Research Signpost
37/661 (2), Fort P.O., Trivandrum-695 023, Kerala, India

Recent Res. Devel. Microbiology, 9(2005): 237-269 ISBN: 81-7736-243-7

10 Thiol disulfide oxidoreductases in bacteria

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1. Protein production

Proteins form the key building blocks of living cells and are involved in all processes of life. The ability to produce proteins in a functional fashion forms an important prerequisite for many medical, industrial, and economical applications. Proteins nowadays find their way in a diverse range of applications varying from sophisticated therapeutics to nanoscale 'machines'. Proteins themselves can be the consumer product, but often they are used as catalysts to produce certain end products. In this respect one can think of the use of enzymes for

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the synthesis of fine chemicals and pharmaceuticals, which often display chirality. As enzymes are well equipped to catalyze reactions in an enantioselective manner one of the two stereo isomers can be made exclusively and as such, the generation of unwanted side products can be prevented. An example is given by lipases, which can catalyze both the hydrolysis and the synthesis of long-chain acylglycerols in an enantioselective fashion [1]. Studies are therefore also undertaken to improve these enzymes, such as the directed evolution of lipase A by using phage display [2].

Short peptides can be chemically synthesized, but when it comes to larger proteins, chemical synthesis is not favourable, because of the many unwanted side products that are being generated during the production process. Cell-based systems form a good alternative for the chemical production. Examples of such cell factories are the micro-organisms *Escherichia coli*, *Bacillus subtilis*, and *Bacillus brevis* (Table I).

Bacterial systems are especially interesting as these, compared to eukaryotic cells, are easy to cultivate in inexpensive media, and they can produce large amounts of protein both intracellular as well as extracellular. In recent years much knowledge has been gained about the secretion machinery and other components that are involved in the production of (heterologous) proteins. However, bottlenecks still exist. One of these bottlenecks is encountered when trying to produce heterologous proteins that contain one or more disulfide bonds. Many proteins that are of interest for the medical and pharmaceutical industry are dependent on the formation of specific disulfide bonds for their function and stability. Well known examples include proinsulin [3], brain-derived neurotrophic factor [4], insulin-like growth factor [5], nerve growth factor [6], lipases [7], and antibody fragments [8-10].

About fifteen years ago there was a general believe that formation of disulfide bonds within proteins produced by prokaryotes was a process that occurred spontaneously. Indeed, there are some examples like CrtJ in *Rhodobacter capsulates* [11], where a disulfide bond is correctly formed when it is exposed to oxygen. Also it has been reported that disulfide bonds can be generated during secretion *via* the periplasmic-independent type I secretion system [12] without involvement of enzymes. Nowadays, the so-called thiol-disulfide oxidoreductases are known to be indispensable for the formation and isomerization of disulfide bonds [13-17]. In this review, this family of disulfide bond forming enzymes will be discussed. Special emphasis will be focused on their role in the biogenesis of components of the bacterial cell, but also the effect on folding of some eukaryotic proteins will be highlighted.

Table I. Examples of micro-organisms and their applications as cell factories.

Organism	Product	References
Escherichia coli	single-chain Fv antibodies	[8,237-239]
Escherichia coli	human leptin	[240]
Escherichia coli	bovine enterokinase subunits	[241]
Escherichia coli	bivalent humanized antibody fragments	[242]
Escherichia coli	chimeric antibody fragments	[243]
Escherichia coli	mini-antibody fragments	[244]
Escherichia coli	proinsulin	[245]
Escherichia coli	brain-derived neurotrophic factor	[4]
Escherichia coli	insulin-like growth factor	[5]
Escherichia coli	nerve growth factor	[6]
Escherichia coli	lipases	[7]
Bacillus subtilis	proteases	[183,246,247]
Bacillus subtilis	cyclomaltodextrin glucanotransferase	[248]
Bacillus subtilis	insecticidal toxins	[249]
Bacillus subtilis	single-chain antibodies	[9,10]
Bacillus subtilis	poly-gamma-glutamate	[250]
Bacillus subtilis	staphylokinase	[251]
Bacillus brevis	single-chain antibodies	[156]

2. Thiol-disulfide oxidoreductases in *E. coli*

The thiol-disulfide oxidoreductase family of *E. coli* belongs to the best studied among all organisms. *E. coli* has six known thiol-disulfide oxidoreductases that act in the periplasm outside the reducing environment of the cytoplasm: DsbA, DsbB, DsbC, DsbD, DsbE, and DsbG [15,16,18]. The cytoplasm of *E. coli* is kept reduced by the thioredoxin and the glutaredoxin systems [19-24]. The thioredoxin system is made up of thioredoxin 1 (TrxA),

thioredoxin 2 (TrxC), and thioredoxin reductase (TrxB), whereas the glutaredoxins GrxA, GrxB, and GrxC constitute the glutaredox system together with the glutathione synthethases GshA and GshB, and the glutathione reductase (GorA).

Despite the reducing environment of the cytoplasm, there are some cytosolic proteins that can contain disulfide bonds. Examples are Hsp33, OxyR, and ribonucleotide reductase. In the latter protein, that synthesizes the building blocks for DNA, a disulfide bridge is formed during the catalytic cycle. The active site disulfide bond is subsequently reduced again by the action of thioredoxin [25-27]. Hsp33 is a cytoplasmically localized chaperone protein with highly reactive cysteines that respond quickly to oxidizing conditions, which leads to the activation of its chaperone function [28]. Like Hsp33, OxyR is a protein that is activated by the formation of a disulfide bond and as such acts as a sensor for the redoxbalance inside the cell. As soon as this intramolecular disulfide bond is generated, OxyR activates the

Table II. CxxC motifs in typical thiol-disulfide oxidoreductases from *E. coli,B. subtilis,* and *Saccharomyces cerevisiae.*

Organism	Enzyme	CxxC	Comments
E. coli	TrxA	CGPC	Thioredoxin 1
E. coli	TrxB	CATC	Thioredoxin reductase
E. coli	TrxC	CGRC	Thioredoxin 2
E. coli	GrxA	$F(X_4)CPYC$	Glutaredoxin A
E. coli	GrxB	CPYX	Glutaredoxin B
E. coli	GrxC	CPYC	Glutaredoxin C
E. coli	DsbA (PpfA)	CPHC	Thiol-disulfide oxidoreductase, Oxidase
E. coli	DsbB	CVLC	Thiol-disulfide oxidoreductase, DsbA Oxidase
E. coli	DsbC (XprA)	$F(X_4)CGYC$	Thiol-disulfide oxidoreductase, Isomerase
E. coli	DsbD	CPSC/CVAC	Thiol-disulfide oxidoreductase, DsbC Reductase
	(CutA2/DipZ)		
E. coli	DsbE	CPWC	Thiol-disulfide oxidoreductase, Cytochrome
	(CycY/CcmG)		Reductase
E. coli	DsbG	CPYC	Thiol-disulfide oxidoreductase, Isomerase
B. subtilis	TrxA	$F(X_4)CGPC$	Thioredoxin
B. subtilis	TrxB	CAVC	Thioredoxin reductase

