strains, besides the 'classical' *dsbA*, possess a second gene which product is classified *in silico* to the DsbA family (Table 2). It is localized before the 5' end of the *dsbB* gene and encodes, depending on the strain, proteins of different length. The shorter (119 aa) protein, present in *C. jejuni* 11168, 84–25 and CF93.6 strains, exhibits homology to the C-terminal fragment of DsbA (Cj0872 and CJJ81176_0883), but does not contain an active center (CXXC) motif characteristic for Dsb proteins. Thus, it is conceivable that the protein does not participate in the formation of disulfide bonds. The second, longer, protein (usually 220 aa in length) was found in proteomes of other *Campylobacter* strains, e.g. the 81–176 strain (CJJ81176_0880). It contains the CXXC motif and its amino

acid sequence exhibits 52% identity (over the length of 191 aa) to 'classical' DsbA (CJJ81176_0883). Comparison of the nucleotide sequence of the 'longer' *dsbA*–CJJ81176_0880 and 'shorter' *dsbA*–cj0864 of the 81–176 strain, shows a deletion of 303 bp in the latter gene (A.M. Łasica & E.K. Jagusztyn-Krynicka, unpublished data).

Interestingly, genomes of some bacteria, e.g. *H. hepaticus*, *Wolinella succinogenes* and *T. denitrificans*, all lack the well-characterized genes of the oxidation pathway implied in disulfide bond formation in periplasmic space.

Role of Dsb protein in biogenesis of flagella and adhesion fimbriae

Bacterial mutants in *dsb* genes of the DsbA/DsbB oxidation pathway are nonmotile and lack functional flagella. The flagellum of Gram-negative bacteria is composed of three main elements: the basal body, hook and filament. The basal body is the most complex structure of the flagellum. It consists of several rings that act as bearings, which join the inner and outer cell membrane. Flagellum biogenesis occurs in several stages. It begins with the formation of the MS ring in the cytoplasmic membrane which is followed by the transport and assembly of flagellum parts. Formation of a functional motility organellum exerts the expression of over 50 genes that encode structural and regulatory proteins as well as proteins engaged in the motility and chemotaxis process. In Gram-negative bacteria, the structural proteins of the flagellum machinery are expressed in a hierarchical manner, according to the order in which they are joined to form a functional organellum. This process is regulated at both the transcription (affinity of RNAP with different sigma factors to promoter sequences and by two-component systems) and the posttranslational level (Aldridge & Hughes, 2002; Macnab, 2003).

Formation of the flagellum in *E. coli* depends on the FlgI protein, which contains one disulfide bridge and is a component of the P ring, localized in the periplasmic space. As anticipated, comparison of the periplasmic subproteome of *E. coli dsbA*[−] mutants and the wild type strain showed that FlgI is lacking among the periplasmic proteins in the Dsb system-deficient strain. Another component of the flagellum, the FliC flagellin, was also absent in the periplasmic subproteome of the *dsbA*[−] mutant. The FliC protein does not contain cysteine residues; yet, its integration into the organellum is most probably impossible due to the defects in the earlier biogenesis stages (Hiniker & Bardwell, 2004). In *S. enterica* sv. Typhi, FliC (flagellin) and FliP (component of the export apparatus; IM protein) were not detected among the periplasmic proteins of the *dsbA*[−] mutant, but were present in the subproteome of the wild-type strain. Both proteins lack cysteine residues. It is known that the

Table 2. Dissemination of DsbI, DsbB and DsbA proteins in *Campylobacter* proteomes

