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Thiol-disulfide oxidoreductases are required for disulfide bond formation in proteins that are exported from the cytoplasm. Four enzymes of this type, termed BdbA, BdbB, BdbC, and BdbD, have been identified in the Gram-positive eubacterium *Bacillus subtilis*. BdbC and BdbD have been shown to be critical for the folding of a protein required for DNA uptake during natural competence. In contrast, no function has been assigned so far to the BdbA and BdbB proteins. The *bdbA* and *bdbB* genes are located in one operon that also contains the genes specifying the lantibiotic sublancin 168 and the ATP-binding cassette transporter SunT. Interestingly sublancin 168 contains two disulfide bonds. The present studies demonstrate that SunT and BdbB, but not BdbA, are required for the production of active sublancin 168. In addition, the BdbB paralogue BdbC is at least partly able to replace BdbB in sublancin 168 production. These observations show the unprecedented involvement of thiol-disulfide oxidoreductases in the synthesis of a peptide antibiotic. Notably BdbB cannot complement BdbC in competence development, showing that these two closely related thiol-disulfide oxidoreductases have different, but partly overlapping, substrate specificities.

Bacillus subtilis is a Gram-positive soil bacterium that is particularly well known for its high protein secretion potential (1, 2). A small group of secreted *Bacillus* proteins is formed by lantibiotics, small post-translationally modified peptides that exhibit antimicrobial activity (3–5). In general, lantibiotics are characterized by the presence of the unusual amino acids 2,3-didehydroalanine and/or 2,3-didehydrobutyrine, which are formed by dehydration of serine and threonine residues, respectively (6). With neighboring cysteine residues they can form a lanthionine (2,3-didehydroalanine) or 3-methyllanthionine bridge (2,3-didehydrobutyrine). To date two lantibiotics of *B. subtilis* have been characterized in detail. These are subtilin from *B. subtilis* ATCC 6633 (7, 8) and sublancin 168 from *B. subtilis* 168 (9). In addition, two lantibiotic-like peptides originating from the ericin gene cluster of *B. subtilis* A1/3 were recently described (10). Notably sublancin 168 displays the extraordinary characteristic of having two disulfide bonds in addition to a β-methyllanthionine bridge (Fig. 1).

Sublancin 168, specified by *sunA*, was identified as a type AII lantibiotic by Paik *et al*. (9) in 1998. Presumably it acts by forming pores in the cytoplasmic membrane of a sensitive organism (11). Type AII lantibiotics are characterized by a "double glycine" GG, GA, or GS motif in their leader sequence, GS in sublancin 168, which is preceded by conserved EL or EV and EL or EM sequences. Cleavage occurs immediately behind the double glycine motif during transport by a dual-function transporter that also has leader peptidase activity. The leader is thought to prevent the lantibiotic from becoming active before translocation (12, 13). The *sunT* gene, which is located directly downstream of *sunA*, encodes a protein possessing features of a dual-function ATP-binding cassette transporter with a proteolytic domain and an ATP-binding cassette. These domains are common among lantibiotic transporters (3).

Interestingly the sublancin 168 operon appears to include the *bdbA* and *bdbB* genes (*bdb* for *Bacillus* disulfide bond) downstream of *sunT* (Fig. 2). The corresponding BdbA and BdbB proteins were previously identified by Bolhuis *et al*. (14) and have been implicated in thiol-disulfide redox reactions. BdbA shows sequence similarity to the thiol-disulfide oxidoreductase Bdb of *Bacillus brevis* (15, 16), whereas BdbB shows significant sequence similarity to DsbB of *Escherichia coli* (15 ,17). BdbB was shown to be involved in the folding of PhoA of *E. coli* upon the expression of this secretory protein in *B. subtilis*. As PhoA contains two disulfide bonds, this indicates that BdbB is involved in disulfide bond formation. However, compared with its paralogue BdbC, BdbB was less important for the folding of PhoA (15). These observations prompted us to investigate whether BdbA, BdbB, and BdbC have a role in the production of functional sublancin 168 in particular because this lantibiotic contains two disulfide bonds. The present results show that BdbB and BdbC are involved in the production of active sublancin 168, whereas BdbA is not required. The finding that thiol-disulfide oxidoreductases are required for the synthesis of a peptide antibiotic has never been reported.

EXPERIMENTAL PROCEDURES

Plasmids, Bacterial Strains, and Growth Conditions—Table I lists the plasmids and strains used. TY medium contained Bacto tryptone (1%), Bacto yeast extract (0.5%), and NaCl (0.5%). Antibiotics were used in the following concentrations: ampicillin, 100 μg/ml; chloramphenicol, 5 μg/ml; erythromycin, 2 μg/ml; and kanamycin, 10 μg/ml. Xylose was used at 1% (w/v) concentrations.