Table II. Continued

B. subtilis	BdbA (YolI)	CPPC	Thiol-disulfide oxidoreductase, unknown function
B. subtilis	BdbB (YolK)	CVLC	Thiol-disulfide oxidoreductase, Sublancin 168
B. subtilis	BdbC (YvgU)	CELC	Thiol-disulfide oxidoreductase, Competence/Sublancin 168
B. subtilis	BdbD (YvgV)	$F(X_4)CPSC$	Thiol-disulfide oxidoreductase, Competence
B. subtilis	YneN	$F(X_4)CKPC$	Thiol-disulfide oxidoreductase, unknown function
B. subtilis	YkvV	$F(X_4)CPPC$	Thiol-disulfide oxidoreductase, Sporulation
B. subtilis	ResA	$F(X_4)CEPC$	Thiol-disulfide oxidoreductase, Sporulation
B. subtilis	YolJ	CLNS	Putative thiol-disulfide oxidoreductase, Sublancin 168
B. subtilis	YveR	CLES	Putative thiol-disulfide oxidoreductase, Unknown Function
S. cerevisiae	PDI	CGHC	Protein Disulfide Isomerase
S. cerevisiae	Eug1p	C(L/I)HS	Protein Disulfide Isomerase Homologue

transcription of genes of proteins that counteract the oxidative stress, such as glutaredoxin I (*grxA*) and glutathione reductase (*gorA*) [29]. Subsequently, the cytoplasm starts to become more reduced again, eventually leading to the reduction of the disulfide bond in OxyR, which is thereby inactivated [30-32]. The OxyR regulon also involves *katG*, specifying a hydroperoxidase [33], *ahpCF*, specifying an alkylhydroperoxide reductase, and *dps*, coding for a DNA protection protein. In contrast to the cytoplasm, the periplasm is very oxidizing and it is this cellular compartment where the thiol-disulfide oxidoreductases DsbA, DsbB, DsbC, DsbD, DsbE, and DsbG of *E. coli* display their action. These enzymes are characterized by a thioredoxin-like fold and by the presence of a CxxC motif (Table II). In the next paragraphs the thiol-disulfide oxidoreductases of *E. coli* will be discussed in more detail.

2.1 DsbA

The thiol-**d**i**s**ulfide **b**ond oxidoreductase DsbA was discovered in 1991 by Bardwell *et al.* They found that *dsbA* (earlier called *ppfA*) mutant cells still exported β-lactamase, alkaline phosphatase, and the outer membrane protein OmpA, but that these proteins largely lacked disulfide bonds [34,35]. With about 850 molecules per cell [36] the 21 kDa DsbA protein was found to be the major oxidase in the periplasm and important for several cellular functions

like motility [37], DTT-resistance [38], production of the heat-stable enterotoxin II [39], benzylpenicillin-resistance [38], and formate-dependent nitrite reduction under anaerobic conditions [40]. The active site CxxC motif of DsbA forms a very reactive disulfide bond that is a 1000 times more reactive to thiol groups than when it would be normally present in a small peptide [41]. The N-terminal, accessible Cys30 of the Cys30-Pro-His-Cys33 motif has a pK_a of about 3, which explains that it is predominantly present as a thiolate anion at physiological pH [42-45]. Furthermore, the DsbA protein with a disulfide bond is far more unstable then its reduced counterpart [46-48]. This makes DsbA very suitable to donate its disulfide bond to other proteins. In fact DsbA is one of the most oxidizing proteins known to exist.

The amino acids that are present in between the two cysteines are very important for the oxidative properties of DsbA and especially the His32 residue is structurally essential, because it stabilizes the thiolate anion of Cys30 and in this way it promotes reduction of DsbA and consequently the transfer of its disulfide bond [49-51].

In 1993 the crystal structure of DsbA was solved and the 3D-structure was found to resemble that of thioredoxin, despite a very low level of amino acid sequence similarity between these two proteins [52]. The covered active site of DsbA is surrounded by grooves and exposed hydrophobic side chains and makes clear why DsbA binds preferentially to polypeptides explaining why it acts on substrate proteins and far less on small thiol compounds [53,54].

During its action, DsbA forms a so-called mixed disulfide with the substrate protein [46,53,55,56]. This covalent intermediate between the N-terminal Cys30 of DsbA and a cysteine of the substrate then dissolves very rapidly with either a disulfide bond in the substrate or with DsbA returning to its oxidized state. When the disulfide bond is transferred to the substrate, DsbA stays behind in its reduced conformation. Subsequently, DsbB reoxidizes DsbA to make it available for a next round of disulfide bond formation.

2.2 DsbB

In 1993, two years after the identification of DsbA a second thiol-disulfide oxidoreductase was identified, which was named DsbB [38,57]. The proposal that the 20 kDa DsbB protein might be responsible for the reoxidation of DsbA was supported by the observation that *dsbB* mutants are unable to form the L and P rings of the flagellar basal body structure (The P-ring protein FlgI contains a disulfide bond). DsbB was found to be an inner membrane protein containing four transmembrane helices with the N- and C-termini located in the cytoplasm. Both periplasmic domains contain two cysteines that are important for the function of DsbB in protein disulfide bond formation [58]. The cysteines in the first N-terminal periplasmic domain (Cys41 and Cys44)

are located in the typical CxxC configuration, whereas the cysteines in the second periplasmic domain (Cys104 and Cys130) are separated by 25 residues. Guilhot *et al.* showed that Cys104 is critical for the formation of a complex between DsbA and DsbB. A bond is first formed between Cys104 of DsbB and Cys30 of DsbA, which then is passed on to Cys30 and Cys33 of DsbA, in this way leading to the reoxidized form of the latter protein [59-62].

Under aerobic conditions, DsbB is present in an oxidized state with two intramolecular disulfide bonds present between Cys41 and Cys44 and between Cys104 and Cys130 [63,64]. The latter disulfide bond is only present when the first disulfide bond between Cys41 and Cys44 is formed. The Cys41-Xaa-Xaa-Cys44 motif is so strongly oxidized that it is resistant against the reducing agent dithiothreitol (DTT) [65]. Two kinds of quinines, ubiquinone and menaquinone, are important for DsbB to stay oxidized and to perform its catalytic activity [66,67]. Under aerobic growth conditions, reduced ubiquinone is reoxidized by cytochrome bo oxidase or cytochrome bd oxidase, which transfer electrons to molecular oxygen [68], whereas under anaerobic conditions the final electron acceptors are formed by molecules like fumarate or nitrite [66,69]. Ala-substitutions of the four amino acid residues on the C-terminal side of the Cys41-Xaa-Xaa-Cys44 motif, showed that the Arg48Ala mutant had a decreased DsbA oxidizing activity. However, all mutants were still DTT resistant and although Arg48 may be important, none of the four residues was found to be crucial for the coupling between the respiratory chain and DsbB [65].

