

Modulation of Thiol-Disulfide Oxidoreductases for Increased Production of Disulfide-Bond-Containing Proteins in *Bacillus subtilis*[∇]

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Disulfide bonds are important for the correct folding, structural integrity, and activity of many biotechnologically relevant proteins. For synthesis and subsequent secretion of these proteins in bacteria, such as the well-known “cell factory” *Bacillus subtilis*, it is often the correct formation of disulfide bonds that is the greatest bottleneck. Degradation of inefficiently or incorrectly oxidized proteins and the requirement for costly and time-consuming reduction and oxidation steps in the downstream processing of the proteins still are major limitations for full exploitation of *B. subtilis* for biopharmaceutical production. Therefore, the present study was aimed at developing a novel *in vivo* strategy for improved production of secreted disulfide-bond-containing proteins. Three approaches were tested: depletion of the major cytoplasmic reductase TrxA; introduction of the heterologous oxidase DsbA from *Staphylococcus carnosus*; and addition of redox-active compounds to the growth medium. As shown using the disulfide-bond-containing molecule *Escherichia coli* PhoA as a model protein, combined use of these three approaches resulted in secretion of amounts of active PhoA that were ~3.5-fold larger than the amounts secreted by the parental strain *B. subtilis* 168. Our findings indicate that *Bacillus* strains with improved oxidizing properties can be engineered for biotechnological production of heterologous high-value proteins containing disulfide bonds.

Disulfide bonds play pivotal roles in the folding, structural integrity, and activity of numerous proteins found in nature. Without the correct thiol oxidation that links their cysteines to disulfide bonds, these proteins are neither fully stable nor active (6, 32). Importantly, many eukaryotic proteins of biopharmaceutical interest contain multiple disulfide bonds. As the demand for such proteins is growing, there is a clear need for cost-effective and high-quality production platforms. Bacterial cell factories, such as *Bacillus subtilis*, can fulfill these criteria very well, but so far their use in biopharmaceutical production has been limited by their relatively poor performance in the production of proteins with disulfide bonds (5, 33, 47).

The formation of disulfide bonds can occur spontaneously, but this process is slow and nonspecific (2). For this reason, so-called thiol-disulfide oxidoreductases (TDORs) that catalyze the formation (oxidation) of disulfide bonds *in vivo* have evolved. Notably, the TDORs also include enzymes that break (reduce) or isomerize disulfide bonds. Cytoplasmic TDORs generally function as reductases, while the extracytoplasmic equivalents are oxidases or isomerases (9, 32, 39). The enzyme-dependent formation of disulfide bonds is a prime reason why proteins containing such bonds are still difficult to produce in bacterial cell factories. Slow and/or nonspecific oxidation of

overproduced proteins often results in slow and incorrect folding of these proteins, making them vulnerable to proteolytic degradation (33). For this reason, we addressed the question of how to increase the oxidative power of *Bacillus* species during protein production in order to produce disulfide-bond-containing proteins more efficiently.

The gram-positive bacterium *B. subtilis* is a preferred organism for secretory protein production, because proteins transported across the cytoplasmic membrane are directly released into the growth medium (42, 43). Other advantages of *B. subtilis* are its high genetic amenability and the fact that it is generally recognized as safe (5, 16, 21, 50). Previous studies on disulfide bond formation in *B. subtilis* have shown that this organism contains at least four TDORs with presumed oxidase activity. These proteins were designated Bdb (*bacillus disulfide bond*) proteins and annotated BdbA to BdbD (7, 8, 25). Especially BdbC and BdbD have major roles in the folding of a secreted heterologous model protein by *B. subtilis*, the PhoA alkaline phosphatase of *Escherichia coli* (4, 7, 18, 25). This is related to the fact that *E. coli* PhoA contains two disulfide bonds that are indispensable for both the enzymatic activity and stability of the protein (38). BdbC and BdbD seem to cooperate as a redox pair in an oxidation pathway in *B. subtilis* (33), similar to the DsbA-DsbB redox pair of *E. coli* (15, 30, 31). It has been proposed that BdbD is the major oxidase that facilitates the formation of disulfide bonds in secretory proteins. Upon oxidation of a substrate, reduced BdbD is reoxidized by the quinone reductase homologue BdbC. To become reoxidized for the next catalytic reaction, BdbC donates its electrons to quinones in the electron transport chain.

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TABLE 1. Plasmids and strains used in this study

Plasmid or strain	Relevant properties	Reference or source
Plasmids		
pMutin2mcs	pBR322-based integration vector for <i>B. subtilis</i> ; contains a multiple cloning site downstream of the P _{spac} promoter and a promoterless <i>lacZ</i> gene; Ap ^r Em ^r	44
pUC18	Ap ^r	45
pDG1727	pMTL23 derivative; Ap ^r Sp ^r	12
pTOPO	pCR-Blunt II-TOPO vector; Km ^r	Invitrogen Life Technologies
pXTC	pX derivative containing a tetracycline resistance marker instead of a chloramphenicol resistance marker; Ap ^r Tc ^r	7
pXTC-ScdsbA	pXTC carrying <i>dsbA</i> of <i>S. carnosus</i> fused to the signal sequence and ribosome binding site of <i>mntA</i> of <i>B. subtilis</i> under transcriptional control of the <i>xylA</i> promoter; Ap ^r Tc ^r	This study
pXTC-SadsbA	pXTC carrying <i>dsbA</i> of <i>S. aureus</i> fused to the signal sequence and ribosome binding site of <i>mntA</i> of <i>B. subtilis</i> under transcriptional control of the <i>xylA</i> promoter; Ap ^r Tc ^r	18
pPSPhoA5	Plasmid carrying the <i>E. coli</i> <i>phoA</i> gene fused to the pre-pro region of the lipase gene from <i>S. hyicus</i> ; Cm ^r	7
pKTH10	Encodes AmyQ of <i>B. amyloliquefaciens</i> ; Km ^r	29
<i>B. subtilis</i> strains		
168	<i>trpC2</i>	21
<i>trxA</i>	Derivative of 168; contains an integrated copy of plasmid pMutin2 in the <i>trxA</i> gene; <i>trxA-lacZ</i> Em ^r P _{spac} - <i>trxA</i> ; IPTG dependent	34
<i>ybdE</i>	Derivative of 168; contains an integrated copy of plasmid pMutin2 in the <i>ybdE</i> gene; <i>ybdE-lacZ</i>	16
<i>ydfQ</i>	Derivative of 168; contains an integrated copy of plasmid pMutin2 in the <i>ydfQ</i> gene; <i>ydfQ-lacZ</i>	16
<i>ydbP</i>	Derivative of 168; contains an integrated copy of plasmid pMutin2 in the <i>ydbP</i> gene; <i>ydbP-lacZ</i>	16
<i>ykuV</i>	<i>trpC2 ykuV::Sp^r</i> (MunI-NgoMIV integration)	This study
<i>ytpP</i>	<i>trpC2 ytpP::Sp^r</i> (BsaAI integration)	This study
<i>yusE</i>	<i>trpC2 yusE::Sp^r</i> (BclI-BclI integration)	This study
<i>stoA</i> (<i>ykvV</i>)	Derivative of 168; contains an integrated copy of plasmid pMutin2 in the <i>stoA</i> gene; <i>stoA-lacZ</i>	16
<i>yneN</i>	Derivative of 168; contains an integrated copy of plasmid pMutin2 in the <i>yneN</i> gene; <i>yneN-lacZ</i>	16
SPβ	<i>trpC2</i> ; ΔSPβ; sublancin 168 sensitive; laboratory name, CBB312	8
<i>bdbC</i>	<i>trpC2 bdbC::Km^r</i>	8
<i>trxA bdbC</i>	<i>trpC2 trxA::pMutin2 bdbC::Km^r</i> ; IPTG dependent	This study
X-ScdsbA	<i>trpC2; amyE::XTC-ScdsbA</i>	This study
<i>trxA</i> X-ScdsbA	<i>trpC2; P_{spac}-trxA; amyE::XTC-ScdsbA</i>	This study
X-SadsbA	<i>trpC2; amyE::XTC-SadsbA</i> ; also known as XdsbA	18
<i>trxA</i> X-SadsbA	<i>trpC2; P_{spac}-trxA; amyE::XTC-SadsbA</i>	This study
<i>S. carnosus</i> TM300	Source of <i>dsbA</i> gene used in the present study	35
<i>S. aureus</i> RN4220	Restriction-deficient derivative of NCTC 8325, cured of all known prophages	19
<i>E. coli</i> DH5α	<i>supE44 hsdR17 recA1 gyrA96 thi-1 relA1</i>	13