DNA Techniques—Procedures for DNA purification, restriction, liga-

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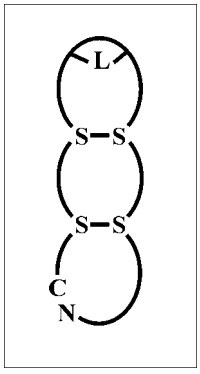

FIG. 1. **Schematic representation of the lantibiotic sublancin 168 as proposed by Paik *et al.* (9).** *C*, carboxyl terminus; *N*, amino terminus; *S–S*, disulfide bond; *L*, lanthionine bridge.

tion, agarose gel electrophoresis, and transformation of competent *E. coli* DH5α cells were carried out as described by Sambrook *et al.* (18). Enzymes were from Invitrogen. *B. subtilis* was transformed as described by Kunst and Rapoport (19). The nucleotide sequences of primers (5′–3′) used for PCR are listed below; nucleotides identical to genomic template DNA are printed in capital letters, restriction sites used for cloning are underlined, and nucleotides used for PCR-mediated coupling are in bold.

To construct *B. subtilis* Δ*bdbA*, splicing by overlap extension (20) was used. Two fragments flanking the *bdbA* gene were amplified and ligated into the chromosomal integration and excision plasmid pORI280 (21) after PCR-mediated coupling or ligation. The upstream fragment *bdbA*fr of 811 nucleotides was amplified using the primers *bdbA*fr1 (GCA ATC AGA TCT TCA GCA GGC AC) and *bdbA*fr2 (**gtt tca tac tag tta gct aat taa tca TAT AGA ATA C**TC CTT ATT TTC CGA GTA GCT CG). The downstream fragment *bdbA*bk of 1034 nucleotides was amplified with the primers *bdbA*bk1 (**gta ttc tat atg att aat tag cta act agt ATG AAA C**TG AGT GAT ATT TAT TTG G) and *bdbA*bk2 (CAA AAT TGC AGA TCT AAA GTA ATC AAC). The resulting plasmid, pORI*bdbA*, was first inserted into the chromosome of *B. subtilis* 168 by a Campbell-type integration. Upon growth in the absence of erythromycin, *B. subtilis* Δ*bdbA* was obtained due to the spontaneous excision of the plasmid from the chromosome together with the *bdbA* gene. Correct integration and excision was verified by Southern hybridization. Note that the *bdbA* deletion is designed in such a way that the *sunT* gene remains intact despite the fact that these genes overlap with four nucleotides.

To construct *B. subtilis* Δ*bdbA*$^{s+}$, the carboxyl-terminal part of the *sunT* gene was amplified with the oligonucleotides sunTrec1 (cgc aca agc tTG TAG CAA AGG CAG TTA TTA GC) and sunTrec2 (CAA TCC gga tcc TCA TAT AGA ATA CTC CTT ATT TTC CG) and ligated into pMutin2 to construct the plasmid pMsunTrec. pMsunTrec was integrated into the chromosome of *B. subtilis* at the *sunT* locus by a Campbell-type integration (single crossover) resulting in *B. subtilis* Δ*bdbA*$^{s+}$.

To construct *B. subtilis bdbB*-X*bdbB*, the *bdbB* gene was amplified using the primers yolK1 (CTC CAC tcT Aga GAA CAC GTC CTG AAA GGA ATT GAA GTA TG) and yolKm2 (cgg att acc gga tcc tca gtt cag gtc ctc ctc gct gat aag ttt ttg ttc ATT ATA TAC ATG TTG ATT TTG TTT T). The amplified fragment was ligated into plasmid pX downstream of the *xylA* promoter. This vector was integrated into the *amyE* locus of *B. subtilis bdbB* by double crossover recombination resulting in the strain *B. subtilis bdbB*-X*bdbB*.

To construct *B. subtilis bdbC*-Km,[1] first a pUC18 construct containing the *bdbC* gene was made. Primers yvgU1 (GAA ATt ctA GAG ACA ATA GAA AAA GAG CTG AAA GGG AAG TAA C) and yvgU3 (GCG CCC GGg ATc CGC GGG CGC TTT TTT TGT TAT TCA GAT TTT TCG CCT TTC AGC AGG CAC) were used to amplify the *bdbC* gene. This fragment was ligated into the multiple cloning site of pUC18 resulting in pUC18bdbC. The *Nsi*I site within the *bdbC* gene was then used to insert a Km^R marker that was isolated from pKM1 (laboratory collection; Jan Kiel) using *Hin*cII. In this way pUC18bdbCKm was obtained, and this plasmid was subsequently used to transform *B. subtilis* 168. A double crossover event then led to *B. subtilis bdbC*-Km in which the *bdbC* gene is disrupted by the Km^R cassette. Correct integration was verified using Southern hybridization. To construct the double mutant *B. subtilis bdbB bdbC*-Km, the *B. subtilis bdbB* strain was transformed with chromosomal DNA of *B. subtilis bdbC*-Km.

