

# Applications of thiol-disulfide oxidoreductases for optimized in vivo production of functionally active proteins in *Bacillus*

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**Abstract** *Bacillus subtilis* is a well-established cellular factory for proteins and fine chemicals. In particular, the direct secretion of proteinaceous products into the growth medium greatly facilitates their downstream processing, which is an important advantage of *B. subtilis* over other biotechnological production hosts, such as *Escherichia coli*. The application spectrum of *B. subtilis* is, however, often confined to proteins from *Bacillus* or closely related species. One of the major reasons for this (current) limitation is the inefficient formation of disulfide bonds, which are found in many, especially eukaryotic, proteins. Future exploitation of *B. subtilis* to fulfill the ever-growing demand for pharmaceutical and other high-value proteins will therefore depend on overcoming this particular hurdle. Recently, promising advances in this area have been achieved, which focus attention on the need to modulate the cellular levels and activity of thiol-disulfide oxidoreductases (TDORs). These TDORs are enzymes that control the cleavage or formation of disulfide bonds. This review will discuss readily applicable approaches for TDOR modulation and aims to provide leads for further improvement of the *Bacillus* cell factory for production of disulfide bond-containing proteins.

**Keyword** *Bacillus* · Bdb · DsbA · PhoA · TDOR · Disulfide bond

## Protein production

Proteins are the key operators and driving force of all cellular activity (Alberts et al. 2008). In the last decades there has been a clear trend towards direct commercial production of specific proteins of interest that show desired biological activities. These protein products range from enzymes for household detergents and textile cleaning to food enzymes, antibiotics, and pharmaceuticals (Meima et al. 2004; Schallmeyer et al. 2004; Zeigler and Perkins 2008). The ability to produce these products is thus an important prerequisite for many industrial and medical applications. Although peptides and proteins can be synthesized chemically, this is in many cases not an option for commercial production due to high costs and technical limitations. An excellent alternative to circumvent this problem is the use of microorganisms as cell-based factories. In this approach, microorganisms are selected or genetically modified to enable and optimize the production of a particular protein of interest. There are various microbial cell factories used in industry, the most common ones being the yeast *Saccharomyces cerevisiae*, the fungus *Aspergillus niger* and the bacteria *Escherichia coli* and *Bacillus subtilis*. Each of these microorganisms has a good track record for protein production. Therefore, the choice of the production host is often determined by specific characteristics of the protein that needs to be produced. This minireview focuses on the use of *B. subtilis* as a cell factory, with special emphasis on disulfide bond formation in secreted proteins.

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## *Bacillus subtilis*

The Gram-positive bacterium *B. subtilis* naturally thrives in the soil and the plant rhizosphere where it feeds on decaying plant material (Earl et al. 2008). To facilitate these breakdown processes, it produces and secretes a wide variety of enzymes, such as proteases, amylases, and lipases, of which many have found industrial applications. Importantly, *B. subtilis* is able to produce these enzymes in high amounts and to secrete them directly into the growth medium. Accordingly, the majority of *B. subtilis* proteins that are transported across the cytoplasmic membrane end up directly in the extracellular milieu (Tjalsma et al. 2000; van Wely et al. 2001). This is in marked contrast to Gram-negative bacteria, such as *E. coli*, which have an outer membrane that retains many proteins in the periplasm. The high capacity for protein secretion makes *B. subtilis* an extremely attractive organism to investigate the total flow of proteins from the cell into the environment by proteomic techniques, and greatly facilitates the down-stream purification in industrial protein productions (Antelmann et al. 2003; Tjalsma et al. 2004). Other useful aspects of *B. subtilis* include its good fermentation capabilities, an awarded Generally Recognized as Safe (GRAS) status by the US food and drug administration and the great amenability of this organism for genetic modification studies (Chen et al. 2006; Dubnau and Lovett 2001). These factors combined have triggered a substantial amount of research on *B. subtilis* in the last decades, and they have made this organism a paradigm for applied research on protein secretion mechanisms and a popular host for industrial protein production (Bron et al. 1999; Meima et al. 2004; Zeigler and Perkins 2008).