Despite the presence of BdbC and BdbD, the total oxidative power of *B. subtilis* is rather limited (33). To increase the thiol-oxidizing capacity, attempts to overexpress individual Bdb proteins or combinations of several Bdb proteins were made. However, this did not result in significantly improved production of proteins with disulfide bonds (7, 8, 25; our unpublished observations). Therefore, in the present study we searched for alternative strategies that could increase the thiol-oxidizing power of *B. subtilis*. Decreasing the levels of a cytoplasmic TDOR with a reductase, TrxA, resulted in increased yields of secreted *E. coli* PhoA. The yields of this protein could be further improved by introduction of staphylococcal DsbA, which is known to be one of the strongest bacterial thiol oxidases (10). Additional improvement was obtained by including redox-active compounds in the growth medium of DsbA-producing strains. Together, our observations provide proof of principle that *Bacillus* strains with optimized oxidative properties can be engineered for the production proteins with disulfide bonds.

MATERIALS AND METHODS

Sequence comparisons and predictions. Thioredoxin amino acid sequences of *B. subtilis* or *E. coli* were used for a BLASTP search of the SubtiList *B. subtilis* sequence database (<http://genolist.pasteur.fr/SubtiList/>) with the algorithms described by Altschul et al. (1). An arbitrary E value less than or equal to 10⁻³ was used to limit the number of sequences for further analysis. After the first run, using either the *B. subtilis* TrxA or *E. coli* TrxA or TrxC sequence as the query sequence, the sequences found were used in turn as query sequences for a BLASTP search of the SubtiList database. Multiple alignments were constructed using ClustalX 1.81 (41). Various protein weight matrices were used with pairwise and multiple alignment parameters. The best alignment, defined by the lowest score, was obtained with the PAM350 matrix (gap opening, 10) for both pairwise (gap extension, 0.1) and multiple (gap extension, 0.2) alignment parameters. The presence of possible signal peptidase I cleavage sites was analyzed using the algorithms described by Nielsen et al. (28). The presence of possible transmembrane segments was analyzed using the algorithms described by Krogh et al. (20).

Bacterial strains, plasmids, and growth media. Bacterial strains and plasmids used in this study are listed in Table 1. Strains were grown with agitation at 37°C in Luria-Bertani (LB) medium consisting of 1% tryptone, 0.5% yeast extract, and 1% NaCl (pH 7.4). When necessary, media were supplemented with antibiotics at the following concentrations: ampicillin, 100 µg/ml (*E. coli*); chloramphenicol,

5 µg/ml (*B. subtilis*); erythromycin, 50 µg/ml (*E. coli*) or 2 µg/ml (*B. subtilis*); kanamycin, 20 µg/ml (*E. coli* and *B. subtilis*); spectinomycin, 100 µg/ml (*E. coli* and *B. subtilis*); and tetracycline, 10 µg/ml (*B. subtilis*). To visualize α -amylase activity (encoded by the *amyE* gene), LB medium plates were supplemented with 1% starch. When required, cystine or cysteine was added to the growth media at a concentration of 100 µg/ml.

Construction of mutant strains. DNA techniques were performed as previously described (18). All TDOR mutant strains were based on *B. subtilis* 168. Strain *ItrxA bdbC* was obtained by transformation of *B. subtilis* *ItrxA* with chromosomal DNA of a *bdbC::Km^r* mutant and subsequent selection of isopropyl- β -D-thiogalactopyranoside (IPTG)-dependent and kanamycin-resistant transformants. It should be noted that *trxA* is an essential gene (34). TrxA can be depleted in *ItrxA* mutant strains because the *trxA* gene of these strains is under transcriptional control of the IPTG-inducible *P_{spac}* promoter (37).

The *B. subtilis* *yusE* strain was constructed as follows. A 1,384-bp DNA fragment starting in the *yusG* gene and ending in the *yusD* gene was amplified using primers GGGGAATTCATAAGACAGCCGATGTGGTC and GGGGGATCCGTAGAATAGCTCGCGAATG, which contain EcoRI and BamHI restriction sites, respectively. This fragment was subsequently cleaved with EcoRI and BamHI and ligated to EcoRI-BamHI-cleaved pUC18. The spectinomycin resistance cassette from plasmid pDG1727 was excised with BamHI and XhoII and used to replace an internal BclI fragment of the pUC18-borne copy of *yusE*. The resulting plasmid, pUSE-Spec, was used to transform *B. subtilis* 168. As shown by PCR, the *yusE* gene of all spectinomycin-resistant transformants tested (*B. subtilis* *yusE*) was disrupted with the spectinomycin resistance cassette of pUSE-Spec as a result of a double-crossover recombination event.