Strain	DsbI	DsbB	DsbA
<i>Campylobacter jejuni</i> ssp. <i>jejuni</i> NCTC 11168	+	+	+ ¹
<i>Campylobacter jejuni</i> RM1221	+	+	+ ²
<i>Campylobacter jejuni</i> ssp. <i>jejuni</i> 81–176	+	+	+ ²
<i>Campylobacter jejuni</i> ssp. <i>jejuni</i> 260.94*	+	+	+ ²
<i>Campylobacter jejuni</i> ssp. <i>jejuni</i> 81116* [†]	+	+	+ ²
<i>Campylobacter jejuni</i> ssp. <i>jejuni</i> 84–25*	+	+	+ ¹
<i>Campylobacter jejuni</i> ssp. <i>jejuni</i> HB93–13*	+	+	+ ²
<i>Campylobacter jejuni</i> ssp. <i>jejuni</i> CF93.6*	+	+	+ ¹
<i>Campylobacter jejuni</i> ssp. <i>doylei</i> 269.97*	+	+	+ ²
<i>Campylobacter coli</i> RM2228*	+	+	+
<i>Campylobacter lari</i> RM2100*	++	–	+ ²
<i>Campylobacter upsaliensis</i> RM3195*	+	+	+
<i>Campylobacter concisus</i> 13826*	+	+	+ ²
<i>Campylobacter curvus</i> 525.92*	+	+	+ ²
<i>Campylobacter fetus</i> ssp. <i>fetus</i> 82–40	+	+	+ ²

*Unfinished genomes (<http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi>).

DsbA designation:

[†]Personal communication B. Pearson, Institute of Food Research, Norwich, UK.

DsbA designation:

+ 'Classical' DsbA: homolog of Cj0872 *C. jejuni* NCTC 11168 or CJJ81176_0883 *C. jejuni* 81–176.

+¹ 'Shorter' DsbA: homolog of Cj0864 *C. jejuni* NCTC 11168.

+² 'Longer' DsbA: homolog of CJJ81176_0883 *C. jejuni* 81–176.

active FliP is necessary for the transport of FliC; but the mechanism by which the Dsb system influences FliP stipulates further studies (Agudo *et al.*, 2004).

In *C. jejuni*, mutations in the genes of the Dsb oxidation pathway (*dsbB* and *dsbI*) can also influence cell motility. Whereas the *dsbB*⁻ strain demonstrates similar motility as the *C. jejuni* wild-type 81–176 strain, the *dsbI*⁻ mutant exhibits an evidently elevated motility and the *dsbI dsbB* double mutant does not form flagella at all (Raczko *et al.*, 2005). The genome of *C. jejuni* NCTC 11168 contains over 50 genes localized in 32 loci, which were found to participate in formation of the bacterial flagellum (Parkhill *et al.*, 2000). As flagellin A of *C. jejuni* does not contain cysteine residues, most probably the Dsb proteins influence other components of the structure, transport or regulation of the flagella assembly process, as was observed for *E. coli* and *S. enterica* sv. Typhi proteomes (Agudo *et al.*, 2004; Hiniker & Bardwell, 2004).

Type IV fimbriae are a relevant virulence factor of many pathogenic microorganisms, which are responsible for the first stage of colonization (adhesion to eukaryotic cells) or biofilm formation. They also participate in the motility of microorganisms on solid medium (twitching motility). Biogenesis of type IV pili involves the activity of over 40 genes, the products of which are often homologous to proteins forming the type II secretion system (GSP – general secretory pathway) (Filloux, 2004). The disulfide bond in the C-terminal part of the protein plays a substantial role in the structure and function of piline. Lack or abnormal structure of the main unit forming the pili causes attenuation of such pathogenic strains as EPEC *E. coli* (BfpA) (Zhang & Donnenberg, 1996), *V. cholerae* (TcpA) (Peek & Taylor, 1992) or *N. meningitidis* (Tinsley *et al.*, 2004). Type IV fimbriae in *N. meningitidis* and *V. cholerae dsb* gene mutants are present on the cell surface, but are dysfunctional in the adhesion and colonization processes (Sun *et al.*, 1997; Tinsley *et al.*, 2004). This phenomenon is not related with a specific resistance of these structures to degradation, as fimbriae of enteropathogenic *E. coli* are formed by the so-called aggregation protein (BfpA; *bundlin*), which undergoes complete degradation in the *dsbA*⁻ mutant (Zhang & Donnenberg, 1996).