The general believe is that the quinones oxidize the Cys41-Xaa-Xaa-Cys44 motif in the first periplasmic domain. The disulfide bond between Cys104 and Cys130 is then formed by the action of Cys41-Xaa-Xaa-Cys44 and can subsequently be used to oxidize DsbA [70]. This model was questioned to some extent by Regeimbal and Bardwell (2002), who found that the redox potentials of the two disulfides in DsbB are considerably less oxidizing than the redox potential of the disulfide of DsbA. In fact, *in vitro* studies showed that DsbA can form a disulfide bond between Cys104 and Cys130 of DsbB. Next to that, they showed that DsbA is oxidized in strains that express a mutant DsbB, containing only one pair of cysteines or no cysteine pairs at all [71]. In this view it would seem unlikely that DsbB functions as the oxidizing protein of DsbA, but it does not explain the earlier findings in which *dsbB* mutants showed phenotypes comparable to *dsbA* mutants. The solution to this paradoxical situation was recently reported by Grauschopf *et al.* They found that, indeed, the Cys104-Cys130 disulfide was less oxidizing than that of DsbA. However, the Cys41-Xaa-Xaa-Cys44 motif in DsbB, that is only accessible to ubiquinone and not to DsbA, has a redox potential of –69 mV (compared to –120 mV of the DsbA CxxC motif) and therefore forms the most oxidizing protein disulfide bond of the thiol-disulfide

oxidoreductases that has been reported up to this moment. It is this redox potential of Cys41-Xaa-Xaa-Cys44 in the first periplasmic domain of DsbB that drives the oxidation of DsbA *via* the disulfide bond in the second periplasmic domain of DsbB, which is immediately oxidized again after it has been reduced [72].

2.3 DsbC

The DsbC (XprA) protein was first characterized by Missiakas *et al*. in 1994. They reported that this 23.3 kDa periplasmic protein is essential for DTT-resistance and that in its absence a variety of reduced disulfide bond-containing periplasmic proteins accumulate in the periplasm [73]. Comparison of DsbC from *Erwinia chrysanthemi* and DsbC of *E. coli* that both contain the active site FxxxxCxxC motif, led already to the assumption that these proteins might belong to a new subfamily of disulfide isomerases [74]. The DsbC protein is able to rearrange incorrectly formed disulfide bonds [75,76]. DsbC of *E. coli* is a stable V-shaped dimer of identical subunits, with each subunit containing an N-terminal dimerization domain (residues 1-65) and a C-terminal thioredoxin domain (residues 78-216) with a Cys98-Gly-Tyr-Cys101 motif and two cysteine residues at positions 141 and 163. Only Cys98 is accessible and Cys141 and Cys163 form a structural, buried disulfide bond [76,77].

DipZ (later called DsbD), a cytoplasmic membrane protein with a thioredoxinlike fold, was found to be important together with thioredoxin to guarantee the disulfide bond isomerase activity of DsbC [78,79]. Production of the horseradish peroxidase isozyme C, which contains complex disulfide bonds and tends to aggregate in *E. coli*, was substantially increased by overexpression of DsbC [80] as was the production of the Ragi bifunctional inhibitor, a 13.1 kDa protein with five overlapping disulfide bonds [81].

In contrast to the CxxC motif in DsbB, which is always oxidized *in vivo*, the CxxC motif of DsbC is reduced at all times in the steady state. Reduction of the CxxC motif is crucial for the isomerase activity of DsbC. For maintaining this reduced state DsbD is essential [82]. DsbD transfers the electrons from thioredoxin across the membrane to DsbC, while thioredoxin is reduced again by thioredoxin reductase [19,83]. The amino acids that make up the center of the CxxC motif are crucial for the isomerase activity of DsbC. By changing amino acid residues within the CxxC motif, Bessette *et al*. were able to improve production of murine urokinase (containing 12 disulfides) and a truncated form of human tissue plasminogen activator (containing 9 disulfides) [84,85].

Chen *et al*. showed that DsbC is not only an isomerase, but also a chaperone. They demonstrated that DsbC promotes the *in vitro* reactivation of D-glyceraldehyde-3-phosphate dehydrogenase and that it suppresses its aggregation, even when Cys98 of the Cys98-Gly-Tyr-Cys101 active site is

carboxymethylated [86]. This was also shown by Liu and Wang, who confirmed the importance of the disulfide bond between Cys141 and Cys163 for this chaperone activity [87]. They found that this disulfide bond is not only important for the folding of DsbC, but also contributes to a large uncharged surface of the V-shape cleft formed by the two complexed DsbC monomers [77]. Lack of this disulfide bond leads to the formation of aggregates of these mutant DsbC proteins in the periplasm [87].

Of all Dsb proteins known so far in *E. coli*, DsbC shows the highest homology to the protein disulfide isomerase (PDI) that is found in eukaryotes [73,74,76,86,88]. Like DsbC, PDI is a dual function protein in displaying isomerase activity as well as chaperone functions [89-91].

2.4 DsbD

In 1995, the search for mutations that could compensate for the *dsbA* mutation led to the discovery of a new gene, which was designated *dsbD* [92]. The *dsbD* gene was earlier identified as CutA2, and implicated in copper homeostasis [93]. Around the same time DsbD was recognized as DipZ, a protein found to be important for the biogenesis of c-type cytochromes and for formate-dependent nitrite reduction (Nrf activity [94]). Cytochromes are proteins that contain heme as a prosthetic group and function in electron transfer [95]. In the biogenesis of c-type cytochromes, DsbD is involved in the attachment of heme to the correct pairs of cysteine residues of apocytochrome c molecules [40,94,96]. Furthermore, the DsbD protein was found to be essential for bacterial growth at temperatures above 42 °C [92].

The DsbD protein is made up from an N-terminal part with eight transmembrane domains and a C-terminal hydrophilic, periplasmic domain with a protein disulfide isomerase-like motif [97-99]. Next to its role in copper homeostasis and c-type cytochrome synthesis, DsbD was found to be needed for the periplasmic DsbC and DsbG proteins to display their isomerase activity [78,82,100,101]. Likewise, DsbE is dependent on DsbD for its function in cytochrome c maturation [97,102,103]. As indicated in the previous paragraph, DsbC is kept reduced under normal circumstances, but in a *dsbD* null mutant, DsbC accumulates in the oxidized form that is able to promote disulfide bond formation, but not isomerisation [25]. Similarly, overexpression of DsbC also leads to disulfide bond formation by DsbC, because DsbC now cannot be reduced by the normal levels of DsbD [73]. Under normal conditions, DsbD vouches for the reduced state of the CxxC motif of DsbC by funneling electrons from the cytoplasmic thioredoxin across the inner membrane to DsbC [83]. Both cysteines in the Cys32-Gly-Pro-Cys35 motif of thioredoxin are needed for this action [104]. Thioredoxin in turn is reduced again by thioredoxin reductase, which receives its reducing equivalents from the cytoplasmic electron donor NADPH [19,20,22].

Stewart and colleagues showed that six out of seven cysteine residues in mature DsbD are conserved and required for the transfer of electrons from the cytoplasm to the *E. coli* periplasm [105]. Gordon *et al*. and Chung *et al*. demonstrated independently that this protein is processed to a 546 residue protein after removal of the 19-residue signal sequence. This was a surprising result, because the large majority of membrane proteins of *E. coli* and other bacteria normally lack cleavable signal peptides [106]. Both the N- and C-terminal domains of DsbD are located in the periplasm, separated by eight transmembrane helices [97,98].