### Product folding and stability

Despite the extensive use of *B. subtilis* for biotechnological purposes, its deployment in protein production is largely limited to homologous proteins, since several bottlenecks in the use of *B. subtilis* for heterologous protein production still exist. These complications can relate to: (1) inefficient targeting and translocation of the foreign proteins to and across the plasma membrane, (2) inefficient posttranslocational folding and release of the exported proteins from the cell surface into the surrounding medium, and (3) degradation of the secreted proteins (Meens et al. 1997; Puohiniemi et al. 1992; Saunders et al. 1987; Bolhuis et al. 1999a; Brockmeier et al. 2006). Product degradation still remains a major potential complication, especially in the use of *B. subtilis* for heterologous protein production. Due to its innate ability to produce and secrete multiple proteases, the extracellular environment of *B. subtilis* is highly proteolytic

(Craddock et al. 2008). Consequently, unfolded or incorrectly folded exported proteins are very quickly degraded before they have a chance to (re)fold into their correct, protease-resistant conformation (Westers et al. 2006a). This is especially problematic for complex recombinant proteins, which often fold only slowly. The issue can to some extent be resolved by using either protease-deficient strains (Wu et al. 1991, 1993; Ye et al. 1999), or by addition of protease inhibitors to *B. subtilis* cultures, as was shown recently for the pharmaceutically relevant recombinant protein human Interleukin-3 (hIL-3; (Westers et al. 2006b, 2008)). However, there seems to be a limit to the level of degradation that can be prevented by such methods and it also raises other possible complications, such as the use of undesirable and/or expensive growth medium additives, and the accumulation of unwanted proteins of *B. subtilis* that would otherwise be degraded by the secreted proteases (Westers et al. 2008). Furthermore, although the final protein yields obtained from such a strategy can be higher, a significant fraction of these proteins may not be correctly folded and will therefore not display the desired biological activity. A more constructive solution to these problems is, therefore, to not only prevent the degradation of slowly folding or unfolded high-value proteins, but also to facilitate a better or faster folding of these proteins, which will render them less vulnerable to proteolysis in a shorter period of time. In turn, this will not only ensure that less of the produced proteins are degraded upon production in *B. subtilis*, but it will also guarantee the high quality of produced proteins for which the *Bacillus* cell factory has a strong track record. In order to achieve this ambitious goal, it is necessary to know why many heterologous proteins actually fold slowly or incorrectly in *B. subtilis*. In the case of proteins with disulfide bonds, this focuses attention on the mechanism of disulfide bond formation in this organism.

### Disulfide bonds

One of the most significant reasons why heterologous and especially eukaryotic proteins often fold with difficulty in *B. subtilis* is the inefficient formation of disulfide bonds (Westers et al. 2004). Disulfide bonds consist of intra- or intermolecular bridges that are formed during oxidation of the thiol groups of two cysteine residues. These bonds play an essential role in the correct folding and structural integrity of numerous proteins found in nature. Importantly, many eukaryotic proteins of biopharmaceutical interest contain multiple disulfide bonds. Well known examples include human insulin, insulin-like growth factor, human growth hormone, brain-derived neurotrophic factor, nerve growth factor, lipases, Bowman–Birk protease inhibitor, and antibody fragments (Wu et al. 1998; Hoshino et al.

2002; Qi et al. 2005; Winter et al. 2002). Without the correct thiol-oxidation that links their cysteines into disulfide bonds, these proteins will neither be fully stable nor active (Collet and Bardwell 2002; Ritz and Beckwith 2001). Heterologously produced proteins in *B. subtilis* in which the disulfide bonds have not been correctly formed are thus quickly degraded. The selection of a cellular host strain capable of efficient and correct formation of disulfide bonds is therefore an important consideration for heterologous protein production.