To construct the *B. subtilis* *ykuV* strain, a 1,516-bp DNA fragment starting in the *ykuU* gene and ending in the *rok* gene was amplified using primers GGGGATCCCGCAAAGTAAGTCTTGAGG and GGGGTCGACATTGTCTTAACCGCAAGCGC, which contain BamHI and SalI restriction sites, respectively. The amplified fragment was cleaved with BamHI and SalI and ligated to BamHI-SalI-cleaved pUC18. The spectinomycin resistance cassette from pDG1727 was excised using EcoRI and BsaWI and used to replace an internal MunI-NgoMIV fragment of the pUC18-borne copy of *ykuV*. The resulting plasmid, pKUV-Spec, was used to transform *B. subtilis* 168. As shown by PCR, the *ykuV* gene of all spectinomycin-resistant transformants tested (*B. subtilis* *ykuV*) was disrupted with the spectinomycin resistance cassette of pKUV-Spec as a result of a double-crossover recombination event.

The *B. subtilis* *ytpP* strain was constructed as follows. A 1,386-bp DNA fragment starting in the *ytpP* gene and ending in the *ytpQ* gene was amplified using primers GGGGGTACCCATTGCCGTGTTCCACTGT and GGGCTGCAGGGCAACCGTATCTCTTTGA, which contain KpnI and PstI restriction sites, respectively. The amplified fragment was cleaved with KpnI and PstI and ligated to KpnI-PstI-cleaved pUC18. The spectinomycin resistance cassette from pDG1727 was excised with HincII and StuI and cloned in the unique BsaAI restriction site in the middle of the *ytpP* gene. The resulting plasmid, pTPP-Spec, was used to transform *B. subtilis* 168. As shown by PCR, the *ytpP* gene of all spectinomycin-resistant transformants tested (*B. subtilis* *ytpP*) was disrupted with the spectinomycin resistance cassette of pTPP-Spec as a result of a double-crossover recombination event.

Single *ybdE*, *ydbP*, *ydfQ*, *stoA* (*ykvV*), *yneN*, and *yusE* mutants and a strain that lacks the SP β prophage carrying the *bdbA* and *yosR* genes were obtained from the BSFA or JAFAN strain collections (16). The correct chromosomal integration of plasmids or antibiotic resistance markers was verified by PCR.

To overexpress the oxidative TDOR DsbA from *Staphylococcus carnosus*, the pXTC expression system was used (7, 18). First, *dsbA* of *S. carnosus* was fused by PCR to the ribosome binding site and signal sequence of *mntA* of *B. subtilis*. For this purpose, a 92-bp fragment containing the ribosome binding site and signal sequence of *mntA* of *B. subtilis* was amplified using primers pXTC_MntA_F (GGGGGACTAGTAAGAGGAGGAGAAATATGAGACAA) and pXTC_MntA_Scar_R (TTTTTGTGAGCATCCCGTTAAAGCAAGGTCGC). A second PCR fragment, which was 566 bp long, resulted from amplification of the *dsbA* gene of *S. carnosus* using primers pXTC_Scar_F (TTAACGGGATGCTCAAAAAGACCCTGATTTA) and pXTC_Scar_R (GGGGGGGATCCTTA TTTTCTAGTAAATCTTTATATCTT). The two resulting PCR products had a 21-nucleotide overlap. Using this overlap, the two different fragments could be fused using 10 PCR cycles without added primers. Next, the fused product was PCR amplified with primers pXTC_MntA_F and pXTC_Scar_R using 20 additional PCR cycles. The resulting 637-bp product was cloned into pTOPO (Invitrogen Life Technologies). After sequence verification, the fused *dsbA* gene was excised from this plasmid with BamHI and SpeI and ligated into the same restriction sites of plasmid pXTC downstream of *P_{xytA}*, resulting in plasmid pXTC-ScdsbA.

Plasmid pXTC-ScdsbA was used to integrate the *P_{xytA}* *ScdsbA* cassette together with the tetracycline resistance marker of pXTC (referred to below as the XTC-*ScdsbA* cassette) into the *amyE* locus of *B. subtilis* 168 and *B. subtilis* *ItrxA* by double-crossover recombination. Selection for tetracycline resistance and screening for an AmyE-negative phenotype on starch-containing plates enabled us to obtain *B. subtilis* strains X-ScdsbA and *ItrxA* X-ScdsbA, respectively.

Plasmid pXTC-SadsbA (also designated pXTC-dsbA) was obtained from previous studies (18). This plasmid carries the *Staphylococcus aureus* *dsbA* gene fused to the ribosome binding site and signal sequence of *mntA* of *B. subtilis* under transcriptional control of the *xytA* promoter (*P_{xytA}*). pXTC-SadsbA was used to integrate the XTC-SadsbA cassette into the *amyE* loci of *B. subtilis* 168 and *B. subtilis* *ItrxA* by double-crossover recombination. Selection for tetracycline resistance and screening for an AmyE-negative phenotype on starch-containing plates yielded *B. subtilis* strains X-SadsbA and *ItrxA* X-SadsbA, respectively.

Alkaline phosphatase activity assay. The alkaline phosphatase assay was performed with growth medium samples as previously described (7). PhoA activity was expressed in U/ml/unit of optical density at 600 nm. All experiments were repeated at least three times.

SDS-PAGE and Western blotting. The presence of TrxA, PhoA, AmyQ, BdbD, and DsbA in growth medium samples and/or cell lysates was detected by Western blotting. Cellular or secreted proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (using precast Nu-PAGE gels from Invitrogen), and proteins were then semidry blotted (1.25 h at 100 mA per gel) onto a nitrocellulose membrane (Protran; Schleicher & Schuell). Subsequently, the TrxA, PhoA, AmyQ, BdbD, and DsbA proteins were detected with specific polyclonal antibodies raised in rabbits (Eurogentec). These antibodies were detected either with horseradish peroxidase-conjugated immunoglobulin G secondary antibodies and the Super Signal West Dura extended duration substrate (Pierce) in combination with the ChemiGenius XE bioimaging system (Syngene) or with fluorescent immunoglobulin G secondary antibodies (IRDye 800 CW goat anti-rabbit antibodies from LiCor Biosciences) in combination with the Odyssey infrared imaging system (LiCor Biosciences). In the latter case, fluorescence at 800 nm was recorded. Densitometric image analysis to quantify relative amounts of proteins as detected by SDS-PAGE and Western blotting was performed with the program ImageJ (<http://rsbweb.nih.gov/ij/>).