Sublancin 168 Activity Assay—A halo assay was performed on plates with *B. subtilis* ΔSPβ as indicator strain. This strain was constructed using the sequences with the NCBI accession numbers M81760 and M81762 as described by Lazarevic *et al.* (22). SPβ-cured *B. subtilis* strains were previously shown to be sensitive to a bacteriocin (betacin) specified by this prophage (23). Sequencing of the SPβ region of the *B. subtilis* 168 chromosome indicated that this bacteriocin is specified by a gene (*yolG*; Ref. 22), which is now known as the gene for sublancin 168 (*sunA*). Thus, the SPβ-cured strain lacks the *sunA* gene and the as yet unidentified sublancin 168 resistance gene(s). The indicator strain and the mutant strains were grown overnight on TY with the appropriate antibiotic(s). The overnight culture of the indicator strain was then diluted 100-fold in TY, and 100 μl was subsequently plated. After drying the plate, 1-μl aliquots of the overnight cultures of the relevant mutant strains were spotted, and the plates were incubated overnight. The next day plates were analyzed for halo formation. In this assay, the presence of antibiotics in the overnight cultures did not result in halo formation. Importantly variations in the halo size of different colonies of the same strain were insignificant, in particular when these colonies were present on one plate.

Delayed Extraction-Matrix-assisted Laser Desorption Ionization-Time of Flight Mass Spectra (DE-MALDI-TOFMS)—DE-MALDI-TOFMS were recorded on a Voyager-RP-DE instrument (PerSeptive Biosystems) using a 337 nm nitrogen laser for desorption and ionization. All experiments were carried out with the linear positive ion mode. The total acceleration voltage was 25 kV; 23.6 kV was used on the first grid. The delay time was 250 ns. 1-ml aliquots of culture supernatants and media were extracted with 200 μl of 1-butanol. 150 μl of the butanolic phase was dried in a Speed-Vac evaporator, and extracted peptides were dissolved in 20 μl of solvent A (0.1% trifluoroacetic acid, 20% acetonitrile in water (v/v)) and adsorbed to 1 μl of POROS 50 R2 (PerSeptive) beads prepared as a microcolumn as described by Kussmann *et al.* (24). After washing with 20 μl of solvent A, the peptides were eluted with 4 μl of a mixture of 70% acetonitrile, 0.1% trifluoroacetic acid in water (v/v). Sample preparation for MALDI was performed with the solution phase nitrocellulose method described by Landry *et al.* (25). Between 100 and 250 single scans were accumulated for each mass spectrum.

Sequence Similarity Searches and Prediction of Transmembrane Regions—Similarity searches were performed with the standard protein-protein BLAST algorithm (www.ncbi.nlm.nih.gov/BLAST/) at NCBI (26) using Swiss-Prot as the data base. Transmembrane segments in SunT were predicted using the TMHMM algorithm version 2.0 (www.cbs.dtu.dk/services/TMHMM-2.0/) from the Center for Biological Sequence Analysis (CBS) (27).

RESULTS

SunT Is Required for Sublancin 168 Production—As a first approach to characterize the factors involved in the production of sublancin 168, the production of this lantibiotic by a *sunT* mutant strain was tested. It has to be noted that the *sunT* strain contains a pMutin2 disruption in the 3′ end of *sunT*, placing the downstream *bdbA*, *yolJ*, and *bdbB* genes under the control of the isopropyl-1-thio-β-D-galactopyranoside-inducible P_{spac} promoter (Fig. 3). Aliquots of an overnight culture of *B. subtilis sunT* were spotted on a plate with the sublancin 168-sensitive indicator strain *B. subtilis* ΔSPβ. It was found that *B. subtilis sunT* completely lacked sublancin 168 activity (Fig. 4) also after induction of *bdbA*, *yolJ*, and *bdbB* transcription by the addition of isopropyl-1-thio-β-D-galactopyranoside (data not shown). The latter observation shows that the lack of sublancin 168 production is not caused by a polar effect on the transcription of the genes located downstream of *sunT*. Taken together these results demonstrate that SunT is essential for the production of active sublancin 168. In what follows, this conclusion is corroborated by mass spectrometry.

BdbB Has a Major Role in Sublancin 168 Production—To investigate whether BdbB is involved in the production of sublancin 168, the halo assay with the ΔSPβ indicator strain was performed as described above. Indeed, *B. subtilis bdbB* showed

[1] The abbreviations used are: Km, kanamycin; DE-MALDI-TOFMS, delayed extraction-matrix-assisted laser desorption ionization-time of flight mass spectra.

