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Mutations in the Thiol-Disulfide Oxidoreductases BdbC and BdbD Can Suppress Cytochrome *c* Deficiency of CcdA-Defective *Bacillus subtilis* Cells

Lýður S. Erlendsson and Lars Hederstedt*

Department of Microbiology, Lund University, SE-223 62 Lund, Sweden

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Cytochromes of the *c* type in the gram-positive bacterium *Bacillus subtilis* are all membrane anchored, with their heme domains exposed on the outer side of the cytoplasmic membrane. They are distinguished from other cytochromes by having heme covalently attached by two thioether bonds. The cysteinyls in the heme-binding site (CXXCH) in apocytochrome *c* must be reduced in order for the covalent attachment of the heme to occur. It has been proposed that CcdA, a membrane protein, transfers reducing equivalents from thioredoxin in the cytoplasm to proteins on the outer side of the cytoplasmic membrane. Strains deficient in the CcdA protein are defective in cytochrome *c* and spore synthesis. We have discovered that mutations in the *bdbC* and *bdbD* genes can suppress the defects caused by lack of CcdA. BdbC and BdbD are thiol-disulfide oxidoreductases. Our experimental findings indicate that these *B. subtilis* proteins functionally correspond to the well-characterized *Escherichia coli* DsbB and DsbA proteins, which catalyze the formation of disulfide bonds in proteins in the periplasmic space.

Gram-negative bacteria, such as *Escherichia coli*, have an inner and an outer membrane that confine the periplasmic space. *Bacillus subtilis* is a gram-positive bacterium with no outer membrane but a periplasm-like compartment confined by a thick cell wall and the cytoplasmic membrane (30, 33). Formation and disruption of disulfide bonds in proteins are important reactions in the periplasm of bacteria (12, 49). Disulfide bonds can form spontaneously at a slow rate. Formation of disulfide bonds in vivo must occur more efficiently than in vitro and is catalyzed by thiol-disulfide oxidoreductases. The available genome sequence data of bacteria belonging to, e.g., the genera *Bacillus* (26) and *Mycobacterium* (11) indicate that gram-positive bacteria contain several membrane-bound proteins involved in thiol redox chemistry in the cell envelope. Very little is known, however, about the physiological role and importance of these proteins.

Several proteins that function in formation and disruption of disulfide bonds in the periplasm of *E. coli* are known in considerable detail (for a review see reference 13). These proteins are located in the cytoplasmic membrane or the periplasmic space and contain thioredoxin sequence motifs (CXXC). DsbA and DsbB constitute an oxidative branch catalyzing formation of disulfide bonds (2, 3, 12). DsbA is a small, water-soluble periplasmic protein that directly oxidizes the substrate protein (23, 50). Reoxidation of DsbA is catalyzed by DsbB, which is an integral membrane protein (2, 20, 24). DsbD has a central function in a reductive branch. This protein transfers reducing equivalents from thioredoxin in the cytoplasm to various thiol-disulfide oxidoreductases in the periplasm (7, 14, 25, 31, 34, 42). The reducing equivalents are needed for isomerization of disulfide bonds (involves DsbC and DsbG) and for reduction of apocytochromes of the *c* type prior to ligation of heme (involves CcmG and CcmH). In cytochrome *c* the heme is covalently attached to the protein by two thioether bonds. Reduced cysteinyls at the heme-binding site (CXXCH) in the apocytochrome are necessary for the attachment of the heme cofactor (4).

Vegetative *B. subtilis* cells are known to contain three proteins with disulfide bonds. Two of them, ComGC and ComGG, are competence proteins. ComGC has one intramolecular disulfide bond, and ComGG has one intermolecular bond (9). The third protein is sublancin 168, which is a lantibiotic containing two intramolecular disulfide bonds and one proposed thioether lanthionine bond (32).

B. subtilis contains four *c*-type cytochromes, which are all membrane bound (6, 45, 46, 48). Proteins CcdA, ResB, and ResC are important for the synthesis of these cytochromes (28, 39). The exact functions of ResB and ResC have not been established. CcdA is an integral membrane protein functionally related to *E. coli* DsbD. CcdA is required for a late step in cytochrome *c* biogenesis, probably in keeping the two critical cysteinyls in apocytochrome reduced (38). Strains lacking CcdA are also defective in spore synthesis, and this is not a secondary effect due to the absence of cytochrome *c* (37).