Involvement of Dsb proteins in secretion machineries

Pathogenic microorganisms with a damaged Dsb system exhibit a reduced level of virulence, which may be due to impairment of the extracytoplasmic protein transport machinery. So far, five protein transport systems were described for Gram-negative bacteria. Three of them – systems I, III and, in great part, IV – transport proteins directly from the cytoplasm to the medium or the cytosol of eukaryotic cells,

omitting the periplasmic space. All five secretion systems mentioned above mainly transport proteins responsible for the virulence of pathogenic microorganisms (Hueck, 1998; Henderson *et al.*, 2004; Christie *et al.*, 2005; Cianciotto, 2005; Troisfontaines & Cornelis, 2005).

The type III transport system operates in cells of many pathogenic bacteria of both animals and humans. It is composed of over 20 gene products, which participate in the transport of effector proteins to the cytosol of eukaryotic cells, where they modulate, in various ways, the metabolism of the infected cells. The phylogeny of the TTSS is controversial. Some members of the scientific community have claimed that TTSS arose by duplication and subsequent transformation of the genes involved in bacterial flagellum biogenesis machinery, whereas others have argued that these two systems are sister groups (Ghosh, 2004; He *et al.*, 2004; Pallen & Matzke, 2006). Disruption of specific components of this secretion machinery due to the impairment of the Dsb protein system was shown to lead to the avirulence of such species as *S. enterica* sv. Typhimurium, *Yersinia pestis*, *Shigella flexneri*, and many others. For *Salmonella*, the *dsbA*⁻ and *srgA*⁻ mutant cells are defective in TTSS structure proteins, SpiA and InvH. Absence of these proteins impairs the invasion process and survival of the pathogen in eukaryotic cells (as described above). In *Yersinia*, effector proteins transported by the type III secretion machinery (Yop proteins) block the process of phagocytosis and induce the apoptosis of macrophages, mainly by influencing the cytoskeletal changes in eukaryotic cells. The *Y. pestis* YscC secretin, which is a homolog of SpiA of *S. enterica*, contains four cysteine residues forming two disulfide bridges, and is responsible for the formation of ring-shaped multimeric complexes in the bacterial cell outer membrane. Point mutations in nucleotide triplets of the secretin gene that encode cysteine residues led to the inhibition of secretion of Yop proteins and to reduction of bacterial virulence (Hueck, 1998; Jackson & Plano, 1999).

The observed effect of TTSS impairment due to improper functioning of the Dsb system is dependent on the oxidoreductase substrate. In the *dsbA*⁻ mutant of *S. flexneri* the Spa32 protein (component of the Mxi-Spa secretion machinery) does not take on a proper conformation. As a result, Ipa effector proteins, which normally are active in eukaryotic cells, remain 'trapped' on the surface of the pathogen (Watarai *et al.*, 1995; Yu *et al.*, 2000). The Spa32 protein, homologous to InvJ of *Salmonella* and to YscP of *Yersinia*, controls the structure of the so-called molecular needle of the secretion syringe machinery, which connects the prokaryotic cell wall to the surface of the eukaryotic cell (He *et al.*, 2004). *Shigella* Ipa participates in invasion and cell-to-cell spread of the pathogen. During the first step of invasion, Ipa proteins induce endocytosis of the pathogen followed by the lysis of the endosome membrane and release

of bacteria to the cytosol, which then multiply and spread to neighboring cells (Nhieu *et al.*, 2005). It seems that the incorrect conformation of Spa32 does not affect *Shigella* internalization into the epithelial cells, but significantly decreases cell-to-cell spread of this pathogen (Yu *et al.*, 2000).

