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The *bdbDC* Operon of *Bacillus subtilis* Encodes Thiol-disulfide Oxidoreductases Required for Competence Development*

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The development of genetic competence in the Gram-positive eubacterium *Bacillus subtilis* is a complex post-exponential process. Here we describe a new bicistronic operon, *bdbDC*, required for competence development, which was identified by the *B. subtilis* Systematic Gene Function Analysis program. Inactivation of either the *bdbC* or *bdbD* genes of this operon results in the loss of transformability without affecting recombination or the synthesis of ComK, the competence transcription factor. BdbC and BdbD are orthologs of enzymes known to be involved in extracytoplasmic disulfide bond formation. Consistent with this, BdbC and BdbD are needed for the secretion of the *Escherichia coli* disulfide bond-containing alkaline phosphatase, PhoA, by *B. subtilis*. Similarly, the amount of the disulfide bond-containing competence protein ComGC is severely reduced in *bdbC* or *bdbD* mutants. In contrast, the amounts of the competence proteins ComGA and ComEA remain unaffected by *bdbDC* mutations. Taken together, these observations imply that in the absence of either BdbC or BdbD, ComGC is unstable and that BdbC and BdbD catalyze the formation of disulfide bonds that are essential for the DNA binding and uptake machinery.

In the Gram-negative bacteria, the efficient and correct formation of disulfide bonds, mostly in periplasmic proteins, requires the activity of thiol-disulfide oxidoreductases (1). In the Gram-positive bacterium *Bacillus subtilis*, the genes encoding several such enzymes have been studied, namely *bdbA*, *bdbB*, and *bdbC* (2). It was shown that BdbB and BdbC play roles in the folding of secreted proteins at the cell surface and that BdbC was required for the development of competence for genetic transformation.

Transformation in *B. subtilis* requires a unique set of gene products that mediate the binding and uptake of macromolecular DNA (reviewed in Ref. 3). These include the products of the *comG* operon and of *comC*, which encode proteins with similarity to components of the type II secretion machinery of Gram-negative bacteria as well as to proteins required for the assembly of type IV pili. ComC is a signal peptidase that cleaves several N-terminal residues from ComGC, ComGD, ComGE, and ComGG, all of which are similar to type IV prepilins. The ComG and ComC proteins are essential for the binding of transforming DNA to the competent cell, although they do not themselves appear to be DNA binding proteins. Instead, the pilin-like ComG proteins, which are translocated to the cell wall and the exterior surface of the membrane after processing, appear to permit contact between transforming DNA and the membrane-localized DNA receptor ComEA (4). It has been shown that ComGC contains an intramolecular disulfide bond and that a minor fraction of ComGG molecules exist as dimers, stabilized by intermolecular disulfide bonds (5). The expression of the genes encoding these transformation proteins is regulated by a complex signal transduction mechanism that culminates in the synthesis of ComK, a factor required for the transcription of the DNA transport genes (6). In fact, the transcription of *comC* and of the *comG* operon is completely dependent on ComK.

When the genome sequence of *B. subtilis* was published in 1997, it represented the first complete sequence of a Gram-positive bacterium (7). Well before the completion of the genome sequence, a program was initiated aimed at the analysis of *B. subtilis* genes with unknown function. This *B. subtilis* Systematic Gene Function Analysis Project was started at the end of 1995 in both Europe and Japan and involved some 30 research laboratories. So far, about 1300 mutants have been tested; some 30 previously unknown essential genes have been identified, and well over 500 mutant strains have been assigned single or multiple phenotypes.[1]

Among other phenotypes, these mutant strains have been analyzed for DNA recombination and competence. In the present paper, we describe the identification of a novel operon required for the late stages of competence development in *B. subtilis*. Disruption of the first gene in this operon, *yvgV*, re-

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[1] The Micado data base is available on the World Wide Web at locus.jouy.inra.fr/cgi-bin/genmic/madbase_home.pl.