Mutations that suppress the defects caused by CcdA deficiency in *B. subtilis* have been isolated but not identified (37). Strains containing such suppressor mutations sporulate like the wild type and emerge as microcolonies within lysed colonies of a strain with *ccdA* deleted on plates incubated for several days at room temperature. In this work we have isolated CcdA suppressor mutations using transposon mutagenesis. The suppressor mutations in both the new and previous isolates were identified. They are all positioned in the *yvgUV* locus. The *yvgU* gene has recently been designated *bdbC* (*Bacillus* disulfide bond) (8). The *yvgV* gene is named *bdbD*. The physiological

* Corresponding author. Mailing address: Department of Microbiology, Lund University, Sölvegatan 12, SE-223 62 Lund, Sweden. Phone: 046 (46) 2228622. Fax: 046 (46) 157839. E-mail: Lars.Hederstedt@mikrbiol.lu.se.

TABLE 1. Bacterial strains and plasmids used in this work

Strain or plasmid	Description[a]	Source or reference
B. subtilis strains		
1A1	*trpC2*	BGSC[b]
LU60A1	*trpC2* Δ*ccdA*::*ble* Pm^r	37
LU62A1	*trpC2* Δ(*ccdA-yneI-yneJ*)::*ble* Pm^r	37
LUL3	*trpC2 bdbC*::*pLLE21* Em^r	This work
LUL4	*trpC2* Δ*ccdA*::*ble bdbC*::*pLLE21* Pm^r Em^r	This work
LUL7	*trpC2* Δ*ccdA*::*ble, bdbD*ΩTn*10*$_{(1)}$ Pm^r Sp^r	This work (Fig. 1)
LUL8	*trpC2* Δ*ccdA*::*ble bdbD*ΩTn*10*$_{(2)}$ Pm^r Sp^r	This work (Fig. 1)
LUL10	*trpC2 bdbD*ΩTn*10*$_{(1)}$ Sp^r	This work
LU62A1R$^{\#1}$	*trpC2* Δ(*ccdA-yneI-yneJ*)::*ble bdbC1* Pm^r	37
LU62A1R$^{\#3}$	*trpC2* Δ(*ccdA-yneI-yneJ*)::*ble bdbD3* Pm^r	37
LU62A1R$^{\#10}$	*trpC2* Δ(*ccdA-yneI-yneJ*)::*ble bdbD10* Pm^r	37
Plasmids		
pHP13	Shuttle vector; Em^r Cm^r	17
pHPSK	Shuttle vector; Em^r Cm^r	21
pHPKS	Shuttle vector; Em^r Cm^r	21
pIC333	Vector for transposon mutagenesis; Em^r Sp^r	41
pMutin2	Integration vector for *B. subtilis*; Em^r Am^r	44
pLLE21	101-bp internal fragment from *bdbC* in pMutin2; Em^{r}Am^r	This work (Fig. 1)
pLLE26	*dbdD* on 0.8-kb fragment in pHPSK; Em^r Cm^r	This work (Fig. 1)
pLLE27	*bdbDC* operon on 1.5-kb fragment in pHPKS; Em^r Cm^r	This work (Fig. 1)
pCPC23	*cccA-phoA* fusion on 3.9-kb fragment in pHP13; Em^r Cm^r	38

[a] Pm^r, Em^r, Am^r, Sp^r, and Cm^R, resistance to phleomycin, erythromycin, ampicillin, spectinomycin, and chloramphenicol, respectively.
[b] *Bacillus* Genetic Stock Center, University of Ohio, Columbus.

role of the BdbC and BdbD proteins in *B. subtilis* has been analyzed. Functions of different *B. subtilis* putative thiol-disulfide oxidoreductases in the cytoplasmic membrane are proposed.

MATERIALS AND METHODS

Bacterial strains and plasmids. The *B. subtilis* strains and the plasmids used in this work are listed in Table 1. *B. subtilis* LUL10 was obtained by transformation of strain 1A1 with LUL7 chromosomal DNA. *E. coli* strain JM109 (*recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi* Δ[*lac-proAB*] F′[*traD36 proAB*$^+$ *lacI*^{q} *lacZ*Δ*M15*]) was used for propagation of plasmids.

Media and growth conditions. *E. coli* cells were grown at 37°C in Luria-Bertani (LB) medium or on LB plates (35). *B. subtilis* strains were cultivated at 37°C in LB or nutrient sporulation medium with phosphate (NSMP) (15) or on tryptose blood agar base (TBAB) plates (Difco). Antibiotics were used at various concentrations when appropriate; for *B. subtilis*, spectinomycin (150 mg/liter), erythromycin (1 mg/liter), and chloramphenicol (4 to 5 mg/liter) were used; for *E. coli*, ampicillin (50 mg/liter) and chloramphenicol (12.5 mg/liter) were used.