AMS labeling of reduced proteins. The cellular levels of oxidized and reduced BdbD were monitored by labeling proteins with 4-acetamido-4'-maleimidylstilbene-2,2'-disulfonate (AMS) (Molecular Probes), essentially as described by Kobayashi et al. (17). Cells were grown in LB medium, collected by centrifugation, and resuspended in 25 mM Tris-HCl, 10 mM EDTA, 0.5 M glycerol, 0.25 mg/ml lysozyme (pH 8.0), 15 mM AMS. After 30 min of incubation at 37°C, the samples were mixed with a loading buffer for SDS-PAGE that lacked reducing agents such as β -mercaptoethanol or 1,4-dithiothreitol. Interestingly, when we tested various protocols for trichloroacetic acid precipitation, which was used by Kobayashi et al. (17) to quench the redox state of DsbA of *E. coli*, we determined that in the case of *B. subtilis* this step was not needed. Colabeling during cell lysis was sufficient to distinguish between the different redox states of BdbD. Notably, AMS binds covalently only to free thiol groups in reduced BdbD molecules. After boiling of the samples for 7 min, BdbD molecules with or without bound AMS were separated by nonreducing SDS-PAGE. Finally, the different BdbD species were visualized by Western blotting and immunodetection with specific polyclonal antibodies. The relative amounts of BdbD species with or without bound AMS were determined using the ChemiGenius XE² bioimaging system and the GeneTools Analysis software package (SynGene). All experiments were repeated at least four times.

RESULTS

Cellular levels of TrxA determine the level of PhoA secretion. In the first attempt to increase the oxidative power of *B. subtilis* for more efficient secretion of disulfide-bond-containing proteins, we focused on potentially reductive systems of this organism. Our assumption was that deletion or decreased expression of the corresponding genes would make *B. subtilis* less reductive. In turn, this might improve the folding of proteins with disulfide bonds. For this purpose, three possible systems from other organisms could be excluded a priori. First, *B. subtilis* lacks homologues of the enzymes that are required for the synthesis of glutathione in gram-negative bacteria and

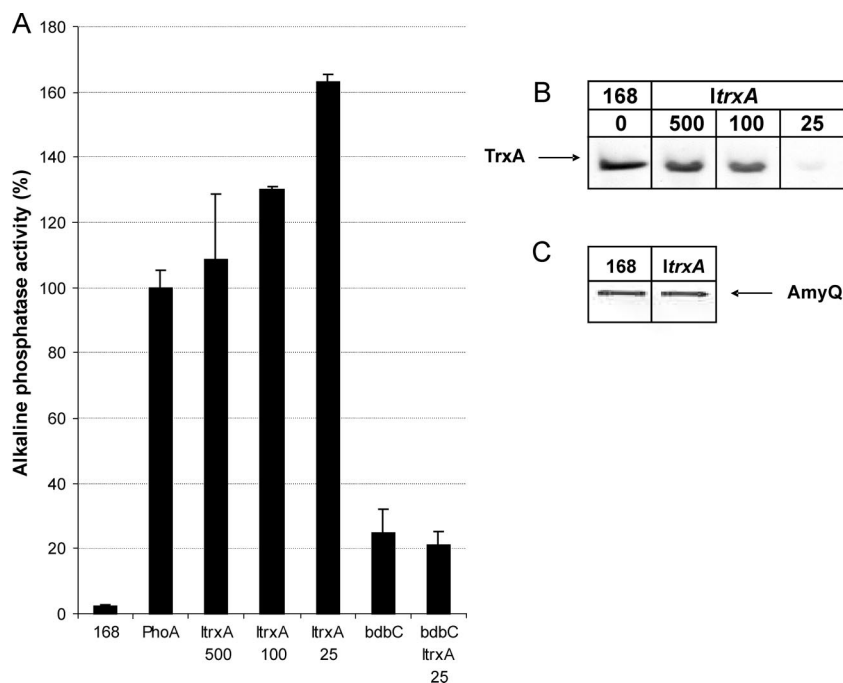


FIG. 1. BdbC-dependent secretion of PhoA by *B. subtilis* *ItrxA*. (A) Alkaline phosphatase activity was assayed using the growth media of *B. subtilis* 168(pPSPHoA5) (PhoA), *B. subtilis* *ItrxA*(pPSPHoA5) grown in the presence of 500, 100, or 25 μ M IPTG (*ItrxA* 500, *ItrxA* 100, and *ItrxA* 25, respectively), *B. subtilis* *bdbC*(pPSPHoA5) (*bdbC*), and *B. subtilis* *ItrxA bdbC*(pPSPHoA5) grown in the presence of 25 μ M IPTG (*bdbC ItrxA* 25). *B. subtilis* 168 (168) was used as a negative control. All strains were grown overnight in LB medium at 37°C. (B) Western blot analysis of TrxA production in *B. subtilis* *ItrxA* and parental strain 168. Cells were grown overnight at 37°C in LB medium. Cellular proteins were separated by SDS-PAGE, and TrxA was visualized by Western blotting. The IPTG concentrations in the medium (0, 25, 100, and 500 μ M) are indicated above the blots. (C) Secretion of AmyQ by *B. subtilis* *ItrxA*. Cells of *B. subtilis* *ItrxA*(pKTH10) (*ItrxA*) and *B. subtilis* 168(pKTH10) (168) were grown in LB medium at 37°C in the presence of 25 μ M IPTG. The presence of AmyQ in growth medium fractions was analyzed by SDS-PAGE and Western blotting.

eukaryotes; second, *B. subtilis* lacks the reducing agent mycothiol, which can be found in the cytoplasm of *Streptomyces* species and fungi (27); and third, *B. subtilis* lacks the proteins involved in isomerization pathways, like the pathway found in *E. coli*. Thus, we focused on thioredoxins and thioredoxin-like proteins. BLASTP searches revealed that the *B. subtilis* 168 genome encodes 12 thioredoxin(-like) proteins (data not shown). These proteins include four membrane proteins (BdbA, ResA, StoA/SpoIVH, and YneN) and eight predicted cytoplasmic proteins (TrxA, YbdE, YdbP, YdfQ, YkuV, YosR, YtpP, and YuseE), which are very similar to known thioredoxins and/or to ResA (11, 51).