TABLE I
Bacterial strains and plasmids

Strains (number) and plasmids	Relevant genotype	Source
Strains		
E. coli		
DH5α	*supE44*; *hsdR17*; *recA1*; *gyrA96*; *thi-1*; *relA1*	Ref. 35
MC1061	F^- *araD139 Δ(ara-leu)7696 galE15 galK16 Δ(lac)X743 rpsL hsdR2 mcrA mcrB1*	Ref. 36
B. subtilis		
168	*trpC2*	Ref. 37
OG1	Prototrophic	Ref. 9
168*sfp*$^+$ (BV02J07)	*trpC2*; *sfp*$^+$ Km^R	L. W. Hamoen, laboratory collection
BRB689	*trpC2 amyQ*$^+$ Cm^R	Ref. 38
bdbC	*trpC2*; *bdbC*::pMutin2*mcs*; *bdbC-lacZ*	Ref. 2
bdbD (BFA1074)	*trpC2*; *bdbD*::pMutin2*mcs*; *bdbD-lacZ*; IPTG-dependent transcription of *bdbC*	This study
Δ*comK* (8G32)	*trpC2*; *tyr-1*; *his*; *ade*; *met*; *rib*; *ura*; *nic*; Δ*comK*; Km^R	Ref. 39
Δ*comK*::*Sp* (BV2005)	*trpC2*; *comK*::*Sp*	L. W. Hamoen, laboratory collection
Δ*mecA* (QB4650)	*trpC2*; Δ*mecA*; Km^R; ComK overproducer	Ref. 40
X*bdbD* (BV2007)	*trpC2*; *amyE*::pXV	This study
X*bdbC*	*trpC2*; *amyE*::pXC	This study
bdbD-X*bdbD* (BV2008)	*trpC2*; *amyE*::pXV *bdbD*::pMutin2*mcs*	This study
bdbC-X*bdbD* (BV2009)	*trpC2*; *amyE*::pXV *bdbC*::pMutin2*mcs*	This study
bdbD-X*bdbC* (BV2010)	*trpC2*; *amyE*::pXC *bdbD*::pMutin2*mcs*	This study
bdbC-X*bdbC*	*trpC2*; *amyE*::pXC *bdbC*::pMutin2*mcs*	This study
bdbC-Δ*comK*	*trpC2*; *bdbC*::pMutin2*mcs* Δ*comK*	This study
BD2528	*his leu met* (*pMCcomS*)	Ref. 30
BD2999	*his leu met*; *bdbD*::*pMutin2mcs* (*pMCcomS*)	This study
BD3000	*his leu met*; *bdbC*::*pMutin2mcs amyE*::*pXC* (*pMCcomS*)	This study
BD3002	*his leu met*; *bdbC*::*pMutin2mcs* (*pMCcomS*)	This study
BD3355	*his leu met*; *bdbD*::*pMutin2mcs amyE*::*pXC* (*pMCcomS*)	This study
Plasmids		
pMutin2*mcs*	pBR322 derivative carrying a *spoVG-lacZ* fusion preceded by a P_{SPAC} promoter and MCS, for insertional inactivation of *B. subtilis* genes; carrying the T_1T_2 terminator from the *E. coli rrnB* operon for improved termination upstream of P_{SPAC}; 8.6 kb	
pSC5	*pMutin2mcs* carrying a 233-bp internal fragment of the *bdbD* gene, generated by PCR, inserted into the *Hin*dIII and *Bam*HI sites	This study
pX	Integrative vector for xylose-inducible expression of genes in *B. subtilis*; 7.5 kb, Ap^R Cm^R	Ref. 11
pXV	pX carrying the wild-type *bdbD* gene	This study
pXC	pX carrying the wild-type *bdbC* gene	This study
pPSPphoA5	Plasmid carrying the *E. coli phoA* gene fused to the prepro region *S. hyicus* lipase gene; Cm^R	J. Meens and R. Freudl, unpublished
pKTH10	pUB110 derivative carrying the *B. amyloliquefaciens amyQ* gene	Ref. 21

sulted in a complete loss of transformability. Interestingly, the YvgV protein shows significant similarity to several known DsbG-like thiol-disulfide oxidoreductases, which catalyze the formation of disulfide bonds in proteins that are exported from the cytoplasm (8). The second gene in the operon is the above mentioned *bdbC* (2). By analogy to *bdbC* and two other genes for proteins implicated in *Bacillus* disulfide bond formation (*i.e. bdbA* and *bdbB*) (2), the *yvgV* gene has been renamed *bdbD*. We show here that both BdbC and BdbD are essential for the stability of disulfide bond-containing transformation proteins and for the secreted protein PhoA. Accordingly, we hypothesize that these typical thiol-disulfide oxidoreductases act as a redox pair, required for the functionality of the DNA-binding and uptake machinery of *B. subtilis*.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Plasmids—The bacterial strains and plasmids used in this study are listed in Table I.

Chemicals and Enzymes—All chemicals used were of analytical grade and, unless indicated otherwise, obtained from Merck or J. T. Baker. Enzymes for molecular biology were purchased from Roche Molecular Biochemicals and used according to the supplier's instructions.

Media and Growth Conditions—*B. subtilis* minimal salts consisted of (per liter): 2 g of K_2SO_4, 10.8 g of K_2HPO_4, 6 g of KH_2PO_4, 1 g of sodium citrate, and 0.02 g of $MgSO_4$. After adjustment of the pH to 7.0 and sterilization, the following components were added to complete the minimal medium used in transformation experiments (per 50 ml): 0.5% glucose, 0.02% casamino acids (Difco), 1.4 mg/ml L-tryptophan, and 2.2 mg/ml ferric ammonium citrate. TY broth consisted of the following (per liter): 10 g of trypton (Difco), 5 g of yeast extract (Difco), and 10 g of NaCl, pH 7.4. Where necessary, media were supplemented with the appropriate antibiotics. Ampicillin and kanamycin (Km) were obtained from Roche Molecular Biochemicals and were used at 50 μg/ml (*Escherichia coli*) and 50 μg/ml (both *E. coli* and *B. subtilis*), respectively. Erythromycin was from Sigma and was used at 150 and 0.4 μg/ml for *E. coli* and *B. subtilis*, respectively; chloramphenicol and spectinomycin were purchased from Sigma and routinely used at 5 μg/ml (*B. subtilis*) and 100 μg/ml (both *E. coli* and *B. subtilis*), respectively.

DNA Manipulations—Chromosomal DNA from *B. subtilis* was isolated according to Ref. 9. Minipreparations of plasmid DNA from *E. coli* were obtained by the alkaline lysis method (10). All cloning procedures were carried out according to Ref. 10. PCR products were purified using the Qiaquick PCR purification kit (Qiagen, Hilden, Germany). Southern blot analyses were performed using the nonradioactive ECL labeling and detection system (Amersham Biosciences).

Construction of BFA1074 (bdbD)—Strain BFA1074 was constructed as follows. A 233-bp fragment (coordinates 3437442–3437675 on the *B. subtilis* 168 genome sequence (7) of the *bdbD* gene (coordinates 3437127–3437795, complementary strand), was amplified by PCR using primers carrying a *Bam*HI (5′-cgc gga tCC ATA CTT CTT CAG ATG CAA G-3′) and a *Hin*dIII (5′-gcc gaa gct TCC GGA CAG CCG TCT ATC-3′) restriction site, respectively. The fragment was subsequently digested with both *Bam*HI and *Hin*dIII and ligated into *Bam*HI-*Hin*dIII-digested pMutin2*mcs*. The resulting plasmid, pSC5, was used to transform competent *B. subtilis* 168 cells; selection of transformants was performed on TY plates containing erythromycin (0.4 μg/ml). Two of the resulting transformants were selected and analyzed by PCR and

TABLE II

Transformability of strains carrying mutations in the bdbDC operon

Transformability was expressed as the percentage of Cm^R transformants of the total viable count. The BFA1075, BFA1076, BFA1078, BFA1079, BFA1081, and BFA1090 strains represent randomly selected control strains from the BSFA collection.