Unfortunately, *B. subtilis* appears not to be very efficient in the formation of disulfide bonds in its exported proteins (Westers et al. 2004), as was underpinned by the heterologous production of the disulfide bond containing *E. coli* alkaline phosphatase PhoA (Darmon et al. 2006; Bolhuis et al. 1999b). Although *B. subtilis* was able to produce and secrete this protein in high amounts into the growth medium, a large fraction was not correctly folded and quickly degraded. Accordingly, the PhoA-producing cells displayed a so-called secretion stress response, which was exacerbated in the absence of appropriate thiol-disulfide oxidoreductases that are needed for posttranslocational PhoA folding (Darmon et al. 2006). This suggested that a possible solution for overcoming problems in the production of proteins with disulfide bonds should be sought in increasing the capacity of *B. subtilis* for disulfide bond formation.

### Thiol-disulfide oxidoreductases

For a long time, the formation of disulfide bonds was believed to occur spontaneously. Spontaneous oxidation of thiol-groups into disulfide bonds does indeed occur, but this

process is very slow and nonspecific (Anfinsen 1973). For this reason, enzymes called thiol-disulfide oxidoreductases (TDORs) have evolved that catalyzes the formation (oxidation) of disulfide bonds in vivo. Notably, the TDORs also include enzymes that break (reduce) or isomerize disulfide bonds. In essence, these enzymes transfer disulfide bonds to or from their substrates in thiol-disulfide exchange reactions. Cytoplasmic TDORs generally function as reductases while their extra-cytoplasmic equivalents are oxidases or isomerases (Dorenbos et al. 2005; Tan and Bardwell 2004; Ritz and Beckwith 2001; Kouwen and van Dijn 2009). Well known examples of TDORs are the generally conserved cellular reductase TrxA (Holmgren 1985), the eukaryotic protein disulfide isomerase (Sevier and Kaiser 2006), and the Dsb proteins in *E. coli* (Heras et al. 2007; Kadokura et al. 2003; Nakamoto and Bardwell 2004). These TDORs are part of the complex TDOR machinery responsible for handling disulfide bonds in the proteins of the respective organisms.

Whereas disulfide bonds are a common feature of many eukaryotic proteins, only few disulfide-bond containing proteins have thus far been identified in *B. subtilis* despite intensive bioinformatic and proteome-wide searches (Dutton et al. 2008; Sarvas et al. 2004; Tjalsma et al. 2004). This could suggest that *B. subtilis* did not encounter the evolutionary selective pressure to develop a complex TDOR system. Nevertheless, several TDORs have been identified in *B. subtilis* (Table 1). These were named Bdb proteins for *Bacillus* disulfide bond formation (Ishihara et al. 1995). The *B. subtilis* BdbD protein is regarded as the major extracytoplasmic thiol oxidase, which is kept in and oxidized state by the paralogous quinone reductases BdbB and BdbC, with a major role for BdbC in this process. The BdbD–BdbBC system thus resembles the DsbA–DsbB system in *E. coli*,