TABLE I
Plasmids and bacterial strains

	Relevant characteristics[a]	Source/Ref.
Plasmids		
pUC18	Ap^R, ColE1, ϕ80dlacZ, *lac* promoter	44
pUC18bdbC	pUC18 derivative; contains the *bdbC* gene	This work
pUC18bdbCKm	pUC18 derivative; contains the *bdbC* gene that is disrupted with a Km^R marker	This work
pKM1	pUC7 derivative; contains the Km^R marker	Laboratory collection
pMutin2	Integration vector for *B. subtilis*; contains a multiple cloning site downstream of the P_{spac} promoter and a promoterless *lacZ* gene preceded by the ribosome binding site of the *spoVG* gene; Ap^R; Em^R	45
pMsunTrec	pMutin2 derivative; carries the 3′ part of the *sunT* gene	This work
pORI280	Em^R; $LacZ^+$; ori^+ of pWV01, replicates only in strains providing *repA in trans*	21
pORI*bdbA*	Em^R; pORI280 derivative for the excision of the *bdbA* gene from the chromosome	This work
pX	Vector for the integration of genes in the *amyE* locus of *B. subtilis*; integrated genes will be transcribed from the xylose-inducible *xylA* promoter; carries the *xylR* gene; 7.5 kb; Ap^R; Cm^R	46
pX*bdbB*	pX derivative; carries *bdbB* downstream of the *xylA* promoter; 8.0 kb; Ap^R; Cm^R	This work
Strains		
E. coli		
DH5α	F^- ϕ80d*lacZ*Δ*M15 end*A1 *rec*A1 *gyr*A96 *thi*-1 *hsd*R17 (r_K^- m_K^+) *sup*E44 *rel*A1 *deo*R Δ(*lac*ZYA-*arg*F) U169	Invitrogen
EC1000	$RepA^+$ MC1000, Km^R, carrying a single copy of the pWV01 *repA* gene in the *glgB* gene	21
B. subtilis		
168	*trp*C2	47
ΔSPβ	*trp*C2; ΔSPβ; sublancin 168-sensitive; laboratory name CBB312	C. Bruand and S. D. Ehrlich, unpublished
sunT	*trp*C2; *sunT*::pMI-*bdbA*; Em^R; the 3′ end of *sunT* is disrupted due to the use of an internal fragment of *sunT* for the integration of pMutin2; formerly known as I*bdbA*	15
Δ*bdbA*	*trp*C2; Δ*bdbA*; *sunT* remains intact; defective in sublancin 168 production	This work
Δ*bdbA*$^{s+}$	*trp*C2; Δ*bdbA*; *sunT*::pMsunTrec; producing sublancin 168	This work
bdbB	*trp*C2; *bdbB*::pMI-*bdbB*; Em^R	15
bdbB-X*bdbB*	*trp*C2; *bdbB*::pMI-*bdbB*; P_{xylA}-*bdbB*; *amyE*; Em^R; Cm^R	This work
bdbC-Km	*trp*C2; *bdbC*::Km; Km^R	This work
bdbB bdbC-Km	*trp*C2; *bdbB*::pMI-*bdbB*; *bdbC*::Km; Em^R; Km^R	This work

[a] Ap^R, ampicillin-resistant; Km^R, kanamycin-resistant; Cm^R, chloramphenicol-resistant; Em^R, erythromycin-resistant.

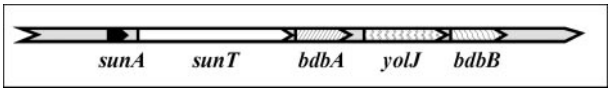

FIG. 2. **Schematic representation of the sublancin 168 operon.** Relative locations of the *sun* genes for sublancin 168 (*sunA)* and sublancin 168 transport (*sunT*), the genes specifying the thiol-disulfide oxidoreductases *bdbA* and *bdbB*, and the gene of unknown function, *yolJ*, are indicated. The distances between the genes are: *sunA-sunT*, 60 nucleotides; *sunT-bdbA*, 4 nucleotides overlap; *bdbA-yolJ*, 2 nucleotides; *yolJ-bdbB*, 1 nucleotide overlaps.