Strain	Relevant genotype	Transformability			
		Viable count	Cm^R colonies	Frequency	Percentage of 168
		$\times 10^6$	$\times 10^3$		%
168	Parental strain	76	1.60	2.1×10^{-3}	100
bdbC	*bdbC*::pMutin2*mcs*	68	0	0	0
bdbD (BFA1074)	*bdbD*::pMutin2*mcs*	72	0	0	0
bdbD (BFA1074) + 100 μM IPTG	*bdbD*::pMutin2*mcs* *bdbC* is transcribed	129	0	0	0
BFA1075	*yvbF*::pMutin2*mcs*	87	3.31	3.8×10^{-3}	181
BFA1076	*yvbG*::pMutin2*mcs*	85	1.38	1.6×10^{-3}	77
BFA1078	*yvbJ*::pMutin2*mcs*	89	3.31	3.7×10^{-2}	177
BFA1079	*yvbK*::pMutin2*mcs*	87	1.54	1.7×10^{-3}	81
BFA1081	*yvbT*::pMutin2*mcs*	95	2.78	2.9×10^{-3}	139
BFA1090	*ypqA*::pMutin2*mcs*	117	3.98	1.6×10^{-3}	81

Southern hybridization to verify that integration had occurred at the desired site. With one of these, the expected PCR fragments and hybridization patterns were obtained (not shown); this strain was designated BFA1074.

Construction of Strains with Ectopic Expression of the bdbC and bdbD Genes—Complementation of insertions in either *bdbC* or *bdbD* was achieved by placing the individual genes under control of the xylose-inducible promoter present on pX (11). For this purpose, the genes were amplified by PCR using primers 5′-gaa att cta ga GAC AAT AGA AAA AGA GCT GAA AGG GAA GTA AC-3′ and 5′-gcg ccc ggg atc cGC GGG CGC TTT TTT TGT TAT TCA GAT TTT TCG CCT TTC AGC AGG CAC-3′ for *bdbC* and 5′-gct cta gaC AAT TGC GAT CCG CTT CT-3′ and 5′-cgg gat ccT AGC GAT AAG AGG CAC AA-3′ for *bdbD*, respectively. These fragments were subsequently cloned into the *Spe*I and *Bam*HI sites of pX, and the resulting constructs were integrated in single copy in the *amyE* locus of the *B. subtilis* chromosome. These constructs were designated X*bdbD* (laboratory collection number BV2007) and X*bdbC*, respectively.

Transformation Assays—*B. subtilis* cells were tested for transformability as follows. Typically, seven mutants were analyzed in parallel, plus the wild-type strain 168 as a control. Cells were grown to competence essentially as described in Ref. 9 and were transformed with chromosomal DNA of strain BRB689 (*amyQ*$^+$ Cm^R; collaboration with the group of M. Sarvas, Public Health Institute, Helsinki, Finland). Transformability was expressed as the percentage of Cm^R transformants of the total viable count. The strains constructed in the present studies were also tested for competence by transformation with chromosomal DNA of *B. subtilis* OG1 (*trp*$^+$) and selection on minimal agar without tryptophan or by transformation with the plasmid pKTH10 and selection of Km^R transformants. To monitor the *srfA* expression and surfactin synthesis, cells were transformed with chromosomal DNA of *B. subtilis* 168sfp$^+$ (Km^R).

Mitomycin C Resistance—The ability of *B. subtilis* strains to repair DNA damage was used as a measure for homologous recombination. To this purpose, resistance to mitomycin C was determined by transfer of colonies to solid media with 60 ng/ml of this mutagen. As a control for mitomycin C sensitivity, the *addAB* knock-out mutant 8GK0 (Δ*addAB*) (12), which does not grow in the presence of 60 ng/ml mitomycin C, was used.

Enzymatic Assays—The assay for alkaline phosphatase activity in growth media and the calculation of PhoA units (per A_{600}) were performed as described in Ref. 13, using *p*-nitrophenyl phosphate (Sigma) as the substrate. To assay cellular β-galactosidase levels, overnight cultures were diluted in fresh medium to an optical density at 600 nm (A_{600}) of 0.05, and samples were taken at hourly intervals for A_{600} readings and β-galactosidase activity determination. The β-galactosidase assay and the calculation of β-galactosidase units (per A_{600}) were performed as described in Ref. 2. 2-Nitrophenyl-β-D-galactopyranoside (Sigma) was used as the substrate.

SDS-PAGE and Western Blot Analyses—The presence of proteins in cell lysates was checked by SDS-PAGE, followed by blotting onto nitrocellulose or polyvinylidene difluoride membranes (Roche Molecular Biochemicals) and subsequent detection of the proteins using appropriate polyclonal antibodies. Membrane and protoplast supernatant fractions were prepared as described previously (14). Exported AmyQ and PhoA of *E. coli* were detected as described previously (2). Chemiluminescent detection of bound antibodies was performed with horseradish peroxidase-conjugated anti-rabbit IgG and the ECL Western blotting analysis system (Amersham Biosciences).

Sequence Comparisons and Predictions—Amino acid sequence similarity searches were carried out using the BLAST algorithms described in Ref. 15 (available on the World Wide Web at www.bork.embl-heidelberg.de/cgi/blast2a). Multiple alignments were performed using ClustalW (available on the World Wide Web at www2.ebi.ac.uk/clustalw/). The presence of possible signal peptidase I cleavage sites was analyzed using the algorithms described in Ref. 16 (available on the World Wide Web at www.cbs.dtu.dk/services/SignalP/).