To test possible effects of the (potential) TDORs mentioned above on the secretion of a disulfide-bond-containing protein, *trxA*, *ybdE*, *ydbP*, *ydfQ*, *ykuV*, *stoA* (*ykvV* or *spoIVH*), *yneN*, *ytpP*, and *yusE* single mutants and a strain that lacks the SP β prophage carrying the *bdbA* and *yosR* genes were transformed with plasmid pPSPHoA5. This plasmid specifies a fusion between the pre-pro region of a lipase from *Staphylococcus hyicus* and the mature PhoA protein (7). *E. coli* PhoA is a sensitive reporter for TDOR activity in *B. subtilis*, because this protein with two disulfide bonds requires oxidative TDORs for folding into a protease-resistant conformation (4, 25). Especially in the absence of BdbC and/or BdbD, unfolded PhoA is readily degraded in the highly proteolytic environments of the *B. subtilis* cell wall and growth medium (33). This basically provides an in vivo protease protection assay for probing the folding efficiency

of secreted PhoA in relation to oxidative TDOR activity. Interestingly, none of the strains lacking an intact *bdbA*, *stoA*, *ybdE*, *ydbP*, *ydfQ*, *ykuV*, *yneN*, *yosR*, *ytpP*, or *yusE* gene were significantly affected in terms of secretion of active PhoA of *E. coli* (data not shown). Unexpectedly, however, depletion of TrxA resulted in increased levels of secretion of PhoA (Fig. 1A). It should be noted that this result could be obtained only with a conditional *trxA* mutant strain (*ItrxA*), because TrxA is essential for the growth and viability of *B. subtilis* (16, 34). In the *ItrxA* strain, the *trxA* promoter region (P_{trxA}) is replaced by the IPTG-dependent P_{spac} promoter. The growth of *B. subtilis* *ItrxA* on plates or in broth is strictly IPTG dependent, unlike the growth of parental strain 168. When cells of *B. subtilis* *ItrxA* are grown in LB broth, wild-type growth rates are observed with an IPTG concentration of 25 μ M or higher (37).

To investigate the importance of the cytoplasmic TrxA level for secretion of active PhoA, the *ItrxA* mutant strain transformed with plasmid pPSPHoA5 was analyzed in detail. The level of secretion of PhoA into the growth medium was determined by alkaline phosphatase activity assays. For this purpose, cells were grown in LB medium supplemented with 25, 100, or 500 μ M IPTG. Interestingly, the results showed that, compared to parental strain 168, the *ItrxA* strain secreted at least 1.5-fold more active PhoA when it was grown in the presence of 25 μ M IPTG (Fig. 1A). Under these conditions, cellular TrxA was barely detectable by Western blotting (Fig. 1B). The level of secreted PhoA was similar to the levels

observed for the parental strain when *B. subtilis ItrxA* was grown in the presence of 500 μ M IPTG (Fig. 1A), which resulted in wild-type levels of cellular TrxA (Fig. 1B). In contrast, PhoA secretion by the parental strain 168 was independent of the IPTG concentration in the growth medium, and the presence of IPTG (25 to 500 μ M) in the growth medium had no detectable influence on the TrxA levels in this strain (not shown). These observations imply that, for the range of IPTG concentrations tested, the amount of active PhoA secreted by the *ItrxA* mutant is inversely proportional to the amount of TrxA in the cells. Notably, TrxA depletion in the *ItrxA* strain did not influence the yields of the secreted α -amylase AmyQ of *Bacillus amyloliquefaciens*, which lacks disulfide bonds. This could be demonstrated by transformation of *B. subtilis ItrxA* with plasmid pKTH10, which encodes AmyQ. Densitometric image analysis of the secreted AmyQ protein bands shown in Fig. 1C revealed that the growth media of the *ItrxA* mutant (25 μ M IPTG) and parental strain 168 contained comparable amounts of AmyQ. These findings suggest that the improved PhoA secretion by the *ItrxA* mutant strain is not due to generally improved synthesis or secretion of proteins but rather to improved posttranslocational folding resulting in protease resistance of the mature PhoA protein. This view was confirmed by the observation that the extracellular proteome of the *ItrxA* mutant strain grown in the presence of 25 μ M IPTG was indistinguishable from the extracellular proteome of parental strain 168 (H. Antelmann and J. Y. Dubois, data not shown).

The observed increase in active PhoA secretion by the *ItrxA* mutant strain grown in the presence of 25 μ M IPTG raised the question of whether this increase required the activity of BdbC. To answer this question, a pPSPHoA5-containing *ItrxA bdbC* double-mutant strain was constructed. Importantly, this double mutant displayed IPTG-dependent growth, showing that the *bdbC* mutation did not suppress the *ItrxA* mutation. As shown in Fig. 1A, PhoA secretion remained strongly BdbC dependent irrespective of the presence of the *ItrxA* mutation. Furthermore, the secretion of PhoA by the *ItrxA bdbC* mutant did not vary when different amounts of IPTG were present in the growth medium (data not shown). These findings suggest that the BdbCD-dependent thiol oxidation pathway (18) is also required for PhoA folding under TrxA depletion conditions.

Cellular levels of TrxA and BdbC influence the redox state of BdbD. To test whether the presence of TrxA can have an impact on the activity of the BdbCD thiol oxidation pathway, we verified the redox state of BdbD with the thiol-specific cross-linking reagent AMS. Due to the molecular mass of AMS, cross-linking of this reagent to reduced cysteine residues in a protein results in a significant reduction in the mobility of the protein during SDS-PAGE (17). In this respect, it is important to note that BdbD contains only two cysteine residues, which are part of the CXXC active site. To study the redox state of BdbD, overnight cultures were used to prepare fresh lysates from the *ItrxA* strain (25 μ M IPTG), the *bdbC* single mutant, the *ItrxA bdbC* double mutant, or parental strain 168. Importantly, the lysates were prepared either in the presence or in the absence of 15 mM AMS. Next, AMS binding to BdbD was analyzed by SDS-PAGE and Western blotting using BdbD-specific antibodies. As shown in Fig. 2, somewhat less than 50% of the BdbD molecules of parental strain 168 were labeled with AMS. By contrast, the ratio of reduced BdbD

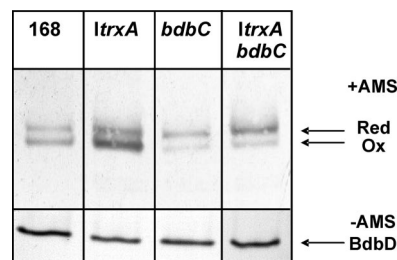


FIG. 2. Redox state of BdbD. Cells of the *B. subtilis ItrxA*, *bdbC*, or *ItrxA bdbC* strain or parental strain 168 were grown overnight in LB medium at 37°C in the presence of 25 μ M IPTG. Cellular proteins were separated by SDS-PAGE, and BdbD was visualized by Western blotting. (Upper panel) Cell extracts prepared in the presence of 15 mM AMS (+AMS). (Lower panel) Cell extracts prepared in the absence of AMS (-AMS). The positions of reduced (Red) and oxidized (Ox) BdbD are indicated on the right.

molecules (i.e., molecules labeled with AMS) to oxidized BdbD molecules (i.e., molecules not labeled with AMS) was significantly shifted toward the oxidized form in the *ItrxA* strain, whereas a significant shift toward the reduced BdbD species was observed in the *bdbC* and *ItrxA bdbC* mutant strains. These differences were observed with both exponentially and postexponentially growing cells of *B. subtilis* 168 and the *ItrxA* and *bdbC* strains (Fig. 2 and results not shown). These findings indicate that significantly more BdbD molecules were oxidized in the *ItrxA* strain than in the parental strain and, conversely, that the *bdbC* mutation resulted in lower numbers of oxidized BdbD molecules. Furthermore, the control experiments performed with lysates of the strains prepared in the absence of AMS showed that BdbD migrated as a single band (Fig. 2). Together, these findings indicate that the cytoplasmic TrxA levels impact the redox state of BdbD in a BdbC-dependent manner.