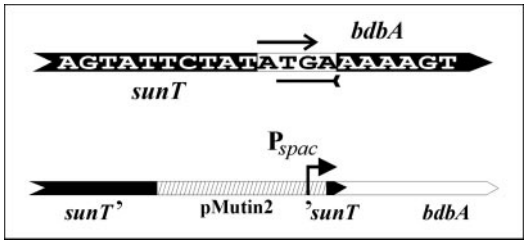

FIG. 3. **Schematic representation of the relative locations of *sunT* and *bdbA* in *B. subtilis* 168 (*top*) and *B. subtilis sunT* (*bottom*).** The Campbell-type integration (single crossover) of pMutin2 leads to the disruption of the 3′ end of the *sunT* gene. Simultaneously the *bdbA* gene is placed under the transcriptional control of the P_{spac} promoter. The relative locations of the overlapping start (ATG) and stop (TGA) codons of *bdbA* and *sunT*, respectively, are marked. The 3′ (*sunT*′) and 5′ (′*sunT*) truncated copies of *sunT* are indicated.

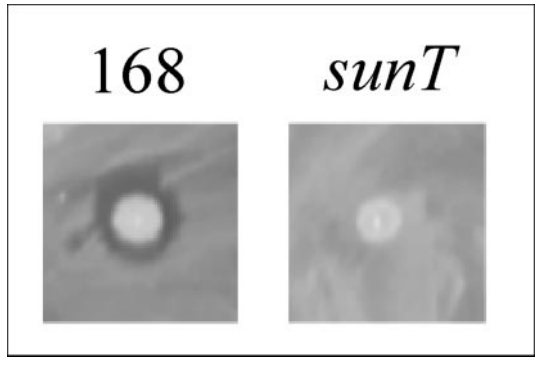

FIG. 4. **Sublancin 168 production in *B. subtilis* 168 and a *sunT* mutant.** From a 100-fold-diluted overnight culture of the *B. subtilis* ΔSPβ indicator strain, 100 μl was plated. After drying of the plate, 1-μl aliquots of overnight cultures of the strains to be tested for sublancin 168 production were spotted, and subsequently the plates were incubated overnight at 37 °C. Sublancin 168 activity is visualized by halo formation.

a significantly decreased cell killing activity compared with the parental strain 168, indicating that BdbB is involved in the production of sublancin 168 (Fig. 5). The effect of the *bdbB* mutation was particularly evident when the cells were grown in the presence of 1% xylose, which is in accord with the observation that xylose affects sublancin 168 production or activity already in the parental strain (Fig. 5, compare the halo size of *B. subtilis* 168 in the presence and absence of xylose). To determine whether the decreased sublancin 168 activity in *B. subtilis bdbB* was a direct consequence of the disruption of *bdbB*, we constructed *B. subtilis bdbB*-X*bdbB*, in which the *bdbB* gene is ectopically expressed from a xylose-inducible promoter. As shown in Fig. 5, the ectopically expressed *bdbB* gene fully restored the production of active sublancin 168 in the presence of xylose. Thus, BdbB is important but not essential for the production of sublancin 168.

BdbC Can Partly Replace BdbB—The residual activity of sublancin 168 upon disruption of the *bdbB* gene suggested that another protein was at least partly able to fulfill the role of

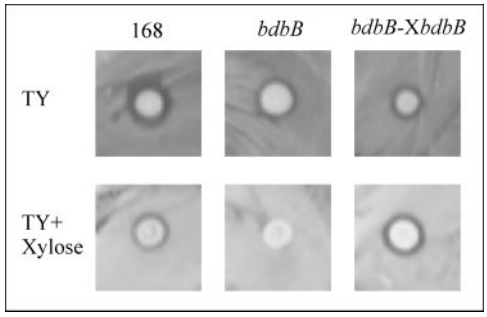

FIG. 5. **Sublancin 168 production in *B. subtilis* 168, *B. subtilis bdbB*, and *B. subtilis bdbB-XbdbB*.** In the latter strain, the ectopic expression of the *bdbB* gene is controlled by a xylose-inducible promoter. TY plates used in the *lower panels* were supplemented with 1% xylose. Sublancin 168 production was tested as described in Fig. 4.

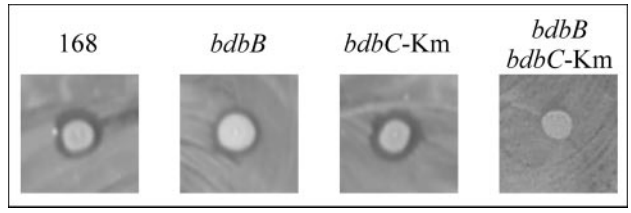

FIG. 6. **Sublancin 168 production in *B. subtilis* 168 and *bdbB*, *bdbC* single and double mutant strains.** Sublancin 168 production was tested as described in Fig. 4.