There are two CxxC motifs present in DsbD: Cys282-Pro-Ser-Cys285 in a predicted transmembrane helix and Cys481-Val-Ala-Cys485 in the C-terminal periplasmic thioredoxin-like fold. Cys282 is not conserved in DsbD-like proteins [98] and can be substituted without affecting the reduced state of DsbC, DsbE, and DsbG [97]. The work of both Chung *et al*. and Gordon *et al*. showed that the six other cysteines in DsbD are critical for its function [97,98]. A model for the functioning of DsbD as proposed by Krupp *et al*. is as follows: An intramolecular disulfide bond between Cys163 and Cys285, which is exposed to the cytoplasm, is resolved by the action of thioredoxin (interaction between Cys32 of thioredoxin and Cys163 of DsbD). These cysteines subsequently donate their electrons to the periplasmic thioredoxin-like motif of DsbD, leading to the reduction of Cys461 and Cys464. The resulting dithiol accordingly reduces an N-terminal disulfide bond between Cys103 and Cys109 and from this point electrons can be transferred to either oxidized DsbC, DsbE, or DsbG [104,107]. This model was corroborated by the work of Goldstone and colleagues, who showed that the N-terminal periplasmic domain of DsbD is directly responsible for the reduction of DsbC [108,109]. Furthermore, Katzen and Beckwith provided evidence for the proposed disulfide bond between Cys163 and Cys285 [110].

The electron transfer from NADPH *via* thioredoxin and DsbD to DsbC and DsbG was reconstituted *in vitro* by using the purified α, β, and γ domains of DsbD and it was shown that the electron flow within DsbD is a thermodynamically driven process [111,112]. DsbD homologues are found in many bacterial kingdoms as well as in archaea and plant chloroplasts. They seem to have evolved in parallel with the DsbB proteins and probably arose from an internal duplication event, which accounts for the many topological types of DsbD, like CcdA in *B. subtilis*, that are found within this family [113].

2.5 DsbE

The 185 residue containing DsbE protein (also known as CycY or CcmG) was first recognized as a product specified by the *ccmG* gene (**c**ytochrome **c** **m**aturation) and shown to be part of the *ccmABCDEFGH* cluster that is essential for the synthesis of c-type cytochromes [114]. In an *E. coli* mutant

lacking all these genes, maturation of the indigenous c-type cytochromes synthesized under anaerobic respiratory conditions, with nitrite, nitrate, or trimethylamine N-oxide as the electron acceptor, was found to be defective. Next to that, the biogenesis of foreign cytochromes, such as the soluble *Bradyrhizobium japonicum* cytochrome c550 and the membrane-bound *Bacillus subtilis* cytochrome c550 was abolished [114]. Metheringham *et al*. suggested that in the folding of apocytochrome, DsbA first oxidizes the cysteines and DsbE (CcmG) is subsequently responsible for reduction of the cysteines to allow the covalent linkage of the pyrrol ring of heme [40,115,116].

Fabianek *et al*. characterized DsbE in *E. coli* for its role in c-type cytochrome maturation. They showed that the protein contains a highly hydrophobic N-terminal segment, which is responsible for anchoring DsbE to the cytoplasmic membrane. The C-terminal domain has a thioredoxin-like fold with the active site Cys-Pro-Trp-Cys and is faced towards the periplasm. Replacement of the active site cysteines demonstrated that both are important for the function of DsbE. Addition of low-molecular-weight thiol compounds could restore the cytochrome c synthesis in the Cys-replacement mutants, but not in the *dsbE* mutant, suggesting a reducing activity of DsbE *in vivo* [102]. This view was supported by the work of Li and colleagues [117,118]. Furthermore, Reid and colleagues demonstrated that *trxA* and *dsbD* mutants are unable to assemble c-type cytochromes or catalyse formate-dependent nitrite reduction, but that these activities could be restored by the addition of the reducing agent 2-mercaptoethanesulphonic acid [119].

According to current models, heme and apocytochrome c are separately transported across the membrane after which disulfide bonds are introduced into apocytochrome c by the action of DsbA. Heme is first covalently coupled to the membrane protein CcmE, which is stabilized in the membrane by CcmD. CcmC is responsible for the transfer and attachment of heme to CcmE. DsbE is thought to reduce the disulfide bonds formed between correctly paired cysteine residues in the cytochrome c apoprotein prior to the heme attachment by CcmF and CcmH to the heme binding site CxxCH of this apoprotein [69,120-122].

Recently, the DsbE protein of *Bradyrhizobium japonicum* was crystallized. The specific interaction between DsbE and apocytochrome c was shown to depend on two features of DsbE. It contains a modified thioredoxin-like fold with an unusually acidic active site and two groove-forming inserts. These two characteristics are thought to account for a high fidelity and very specific reducing activity [123]. DsbE of *E. coli*, which shares 31% sequence identity with DsbE of *B. japonicum*, lacks seven amino acids that correspond to an N-terminal insert forming a groove in *B. japonicum* [124]. Despite this deletion, also DsbE of *E. coli* seems to have the reduction of apocytochrome c as its specific task.

2.6 DsbG

DsbG was identified by Andersen *et al*. in 1997. It contains the characteristic CxxC motif of thioredoxin-like proteins (Cys126-Pro-Tyr-Cys129). When overexpressed, *E. coli* can grow at concentrations of dithiothreitol that are normally lethal for this organism, whereas deletion mutants of *dsbG* are sensitive to dithiothreitol. Replacement of Cys126 by Ala resulted in a total loss of DsbG activity, whereas replacement of Cys129 by Ala led to a decreased activity [100].

DsbG shows sequence similarity to the thiol-disulfide isomerase DsbC and, like DsbC, DsbG forms a stable periplasmic dimer. Van Straaten and colleagues already suggested that the substrate specificity of DsbG might be narrower than that of DsbC [125]. This view was corroborated by the work of Bessette *et al*. They also showed that overexpression of DsbG in a *dsbC* mutant allowed the correct folding of bovine pancreatic trypsin inhibitor, a protein with three disulfides, and to a lesser extent, mouse urokinase with twelve disulfides [101]. As for DsbC, the reduction of the active site thiols of DsbG is totally dependent on DsbD [104,111,112]. Both DsbC and DsbG have chaperone activity that is independent of their isomerase activity as demonstrated by the prevention of misfolding and aggregation of citrate synthase as well as that of luciferase *in vitro* [126]. Notably, overexpression of both DsbC and DsbG was shown to markedly improve the formation of a single-chain Fv antibody [127].

2.7 Other *E. coli* proteins implicated in disulfide bond formation

Apart from the Dsb proteins and thioredoxins 1 and 2, several other proteins of *E. coli* have been implicated in thiol-disulfide oxidoreductase reactions. One example is DnaJ [128], a 41 kDa cytoplasmic heat shock protein that normally is regarded as a chaperone, but for which there is evidence that it can catalyze isomerization reactions [129]. Production of a single-chain Fv antibody fragment and proinsulin could considerably be improved by addition or cosecretion, respectively, of DnaJ [130,131]. Another example of a protein with Dsb-like activity is CcmH, which together with DsbE (CcmG) is involved in the reduction of the CxxCH-motif of apocytochrome c to enable the attachment of heme [122,132,133].

A recent observation of Fomenko and Gladyshev who found that certain proteins that contain a CxxS motif instead of the canonical CxxC motif are also involved in thiol-disulfide oxidoreductase reactions, might yet lead to the discovery of even more proteins that are involved in the making and breaking of disulfide bonds [134]. Two such proteins that might have this potential were recently identified in *B. subtilis* (our unpublished observations) and are

discussed below. It is conceivable that similar proteins will also be discovered in *E. coli* and other organisms.