Transposon insertional mutagenesis. Plasmid pIC333 was used for transposon mutagenesis (41). The plasmid contains an erythromycin resistance gene, a temperature-sensitive *B. subtilis* origin of replication, a transposase gene, a Tn *10* transposon unit with an internal spectinomycin resistance gene, and a pUC origin of replication. *B. subtilis* strain LU60A1 was transformed with the plasmid and grown on TBAB plates with erythromycin at 28°C. Colonies were selected and individually grown at 28°C in LB medium supplemented with spectinomycin. The temperature was then elevated to 37°C, and the cultures were incubated for 4 h. Cultures were then grown for sporulation. Finally the cultures were incubated at 80°C for 15 min, and samples were plated on NSMP plates with spectinomycin. The plates were incubated at 37°C overnight, and colonies were screened for TMPD (*N,N,N′,N′*-tetramethyl-*p*-phenylenediamine) oxidation activity

DNA techniques. Standard DNA techniques were used (35). Plasmid DNA was isolated by using a Quantum prep plasmid miniprep kit (Bio-Rad) or by CsCl density gradient centrifugation. Chromosomal DNA from *B. subtilis* was isolated as described by Marmur (29). *E. coli* was transformed by electroporation, and *B. subtilis* was grown to natural competence essentially as described by Hoch (19). *B. subtilis* strains defective in competence were transformed using protoplast transformation (19).

Fluorescent DNA sequencing was carried out using the BigDye terminator cycle sequencing ready reaction kit (Applied Biosystems) and analyzed on a ABI prism 3100 DNA sequencer.

Construction of plasmids. Plasmid pLLE21 was constructed by amplifying an internal fragment of *bdbC* using primers 5′-AAAAGCTTCTGTGCTGGTACCAGCG-3′ and 5′-AAGGATCCCGAGCACGGCACGCC-3′ (the underlined sequences indicate the restriction sites added via the primers). The PCR product was cut with *Hin*dIII and *Bam*HI and cloned into pMutin2 that had been cut with the same restriction enzymes. Plasmid pLLE26 was constructed using primers 5′-CCATCGATGTTACTTCCCTTTCAGC-3′ and 5′-CGGGATCCAACGCCATGCGCGTCATG-3′. The amplified *bdbD* gene was cut with *Bam*HI and *Cla*I and cloned into vector pHPSK. Plasmid pLLE27 was obtained using primers 5′-CGGGATCCAACGCCATGCGCGTCATG-3′ and 5′-CGGGATCCTTCCTCTTCCATCGCAAC-3′. The amplified *bdbDC* region was cut with *Bam*HI and *Xba*I and cloned into pHPKS.

Spore assay. Cultures were grown in 25 ml NSMP at 30°C in baffled Erlenmeyer flasks for 2 days. The sporulation efficiency of strains was analyzed by heating 5-ml samples at 80°C for 10 min. Serial dilutions of heat-treated and unheated samples were spread on TBAB plates. After incubation of the plates at 37°C overnight, colonies were counted.

Alkaline phosphatase activity and Western blot analysis. The protoplast supernatant subfraction was isolated from *B. subtilis* strains as described previously for strains 3G18 and LU6018 by Schiött et al. (38). Alkaline phosphatase activity was measured using *p*-nitrophenyl phosphate as the substrate as described before (47). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis protein separation was carried out using the Schägger and von Jagow system (36). Proteins were transferred from the gel to a polyvinylidene fluoride blotting membrane using a semidry electroblotter (KemEn Tec Semidry Blotter II). Rabbit antiserum against *E. coli* alkaline phosphatase was used as the primary antibody. A peroxidase-labeled antirabbit antibody from the ECL Western blotting analysis system (Amersham Pharmacia Biotech) was used for visualization of the bound primary antibody.

Cytochrome *c* oxidase activity assay. Isolated membranes from *B. subtilis* strains were added at a final protein concentration of 40 μg/ml to a 40 μM reduced solution of *Saccharomyces cerevisiae* cytochrome *c* in 20 mM MOPS (morpholinepropanesulfonic acid), pH 7.4. Oxidation of the *c*-type cytochrome was measured by a dual-wavelength spectrometer using the wavelength pair 540 and 550 nm (45). An extinction coefficient ($\varepsilon_{550-540}$) of 19.5 mM^{-1} cm^{-1} was used to calculate activities. The measurements were carried out at 30°C using a 3-ml cuvette with a magnetic stirrer

Other methods. Membranes were isolated from strains grown in NSMP at 37°C essentially as described previously (18). Protein concentrations were determined using the bicinchoninic acid protein assay (Pierce Chemical Co.) with bovine serum albumin as the standard. TMPD oxidation by colonies on NSMP