RESULTS

The Competence-null Phenotype of BFA1074 Is Due to an Insertion in bdbD—Of all *B. subtilis* Systematic Gene Function Analysis Project mutants tested, only one (BFA1074) exhibited a complete loss of transformability (Table II). In this particular strain, the chromosomal integration vector pMutin2*mcs* (17) was inserted in the *bdbD* gene (Fig. 1). To verify that the competence defect of strain BFA1074 was due to inactivation of *bdbD*, we transformed the parental strain 168 with chromosomal DNA of BFA1074 and tested the resulting erythromycin-resistant strain for transformability. This strain exhibited the same complete loss of transformability as the original strain BFA1074 (results not shown), confirming that the defect was indeed caused by the pMutin2*mcs* insertion in *bdbD*.

As inferred from the genome sequence (7), *bdbD* forms an operon-like structure with the downstream *bdbC* gene (Fig. 1*A*), which has been implicated in secretion and competence development (Table II) (2). The *bdbDC* operon is flanked by two transcriptional terminators, and *bdbD* and *bdbC* are separated by four nucleotides. A putative σ^A-type promoter (TTGCGA-17 bp-TTTAAA) was found upstream of *bdbD* with the −35 sequence overlapping the proximal arm of the upstream terminator. Consistent with these indications that *bdbD* and *bdbC* form an operon, the expression profiles of both genes (determined with transcriptional *lacZ* gene fusions provided by integrated pMutin2*mcs* plasmids; see Fig. 1*B*) were nearly identical, irrespective of the growth medium used (minimal or TY medium; data not shown).

The bdbDC Operon Encodes Typical Thiol-disulfide Oxidoreductases—Based on computer-assisted analyses, the BdbD protein has a cleavable amino-terminal signal peptide. Thus, it seems likely that, upon translocation, this protein is proteolytically released from the membrane by one of the type I signal peptidases of *B. subtilis* (18, 19). Interestingly, the predicted mature part of BdbD contains a C*XX*C motif, which is typical for thiol-disulfide oxidoreductases involved in the formation or isomerization of disulfide bonds. These enzymes include thioredoxins, protein-disulfide isomerases, and the periplasmic

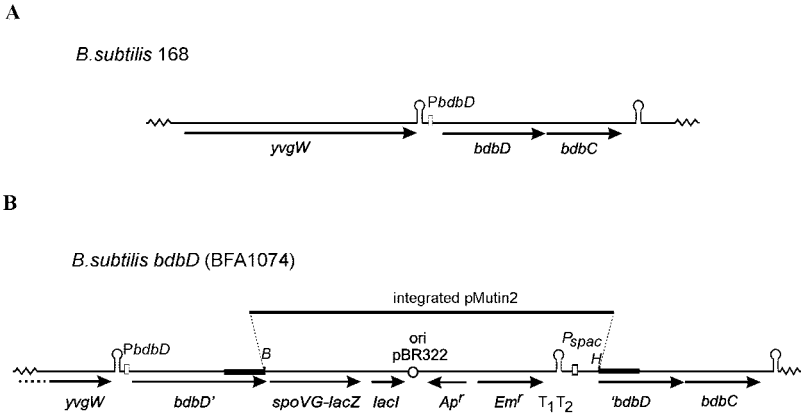

FIG. 1. **Construction of a *bdbD* derivative of *B. subtilis* 168.** *A*, schematic presentation of the *bdbDC* region of *B. subtilis* 168. *B*, by a single-crossover event (Campbell-type integration), the *bdbD* gene was disrupted, and *bdbC* was placed under the transcriptional control of the Pspac promoter of the integrated plasmid pMutin2mcs, which can be repressed by the product of the *lacI* gene. Simultaneously, the *spoVG-lacZ* reporter gene of pMutin2mcs was placed under the transcriptional control of the *bdbDC* promoter. The chromosomal fragment from the *bdbDC* region, which was amplified by PCR and cloned into pMutin2mcs, is indicated with *black bars*. Only the restriction sites relevant for the construction are shown (*B*, *Bam*HI; *H*, *Hin*dIII). *Ori pBR322*, replication functions of pBR322; *Ap*^{r}, ampicillin resistance marker; *Em*^{r}, erythromycin resistance marker; T_1T_2, transcriptional terminators on pMutin2mcs; *bdbD'*, 3′-truncated *bdbD* gene; *'bdbD*, 5′-truncated *bdbD* gene.

DsbA, DsbC, DsbG, and DsbE proteins of *E. coli* (1, 20). In fact, BdbD shows the highest levels of amino acid sequence similarity to DsbA from another Gram-positive bacterium, *Staphylococcus aureus* (Fig. 2)[2] and with the DsbG protein of *Chlamydia trachomatis* (22) (55 and 53% identical residues and conservative replacements in regions of 171 and 176 amino acids, respectively). In addition, we observed sequence similarity, albeit more limited, with DsbG of *E. coli* as well as the DsbA proteins of *Haemophilus influenzae* (23), *Neisseria meningitidis* (24), and *Pseudomonas aeruginosa* (25). A further characteristic of several thiol-disulfide oxidoreductases (but not of DsbA of *S. aureus*), namely a conserved Phe residue at position −5 relative to the C*XX*C motif (8, 26), is also present in the predicted BdbD protein. Thus, like the adjacent *bdbC* gene (2), *bdbD* specifies a typical thiol-disulfide oxidoreductase.