Expression of DsbA increases PhoA secretion. An potential alternative approach for increasing the capacity of *B. subtilis* for thiol oxidation is the overproduction of known thiol oxidases. However, our attempts to increase the levels of BdbC and BdbD have not been successful thus far (data not shown). Therefore, we investigated the possibility of expressing heterologous oxidases. For this purpose, we capitalized on our previous studies with the major oxidase DsbA of *Staphylococcus aureus* (referred to below as SaDsbA) (18). This homologue of *B. subtilis* BdbD was able to complement the loss of both BdbC and BdbD for secretion of active PhoA. Moreover, when SaDsbA was expressed in parental strain 168, an approximately 1.5- to 2.0-fold increase in the secretion of active *E. coli* PhoA was observed, which was similar to the increase observed when TrxA was depleted, as described above. However, since *S. aureus* is known to be a dangerous pathogen (24, 36), the potential use of SaDsbA for biotechnological purposes is limited. For this reason, we searched for a DsbA protein in a nonpathogenic close relative of *S. aureus*. The best source for a *dsbA* gene turned out to be *S. carnosus*, which is well known because of its use as a starter in the fermentation of cheese and dry sausage. Accordingly, this organism has the generally recognized as safe status. Therefore, *B. subtilis* strains containing genes that originated from this staphylococcal species should be more generally acceptable for industrial applications.

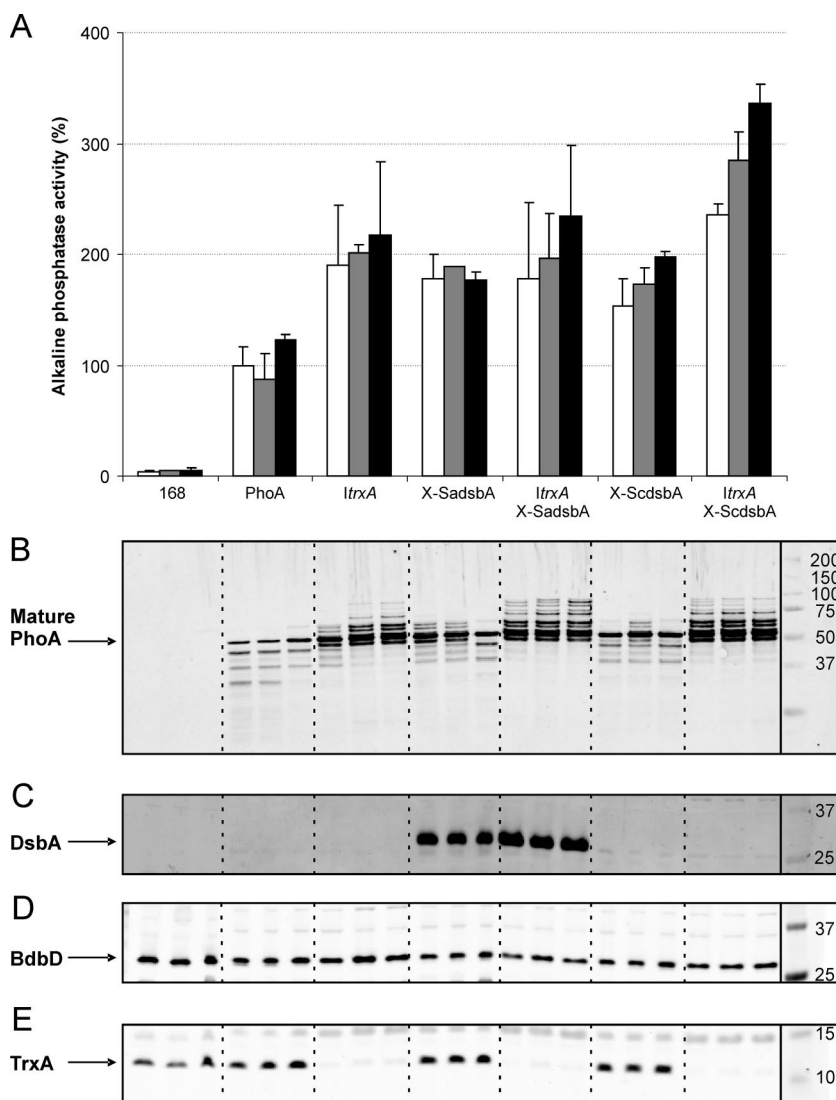


FIG. 3. Increased production of *E. coli* PhoA by engineered *B. subtilis* strains. The *B. subtilis* ItrxA, X-SadsbA, ItrxA X-SadsbA, X-ScdsbA, and ItrxA X-ScdsbA strains and parental strain 168 (PhoA) were transformed with pSPHoA5 for *E. coli* PhoA production. All mutant strains and parental strain 168 (no PhoA production) were grown overnight in LB medium containing 0.5% xylose and 25 μ M IPTG (open bars) and additional 100 μ g/ml cystine (gray bars) or cysteine (black bars). Next, growth medium samples were removed and used for alkaline phosphatase activity assays (A), as well as SDS-PAGE and Western blotting with specific antibodies against PhoA (B). (C to E) Lysates of cells from cultures corresponding to the samples used for panels A and B were analyzed by Western blotting with specific antibodies against *S. aureus* DsbA (C), BdbD (D), or TrxA (E). PhoA activity was expressed in U/ml/unit of optical density at 600 nm relative to the PhoA activity of the parental strain expressing PhoA, which was defined as 100% (6.0 U/ml/unit of optical density at 600 nm). The arrow in panel B indicates the position of mature PhoA. Bands with higher mobility on SDS-PAGE gels are breakdown products of PhoA, and bands with lower mobility are unprocessed forms of proPhoA. The arrows in panels C, D, and E indicate the positions of DsbA, BdbD, and TrxA, respectively. Note that the antibody against *S. aureus* DsbA is cross-reactive with *S. carnosus* DsbA. The positions of molecular weight markers are indicated on the right.