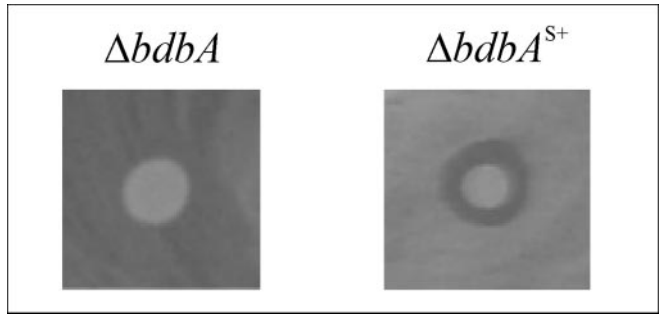

FIG. 7. **Sublancin 168 production in *B. subtilis* Δ*bdbA* and *B. subtilis* Δ*bdbA*$^{s+}$.** Sublancin 168 production was tested as described in Fig. 4.

BdbB. Especially BdbC was a likely candidate because of its high degree of sequence similarity with BdbB (15). Therefore, the possible effect of a *bdbC* disruption on sublancin production was tested. In contrast to the *bdbB* mutant, the *bdbC* mutant produced sublancin 168 at levels that were comparable to those produced by the parental strain (Fig. 6). However, the strain with the *bdbB* and *bdbC* genes both disrupted did not display any sublancin 168 activity. This leads to the conclusion that BdbC is not required for production of sublancin 168 when BdbB is present. Nevertheless, BdbC can partly replace BdbB in the absence of the latter.

BdbA Is Not Required for Sublancin 168 Production—To investigate the role of BdbA in sublancin 168 production a strain with a clean *bdbA* deletion was constructed and subsequently tested for sublancin 168 production. This strain did not show any sublancin 168 activity (Fig. 7). However, also when this strain was provided with a *bdbA* gene that was ectopically expressed from a xylose-inducible promoter, sublancin 168 production was not restored (data not shown). This indicated that *bdbA*, unlike *sunT*, is not essential for this process. As shown in Fig. 3, the *sunT* and *bdbA* genes are partially overlapping. Thus, the 3′ end of the *sunT* gene might have been damaged during the construction of the clean *bdbA* deletion strain. Alternatively the deletion of *bdbA* might interfere with the expression of its downstream genes resulting in reduced levels of active sublancin 168. Therefore, a pMutin2-based integration plasmid containing the 3′ end of *sunT* was constructed. Upon a Campbell-type integration of this plasmid (pMsunTrec), the 3′ end of *sunT* was replaced with the experimentally verified correct sequence. Simultaneously the downstream genes of *bdbA* were placed under the control of the P_{spac} promoter. Subsequently we determined whether sublancin 168 activity in the resulting strain Δ*bdbA*$^{s+}$ was restored. This was indeed the case as depicted in Fig. 7. The observed sublancin 168 production by this strain, which lacks *bdbA*, implies that BdbA is dispensable for the production of this lantibiotic.

Subtilosin Production Is Not Affected in bdb Mutants—In addition to sublancin 168, another bacteriocin of *B. subtilis* 168 is known for which the presence of a disulfide bond has been proposed initially: subtilosin (28). Subtilosin (29) is composed of 35 amino acids, including three cysteines. Despite the presence of these cysteines, a recent structural analysis of subtilosin by two-dimensional ^{1}H NMR provided no evidence for disulfide bond formation in this bacteriocin (30). To investigate whether the Bdb proteins might, nevertheless, be involved in the synthesis of subtilosin, DE-MALDI-TOF mass spectrometric analyses were performed. Notably this allowed the parallel monitoring of the presence of both subtilosin and sublancin 168 in supernatants of *bdb* mutant strains. As shown in Fig. 8, subtilosin was identified by its proton-adduct at *m/z* 3403.3 (average mass; Ref. 30). The presence of sublancin 168 is indicated by a signal at *m/z* 3881.2 (average mass), which is in good accordance with *m/z* 3877.8 observed for purified sublancin (Ref. 9; data not shown). The data demonstrate that subtilosin production is not affected in the *bdb* mutant. Furthermore, the mass spectrometry results concerning sublancin 168 are in accord with the results from the halo assays. Interestingly supplementation of the medium with xylose leads to the appearance of additional signals with appreciably higher masses (+132 and +264 Da) than observed in medium without xylose. This observation can be interpreted with a covalent modification of subtilosin and sublancin 168, suggesting that both bacteriocins are modified under these conditions. In conclusion, the present observations show that SunT and BdbB are major determinants for the production of sublancin 168, whereas the production of subtilosin does not depend on these proteins.