3. Thiol-disulfide oxidoreductases in *B. subtilis*

3.1 *Bacillus subtilis*

The rod-shaped eubacterium *Bacillus subtilis* can be regarded as the paradigm for Gram-positive organisms. In the past decade, much research has been focused on *B. subtilis* to exploit its high potential for protein secretion that allows the production of various secretory proteins at gram per liter amounts (For reviews see: [135-141]). Although *B. subtilis* is generally regarded as an aerobic bacterium, it has been shown that this organism is also able to grow in an anaerobic environment in the presence of nitrate or nitrite [142-146]. In 1997 its complete genome of 4,215 kbp was annotated and shown to contain about 4,100 protein-coding sequences [147]. In recent years, much effort has been put into the elucidation of the function and importance of the proteins specified by these identified genes [148,149].

B. subtilis can be regarded as an interesting model organism for other Grampositive spore-forming bacteria, such as the pathogenic *Bacillus anthracis* [150], *Bacillus cereus* [151], *Clostridium botulinum* [152], *Clostridium difficile* [153], and *Clostridium perfringens* [154], the industrially important *Clostridium acetobutylicum* [155], and *Bacillus brevis* [156], and the extremophiles *Bacillus halodurans* [157] and *Oceanobacillus iheyensis* [158].

3.2 The cytoplasm of *B. subtilis*

Like in *E. coli*, the cytoplasm of *B. subtilis* is a reducing environment, that is kept in a reduced state by the thioredoxin system. Unlike *E. coli*, *B. subtilis* does not contain a glutaredoxin system, although it contains a glutaredoxin homologue YtnI [149]. Furthermore, the reducing agent mycothiol that can be found in fungi and *Streptomyces* species [159] is not present in *B.subtilis*. Thus, *B.subtilis* seems to be solely relying on the thioredoxin- and thioredoxin reductase-like proteins to keep its cytoplasm reduced. The fact that TrxA, unlike its *E. coli* counterparts TrxA and TrxC, was found to be essential for viability, underscores the dependence of *B. subtilis* on this system [160]. Dubois and colleagues demonstrated that TrxA is involved in the important processes of protein secretion, competence development, and sporulation [161]. They also showed that *B. subtilis* contains the genes for at least seven other cytoplasmic thioredoxin-like proteins: YbdE, YdbP, YdfQ, YkuV, YosR, YtpP, and YusE. Next to the thioredoxin reductase TrxB, also *ycgT, ypdA* and *yumC* specify thioredoxin reductase-like proteins [147].

In order to maintain a reducing environment inside the cell under conditions of oxidative stress, *B. subtilis* can employ a variety of mechanisms. The *B. subtilis* spore DNA for example, is protected against oxidative damage

by α- and ß-type small acid soluble proteins [162,163] and the protein KatX has a role in hydrogen peroxide detoxification during germination [164,165]. Hydrogen peroxide can arise from the action of NADPH oxidase that generates superoxide radicals (O_2^-), which contain one free electron. On itself they are not very toxic, but the superoxide can be converted to hydrogen peroxide (H_2O_2), which in turn can oxidize chloride ions to form the extremely toxic hypochlorous acid (HOCl) [166,167]. It is therefore imperative that *B. subtilis* scavenges away the molecules that lead to these compounds or prevents their generation. SodA, a superoxide dismutase, and CysK, an enzyme of the cysteine biosynthetic pathway, which are induced by paraquat [168,169], seem to fulfill important roles in this protection [165].

In *B. subtilis* there is also a general oxidative stress response that is mediated by the alternative sigma factor SigB [170,171]. Next to the induction by hydrogen peroxide, the SigB regulon is induced by a variety of stress factors, such as heat, ethanol, acids, salt, and starvation. Especially glucose starvation was found to trigger the general oxidative stress response of aerobically growing cells [172,173]. The genes for TrxA and the general stress protein Dps are members of the SigB regulon [160,169]. Dps belongs to the same group as MrgA (a *dps* homologue) that is PerR regulated and, like MrgA, is involved in DNA protection [169]. For *B. subtilis* it is hypothesized that PerR has a role in repressing the genes encoding catalase (*katA*), alkylhydroperoxide reductase (*ahpCF*) and MrgA. Expression can be induced by addition of hydrogen peroxide or metal ion limitation. PerA is thought to have a DNA-binding domain and a metal-binding domain, and the latter is suggested to be involved in the regulation, which is effectuated by the reduction and oxidation of the metal ion. Especially the divalent ions of manganese and iron seem to be fulfilling this function [174]. Manganese is known to be one of the key players in protecting *B. subtilis* against superoxide radicals [175].

In conclusion it can be stated that *B. subtilis* has a whole battery of reducing mechanisms in the cytoplasm. Despite this, like in *E. coli*, there are some examples of proteins that transiently contain a disulfide bond. These include ribonucleotide reductase [26,176], phosphoadenosyl phosphosulfate reductase, and methionine sulfoxide reductase [163].

3.3 Identification of thiol-disulfide oxidoreductases in *Bacillus*

The cell envelope of the Gram-positive *B. subtilis* is composed of a cytoplasmic membrane surrounded by a thick cell wall composed of peptidoglycan and the anionic polymers teichoic acid and teichuronic acid [177-179]. Merchante and colleagues have proposed that the extracytoplasmic

compartment, which has the membrane and the cell wall as its boundaries is equivalent to the periplasm of *E. coli* both quantitatively and qualitatively [180,181]. It appears that this confined space, like the periplasm in *E. coli* is oxidizing and that it forms a subcellular compartment where thiol-disulfide oxidoreductases of *B. subtilis* display their action. Disulfide bond formation in a certain protein is believed to take place immediately after translocation of the substrate across the membrane. Translocated proteins that do not assume their correct conformation are prone to degradation by proteases that are present in the cell wall and the medium [138,182,183]. The most effective way to accomplish disulfide bond formation immediately after translocation is to have the thiol-disulfide oxidoreductases located in the cytoplasmic membrane, in close proximity to the exit site of the translocation machinery.

The first thiol-disulfide oxidoreductase identified in a *Bacillus* species was the Bdb protein of *Bacillus brevis* (***B****acillus* **d**isulfide **b**ond oxidoreductase [184], which was shown to complement a *dsbA* mutation in *E. coli* with respect to motility and alkaline phosphatase (PhoA) activity [184]. The first thiol-disulfide oxidoreductases in *B. subtilis* were reported by Bolhuis and co-workers in 1999 and named BdbA, BdbB, and BdbC [185]. They were identified on the basis of their sequence similarity to other thiol-disulfide oxidoreductases through data base searches. The current knowledge concerning disulfide bond forming enzymes in *B. subtilis* will be reviewed in the paragraphs below.

3.4 BdbA and BdbB

BdbA (YolI) of *B. subtilis* was identified by its similarity (43 %) to Bdb of *Bacillus brevis*. It is a 137 residue protein with a predicted signal sequence of 26 amino acids [185]. Despite the absence of transmembrane domains and known cell wall retention signals like cell wall-binding repeats [186], or LPxTG and NPQTN motifs [187], BdbA does appear to be retained in the cell envelope (our unpublished observations), a localization that was as well predicted for Bdb in *B.brevis* [184].