An *S. carnosus* homologue of *S. aureus* DsbA (referred to below as ScDsbA) was identified by BLASTP searches (51% identical amino acids) using the sequenced genome of *S. carnosus* strain TM300 (R. Rosenstein and F. Götz, personal communication). Like SaDsbA, the ScDsbA protein is homologous to *B. subtilis* BdbD (36% identical amino acids overall). In order to express *S. carnosus* ScDsbA in *B. subtilis*, the same xylose-inducible pXTC expression system that was used to express *S. aureus* SaDsbA (18) was used. For this purpose, we fused the sequence encoding the mature ScDsbA lipoprotein to the ribosome binding site and signal sequence of the *B.*

subtilis *mntA* gene, which codes for an abundantly expressed lipoprotein in this organism (3). Upon integration of the XTC-ScdsbA cassette containing the hybrid ScdsbA gene into the *B. subtilis* 168 chromosome, xylose-inducible expression of cell-associated ScDsbA was obtained (data not shown) (below the strains containing this cassette are referred to as X-ScdsbA strains). For immunodetection of ScDsbA we used antibodies against SaDsbA that also showed cross-reactivity with ScDsbA (Fig. 3). As expected, the cellular levels of ScDsbA depended on the amount of xylose added to the growth medium. The highest levels of cellular ScDsbA were observed when the

X-ScdsbA cells were induced with a concentration of xylose of 1.0% or higher, whereas no ScDsbA was detectable when cells were grown in the absence of xylose (data not shown).

To assess the effect of ScDsbA expression on the extracellular accumulation of active *E. coli* PhoA, *B. subtilis* strain X-ScdsbA was transformed with plasmid pPSPHoA5. For comparison, PhoA production by the single-mutant strains X-SadsbA and *Itrx4* and the double-mutant strains X-SadsbA *Itrx4* and X-ScdsbA *Itrx4* was assayed in parallel (all these strains were transformed with plasmid pPSPHoA5; *Itrx4* strains were grown in the presence of 25 μ M IPTG). The results indicate that expression of ScDsbA indeed led to increased secretion of active PhoA (Fig. 3A) compared to the secretion by the parental strain. The extent to which ScDsbA expression increased the level of extracellular PhoA was comparable to the extent to which SaDsbA expression increased the level of extracellular PhoA, indicating that these proteins are functionally interchangeable when they are expressed in *B. subtilis*. Furthermore, the expression of ScDsbA or SaDsbA and the depletion of TrxA resulted in comparable increases in the extracellular levels of active PhoA (approximately 1.5- to 2.0-fold increases). When TrxA depletion was combined with ScDsbA or SaDsbA expression, even higher levels of active extracellular PhoA were observed, especially with the X-ScdsbA *Itrx4* strain (Fig. 3A).

Importantly, the levels of PhoA activity in the different growth medium samples correlated well with the levels of mature PhoA protein detected in the corresponding samples, as shown by Western blotting (Fig. 3B). Interestingly, compared to parental strain 168 expressing PhoA, significantly smaller amounts of extracellular breakdown products of mature PhoA were observed for all mutant strains tested. The smallest amounts of degradation products of mature PhoA were observed for *Itrx4* strains with TrxA depleted. Furthermore, multiple PhoA products with lower SDS-PAGE mobility than mature PhoA, probably representing intermediate processing products of pro-PhoA, were detectable in growth media of strains containing the *Itrx4* mutation, and to a lesser extent, this was also the case for the X-SadsbA and X-ScdsbA strains. The largest amounts of PhoA protein were observed in medium fractions of the X-ScdsbA *Itrx4* strain in which TrxA was depleted. Taken together, these results show that modulation of cytoplasmic TrxA and/or extracytoplasmic TDOR levels can result in increased levels of secreted active PhoA.

Optimized levels of secreted PhoA facilitated by redox-active medium compounds. Recently, we reported that the activity of staphylococcal DsbA depends on redox-active compounds in the growth medium. This was shown by growing DsbA-producing *B. subtilis* in synthetic media with or without cysteine/cystine and subsequently measuring the extracellular accumulation of active *E. coli* PhoA (18). Therefore, we investigated whether this DsbA activity could also be enhanced by addition of excess redox-active compounds, such as cysteine or cystine, to the rich LB growth medium. For this purpose, we grew all TrxA depletion and/or DsbA-expressing strains in parallel cultures in the presence of 100 μ g/ml added cystine (i.e., oxidized cysteine) or cysteine. The results show that addition of either cystine or cysteine to the *Itrx4* X-ScdsbA strain resulted in strongly increased levels of active extracellular PhoA (Fig. 3A). The increases in extracellular PhoA activity were most pronounced (about 3.5-fold) when cysteine was added. Quantifi-

cation of the Western blot data in Fig. 3B indicated that the amount of mature PhoA was about fourfold larger in the *Itrx4* X-ScdsbA strain when it was grown in the presence of cysteine. Furthermore, this positive trend was also observed for the X-ScdsbA strain, but the degree of stimulation was lower than that in the *Itrx4* X-ScdsbA strain. For the other strains addition of cysteine or cystine to the growth medium did not result in statistically significant increased extracellular PhoA levels under the conditions tested. The PhoA activity data were confirmed by Western blotting, which showed that all observed increases in activity correlated with increased PhoA protein levels (Fig. 3B). Moreover, especially the addition of cysteine seemed to result in reduced extracellular accumulation of degradation products of mature PhoA, even in parental strain 168. To investigate whether cystine or cysteine in the growth medium had an influence on known TDORs that impact the secretion of active PhoA, we investigated the cellular levels of DsbA, BdbD, and TrxA. As shown in Fig. 3C to E, the levels of these proteins were not affected by the presence of added cystine or cysteine.

Taken together, our study aimed at increasing the thiol-oxidizing power of *Bacillus* yielded three approaches leading to stepwise improvements in the yields of secreted active *E. coli* PhoA.

DISCUSSION

In the present study we developed novel concepts for optimized production of disulfide-bond-containing proteins in *Bacillus*. In essence, this goal can be achieved by (i) depletion of the major cytoplasmic disulfide bond reductase TrxA; (ii) co-expression of a staphylococcal thiol oxidase (DsbA) with the disulfide-bond-containing protein that is to be produced; (iii) use of growth medium supplemented with a redox-active compound, such as cysteine; and (iv) a combination of these three approaches. Proof of principle was obtained by combined use of these three approaches for optimized secretion of the disulfide-bond-containing protein PhoA from *E. coli*. This resulted in approximately 3.5-fold-larger amounts of active PhoA protein in the growth medium. Our findings underpin the fact that significant amounts of high-value proteins are degraded after heterologous production in *Bacillus* species and that significant amounts of such "lost proteins" can be rescued by cell factory engineering.