Both bdbD and bdbC Are Required for Competence Development—As the mutant strain BFA1074 was obtained through the integration of pMutin2*mcs* in *bdbD*, the competence defect of this strain may be due to a polar effect on the expression of *bdbC* rather than to the disruption of *bdbD* itself. To test this possibility, the transformability of the *bdbD* mutant strain BFA1074 was tested in the presence of IPTG[3] in order to induce *bdbC* transcription from the Pspac promoter of the integrated pMutin2*mcs* (see Fig. 1*B*). As shown in Table II, IPTG-induced expression of *bdbC* did not restore the transformability of BFA1074, and therefore, the competence-null phenotype seemed to be caused by disruption of the *bdbD* gene itself. To verify this, *bdbC* or *bdbD* was ectopically expressed in the *amyE* locus under control of a xylose-inducible promoter, resulting in the construct X*bdbC* or X*bdbD*, respectively. These constructs were then combined with the *bdbC* or *bdbD* mutations, and the transformability of the resulting strains was tested. As shown in Table III, competence was almost completely restored by the xylose-induced ectopic expression of *bdbC* in the *bdbC*-X*bdbC* strain. In fact, competence of the latter strain was even restored in the absence of xylose induction, which must be attributed to leakiness of the xylose-inducible promoter (see Ref. 18). In contrast, no complementation of the competence defect was observed when expression of one or both of the genes was lacking. This was the case for the following strains: *bdbD*-X*bdbD* (BdbC$^-$ in the absence of IPTG), *bdbC*-X*bdbD* (under all conditions BdbC$^-$), or *bdbD*-X*bdbC* (under all conditions BdbD$^-$), irrespective of the presence of xylose (Table III). These observations show that both BdbD and BdbC are essential for competence development.

BdbD and BdbC Are Required for the Transformation Process—Next, we asked which step(s) in the molecular cascade leading to competence development were affected in the *bdbD* mutant. The production of the competence transcription factor ComK was monitored by Western blotting in cells of the *bdbD* strain, which lacks BdbD and BdbC in the absence of IPTG (see Tables II and III) and in the parental strain 168. As shown in Fig. 3, the synthesis of ComK in the *bdbD* strain (BFA1074) was not reduced compared with the wild type strain, in either the presence or absence of IPTG, indicating that BdbD and BdbC do not affect the synthesis or stability of ComK. ComS, an essential molecule for the induction of ComK synthesis (27), is encoded on a small open reading frame within the *srfA* transcript. In independent experiments, we have shown that the expression of *srfA* is also not affected by the inactivation of *bdbD* or *bdbC* (not shown). Thus, ComS is most likely synthesized in both mutant strains, which is consistent with the absence of an effect on ComK synthesis.

ComK is the key activator for transcription of the genes required for both DNA binding and uptake and the incorporation of incoming DNA into the *B. subtilis* chromosome by homologous recombination. Since the cellular level of ComK was apparently not affected by inactivation of the *bdbDC* operon, it appears that the defect in transformability was probably not due to a regulatory defect. To confirm that the effect was not regulatory, we performed Western blots using antiserum against ComEA and ComGA, two essential proteins required for DNA binding to the cell surface and both completely dependent on ComK for their synthesis (4). Fig. 4 demonstrates that similar levels of these proteins are produced in the parental strain and *bdbD* mutant.

The possibility that inactivation of *bdbDC* caused a defect in homologous recombination was investigated by testing resistance to the DNA-damaging agent mitomycin C. The repair of mitomycin C damage is defective in the absence of recombination, and mutants deficient in recombination therefore exhibit mitomycin C sensitivity. Both the *bdbD* (BFA1074) strain and the *bdbC* strain showed wild-type resistance to mitomycin C (data not shown), indicating that the failure to obtain transfor-

[2] A. Dumoulin, direct submission to GenBank™, accession number AAG41993.

[3] The abbreviation used is: IPTG, isopropyl-1-thio-β-D-galactopyranoside.

```
BdbD_Bs      MKKKQQSSAKFAVILTVVVVVLLAAIVIINNKTEQGNDAVSGQPSIKGQPVLGKDDAPVT  60
DsbA_Sa      --------MTKK--LLTLFIVSMLILTACGKKESATTSSKNGKP---------------L
                        *  : :* :  :    :*      :  *:*

                     #  #
BdbD_Bs      VVEFGDYKCPSCKVFNSDIFPKIQKDFIDKGDVKFSFVNVMFHGKGSRLAALASEEVWKE 120
DsbA_Sa      VVVYGDYKCPYCKELDEKVMPKLRKNYIDNHKVEYQFVNLAFLGKDSIVGSRASHAVLMY
             ** :****** ** ::  ::**::*::**:  *:: ***: * ** * : : **  *

BdbD_Bs      DPDSFWDFHEKLFEKQPDTEQEWVTPGLLGDLAKSTTKIKPETLKENLDKETFAS----Q 180
DsbA_Sa      APKSFLDFQKQLFAAQQDENKEWLTKELLDKHIKQLHLDKETENKIIKDYKTKDSKSWKA
                * ** **::**  * * ::**:*  **    *     *    *   * :*  *

BdbD_Bs      VEKDSDLNQKMNIQATPTIYVNDKVIKNFADYDEIKETIEKELKGK 226
DsbA_Sa      AEKDKKIAKDNHIKTTPTAFINGEKVEDPYDYESYEKLLKDKIK--
              ***  : :  :*::*** ::* : :::  **:  :: :: ::*
```

FIG. 2. **Alignment of the deduced amino acid sequence of BdbD and DsbA of *S. aureus*.** The *upper line* shows the *B. subtilis* (*Bs*) BdbD protein; in the *lower line*, the amino acid sequence of the *S. aureus* (*Sa*) DsbA protein is presented. Identical residues (*), conservative replacements (:), and the two cysteine residues (#) of the potential active site are marked.

TABLE III
Requirement of bdbD and bdbC for competence development

Mutant strains *bdbD* (BFA1074) and *bdbC*, carrying additional insertions of either *bdbD* or *bdbC* under control of a xylose-inducible promoter in the *amyE* locus, were grown in minimal medium in the absence or presence of 2% xylose. The strains were tested for transformation with chromosomal DNA of *B. subtilis* OG1 (*trp*$^+$). Transformability was expressed as the percentage of *trp*$^+$ transformants of the total viable count.