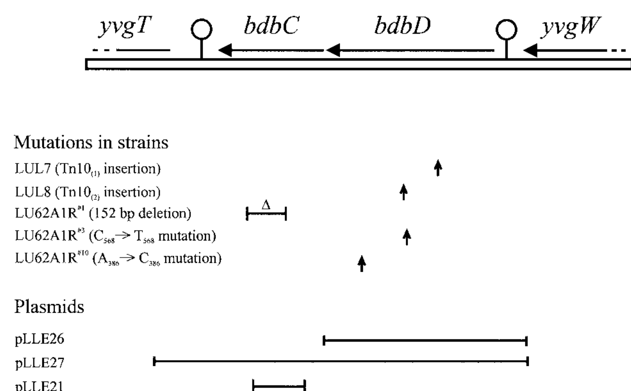

FIG. 1. Map of the *bdbD-bdbC* region in the *B. subtilis* chromosome. The positions of mutations identified in this work are indicated. Shown also are DNA fragments cloned in plasmids and used for complementation analysis (pLLE26 and pLLE27) or disruption of the *bdbC* gene (pLLE21).

plates was assayed as described by Le Brun et al. (28) except when cells were grown on NSMP plates containing dithiothreitol (DTT) or L-cystine. In these cases an Immobilon-NC Triton X-100-free MCE filter (pore size, 0.45 μm) disk (Millipore) was laid on the surface of the agar in the plate. The bacterial strains were streaked directly on the filter, and the plate was then incubated at 37°C overnight. Next day, prior to the TMPD oxidation activity staining, the filter was removed from the plate and washed by floating it on distilled water. The same procedure and the TMPD staining solution used for plates were used for the filters except that the agar solution was replaced by water.

RESULTS

Isolation of CcdA suppressor mutations using Tn *10*. It was previously observed that strains containing CcdA suppressor mutations are proficient in sporulation (37). This property was exploited to isolate a new set of mutants. *B. subtilis* strain LU60A1, with the *ccdA* gene deleted, was subjected to random Tn*10* insertion mutagenesis using pIC333. To select clones containing CcdA suppressor mutations, mutagenized cells were grown for sporulation. The cultures were heat treated, to kill vegetative cells, and samples were spread on NSMP plates. Colonies resulting after overnight growth at 37°C were screened in situ for TMPD oxidation activity. This activity is dependent on a functional cytochrome caa_3 in the cytoplasmic membrane (45). CcdA-deficient strains lack cytochrome *c* and are therefore TMPD oxidation negative. Chromosomal DNA was isolated from independent TMPD oxidation-positive clones and used to transform LU60A1 to spectinomycin resistance to select for the transposon. TMPD oxidation-positive transformants LUL7 and LUL8 were analyzed. Both strains were found to have the *bdbD* gene disrupted by insertion of the transposon (Fig. 1), as determined by sequence analysis of the DNA flanking Tn *10*.

Mutations in *bdbC* and *bdbD* can both suppress CcdA deficiency. The *bdbD* gene is located in a dicistronic operon, *bdbDC* (Fig. 1), as deduced from the *B. subtilis* genome sequence (26). This gene organization and the fact that BdbD and BdbC are apparent orthologs of *E. coli* DsbA and DsbB, respectively, indicate that BdbC and BdbD function as a pair. The *bdbC* gene in strain LU60A1 was therefore inactivated by integration of the pMutin2 derivative pLLE21 (Fig. 1). Colonies of the resulting strain, LUL4, on NSMP plates oxidized TMPD. This showed that inactivation of *bdbC* also suppresses the cytochrome *c* synthesis defect of CcdA-deficient mutants.

To determine if previously isolated CcdA-suppressor strains are mutated in the *bdbDC* locus, LU62A1R$^{\#1}$ and LU62A1R$^{\#10}$ were transformed with plasmid pLLE27, carrying the *B. subtilis bdbDC* gene cluster, and pHP13, which is the corresponding vector. Colonies of the strains transformed with pHP13 were TMPD oxidation positive, as expected. However, colonies obtained after transformation with pLLE27 were TMPD oxidation negative. Thus, in the presence of the wild-type *bdbDC* locus on a plasmid the suppression of the CcdA deficiency in these strains was reversed. The suppressor phenotype of LU62A1R$^{\#10}$ was also reversed by pLLE26 carrying only *bdbD*. The results suggested that the previously isolated strains carry mutations in the *bdbCD* locus and that these mutant genes are recessive relative to the wild-type locus.

Amplification of the *bdbDC* region of strains LU62A1R$^{\#1}$, LU62A1R$^{\#3}$, and LU62A1R$^{\#10}$ by PCR and subsequent DNA sequence analysis demonstrated mutations in *bdbD* and *bdbC* (Fig. 1). Strain LU62A1R$^{\#1}$ was found to contain a 152-bp deletion in the *bdbC* gene (bases G_{138} to C_{290}). LU62A1R$^{\#3}$ has a C_{568}-to-T_{568} transition in *bdbD*, resulting in a nonsense mutation (Gln_{190}→amber stop codon). LU62A1R$^{\#10}$ has an A_{386}-to-C_{368} transversion, changing His_{129} in BdbD to a Pro residue.