**Table 1** Thiol-disulfide oxidoreductases described in this paper

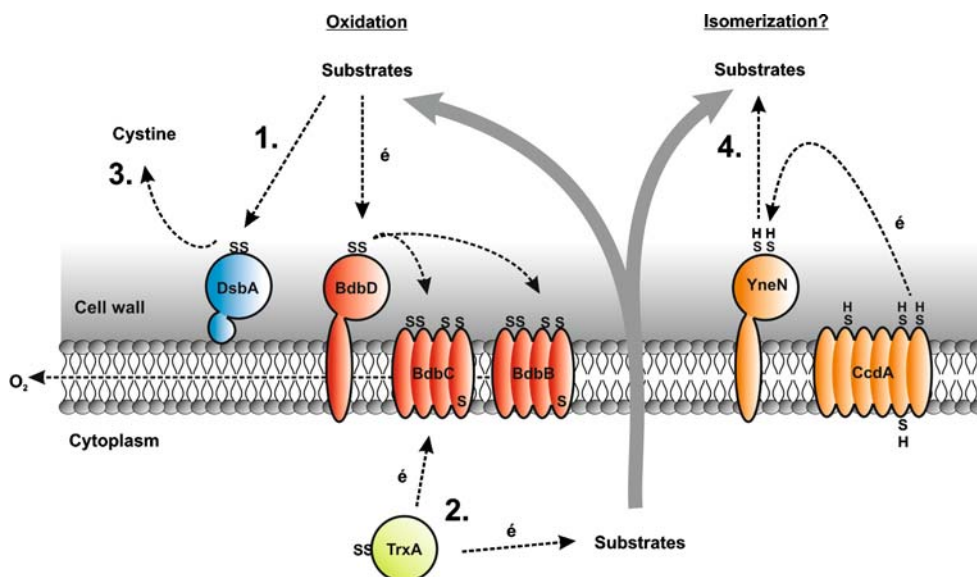
Organism	Gene	Product	Reference
<i>Bacillus subtilis</i>	<i>bdbB</i>	Membrane-embedded thiol oxidase/quinone reductase ( <i>Bacillus</i> disulfide bond protein B)	(Dorenbos et al. 2002)
	<i>bdbC</i>	Membrane-embedded thiol oxidase/quinone reductase ( <i>Bacillus</i> disulfide bond protein C)	(Bolhuis et al. 1999b)
	<i>bdbD</i>	Membrane-associated extracytoplasmic thiol oxidase ( <i>Bacillus</i> disulfide bond protein C)	(Meima et al. 2002)
	<i>ccdA</i>	Membrane-embedded disulfide reductase (cytochrome <i>c</i> deficiency protein A)	(Schiott et al. 1997)
	<i>resA</i>	Membrane-associated extracytoplasmic disulfide reductase (respiratory protein A)	(Erlendsson et al. 2003)
	<i>stoA (spoIVH)</i>	Membrane-associated extracytoplasmic disulfide reductase (sporulation thiol-disulfide oxidoreductase A)	(Erlendsson et al. 2004)
	<i>trxA</i>	Cytoplasmic disulfide reductase (thioredoxin A)	(Kouwen et al. 2008)
<i>Staphylococcus aureus</i>	<i>dsbA</i>	Membrane-associated extracytoplasmic thiol oxidase (disulfide bond protein A)	(Dumoulin et al. 2005)
	<i>catA</i>	CcdA-associated TDOR	(Tanaka et al. 2003)

(Kouwen et al. 2007; Kouwen and van Dijl 2009). Several studies on the secretion of the heterologous disulfide bond-containing protein PhoA of *E. coli* by *B. subtilis* revealed that BdbB, BdbC, and BdbD contribute to the folding of exported PhoA into a stable and active conformation (Bolhuis et al. 1999b; Dorenbos et al. 2002; Meima et al. 2002). *BdbB*, *bdbC* or *bdbD* mutant strains secreted significantly lower amounts of active PhoA than the parental strain 168. Since the rates of synthesis and signal peptide processing of the PhoA precursor were not negatively affected in *bdb* mutant strains, it was concluded that this protein was degraded after membrane translocation due to a folding defect that was caused by the absence of the different Bdb proteins. Most likely, this folding defect would relate to ineffective disulfide bond formation. Notably, however, substantial amounts of overexpressed PhoA were also degraded in the Bdb proficient parental strain 168, indicating that the activity of TDORs such as BdbBCD might be limiting in *B. subtilis* (Darmon et al. 2006; Kouwen et al. 2008). Based on these findings, several strategies were developed to overcome the apparent TDOR limitation. These are schematically represented in Fig. 1 and will be discussed in the following sections.