Strain	Xylose induction	Relevant phenotypes	Transformability	
			Frequency	Percentage of 168
				%
168	−	Parental strain	6.3×10^{-2}	100
X*bdbD* (BV2007)	−	BdbD$^+$ BdbC$^+$	6.2×10^{-2}	98
X*bdbD* (BV2007)	+	BdbD$^+$ BdbC$^+$	7×10^{-2}	111
bdbD-X*bdbD* (BV2008)	−	BdbD$^-$ BdbC$^-$	$<10^{-5a}$	<0.001
bdbD-X*bdbD* (BV2008)	+	BdbD$^+$ BdbC$^-$	$<10^{-5a}$	<0.001
bdbC-X*bdbD* (BV2009)	−	BdbD$^-$ BdbC$^-$	$<10^{-5a}$	<0.001
bdbC-X*bdbD* (BV2009)	+	BdbD$^+$ BdbC$^-$	$<10^{-5a}$	<0.001
bdbD-X*bdbC* (BV2010)	−	BdbD$^-$ BdbC$^-$	$<10^{-5a}$	<0.001
bdbD-X*bdbC* (BV2010)	+	BdbD$^-$ BdbC$^+$	$<10^{-5a}$	<0.001
bdbC-X*bdbC*	−	BdbD$^+$ BdbC^{+b}	2.9×10^{-2}	48
bdbC-X*bdbC*	+	BdbD$^+$ BdbC$^+$	3.2×10^{-2}	53

[a] No transformants were obtained under our test conditions, after overnight growth at 37 °C.
[b] Due to leakiness of the xylose-inducible promoter (18), the relevant phenotype is BdbC$^+$, even in the absence of xylose induction.

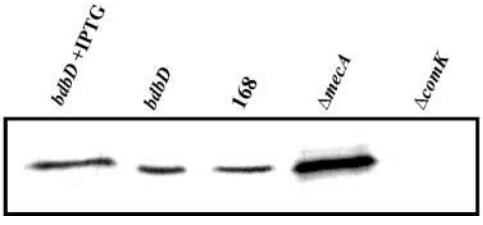

FIG. 3. **Immunological detection of the competence transcription factor ComK.** Proteins were separated by SDS-PAGE and blotted onto polyvinylidene difluoride membranes. The ComK protein was detected using ComK-specific antisera and chemiluminescence. As a positive control, the lysate of a ComK-overproducing *mecA* strain (QB4650) was used; as a negative control, the lysate of a *comK* deletion mutant (8G32) was used.

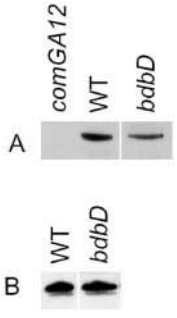

FIG. 4. **Inactivation of *bdbDC* does not eliminate ComEA or ComGA synthesis.** Membrane preparations from a wild-type strain carrying a plasmid that overexpresses *comS* and an isogenic *bdbD* strain that cannot produce either BdbC or BdbD were analyzed by Western blotting with antiserum raised against ComGA (*A*) or ComEA (*B*). Also included in *A* as a negative control is a membrane extract from an isogenic comGA12 strain, carrying a polar mutation that inactivates the *comG* operon.

mants with these strains is not caused by a defect at the level of recombination. Consistent with this view, the efficiency of transformation of *bdbD* and *bdbC* strains with the autonomously replicating plasmid pKTH10 was shown to be affected to similar extents as the transformation with chromosomal DNA (data not shown). Taken together, these observations show that the competence-null phenotype displayed by the *bdbD* and *bdbC* strains is due to a defect in the DNA uptake process rather than in the regulatory or recombination mechanisms.

The Secretion of E. coli PhoA Is BdbD-dependent—To investigate whether BdbD could be involved in the formation of disulfide bonds in secreted proteins, as previously shown for BdbC (2), the alkaline phosphastase PhoA of *E. coli* was used as a reporter, first because PhoA contains two intramolecular disulfide bonds, which are essential for its activity and stability (28), and, second, because the formation of these disulfide bonds requires the activity of an oxidase, such as DsbA of *E. coli* (see Ref. 20) (29). For this purpose, *B. subtilis bdbD* (BFA1074) was transformed with plasmid pPSPphoA5, specifying PhoA of *E. coli*, fused to the signal peptide and pro region of a lipase from *Staphylococcus hyicus*. The activity of *E. coli* PhoA in the growth medium of the *bdbD* strain (2.37 ± 0.35 units/A_{600}) was reduced about 5-fold compared with the paren-

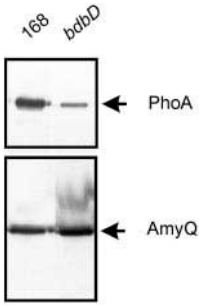

FIG. 5. ***B. subtilis bdbD* secretes reduced amounts of *E. coli* PhoA.** The presence of *E. coli* PhoA (containing two disulfide bonds; *upper panel*) or AmyQ of *B. amyloliquefaciens* (lacking disulfide bonds; *lower panel*) in the growth media of the *bdbD* mutant (BFA1074) or the parental strain 168 was monitored by Western blotting. For this purpose, cells containing plasmid pPSPphoA5 for PhoA production or pKTH10 for AmyQ production were used.