Inactivation of *bdbC* and *bdbD* in a wild-type genetic background (strains LUL3 and LUL10) did not affect the TMPD oxidation activity of colonies on NSMP plates. Light-microscopic observations of LUL3 and LUL10 showed no apparent differences compared to the parental strain, 1A1, in terms of cell size, shape, or motility.

Cytochrome *c* oxidase activity of suppressor-containing strains. The TMPD oxidation phenotype of strains with *ccdA* deleted and carrying *bdbC* or *bdbD* mutations was found to be dependent on the growth medium. Compared to colonies of the wild-type strain, colonies of suppressor-containing strains showed very low TMPD oxidation activity when grown on TBAB plates but high activity when grown on NSMP plates. This difference can be explained by variations in enzyme content and by the fact that TMPD oxidation activity is not apparent if the cytochrome caa_3 content is below a certain threshold level. *B. subtilis* cells grown on NSMP contain about five times more cytochrome caa_3 than cells grown on TBAB (J. Bengtsson and L. Hederstedt, unpublished data). To determine the extent of suppression of the CcdA deficiency, cytochrome *c* oxidase activity of isolated membranes from cells grown on NSMP was analyzed (Table 2). A strain with *ccdA* deleted completely lacked oxidase activity but showed 7 to 8% of wild-type activity when *bdbC* or *bdbD* was inactivated. This result showed that a lack of BdbC or BdbD only partially overcomes the defect in cytochrome *c* oxidase activity caused by CcdA deficiency.

Competence development and sporulation efficiency of BdbC- and BdbD-deficient strains. It has been noted previously that strain LU62A1R$^{\#3}$ (37) and a *B. subtilis* BdbC-deficient strain (8) do not develop competence. We found that all our strains carrying mutated *bdbD* or *bdbC* (Table 1) were defective in competence irrespective of whether CcdA was present or absent in the strains.

TABLE 2. TMPD oxidation phenotypes of colonies on NSMP plates and cytochrome *c* oxidase activities of isolated membranes

Strain	Relevant genotype	TMPD phenotype	Cytochrome *c* oxidase activity (%)[a]
1A1	Wild type	Positive	100
LU62A1	Δ(*ccdA-yneI-yneJ*)::*ble*	Negative	<0.1
LU62A1R[#1]	Δ(*ccdA-yneI-yneJ*)::*ble bdbC1*	Positive	8.3
LU62A1R[#10]	Δ(*ccdA-yneI-yneJ*)::*ble bdbD10*	Positive	6.9

[a] Cytochrome *c* oxidase activities of isolated membranes are shown as percentages of activity compared to that for 1A1. The activity of 1A1 membranes was 0.21 μmol of cytochrome *c* oxidized per min per mg of protein.

The ability of strains to form heat-resistant spores during growth in NSMP for 2 days at 30°C was assessed (Table 3). The results showed that BdbC and BdbD are not important for sporulation. They also demonstrated that, in the absence of functional BdbC or BdbD, the CcdA protein is no longer required for efficient synthesis of spores.

BdbC- and BdbD-deficient strains are defective in the synthesis of a disulfide bond-containing protein. To determine if *bdbD* and *bdbC* are involved in disulfide bond formation, we looked at the abilities of different strains to produce active *E. coli* alkaline phosphatase (PhoA). This enzyme requires formation of two intramolecular disulfide bonds to be active (40).

Alkaline phosphatase was expressed from pCPC23 as a CccA-PhoA fusion protein (38). The CccA part corresponds to the first 104 residues of the *B. subtilis* cytochrome c_{550}. It replaces the native *E. coli* N-terminal signal sequence and directs the excretion of PhoA (47). With CccA-PhoA in *B. subtilis*, it has been demonstrated that the major portion of the alkaline phosphatase activity is found in the protoplast supernatant subfraction (38). It appears as if the PhoA part needs to be cleaved off from the CccA-PhoA polypeptide to become active. The activities of soluble alkaline phosphatase produced by different strains containing pCPC23 or plasmid vector pHP13 are shown in Table 4.