### Overexpression of (heterologous) oxidative TDORs

The production of secretory proteins at high levels may result in a saturation of the cellular machinery of *B. subtilis*

that is required for the secretion and subsequent folding of these proteins (Hyyrylainen et al. 2001; Sarvas et al. 2004). This might apply also to the availability of Bdb proteins in cells overproducing the *E. coli* PhoA protein or other proteins with multiple disulfide bonds. The most straightforward solution to overcome this bottleneck would be to produce oxidative TDORs at elevated levels, and in concert with the produced heterologous proteins. Indeed, such attempts have previously been made with the Bdb proteins (Darmon et al. 2006; Kouwen et al. 2007). This however did not result in improved protein folding and secretion of *E. coli* PhoA. Even the combined expression of different Bdb proteins was shown to have no beneficial effects on PhoA production (our unpublished observations). One possible explanation for this might be that another non-overexpressed TDOR of the oxidative pathway was still a limiting factor. Also it turned out difficult to overproduce the *B. subtilis* Bdb proteins in *B. subtilis* (Kouwen et al. 2007). Therefore, the possibility of expressing heterologous oxidases in *B. subtilis* was recently investigated (Kouwen et al. 2008). The oxidative TDOR DsbA of *Staphylococcus aureus* seemed a good candidate for this purpose since this homologue of *B. subtilis* BdbD was shown to be one of the strongest thiol oxidases known (Dumoulin et al. 2005). Expression of the *S. aureus* DsbA in *B. subtilis* was shown to complement for the loss of both BdbC and BdbD in the secretion of active PhoA (Kouwen et al. 2007). Interestingly, when *S. aureus* DsbA was expressed in Bdb proficient *B. subtilis* 168, an increase in the secretion of



**Fig. 1** Schematic representation of different strategies for improved disulfide bond formation in *B. subtilis*. The diagram shows known TDORs and their respective subcellular topologies. Optimized disulfide bond formation can already be achieved by: (1) introduction of strong thiol-oxidases like staphylococcal DsbA, (2) depletion of the cytoplasmic disulfide reductase TrxA, and (3) addition of redox-active

compounds to the growth medium. Further improvement can possibly be achieved by (4) the modulation of the yet hypothetical CcdA–YneN pathway for disulfide isomerization in *B. subtilis* as was described for *B. choshinensis*. Dashed arrows indicate the flow of electrons. The large gray arrows indicate protein export from the cytoplasm. See text for further details



active *E. coli* PhoA of about 1.5–2.0-fold was observed and, at the same time, less PhoA degradation was detectable. This therefore seems a useful strategy to increase the potential of *B. subtilis* to produce proteins with disulfide bonds. Notably, it may not be desirable to use genes derived from pathogenic bacteria such as *S. aureus dsbA* in the production of, in particular, pharmaceutical proteins. To avoid this potential problem, the homologous *dsbA* gene from the food-grade nonpathogenic bacterium *Staphylococcus carnosus* was cloned and expressed in *B. subtilis* (Kouwen et al. 2008). Like DsbA from *S. aureus*, the *S. carnosus* DsbA allowed the secretion of active PhoA at elevated levels. Evidently, the co-expression of appropriate TDORs with secreted disulfide bond-containing proteins is a very promising route to be followed for obtaining increased yields of correctly folded proteins with disulfide bonds.

### Depletion of reductive TDORs

An alternative strategy to achieve better folding of disulfide bond-containing proteins through TDOR modulation is the removal of inefficient or counter-productive TDORs. This was shown by depleting the major reductive TDOR thioredoxin A (TrxA) from the cytoplasm of cells producing *E. coli* PhoA, which resulted in the extracellular accumulation of active PhoA at elevated levels (Kouwen et al. 2008). Noteworthy though, the depletion of other (potential) cytoplasmic or membrane-associated reductive TDORs did not result in higher levels of active PhoA. The finding that depletion of TrxA did contribute to a more effective folding of PhoA was actually counter-intuitive since TrxA is a cytoplasmic protein while PhoA folding and disulfide bond formation take place after export from the cytoplasm at the trans side of the membrane. An explanation was found when the redox state of BdbD under conditions of TrxA limitation was examined (Kouwen et al. 2008). The depletion of TrxA was shown to result in relatively higher levels of oxidized BdbD. On the contrary, the absence of the quinone reductase BdbC resulted in high levels of reduced BdbD, which is consistent with the fact that mutation of *bdbC* resulted in impaired PhoA folding (Bolhuis et al. 1999b; Kouwen et al. 2008; Meima et al. 2002). A *bdbC* mutation was dominant over the effects of TrxA depletion. Taken together, these findings show that the cytoplasmic reductase TrxA has an impact on the redox state of extracytoplasmic BdbD in a BdbC-dependent manner. This implies that electron transfer from TrxA to BdbC/BdbD can occur either directly from TrxA to BdbC to BdbD, or indirectly through other as yet unidentified components. Consistent with this idea, recent studies indicate that TrxA is the primary electron donor to several