A *bdbA* mutant has no strong phenotype as the strain is still viable, motile, able to sporulate, competent for DNA binding and uptake, and resistant against oxidizing (methyl viologen, paraquat) and reducing agents (dithiothreitol). Although initial studies with a conditional *bdbA* mutant indicated that BdbA has no role in the secretion of active PhoA of *E. coli* [185], more recent studies with a *bdbA* deletion mutant indicate that PhoA secretion in the absence of BdbA is slightly decreased. Notably, PhoA of *E. coli* needs two disulfide bonds for its activity and stability [100,188,189]. Despite the fact that the *bdbA* gene is part of a locus that seems to be devoted to the production of the lantibiotic sublancin 168 [190], BdbA has apparently no essential role in the production of this bacteriocin [191].

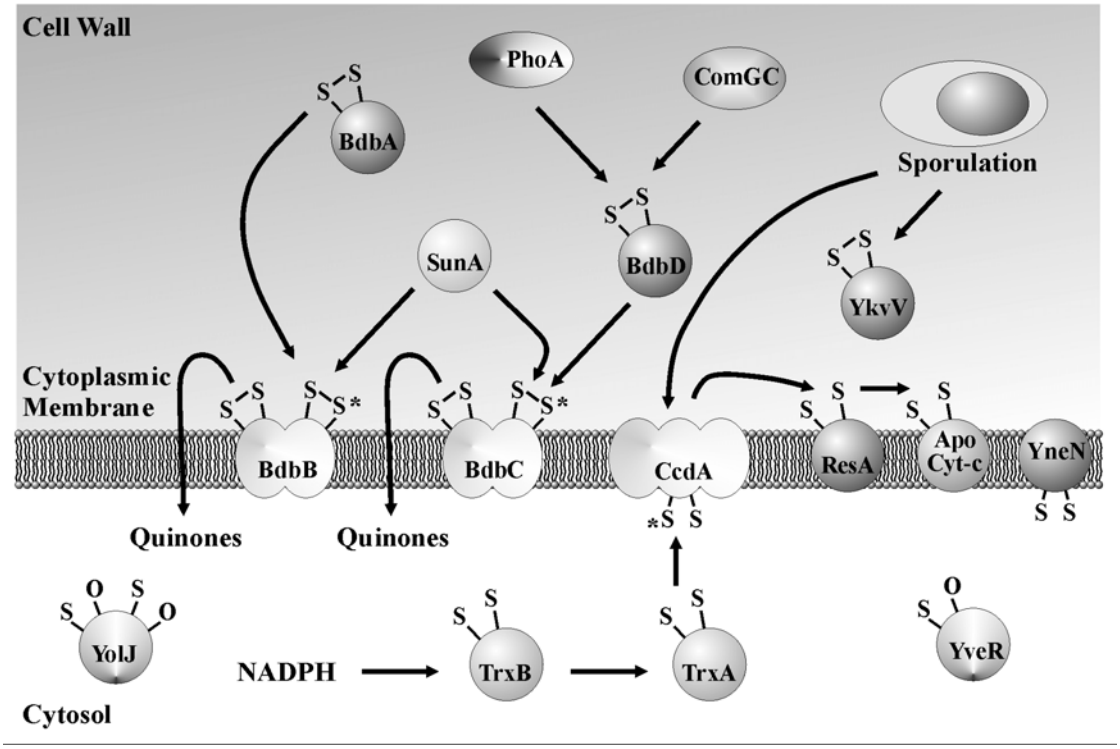

Figure 1. Model of the Bdb system in *B. subtilis*. BdbA/B and BdbC/D are depicted as oxidative pathways. CcdA and ResA are thought to be responsible for the reduction of apocytochrome-c (Apo Cyt-c). CcdA and YkvV have been implicated in sporulation. Arrows indicate the most likely direction of electron flow during redox reactions. Furthermore, the most likely *in vivo* state of the relevant cysteine residues in each protein is indicated. Thus, BdbD is depicted with a disulfide bond (S-S), whereas the cysteines in TrxA and ResA are predominantly reduced (S). The CxxS motifs in YolJ and YveR are indicated with "S O", because intramolecular disulfide bonds can not be formed in these proteins. The cysteine residues in S-S bonds indicated with a * are <u>not</u> present in CxxC motifs.

BdbB (YolK) was identified as a homologue of the *E. coli* thiol-disulfide oxidoreductase DsbB [185]. It is a 148 residue protein with four predicted transmembrane helices and with the N- and C-termini located in the cytoplasm. It shares 47% identical and conserved residues with DsbB and, like DsbB, it contains the Cys-Val-Leu-Cys motif in the N-terminal extracytoplasmic domain and two conserved cysteines in the second extracytoplasmic domain. Different from DsbB, where the two cysteines in the second loop are separated by 25 residues, there are only 6 residues present between these cysteines in BdbB. The distance between the CxxC motif and the predicted transmembrane helix is in both cases four residues, with an arginine residue at the +4 position relative to the second Cys residue of the CxxC motif. This distance is imperative for DsbB function in *E. coli*, probably because of the reaction with quinones in the membrane [65,192]. It is very likely that the same is true for

the reaction of BdbB with quinones. If so, BdbB represents a connection between the electron transport chain and the extracytoplasmic enzymes for disulfide bond formation as described for DsbB in the DsbA-DsbB system. In this view, a system constituting BdbA and BdbB, could be responsible for the introduction of disulfide bonds. This would allow BdbA to oxidize certain substrates that are, so far, unknown.

BdbA and BdbB are both part of the sublancin 168 locus. In contrast to the deletion of the *bdbA* gene, the removal of *bdbB* led to a decrease in sublancin 168 activity, although the activity was not totally abolished [191]. In conclusion, it can be stated that BdbB, like BdbA, is of minor importance for *B. subtilis* because, like in the *bdbA* mutant, viability, motility, sporulation, competence, and resistance to oxidizing and reducing agents are not affected in a *bdbB* mutant of *B. subtilis*. Only the secretion of the heterologous PhoA protein of *E. coli* seems to be mildly affected in the *bdbB* mutant strain [185], similar to the level found in the *bdbA* deletion mutant (our unpublished observations). Thus, the activity of BdbB seems to be largely dedicated to the production of the lantibiotic sublancin 168. Notably, these findings are consistent with the fact that the sublancin locus is part of the SPβ prophage, which is completely dispensable for *B. subtilis* 168.