Strains 1A1 (wild type) and LU62A1 (Δ*ccdA*) containing pCPC23 showed similar alkaline phosphatase activities. BdbC- and BdbD-deficient strains containing pCPC23 produced less alkaline phosphatase activity; the difference in activity was 3- to 3.5-fold. The effects of BdbC and BdbD deficiency were about the same. Western blot analysis was consistent with these results, showing a reduced level of alkaline phosphatase protein in extracts from BdbC- and BdbD-defective strains (blot not shown). The results indicated that disulfide bond formation in

TABLE 4. Alkaline phosphatase activities of the protoplast supernatant fractions of different *B. subtilis* strains containing pHP13 or pCPC23

Strain	Alkaline phosphatase activity (U/ml of culture)[a] for plasmid:	
	pHP13	pCPC23
1A1	<0.01	4.4
LU62A1	<0.01	3.9
LU62A1R[#1]	<0.01	1.3
LU62A1R[#10]	<0.01	1.2
LUL3	<0.01	1.5
LUL10	<0.01	1.4

[a] The values are the averages obtained from three independent experiments. The variation between experiments was less than 15%. One unit corresponds to 1 nmol of phosphoester bonds hydrolyzed per min per amount of cell fraction corresponding to 1 ml of culture.

proteins is impaired in the absence of BdbC or BdbD. We consider it unlikely that the observed decrease in alkaline phosphatase activity of BdbC- and BdbD-deficient strains occurs because these strains have defects in translation of PhoA or export of the polypeptide compared to the parental strain.

Effects of low-molecular-weight redox compounds. CcdA is thought to transfer reducing equivalents from the cytoplasm to thioredoxin-like protein domains on the outer side of the cytoplasmic membrane (37). Consistent with such a function, we found that the defect in TMPD oxidation activity of colonies of CcdA-deficient strains can be suppressed if the reducing thiol reagent DTT is added to the growth medium (Table 5). Strains deficient in CcdA and BdbC or BdbD, in contrast, lost the ability to oxidize TMPD if the oxidizing thiol compound L-cystine was added to the growth medium. Full effect was obtained at 5 mM L-cystine in plates, but a 1 mM concentration of the reagent also had an effect. The presence of L-cystine (5 mM) or DTT (15 mM) did not affect the TMPD oxidation phenotypes of strains defective in only BdbC or BdbD. These results suggest that *B. subtilis* BdbC and BdbD, similar to the *E. coli* orthologs, DsbB and DsbA, catalyze formation of disulfide bonds in proteins on the outer side of the cytoplasmic membrane. Control experiments showed that DTT (15 mM) or L-cystine (5 mM) in the growth medium had no apparent effect on the growth of strains and did not complement the defect in TMPD oxidation activity of colonies of cytochrome caa_3- (Δ*ctaCD*), ResB-, or ResC-deficient strains.

TABLE 3. Sporulation efficiencies of different *B. subtilis* strains

Strain	Relevant genotype	Titer (CFU/ml)		Sporulation efficiency[b]
		Total cell	Spore[a]	
1A1		4.8×10^8	4.4×10^8	92
LU60A1	Δ*ccdA*::*ble*	1.4×10^8	4.2×10^6	3.0
LU62A1	Δ(*ccdA-yneI-yneJ*)::*ble*	4.3×10^8	1.7×10^7	3.5
LU62A1[#1]	Δ(*ccdA-yneI-yneJ*)::*ble bdbC1*	3.7×10^8	3.2×10^8	87
LU62A1[#10]	Δ(*ccdA-yneI-yneJ*)::*ble bdbD10*	4.6×10^8	4.2×10^8	91
LUL3	*bdbC*::pLLE21	3.9×10^8	3.4×10^8	87
LUL10	*bdbD*ΩTn *10*	3.6×10^8	3.0×10^8	82

[a] Titer after 10 min at 80°C.
[b] Sporulation efficiency is calculated as 100 times the spore titer divided by the total cell titer.

TABLE 5. TMPD oxidation activity of colonies on supplemented NSMP plates

Strain	Activity with:		
	No addition	15 mM DTT added	5 mM L-cystine added
1A1	Positive	Positive	Positive
LU62A1	Negative	Positive	Negative
LU62A1R#1	Positive	Positive	Negative
LU62A1R#10	Positive	Positive	Negative

DISCUSSION

In this work we show that the defects in spore and cytochrome *c* synthesis caused by a lack of CcdA protein in *B. subtilis* can be suppressed by mutations in the *bdbC* or the *bdbD* gene. The two genes are organized in a dicistronic operon. Our results from the analysis of BdbC- and BdbD-deficient strains combined with results recently reported by Bolhuis et al. (8) and a comparison with the well-characterized *E. coli* DsbA and DsbB proteins indicate that BdbC and BdbD function as a pair in catalyzing disulfide bond formation in proteins on the outer side of the cytoplasmic membrane in *B. subtilis*.