different TDORs that are active on the extracytoplasmic side of the *B. subtilis* membrane (Moller and Hederstedt 2008). Minimization of this electron transfer route by reduction of the cytoplasmic TrxA to levels that are sufficient to sustain wild-type growth and cell viability will thus result in relatively higher levels of BdbD proteins in an oxidized state, which is necessary for improved oxidation of substrate proteins with disulfide bonds. Possibly, further optimization of the oxidative properties of *B. subtilis* can be achieved once the cytoplasmic and extracytoplasmic TDOR networks and their essential contact points in the cytoplasmic membrane have been elucidated. This would allow a detailed fine-tuning of the electron transfer routes for optimized recycling of BdbD to the oxidized state, thereby facilitating fast and effective substrate oxidation.

### Overexpression of isomerases

In *E. coli*, disulfide isomerases contribute significantly to the final yield of correctly folded disulfide bond containing proteins, by reoxidizing any incorrectly formed disulfide bonds. In contrast to *E. coli*, disulfide isomerases, if at all present, have so far remained undetected in *B. subtilis* (Kouwen and van Dijn 2009). Clearly, *B. subtilis* lacks obvious homologues of *E. coli* isomerases, such as DsbC or DsbG. Nevertheless, *B. subtilis* does contain reductive TDORs in the cytoplasmic membrane, which have been shown to be involved in reducing apocytochrome *c* and spore cortex proteins (Moller and Hederstedt 2006; Erlendsson and Hederstedt 2002; Erlendsson et al. 2003). Interestingly, the central TDOR in this reductive system, CcdA, together with an associated TDOR (ResA or StoA), represents a functional and structural homologue of the *E. coli* DsbD TDOR, which transfers electrons to the periplasmic disulfide isomerase DsbC (Katzen et al. 2002). Previously, the homologue of another CcdA-associated TDOR, YneN (also known as CatA), was shown to have protein disulfide oxidoreductase-like activity in *Brevibacillus choshinensis* (*Bacillus brevis*). Incubation of nonnative human epidermal growth factor (hEGF) containing incorrect disulfide bonds with *B. choshinensis* cells overexpressing YneN, or coexpression of the two proteins, increased the final yield of active hEGF protein (Miyauchi et al. 1999; Tanaka et al. 2003, 2005). However, coexpression or deletion of the *B. subtilis* YneN together with the expression of *E. coli* PhoA by *B. subtilis* did not have any detectable effect on the yield of active PhoA (our unpublished observations). It thus remains to be shown whether *B. subtilis* contains a system for protein disulfide isomerization. An intriguing possibility is that the antagonistic effects of the reductive CcdA–ResA and oxidative BdbCD pathways serve in the isomerization of disulfide

bonds (Kouwen and van Dijl 2009). Conceivably, *B. subtilis* can achieve disulfide isomerization by bringing these pathways together, thereby overcoming the lack of more dedicated disulfide isomerases such as DsbC and DsbG of *E. coli*.