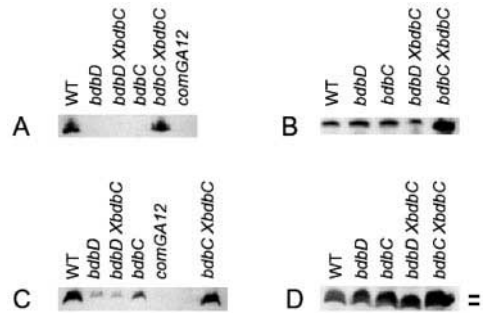

FIG. 6. **Western blot analysis of ComGC and ComGG in wild-type and *bdbDC* mutant backgrounds.** All of the strains overexpressed *comS*. Protoplast supernatant (*A* and *B*) and membrane preparations (*C* and *D*) were isolated from isogenic strains carrying the indicated mutations. *A* and *C* were developed with anti-ComGC antiserum, and *B* and *D* were developed with anti-ComGG antiserum. The *top and bottom bars* in *D* indicate the positions of pre-ComGG and mature ComGG, respectively.

tal strain 168 (13.01 ± 0.48 units/A_{600}). The secretion of active PhoA by the *bdbD* strain was not restored when the transcription of *bdbC* was induced with IPTG (2.48 ± 0.39 units/A_{600}), showing that BdbD, like BdbC (2), assists in the secretion of active PhoA. In contrast, the *bdbD* mutation did not affect the extracellular levels of the α-amylase AmyQ of *Bacillus amyloliquefaciens* (Fig. 5) or *B. subtilis* PhoA,[4] neither of which contain disulfide bonds.

BdbC and BdbD Are Both Required for the Stability of the Pilin-like ComGC Proteins—Since BdbC and BdbD are required for transformation but not for the expression of the competence genes *comEA* and *comGA* and since they are likely to function as thiol-disulfide oxidoreductases, we postulate that BdbC and BdbD are needed for the correct folding of at least one essential transformation protein. ComGC is a disulfide bond-containing protein (5) that is absolutely required for transformation. Since very few transformants were obtained when the *bdbDC* operon was inactivated, this pilin-like protein is an excellent candidate for a BdbD/BdbC substrate. ComGG, another essential, pilin-like competence protein, contains an intermolecular disulfide bond (5). However, only a minor fraction of ComGG is in this disulfide-bonded, dimerized form. We determined by Western blotting whether the amounts of ComGC and ComGG are altered by mutation of *bdbD* or *bdbC*. To do this we moved the *bdbD* and *bdbC* mutations into a strain that overexpresses *comS*. In this strain, nearly all of the cells in the culture become competent, and the Western blot signals of competence proteins are enhanced about 10-fold compared with the wild type (30). In the new background, as in the original strains, the transformation frequencies obtained for the *bdbD* and *bdbC* mutants were less than 10^{-6}, 10,000-fold lower than that of the *comS*-overexpressing strain with intact *bdbD* and *bdbC* genes.

In competent cells, the pilin-like proteins are recovered in two fractions, in the membrane fraction and in the protoplast supernatant (5). The latter probably represents cell wall-associated material. Pre-ComGC, which contains a single predicted membrane-spanning segment, exists as an integral membrane protein with its C terminus facing the cell wall. Upon processing by the ComC signal peptidase, the mature form of ComGC is liberated from the membrane and found in the protoplast supernatant fraction (5, 31). In contrast, some pre-ComGG molecules are present as integral membrane proteins, arranged with their C terminus in the cytosol, while other pre-ComGG molecules are peripheral membrane proteins, exposed on the cytosolic face of the membrane. The mature ComGG is translocated to a position exterior to the membrane and is recovered in the protoplast supernatant. Fig. 6*A* shows that in the *bdbC* and *bdbD* mutants, in contrast to the wild type, there is no detectable ComGC in the protoplast supernatant fraction. In other gels, upon prolonged exposure, a faint ComGC signal was detectable in that fraction (not shown). In the cell membrane fraction, the amount of ComGC is also dramatically lowered in the *bdbD* mutant, although there is a residual signal in this fraction (Fig. 6*C*). Although the effect of the polar *bdbD* mutation on the ComGC signal appears to be more severe than that of the *bdbC* mutation (Fig. 6*C*) in other gels, the effects of these mutations were equivalent (not shown). The decreased amount of ComGC in the *bdbD* mutant relative to that in the wild-type, is not due to a polar effect on *bdbC*, since it cannot be complemented by ectopic expression of the latter (Fig. 6, *A* and *C*). This complementation failure is not due to inadequate expression of the ectopic *bdbC*, since full complementation of the *bdbC* mutant was obtained (Fig. 6, *A* and *C*). ComGG behaves differently: no effect of *bdbDC* inactivation on the ComGG signal was detected (Fig. 6, *B* and *D*). An unprocessed, membrane-associated ComGG band is usually detectable (5) and is visible in Fig. 6*D*. The absence of BdbD and BdbC clearly does not prevent the processing of pre-ComGG. The failure of the *bdbDC* knockout to alter the total ComGG signal is consistent with the presence of the disulfide bond in only a minor fraction of ComGG. Since there is little or no effect of *bdbDC* inactivation on the expression of late competence genes, including the *comG* operon (Fig. 4), we conclude that in the absence of the BdbD or BdbC proteins, ComGC cannot fold correctly and is consequently degraded by a cell surface protease. This provides an adequate explanation for the competence deficiency of the *bdbDC* loss of function mutants.

ComK Is a Regulator of bdbDC Transcription—As BdbD and BdbC play critical roles in the development of competence, we investigated whether ComK is involved in the transcription of the *bdbDC* operon. For this purpose, the transcriptional *bdbC-lacZ* gene fusion in the *bdbC* mutant strain was used. As shown in Fig. 7, the disruption of the *comK* gene in the *bdbC* mutant resulted in a significant decrease of *bdbC* transcription when cells were grown in minimal medium. A comparable result was obtained for *bdbD* and *bdbC* in transcript profiling experiments with DNA arrays.[5] These observations show that ComK is a positive regulator of the *bdbDC* operon and that the *bdbD* and *bdbC* genes can be regarded as late competence genes.