Dsb proteins also regulate secretion of the *Bordetella pertussis* PTX toxin, which is transported atypically in a two-step manner. The PTX toxin is formed by five subunit types (1 × S1, 1 × S2, 1 × S3, 2 × S4 and 1 × S5) differing in amino acid sequence and function. S1 (subunit A) has enzymatic activity and is translocated to the eukaryotic cell, whereas S2–S5 (B subunits) are responsible for receptor recognition (Locht, 1999). In the *B. pertussis dsbA*⁻ mutant, the A (S1) and B (B2) subunits undergo degradation (Stenson & Weiss, 2002). Individual subunits are translocated by Sec proteins through the cytoplasmic membrane to the periplasm where the mature toxin is assembled. The toxin possesses its own protein transport system across the outer membrane, comprising proteins homologous to proteins of the type IV secretion machinery. These proteins are encoded by a cluster of nine *ptl* genes (pertussis toxin liberation), cotranscribed with five genes encoding the toxin subunits (S1–S5) (Weiss *et al.*, 1993; Kotob *et al.*, 1995). Stenson & Weiss (2002) proved that the *dsbC* gene mutation leads to toxin transport inhibition, which, in contrast to mutation in the *dsbA* gene, does not influence the holotoxin assembly. The component of the secretion machinery, which at the same time could be the target of DsbC, has not yet been identified.

Synthesis of type P pili in uropathogenic *E. coli* is a good example of the influence of Dsb proteins on the activity of the type II secretion system. Pili are responsible for adhesion of the pathogen to the epithelium of the urinary tract. Biogenesis of type P pili demands expression of 11 genes (*papA–K*), encoding structural subunits of the pili, and accessory proteins that participate in their formation. Dsb proteins affect the periplasmic PapD chaperonin, which binds to the pili subunits and transports them to the PapC protein localized in the outer membrane. The tertiary structure of PapD contains one disulfide bond, which is catalyzed by the DsbA protein. In the absence of DsbA, which impairs its proper role, PapD adapts an incorrect conformation and becomes prone to proteolytic digestions by the DegP protease. This results in lack of adhesion structures and reduction of strain virulence (Jacob-Dubuisson *et al.*, 1994).

Influence of Dsb proteins on secreted virulence factors

Virulence factors transported by the type II and V secretion systems (autotransporters) are transiently present in the

periplasm. Many of the factors transported by the type II secretion system adapt an active conformation due to disulfide bridge formation, and are subsequently secreted outside the bacterial cell. Listed below are several examples of virulence factors that are targets of Dsb proteins.

Toxins of *V. cholerae* (CT), *E. coli* (LT) and *B. pertussis* (PTX) belong to the AB₅ toxin group. Maturation of these toxins occurs in the periplasm and is promoted by Dsb proteins. Cholera and the LT toxins are formed by five B subunits responsible for the recognition of eukaryotic cell receptors and one A subunit with enzymatic activity. The *V. cholerae dsbA*⁻ mutant is defective in proper folding of B subunits (each of them contains one disulfide bridge) (Yu *et al.*, 1992). Similarly, assembly of the mature, thermosensitive LT holotoxin of *E. coli* depends on the activity of the DsbA protein. Mutation in the *dsbA* gene causes instability in the A subunit and in consequence its degradation (Okamoto *et al.*, 1998). In contrast, the presence of two thiol groups instead of a disulfide bond in the B subunit leads to its stable association with cell membranes, which prevents correct assembly of the toxin (Hardy & Hedges, 1996).