It is suggested that CcdA in the *B. subtilis* cytoplasmic membrane transfers reducing equivalents from thioredoxin in the cytoplasm to an as yet unidentified thiol-disulfide protein(s) in the periplasm. One proposed role of this transmembrane electron transfer pathway involving CcdA is to keep the cysteine residues in the heme-binding site in apocytochrome *c* reduced so that heme can be covalently bound. During transport across the cytoplasmic membrane the cysteine residues in the apocytochrome are most likely in the reduced state. Our results suggest that BdbD and BdbC oxidize the two thiol groups in the heme-binding site of apocytochrome *c* to form a disulfide bond. CcdA, together with other proteins (as discussed below), seemingly counteracts the effect of BdbC and BdbD by breaking (reducing) the disulfide bond in apocytochrome *c* (Fig. 2). This proposed function of CcdA is consistent with our finding that addition of the reducing agent DTT to a Δ*ccdA* strain restores the ability to synthesize cytochrome *c* (Table 5) and also increases the efficiency of sporulation (our unpublished data). Furthermore, the disulfide bond-containing compound L-cystine was found to compensate for the lack of BdbC and BdbD, i.e., in the presence of L-cystine, inactivation of *bdbC* and *bdbD* no longer suppressed the cytochrome *c* defect of a CcdA-deficient strain. L-Cystine and also oxidized glutathione can compensate for the effects seen with *E. coli* DsbA- and DsbB-deficient strains. A concentration of 0.6 mM L-cystine is required for the production of an oxidized λ102MalF-βgal fusion protein in a strain lacking DsbB, but 8.6 mM is required in a strain lacking DsbA (2). We found no clear difference in the levels of L-cystine required to reverse the TMPD oxidation-positive phenotype of different CcdA-deficient *B. subtilis* strains defective in BdbC and BdbD.

The low sporulation efficiency of strains lacking CcdA was increased to the wild-type level by inactivation of *bdbC* or *bdbD*. Synthesis of the spore coat during sporulation involves proteins that are rich in cysteine residues, and many spore coat proteins are heavily cross-linked by disulfide bonds in the final spore (1). BdbD and BdbC do not seem important for spore synthesis, indicating that disulfide bonds may not be essential in spores or that in *B. subtilis* there exists thiol-disulfide oxidoreductases other than BdbD and BdbC. The reason why CcdA is required for efficient sporulation is not known, but it might be needed to break or reorganize disulfide bonds during spore maturation and germination.

Strains deficient in BdbC or BdbD but sufficient in CcdA are not able to develop competence. Two proteins in *B. subtilis* known to have disulfide bonds are the essential competence proteins, ComGC and ComGG. They are located on the cell surface and have a role in binding DNA to the cell surface (9, 10). ComGC is stabilized by BdbDC (D. Dubnau, personal communication). Thus is seems as if BdbC and BdbD catalyze disulfide bond formation in ComGC and ComGG.

Searches for homologues of *E. coli* Dsb proteins in *B. subtilis* using the genome sequence have revealed several candidates. DsbA and *B. subtilis* BdbD show 41% sequence similarity and

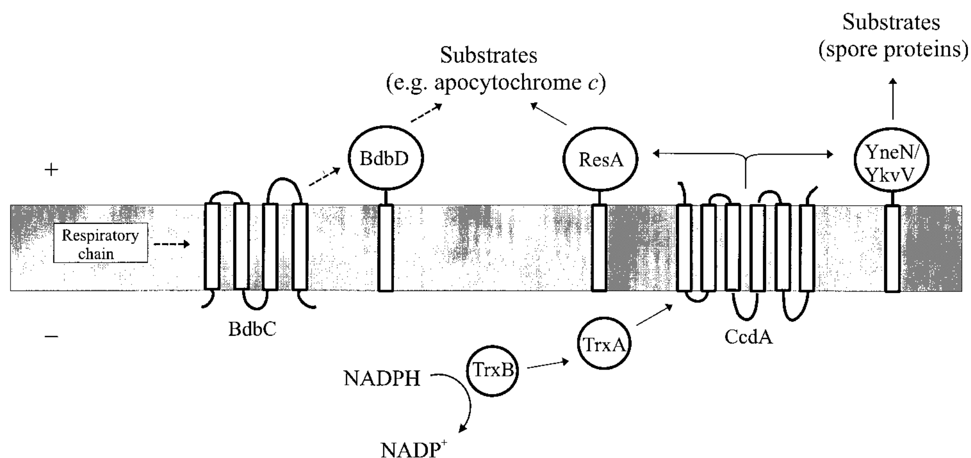

FIG. 2. Suggested function of *B. subtilis* thiol-disulfide oxidoreductases in the cytoplasmic membrane. + and −, positive and negative sides of the membrane. Dashed and solid arrows indicate roles in oxidation and reduction, respectively. The indicated functions of TrxA, ResA, YneN, and YkvV are speculative.