3.5 BdbC and BdbD

In addition to BdbB, a second homologue of the *E. coli* protein was identified in *B. subtilis* and named BdbC (YvgU) [185]. The 138 amino acids long BdbC protein shares 42% identical residues and conservative replacements with DsbB and it also contains four hydrophobic regions. Similarly, it is thought to have two extracytoplasmic loops that both contain two cysteines. The distance between the two cysteines in the C-terminal loop of BdbC comprises only five residues similar to BdbB. In contrast to the CxxC motifs in BdbB and DsbB that comprise Cys-Val-Leu-Cys, the valine in BdbC is replaced by glutamate, resulting in a Cys-Glu-Leu-Cys motif, which may influence the redox potential of the CxxC motif [193]. The gene specifying BdbC forms, together with the gene specifying BdbD, the bicistronic *bdbDC* operon. Most likely, BdbC has a role in the re-oxidation of reduced BdbD [161,194]. BdbD was initially predicted to be a 222 residue pre-protein that is translocated across the cytoplasmic membrane after removal of its putative signal peptide [138]. However, it seems more likely that the hydrophobic residues in the N-terminus of BdbD function as a membrane anchor rather than a signal peptide. Over a region of 171 residues, BdbD shares 55% identical residues and conservative replacements with DsbA of the Gram-positive *Staphylococcus aureus*. Furthermore, similarity can be observed, albeit of a less strong nature, with DsbG of *E. coli* and with the DsbA proteins of *Haemophilus influenza* [195], *Neisseria meningitidis* [196], and *Pseudomonas*

aeruginosa [197]. The *bdbD* gene has initially been identified by the Systematic Gene Function Analysis Project as a gene essential for competence development [149,194]. By analogy with the DsbA-DsbB system, the cysteines in the C-terminal extracytoplasmic loop of BdbC are presumably responsible for the interaction with BdbD, whereas the CxxC-motif in the first loop is oxidized by the quinones of the electron transfer chain in the membrane. It is tempting to speculate that differences between the extracytoplasmic loops of BdbB and BdbC are responsible for preferential interactions of these proteins with either BdbA or BdbD, respectively.

Both BdbC and BdbD are required for competence development and the secretion of PhoA of *E. coli* [185,194]. Meima *et al*. showed that the competence defect of *bdbC* or *bdbD* mutant *B. subtilis* strains can be attributed to a defect in the folding of the ComGC protein to a protease-resistant conformation [194]. The ComGC protein that is similar to type IV pilins is known to require one intramolecular disulfide bond for stability [198,199]. Absence of this disulfide bond presumably makes ComGC prone to degradation, which unfavorably affects competence development. Although the competence of *B. subtilis bdbC* and *bdbD* mutants is severely affected, very small residual levels of competence development can be observed in these strains (our unpublished observations). This suggests that a small portion of the ComGC population is oxidized to the active conformation even in the absence of BdbC or BdbD. This could be due to the activity of other thiol-disulfide oxidoreductases.

3.6 Other *B. subtilis* proteins implicated in disulfide bond formation

3.6.1 CcdA and ResA

The 235 residue CcdA protein was identified as an integral 26 kDa membrane protein involved in the synthesis of c-type cytochromes by Schiott and colleagues in 1997 [200]. CcdA has six α-helical transmembrane segments and its N- and C-termini are both located on the extracytoplasmic side of the membrane [201]. CcdA seems to work together with the proteins ResB and ResC in the synthesis of cytochrome c [202].

Recently, also the putative thiol-disulfide oxidoreductase ResA, a 181 residue protein with one transmembrane segment and a C-terminal thioredoxin-like motif that is located on the outside of the cytoplasmic membrane, was shown to be involved in cytochrome c biogenesis [203]. The membrane bound bacterial c type cytochromes are important for respiration where they function as electron transporters. For their activity a heme group has to be covalently attached to the apocytochrome [95,204,205]. Attachment of the heme group to the cytochrome occurs *via* two thioether bonds between

the vinyl side chains of the heme and the cysteine residues of the CxxCH motif in apocytochrome c [120]. These cysteines have to be reduced for this reaction to happen. The current view is that a co-operation between ResA and CcdA assures the reduced state of these cyteines [203].

CcdA of *B. subtilis* is a homologue of the CcdA protein that is found in *Rhodobacter capsulatus* and it also shows similarity to the central membrane region of DsbD (DipZ) of *E. coli* [96,132,202]. Schiott and co-workers could show that *ccdA* mutants of *B. subtilis* are blocked in a late step in cytochrome c synthesis [206]. Unlike all other thiol-disulfide oxidoreductases, CcdA does not contain the canonical CxxC motif. However, it does contain two conserved cysteine residues. Based on the work of Deshmukh *et al*. who found two conserved cysteines in *R. capsulatus* CcdA to be essential for its role in cytochrome c synthesis [132], Schiott *et al*. propose a similar role for CcdA in transferring electrons across the membrane [105,201]. Therefore, CcdA is thought to work together with ResA in a manner similar to the DsbD-DsbE system in *E. coli* where DsbD guarantees the reduction of DsbE. In turn, DsbE can reduce apocytochrome c. Further evidence for CcdA and ResA involvement in a reducing pathway comes from experiments in which cytochrome c synthesis in either a *ccdA* or a *resA* mutant could be restored by removal of BdbC, BdbD or by the addition of dithiothreitol (DTT) [203,207]. The BdbC and BdbD proteins are thought to catalyze the formation of an intramolecular disulfide bond in the CxxCH motif of apocytochrome and DTT is a strong reducing agent that can break this bond.

3.6.2 YneN

Little is currently known about the 170 residue YneN protein that seems to be a membrane-bound member of the thiol-disulfide oxidoreductase family. Like BdbA, BdbB, BdbC, and BdbD, it contains the characteristic CxxC motif with a lysine and a proline between the two cysteines. However, in contrast to BdbA-D, topology predictions based on the TMHMM algorithm version 2.0 (http://www.cbs.dtu.dk/services/TMHMM-2.0/) [208] indicate that the active site of YneN resides at the cytoplasmic side of the membrane. This is in conflict with the YneN topology predicted by Erlendsson *et al.*, who have suggested that the catalytic domain of this protein is localized at the extracytoplasmic side of the membrane [207]. If the thioredoxin-like domain of YneN is indeed located in the cytoplasm, this opens up the interesting possibility that YneN acts as a mediator in electron transfer from the inside to the outside of the cell, with or without the aid of other components in or close to the membrane.

One gene of the plant pathogen *Xylella fastidiosa* specifying a putative thioredoxin, XF1990, shows 30% identity with YneN of *B. subtilis* [209]. Further evidence supporting the view that YneN is a thiol-disulfide

oxidoreductase was obtained by Di Gennaro and co-workers, who developed a sequence-to-structure-to-function algorithm to reveal, among others, that YneN is a possible Dsb protein. In the same analysis they also identified BdbA, TrxA, YkvV, ResA, and BdbD [210]. Till now, the exact role of YneN remains an enigma, but its undefined membrane topology makes it a very interesting subject for further studies.