Introduction of disulfide bonds is also essential for the virulence of *P. aeruginosa*. Mutants in the *dsbA*⁻ gene exhibit reduced colonization potential, as shown on the *Arabidopsis thaliana* plant model and animal models [mice – full-thickness skin thermal burn mouse model (Rahme *et al.*, 1997) and *Caenorhabditis elegans* – slow killing assay (Tan *et al.*, 1999)]. Reduced virulence is partially due to instability and degradation of such ectoenzymes as elastase or lipase, proteins transported by the type II secretion system and proteins that have disulfide bonds in their native structure. Elastase is synthesized as a preprotein the signal sequence of which is removed during transport across the cytoplasmic membrane. It contains two intermolecular disulfide bridges, which are introduced successively. Formation of the first disulfide bridge allows the protein to adapt a specific conformation necessary for its subsequent autocatalytic processing. Introduction of the second disulfide bond permits translocation of the protein across the outer membrane (Braun *et al.*, 2001; Urban *et al.*, 2001). An avirulent phenotype of the *P. aeruginosa dsbA*⁻ mutant is also associated with the deficiency in transcription and expression of the genes encoding TTSS effector proteins (ExoU and ExoT). ExoU is a phospholipase that modulates signal transduction. ExoT is a bifunctional protein, exhibiting ADP-ribosyltransferase activity, which activates proteins from the Rho family (Hueck, 1998; Barbieri & Sun, 2004; Sato & Frank, 2004; Sato *et al.*, 2005). The ExsA protein is the main regulator that influences expression of genes encoding these two proteins and shows 56% amino acid sequence identity with the *Yersinia* VirF protein (AraC family of transcription regulators) (Hueck, 1998). Ha *et al.*

(2003) have proved that the *exsA* gene in the *dsbA*⁻ mutant of *P. aeruginosa* is not expressed, which results in inhibition of ExsA-dependent gene expression (e.g. ExoU and ExoT effector proteins). Authors speculate that DsbA is responsible for maintaining proper conformation of an unknown periplasmic sensor protein, which regulates the *exsA* gene expression in response to environmental factors (Ha *et al.*, 2003).

Influence of DsbA protein on the stability of virulence factors was observed also for *Erwinia* plant pathogens. *Erwinia chrysanthemi*, classified in the *Enterobacteriaceae*, is a commonly present pathogen of many plant species, causing soft-rot disease. Proteins responsible for the host-pathogen interaction as well as ectoenzymes that degrade structural components of plant cells were shown to be involved in the virulence of this pathogen (Yang *et al.*, 2002). Shevchik *et al.* (1995) proved that pectinases as well as the EGZ cellulase, which are responsible for cellular wall degradation, are prone to quick proteolysis in *dsbA*⁻ mutant cells.

Autotransporters, termed by some authors as the type V transport system, constitute a numerous extracellular protein group, consisting of mainly virulence factors, and do not demand accessory protein activity to pass from the periplasm to the cell surface. They contain only few cysteine residues, which are usually localized closely together. Dsb proteins do not affect the activity of autotransporters (Jacob-Dubuisson *et al.*, 2004). The only known autotransporter protein which contains a disulfide bridge is the *Shigella* IcsA protein (an autotransporter localized at the cell pole). It is involved in motility of the pathogen in the host cell cytoplasm by inducing actin polymerization (Gouin *et al.*, 2005). Yet, DsbA activity is not necessary for the formation of a disulfide bond in this protein (Brandon & Goldberg, 2001). Hence, this may imply that the disulfide bond is formed with the assistance of host cell oxidoreductases after IcsA is exported to the cell surface of the pathogen.

Application of Dsb proteins in biotechnology

The first papers describing possible applications of Dsb proteins in biotechnology appeared in the early 1990s after the mechanism of disulfide bond formation in bacterial cells was described. It was demonstrated that many crucial, from the medical aspect, eukaryotic proteins can be produced in prokaryotic expression systems only after overproduction of certain Dsb proteins. To obtain considerable amounts of protein, not only high gene transcription and resulting mRNA translation levels must be assured but gene products must also undergo proper folding and adapt a correct