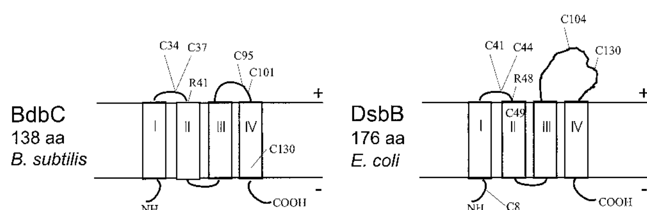

FIG. 3. Comparison of the *B. subtilis* BdbC and *E. coli* DsbB proteins in the cytoplasmic membrane. The predicted topology of DsbB is confirmed by experimental data (20). There is a notable difference in the size of the loop between transmembrane segments III and IV in the two proteins. DsbB has four essential cysteine residues (C_{41}, C_{44}, C_{104}, and C_{130}) facing the periplasmic space (20). The position of a conserved arginine residue important for reoxidation of *E. coli* DsbB (22) is indicated.

21% identity. Each has a thioredoxin motif (CXXC), and the two overlap in the alignment. The crystal structures of both oxidized and reduced DsbA are known (16). DsbA contains two domains: domain A, which is very similar to that of *E. coli* thioredoxin, and domain B. Based on the structural data and sequence comparisons *B. subtilis* BdbD most likely also has two domains. *E. coli* DsbA is a soluble protein located in the periplasm and is synthesized with a N-terminal cleaved export signal sequence. BdbD has a putative signal peptide and a type I signal peptidase (SPase) cleavage site at residue 27 (43). However, since predictions for type I SPase cleavable signal peptides often turn out to be wrong, BdbD might well be membrane bound by an N-terminal single transmembrane segment (Fig. 2). *E. coli* DsbB and *B. subtilis* BdbC show 52% sequence similarity and 21% identity, and the thioredoxin motifs overlap. BdbC is a 138-amino-acid protein that has four predicted transmembrane regions (prediction of transmembrane regions was obtained using the TMHMM server [http://www.cbs.dtu.dk/services/TMHMM-1.0/]). BdbC contains five cysteine residues, and four of them, Cys_{34}, Cys_{37}, Cys_{95}, and Cys_{101}, correspond in position to the four essential cysteines (Cys_{41}, Cys_{44}, Cys_{104}, and Cys_{130}) in DsbB, which also has four transmembrane regions (Fig. 3) (2). An arginine residue in DsbB with a role in the interaction with quinone in the membrane is also conserved in *B. subtilis* BdbC (22).

B. subtilis BdbA and BdbB, are also apparent homologues of *E. coli* DsbA and DsbB. The *bdbA* and *bdbB* genes are located in an operon, *sunA-sunT-bdbA-yolJ-bdbB*, which is part of the prophage SPβ genome (27). This prophage resides in the chromosomes of all the strains used in this work. The YolJ protein is of unknown function, SunA is a sublancin 168 lantibiotic antimicrobial peptide that is secreted into the medium, and SunT is a potential ABC-type transporter with a proteolytic domain and an ATP-binding domain. Sublancin 168 has two disulfide bonds and one thioether lanthionine bond (32). BdbA and BdbB possibly function as thiol-disulfide oxidoreductases specific for this lantibiotic. Deletion of the *bdbA-yolJ-bdbB* genes in *B. subtilis* strain LU60A1 did not suppress the TMPD oxidation-negative phenotype or affect the ability of the cell to develop natural competence (our unpublished data). It has been demonstrated that inactivation of *bdbB* in *B. subtilis* has only a small effect on the yield of active recombinant *E. coli* alkaline phosphatase compared to disruption of the *bdbC* gene (8). Thus, it seems as if BdbC and BdbD constitute a major enzyme system for disulfide bond formation in *B. subtilis*.

There are several proteins in *B. subtilis* that might accept reducing equivalents from CcdA and play a role in keeping the cysteine residues in apocytochrome reduced or function in sporulation. These proteins, ResA, YneN, and YkvV, all have high sequence similarity to CcsX of the gram-negative bacterium *Bordetella pertussis*. CcsX is known to be involved in cytochrome *c* synthesis (5). It has one transmembrane region in the N-terminal part and contains a thioredoxin motif. The *resA* gene in the *B. subtilis* genome sequence in the SubtiList database has an error affecting the reading frame (N. E. Le Brun, personal communication). Based on the corrected sequence, ResA has 179 amino acid residues. The N-terminal part of ResA probably functions both as a signal sequence for export of the rest of the protein and as a membrane anchor. YneN and YkvV each also have one transmembrane region and contain one thioredoxin motif. Deletion of the *yneN* gene in strain 1A1 does not affect the TMPD oxidation phenotype or sporulation efficiency, indicating that YneN does not play an important role in these processes (our unpublished data). ResA is encoded by the same operon as the ResB and ResC proteins, which are required for cytochrome *c* synthesis (28). We predict that ResA functions in cytochrome *c* biosynthesis. The paralogous YneN and YkvV might be involved in sporulation but have overlapping functions, as illustrated in Fig. 2.

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