[4] Z. Pragai, personal communication.

[5] R. Meima, C. Eschevins, S. Fillinger, A. Bolhuis, L. W. Hamoen, R. Dorenbos, W. J. Quax, J. M. van Dijl, R. Provvedi, I. Chen, D. Dubnau, and S. Bron, unpublished observations.

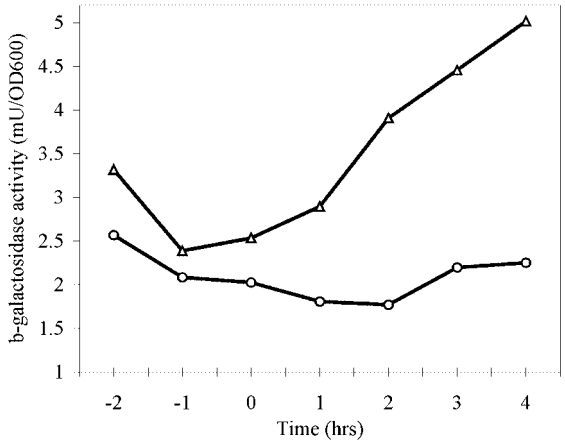

FIG. 7. **ComK-dependent expression of *bdbC*.** The expression of *bdbC* was studied using a transcriptional *lacZ* fusion. For this purpose, *bdbC* (△) or *bdbC comK* (○) mutant strains were grown in minimal medium, and the production of β-galactosidase was monitored at hourly intervals. The time scale indicated on the *x* axis reflects the time relative to the transition from exponential to postexponential growth (T_0).

DISCUSSION

In an attempt to identify novel functions required for genetic competence, *B. subtilis* mutants constructed in the framework of the *B. subtilis* Systematic Gene Function Analysis Project were screened for transformability. Among the nearly 1300 mutants tested, a competence-null phenotype was observed for strain BFA1074, carrying an insertion in the *bdbD* gene. In addition to *bdbD*, the downstream gene, *bdbC*, which apparently forms a bicistronic operon with *bdbD*, is required for competence (2). Both BdbD and BdbC belong to the thioredoxin family of redox proteins, showing the highest levels of similarity to enzymes involved in disulfide bond formation in periplasmic and extracellular proteins of Gram-negative bacteria. The predicted BdbD protein contains the FX_4C*XX*C motif, typical of the active sites of several members of the thioredoxin superfamily, and also shows similarity to DsbA- and DsbG-like proteins from various organisms. Although the overall similarity with these proteins is relatively low, this is common for members of the thioredoxin superfamily, which generally lack overall sequence similarity (32). Like BdbD, BdbC contains a typical active site C*XX*C motif. The similarity of BdbC to several known DsbB proteins is higher than that of BdbD (2).

Our experiments show that the *bdbDC* operon is not needed for the expression of the late competence genes, suggesting strongly that it is instead required for the correct folding of one or more essential transformation proteins. Among the very few examples of translocated proteins known to contain disulfide bonds in *B. subtilis* are ComGC (one intramolecular disulfide bond) and the ComGG homodimer (one intermolecular disulfide bond). These type IV pilin-like proteins form parts of the DNA uptake machinery and are required for DNA binding (5, 33, 34). Our experiments show that in the absence of either BdbD or BdbC, the Western blot signal for ComGC is markedly reduced. An attractive working hypothesis for the role of BdbD and BdbC is that these enzymes facilitate the proper folding of ComGC by catalyzing disulfide bond formation. Presumably, when incorrectly folded, ComGC is unstable. If this hypothesis is correct, the BdbD-BdbC pair could in fact represent a redox system required for the assembly of the DNA uptake apparatus of *B. subtilis*. Accordingly, BdbD might act as an extracytoplasmic oxidase or isomerase catalyzing the formation of the proper disulfide bond in ComGC. Earlier studies on transformation of *H. influenzae* indicated that the DsbA-like Por protein is involved in DNA uptake, presumably because this process involves outer membrane proteins containing disulfide bonds (23). Similar to other known redox couples (*e.g.* DsbA and DsbB of *E. coli*), recycling of BdbD would be achieved by a membrane-bound component, the DsbB ortholog BdbC. In a similar manner, BdbD and BdbC might cooperate in the formation of correct disulfide bonds in heterologous proteins, such as PhoA of *E. coli*. Consistent with the idea that BdbD and BdbC form a functional redox pair, the *bdbDC* operon appears to be conserved in at least one other organism, namely *C. trachomatis* (not shown), although the function of this operon is not known. Interestingly, the absence of the second DsbB ortholog of *B. subtilis*, BdbB, did not detectably affect competence development, although the secretion of PhoA was mildly affected (2). Therefore, it appears that although the specificities of the BdbB- and BdbC-containing redox systems partially overlap, assembly of an active DNA translocase is strictly dependent on the latter.

In conclusion, our results clearly demonstrate that (i) both BdbD and BdbC are similar to thiol-disulfide oxidoreductases, (ii) the stability of a disulfide bond-containing secretory reporter protein is affected by disruption of the *bdbDC* genes, (iii) *bdbD* and *bdbC* are individually required for transformation, and (iv) *bdbD* and *bdbC* are both required for the stabilization of the disulfide bond-containing protein ComGC. The latter observation provides a sufficient explanation for the BdbD and BdbC requirement in competence development. Moreover, the view that *bdbD* and *bdbC* should be regarded as late competence genes is fully supported by the observation that their transcription is significantly enhanced in the presence of ComK when the cells are grown to competence. Although ComGC is likely to be a target for the BdbD-BdbC system, no direct evidence for the role of the BdbD and BdbC proteins in folding of ComGC protein has been obtained so far. Additional experiments will be required to elucidate the precise molecular mechanism by which BdbC and BdbD are involved in the establishment of competence.

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