tertiary structure which is decisive for its biological activity. Proper conformation of many eukaryotic proteins is dependent on disulfide bonds. Joly *et al.* (1998) proved that overexpression of *dsbA* and *dsbC* genes increases twofold the level the insulin-like growth factor I product (IGF-I) in *E. coli* cells. This 70-amino acid hormone protein is a homolog of proinsulin and contains six cysteines which form three disulfide bonds necessary for its activity (Hua *et al.*, 1996). Qiu *et al.* (1998) were successful in obtaining a high level of the human tissue-type plasminogen activator (tPa) in *E. coli* periplasm only when the DsbC protein was overexpressed simultaneously. The tPa protein contains 17 S-S bonds and catalyzes the proteolytic activity of the plasminogen (conversion into plasmin). Periplasmic oxidoreductases of *E. coli* also influence the level of the human nerve growth factor (NGF) β -NGF in the periplasm. NGF, which contains three disulfide bonds, was efficiently produced in its biologically active form only when all main proteins of the Dsb system were overproduced (A-D) (Kurokawa *et al.*, 2001). Many other laboratories obtained similar results documenting the influence of overproduction of Dsb proteins (mainly DsbC) on the solubility and level of heterologous proteins expressed in the periplasm of *E. coli*, e.g. the horseradish peroxidase (four disulfide bonds), mouse urokinase (12 disulfide bonds) or antibodies of the scFv type (single-chain Fv antibodies) (Kondo *et al.*, 2000; Bessette *et al.*, 2001; Zhang *et al.*, 2002). However, not all proteins which contain disulfide bonds were found to require the active DsbC for adapting a proper conformation. This feature seems to be correlated with the position of disulfide bridges. In DsbC-independent proteins, disulfide bonds are formed between two cysteines located consecutively in the primary structure of the protein and are not separated by other disulfide bonds. Studies were performed on the AppA protein (acid phosphatase; phytase) which contains four disulfide bonds, one of which is formed by cysteines localized in positions 155 and 430 of the protein, separated by two other disulfide bridges (Berkmen *et al.*, 2005). Transport across the cytoplasmic membrane also influences proper conformation of recombinant proteins. Substitution of the Sec (secretion) sequence by the twin arginine translocation (TAT) signal sequence made it possible to obtain native tPa and vtPa proteins (shorter forms of tPa with nine S-S bonds) even without DsbC overproduction (Kim *et al.*, 2005).

Moreover, *in vitro* attempts were made to produce recombinant eukaryotic proteins containing numerous disulfide bonds [vtPa, granulocyte-macrophage colony-stimulating factor (GM-CSF)]. To block the SH groups and enable S-S bond formation, oxidative conditions were generated by adding iodine acetamide, specific glutathione-containing buffers and DsbC to the native protein extracts (Yang *et al.*, 2004; Yin & Swartz, 2004).

Concluding remarks

Mutations in *dsb* genes have an overwhelmingly wide and diverse influence on the virulence of Gram-negative pathogens. The most crucial role is performed by components of the DsbA/DsbB oxidation pathway; yet, isomerization of disulfide bonds, catalyzed by DsbC/DsbD, can also play a key function in the proper folding of the proteins essential in pathogenesis. Loss of stability and function due to the lack of disulfide bridges affects both proteins participating in host-pathogen interactions, virulence factors secreted into the medium as well as proteins, which are the structural components of secretion machineries responsible for the translocation of virulence factors across membranes. Indirect influence of Dsb proteins on the activity of regulatory factors was also noted. Elucidation of the interaction networks of Dsb protein in other species than *E. coli* may contribute to the development of new attenuated recombinant strains of such pathogenic bacteria as *Salmonella*, *Shigella* and *Listeria*, which could potentially serve in the prophylaxis of infectious diseases. Many experiments showed that enteropathogenic bacterial strains attenuated at a certain level can serve as effective vaccines not only against diseases they induce but also as vaccine carriers of other antigens or DNA (Xu & Ulmer, 2003; Schoen *et al.*, 2004).

In recent years, the tertiary structure of several Dsb proteins was solved and this probably will initiate studies on their potential application as targets of antibacterial drug activities (Couprie *et al.*, 2000; McCarthy *et al.*, 2000; Stirnimann *et al.*, 2005). The difference between the structure of Dsb proteins of pathogens and bacteria that are part of physiological flora, still needs to be fully understood. In addition, as discussed in the last chapter of the review, bacterial cells overproducing Dsb proteins (mainly DsbC) are successfully used to obtain properly folded eukaryotic proteins, which in turn find application in the therapy of various diseases.

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