DISCUSSION

In this report we show for the first time that the presence of at least one thiol-disulfide oxidoreductase, BdbB or BdbC, is required for the production of active sublancin 168. Notably BdbB is of major importance for this process, whereas the involvement of BdbC is only evident in the absence of BdbB. Whether BdbB and BdbC are directly or indirectly involved in sublancin 168 production has yet to be determined. The presence of two disulfide bonds in this lantibiotic makes it conceivable that BdbB and BdbC are directly involved in its folding. However, alternative indirect effects on sublancin 168 folding or maturation cannot be excluded. For example, it is conceivable that BdbB and BdbC act on SunT because this protein contains 10 cysteine residues. SunT belongs to a large family of ATP-binding cassette transporters involved in the processing and export of lantibiotics and other bacteriocins (31). Consistently our present findings show that SunT is required for the production of sublancin 168. Although most ATP-binding cassette transporters appear to contain six transmembrane segments, the bacteriocin transporters are generally characterized by four transmembrane domains (32). In addition, these transporters have a carboxyl-terminal ATP-binding site (33), which

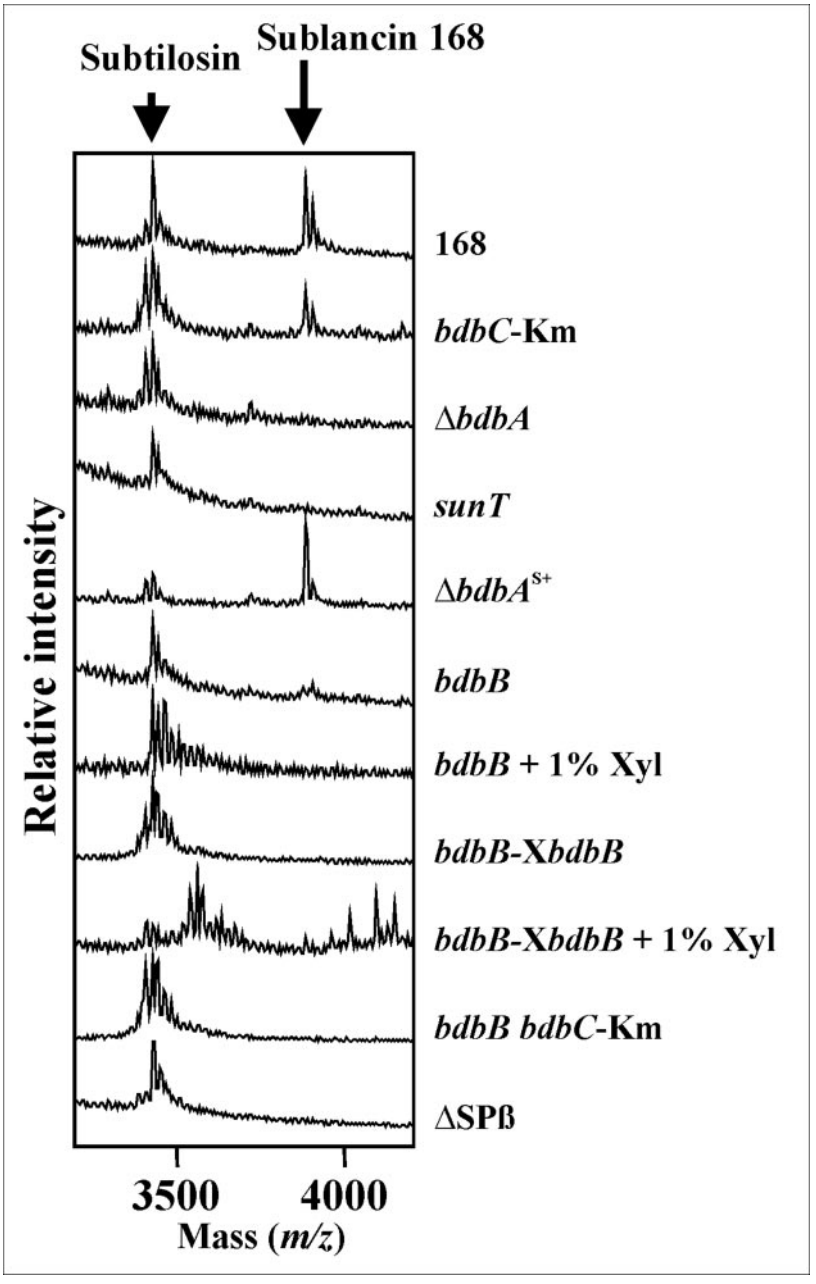

FIG. 8. **Mass spectrometric analysis of subtilosin and sublancin 168 production in different *B. subtilis* strains.** The peak cluster at *m/z* 3400 accounts for the proton-adduct $[M + H]^+$ (*m/z* 3403.3, average mass) and alkali-adducts of subtilosin (30). The signal observed at *m/z* 3881.2 is in accordance with the proton adduct of sublancin 168 (average mass).