3.6.3 YkvV

YkvV was the sixth putative thiol-disulfide oxidoreductases of *B. subtilis* that we identified by paralogue analyses. This 165 residue protein is predicted to be secreted after removal of a 26 residue signal peptide (SignalP; http://www.cbs.dtu.dk/services/SignalP/; [211,212]). Two prolines separate the cysteines in the active site of YkvV. Eichenberger and co-workers showed that transcription of *ykvV* is under the control of the transcription factor SigE, which is known to regulate expression in the mother cell during sporulation. A large group of genes controlled by SigE is involved in the synthesis of the cortex and the coat of the spore [179,213-215]. The *ykvV* mutant showed dark spores under phase contrast microscopy, whereas in the wild-type strains they are bright. This suggests that there is a defect in the maturation of the spore core or the spore cortex [216]. The authors recognized the relationship of YkvV with thiol-disulfide oxidoreductases and proposed a role for YkvV in the space that is formed between the inner and outer membrane of the forespore where the cortex is synthesized [216]. Very recently, the thiol-disulfide oxidoreductase activity of YkvV was confirmed biochemically [217]. A role for YkvV in cortex formation is in agreement with the observation by Imamura and co-workers who revealed with electron microscopy that *ykvV* mutants lack a visible cortex layer. They showed that disruption of the *ykvV* gene resulted in a large decrease in the number of heat resistant spores [218]. Accordingly, the gene was renamed *spoIVH*. Unfortunately, *ykvV* has also been renamed *stoA* (sporulation thioldisulfide oxidoreductase A) [219]. Many of the proteins involved in spore coat formation contain cysteines. Examples are CotA (outer coat) with 4 cysteines, CotC (outer coat) with 4, CotD (inner coat) with 5, CotE (forespore outer membrane) with 2, CotG with 8 (disruption of *cotG* prevents insertion of CotB in the outer coat), CotX (insoluble fraction of the spore coat) with 7, CotY (insoluble fraction of the spore coat) with 15, and CotZ (insoluble fraction of the spore coat) with 10. Whereas many of these proteins were not found to be essential for spore development on their own [213], misfolding of more than one of these proteins, because of the absence of YkvV, could well be the reason for the defects found in sporulation of the *ykvV* mutant. It still has to be elucidated which proteins are the exact substrates of YkvV, but a role as thiol-disulfide oxidoreductase is very likely.

3.6.4 YolJ and YveR

The *yolJ* gene is located between two genes, *bdbA* and *bdbB* in the sublancin 168 locus, that both specify a thiol-disulfide oxidoreductase. Notably, YolJ contains a CxxS motif that was recently implicated in thiol-disulfide oxidoreductase reactions [134]. YolJ is a protein of 422 amino acids with a predicted cytoplasmic localization and it has a paralogue in YveR, a 344 residue protein. YolJ was found to be essential for sublancin 168 production and both YolJ and YveR were shown to impact on the activity of heterologously produced and secreted PhoA of *E. coli* to a similar extent as BdbB (our unpublished observations).

Fomenko and Gladyshev have recently proposed that a conserved CxxS motif, followed by an α-helix, is indicative for a redox function and corresponds to thiol-dependent sites in redox proteins [134]. According to this hypothesis, the α-helix together with the serine could have a role in stabilizing the cysteine thiolate that is involved in the redox reactions. The location of YolJ on the chromosome might indicate that YolJ works closely together with the membrane bound BdbB and the extracytoplasmic BdbA protein. This assumption is underlined by the fact that YolJ and BdbB are both important for the production of the lantibiotic sublancin 168 (Dorenbos *et al*., 2002; our unpublished observations). The natural role of YveR in *B. subtilis* is unknown. There is one report of Branda *et al*. in which YveR is proposed to have a role in biofilm formation [220]. The exact functions of YolJ and YveR, however, remain to be elucidated. If YolJ and YveR indeed are involved in thiol-disulfide oxidoreductase reactions, these proteins would be the first prokaryotic CxxS proteins with such a function.

4. Thiol-disulfide oxidoreductases in other organisms

In recent years thiol-disulfide oxidoreductase-like proteins have also been identified in bacteria other than *E. coli*, *B. subtilis* and their close relatives (Table III). Recent reports point to the presence of thiol-disulfide oxidoreductases in the strict anaerobe *Fibrobacter succinogenes* [221]. Mallick and co-workers provided genomic evidence for disulfide bond-containing intracellular proteins of archaeal microbes [222]. This would imply the existence of a totally different thiol-disulfide oxidoreductase system, because normally intracellular disulfide bond formation is very rare due to the generally reducing environment of the cytoplasm.

In eukaryotes protein disulfide isomerase (PDI) that operates in the endoplasmic reticulum is responsible for the formation and isomerizaton of disulfide bonds [223]. PDI is a large complex 57 kDa protein of five domains [223,224], two of which are homologous to thioredoxin [225]. Each of these

Table III. Examples of thiol-disulfide oxidoreductases in bacteria other than *E. coli* and *B. subtilis.*

Organism	Proteins	References
Pseudomonas aeruginosa	DsbA/DsbC	[197,252]
Erwinia chrysanthemi	DsbC	[74]
Xylella fastidiosa	XF1990	[209]
Shigella flexneri	DsbA	[253]
Staphylococcus aureus	DsbA	[254]
Salmonella enteritidis	Dlp/Dlt	[255]
Salmonella typhi	Dlp/Dlt	[255]
Salmonella enterica serovarTyphimurium	SrgA/DsbA	[256,257]
Bordetella pertussis	DsbA/DsbB/DsbC	[258]
Erwinia carotovora	DsbA/DsbC	[259]
Vibrio cholerae	TcpG	[260]
Burkholderia cepacia	DsbA/DsbB	[261]
Bradyrhizobium japonicum	TlpA	[262]
Paracoccus denitrificans	CcmG	[263]
Haemophilus influenzae	Por	[195]

two domains contains an active site motif Cys-Gly-His-Cys, which is comparable to the Cys-Gly-Pro-Cys sequence that is found in thioredoxin. PDI also has similarities with DsbC of *E. coli* in that it shows chaperone activity [86]. In isolation, the two active site-containing domains of PDI can fulfill the same role as DsbA in that they catalyze disulfide bond formation very efficiently. However, the isomerase activity and reducing capability of the separated domains is severely decreased [226]. Ostermeier and colleagues showed that rat PDI secreted to the periplasmic space of *E. coli* can catalyze the formation of disulfide bonds and complement several of the phenotypes of *dsbA* mutants in a DsbB-dependent manner [227].

Finally, the thiol-disulfide oxidoreductases gain more and more attention also in the medical field. Not only because of organisms that rely on these enzymes for their pathogenicity [195,228-232], but also because the major thiol-disulfide oxidoreductase in eukaryotic species, PDI, is involved in certain diseases like AIDS [233-235] and Alzheimer disease [236]. Because of its

complexity, PDI is a highly challenging but difficult object for study. Studies on the microbial thiol-disulfide oxidoreductases are, therefore, highly suitable to increase our knowledge concerning the more complex thiol-disulfide oxidoreductases in eukaryotes.

5. Outlook

As outlined in the foregoing sections, disulfide bond formation is a critical parameter for the stability and/or activity of many proteins. With the help of their thiol-disulfide oxidoreductases, bacteria are capable of catalyzing the formation of disulfide bonds in certain proteins they export to extracytoplasmic compartments or the growth medium. Nevertheless, in the industrial production of eukaryotic proteins with bacteria, the formation of disulfide bonds has been identified as a major bottleneck, especially if such heterologous proteins contain multiple disulfide bonds. Now that many of the key enzymes and mechanisms involved in bacterial disulfide bond formation have been identified, it is foreseeable that protein production bottlenecks at the level of disulfide bond formation can be removed by tailoring the bacterial machinery for disulfide bond formation and isomerization. This seems very well feasible not only in Gram-negative bacteria, such as *E. coli*, but also in major industrial "workhorses" for biotechnological protein production, such as *B. subtilis*.

Acknowledgements

We thank Jean-Yves Dubois for critically reading the manuscript, and other members of the Groningen and European *Bacillus* Secretion Groups for stimulating discussions. Funding for the project, of which this work is a part, was provided by the CEU projects QLK3-CT-1999-00413, QLK3-CT-1999-00917, LSHC-CT-2004-503468 and LSHG-CT-2004-005257.

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