In previous studies we investigated the roles of membrane-associated TDORs in the secretion of *E. coli* PhoA and showed that BdbC and BdbD were of prime importance for preventing the degradation of PhoA that was translocated across the membrane (4, 25). Our studies also revealed that, despite the PhoA folding activity of BdbCD, substantial amounts of translocated PhoA were degraded (7, 18). This was likely to be due to the known limited capacity for disulfide bond formation of *B. subtilis* (33). At the time, we did not include cytoplasmic TDORs in our studies, because these enzymes are generally believed to function as thiol reductases rather than as thiol oxidases that might facilitate the folding of PhoA. In the present study we investigated how the thiol-oxidizing power of *B. subtilis* could be increased. For this purpose, the cytoplasmic TDORs were interesting, because lowering their cellular levels would decrease the thiol-reducing power and concomitantly

increase the oxidative power. Of the 10 thioredoxinlike proteins of *B. subtilis* tested, only TrxA impacted the secretion of PhoA. Our results indicate that TrxA counteracts the production of secreted active PhoA, most likely due to its general thiol reductase function in the cytoplasm. At present, we do not know why removing the remaining thioredoxin-like proteins had no effect on PhoA secretion, but these proteins either may have very specific functions, as was shown previously for StoA (11), or may not be expressed under the conditions tested.

Thioredoxins are small, heat-stable, ubiquitous TDORs that are involved in a large variety of processes, ranging from enzyme activation to mitochondrion-dependent apoptosis (40). During catalysis, the cysteine residues of their CXXC active site undergo a reversible oxidation-reduction reaction. In the bacterial cytoplasm, thioredoxin is usually present in a reduced state in order to prevent the formation of disulfide bonds in cytoplasmic proteins. Interestingly, depletion of TrxA in *B. subtilis* resulted in specific approximately 1.5- to 2-fold increases in the extracellular levels of *E. coli* PhoA. The increased PhoA levels coincided with the disappearance of PhoA degradation products and the appearance of incompletely processed pro-PhoA in the medium. Notably, the results of our previous DNA array analyses showed that the expression of none of the known genes for major secretion machinery components or proteases of *B. subtilis* is affected by TrxA depletion (37). Taken together, these findings imply that TrxA influences the activity (but not the amounts) of secretion machinery components that are specifically involved in PhoA secretion. Indeed, TrxA was shown to have a significant impact on the redox state of the extracytoplasmic TDOR BdbD since TrxA depletion resulted in increased levels of oxidized BdbD molecules. Our observations indicate, therefore, that diminished reductive power of the cytoplasm as a consequence of TrxA depletion results in increased levels of oxidized BdbD molecules. Thus, TrxA depletion has an effect that is opposite the effect of a *dbbC* mutation, which results in strongly reduced folding of PhoA (4) and, at the same time, significantly increased levels of reduced BdbD. Notably, the improved PhoA secretion by cells with TrxA depleted still depends on the presence of BdbC. Therefore, it seems that the increased levels of oxidized BdbD molecules in cells with TrxA depleted increase the cellular capacity to oxidize exported proteins, such as PhoA. Thus, our data indicate that the improved secretion of PhoA by cells with TrxA depleted can be attributed to improved posttranslocational disulfide bond formation in PhoA rather than to improved pre-PhoA translocation across the membrane.

The observation that addition of cysteine or cystine to the media of strains expressing *S. carnosus* DsbA leads to increased extracellular levels of active PhoA is in agreement with the recently documented dependence of *S. aureus* DsbA on cysteine for activity (18). Most likely, this is related to the fact that all (sequenced) staphylococci lack BdbC-like quinone reductases for DsbA reoxidation during catalysis (18). This requirement for redox-active medium components for DsbA activity is not immediately obvious when cells are grown in LB medium, because this growth medium contains such components. However, our present findings with *S. carnosus* DsbA indicate that limiting amounts of redox-active components are present for optimal activity of this thiol oxidase. It should be

noted that addition of cysteine or cystine to the media had positive effects on the yields of extracellular active PhoA from *S. carnosus* DsbA-expressing cells. Most likely, this was due to the fact that cysteine is readily oxidized to cystine in the presence of molecular oxygen. In fact, addition of cysteine seems to be more effective than addition of cystine, which can be explained at least in part by the poor solubility of cystine. Furthermore, recent studies by Smits et al. suggested that cells with TrxA depleted are auxotrophic for cysteine as growth inhibition of cells with TrxA severely depleted could be reversed by adding cysteine to the growth medium (37). Under these conditions cysteine may actually protect various cytoplasmic proteins against irreversible thiol oxidation (14, 22). Addition of cysteine is therefore preferred over addition of cystine in order to increase the oxidative potential of strains expressing *S. carnosus* DsbA.

A clear outcome of the present study is that the highest extracellular levels of active *E. coli* PhoA are produced when TrxA is depleted and *S. carnosus* DsbA is expressed in a strain growing in medium that is supplemented with cysteine. Specifically, these conditions resulted in a 3.5-fold increase in production of active extracellular PhoA or an estimated increase in the concentration of extracellular mature PhoA protein from ~90 to ~350 mg/liter (7, 46, 48; data not shown). These findings suggest that more than 70% of the PhoA synthesized in parental *B. subtilis* strain 168 is subject to degradation and that the degraded PhoA can be rescued in appropriately modified strains. To date, it is not clear whether further increases can be achieved (for example, by deletion of particular protease genes), but this seems to be a promising approach on the basis of previous studies (23, 26, 47, 49). In this context, it is relevant to note that the membrane-cell wall interface of *B. subtilis* is a highly proteolytic environment in which heterologous translocated proteins, including even α -amylases from other bacilli, are readily degraded due to suboptimal conditions for their folding (33). Clearly, even in the cysteine-supplemented medium of our best-producing strain, PhoA degradation products were still observed. We are therefore convinced that the *B. subtilis* cell factory provides an opportunity for further improvement. This improvement may be achieved by constructing strains with optimized properties for protein folding after translocation across the membrane or reduced proteolytic activity and by using optimized growth protocols. In turn, such strains should make downstream processing of protein products (with or without specific affinity tags) considerably easier. Even though further improvements are conceivable, we are convinced that our present study represents a major step toward the generation of optimized *Bacillus* strains for production of high-value proteins with disulfide bonds.

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