### Addition of redox active compounds to the growth medium

Instead of increasing the expression levels of oxidative TDORs, it may also be possible to increase their activity for substrate oxidation through enhanced reoxidation during catalysis. Whereas the reoxidation rate of the oxidative TDORs in *B. subtilis* seems to be determined by other TDORs and their connection with the electron transfer chain in the membrane, this does not seem to be the case in certain other Gram-positive bacteria, such as *S. aureus*. When expressed in *B. subtilis*, the activity of *S. aureus* DsbA was found to depend completely on the presence of redox active compounds in the growth medium (Kouwen et al. 2007). Thus, the DsbA protein can achieve reoxidation during catalysis by electron transfer to externally added electron acceptors such as cystine, the oxidized form of cysteine. This approach seemed an attractive option for modulating the oxidative properties of *B. subtilis* strains expressing the DsbA proteins of *S. aureus* or *S. carnosus*. We therefore tested its applicability by adding increased amounts of cystine to the growth media of *B. subtilis* cells expressing these DsbA proteins. Indeed, enhanced extracellular levels of folded and active *E. coli* PhoA were observed when DsbA expressing strains were grown in the presence of added cystine (Kouwen et al. 2008). It thus seems that the addition of redox active compounds to the growth medium is a realistic possibility to increase the oxidative properties of engineered *Bacillus* host strains for increased production of proteins with disulfide bonds.

### Outlook—combining the different approaches

The proof-of-principle for the concept that TDOR modulation can result in a significantly improved production of proteins with disulfide bonds, such as *E. coli* PhoA, has been obtained through the combined depletion of TrxA, production of staphylococcal DsbA, and addition of extracellular oxidative compounds that can accept electrons from DsbA. In this case, up to four-fold increased levels of active extracellular PhoA can be observed, indicating a significant improvement of posttranslocational folding and a concomitant reduction of posttranslocational PhoA degradation (Kouwen et al. 2008). Importantly, hardly any breakdown products of PhoA were observed under these

conditions, indicating that most of the PhoA was correctly folded to a protease-resistant conformation. This indicates that it should be possible to challenge and employ the combined strategies described in this review for the production of other useful proteins that contain disulfide bonds as summarized in Fig. 1.

Following the chain of events in the synthesis and secretion of proteins, we envisage several relevant and timely activities. In the first place, the cytoplasmic TDOR network and its impact on secreted disulfide bond-containing proteins must be disentangled. In addition to TrxA, there are at least seven other cytoplasmic TDORS that potentially have a role in cytoplasmic disulfide handling (YbdE, YdbP, YdfQ, YkuV, YosR, YtpP, and YusE; (Erlendsson et al. 2004; Kouwen et al. 2008; Kouwen and van Dijl 2009; Zhang et al. 2006)). While no role for these TDORS has been established as yet, they may impact on the production of proteins with disulfide bonds under particular conditions, for example when the level of TrxA is down-regulated. In this respect, it is noteworthy that TrxA was originally identified as an essential protein in *B. subtilis* (Kobayashi et al. 2003; Scharf et al. 1998). Recent studies however, have shown that TrxA is nonessential when the growth medium is supplemented with cysteine, methionine, and deoxyribonucleosides (Moller and Hederstedt 2008). This suggests that there is further room for TDOR modulation in engineered strains that lack TrxA. Like the cytoplasmic TDOR network, the membrane and transmembrane TDOR networks deserve further investigation in relation to improving the potential of *B. subtilis* for disulfide bond formation. Next to BdbBCD, there are at least five TDORS (BdbA, CcdA, ResA, StoA/SpoIVH, and YneN) that deserve closer investigations with respect to their possible positive or negative roles in extracytoplasmic disulfide bond formation (Erlendsson et al. 2004; Kouwen et al. 2008; Kouwen and van Dijl 2009; Zhang et al. 2006). Clearly, reducing the reductive effects of the CcdA–ResA pathway, which is known to be antagonistic to the BdbCD pathway (Erlendsson and Hederstedt 2002) may be a way to achieve further optimized reoxidation of BdbD during catalysis. Admittedly however, our attempts so far to achieve this goal have not been successful (unpublished observations), suggesting that this cannot be done in a very straightforward manner. Possibly, in combination with heterologous oxidases from Staphylococcus species, the engineering of membrane-associated TDORS of *B. subtilis* will yield applicable results. If so, further progress can be achieved by identifying optimal electron acceptors that can be added to the growth medium. Such growth medium additives would, of course, need to be nontoxic and cheap. Since these are all achievable goals, we are convinced that a combined application and fine-tuning of the strategies described in this review will soon allow

optimal use of the high production potential of *B. subtilis* for high-value proteins with disulfide bonds.

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