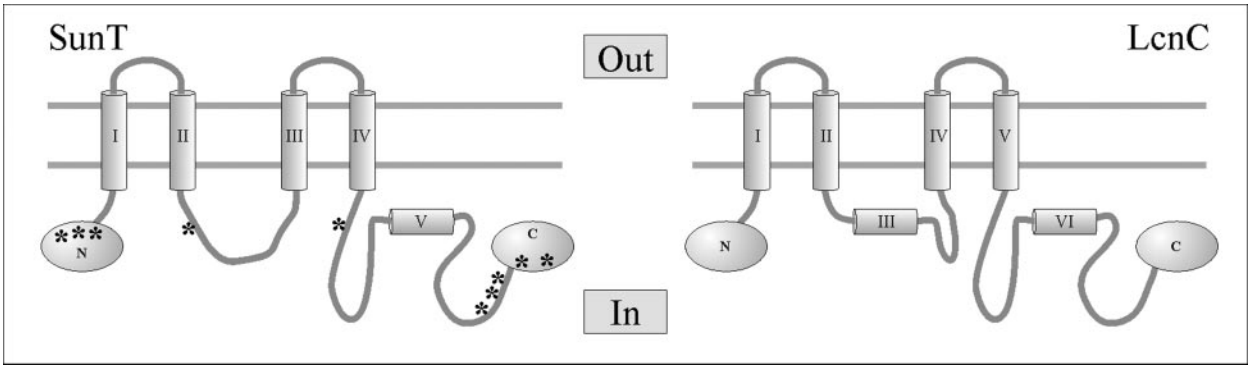

FIG. 9. **Predicted membrane topology of SunT and comparison with the model proposed by Franke *et al.* (36) for the structurally related LcnC protein.** *Stars* (*) indicate the relative location of the 10 cysteines in SunT. *I–VI*, hydrophobic domains I–VI; *N*, amino terminus; *C*, carboxyl terminus.

is located in the cytoplasm. Specifically SunT shows a high level of sequence similarity to MesD of *Leuconostoc mesenteroides* (34) and LcnC of *Lactococcus lactis* (35), the transporters for mesentericin Y105 and lactococcin A, respectively. For LcnC a detailed topology analysis was performed by Franke *et al*. (36) indicating that this protein has four transmembrane

segments. Computer-assisted predictions suggest that SunT, like LcnC, contains four transmembrane sequences, an amino-terminal cytoplasmic peptidase domain (36), and one carboxyl-terminal ATP-binding domain. A fifth hydrophobic domain, which is conserved in LcnC, is probably not spanning the membrane as this would localize the ATP-binding domain at the extracytoplasmic side of the membrane (Fig. 9). Importantly all 10 cysteine residues of SunT have a predicted cytoplasmic localization, which makes them unlikely substrates for BdbB or BdbC, the catalytic domains of which are localized at the extracytoplasmic side of the membrane (15). Consequently an indirect influence of BdbB or BdbC via SunT on sublancin 168 appears to be unlikely.

Apart from the fact that the SPβ prophage is required for immunity against sublancin 168, it is presently not known how this immunity is acquired. In general, two distinct systems for immunity against bacteriocins have been described. The first makes use of dedicated "immunity" proteins, small proteins that are weakly associated with the outer surface of the cytoplasmic membrane thereby preventing pore formation (11, 37, 38). The second is constituted of ATP-binding cassette transporters (39–41). In this light, SunT could represent a sublancin 168 immunity system that actively prevents the accumulation of this lantibiotic in the membrane (22). However, our preliminary data show that the *sunT* mutant used in the present studies is resistant against sublancin 168, which suggests that SunT is not involved. Likewise, BdbA, BdbB, and BdbC are not required for immunity (data not shown).

Although the *bdbA* gene partly overlaps with the upstream *sunT* gene, the deletion of this gene in the $\Delta bdbA^{S+}$ strain did not affect the production of active sublancin 168. This is remarkable because BdbA is the equivalent of Bdb of *B. brevis*, which can replace the major oxidase DsbA of *E. coli* (16). The fact that BdbA is dispensable for sublancin production does not exclude its possible involvement in this process. For example, another as yet unidentified thiol-disulfide oxidoreductase might complement for the absence of BdbA, similar to what we have shown for BdbC. One of the candidates could be BdbD, which cooperates with BdbC in competence development (42). Both proteins, which are encoded by the bicistronic *bdbDC* operon, are required for the folding of ComGC most likely because this component of the DNA uptake machinery contains an intramolecular disulfide bond, which is essential for its role in competence (43).

The fact that subtilosin production is not affected in the mutants studied is fully consistent with the recently published structure of this peptide antibiotic (30). According to this model, subtilosin has three inter-residue bridges in which its cysteine residues are involved. Two cysteines are linked with phenylalanine residues, and one is linked with a threonine. This raises the intriguing question of how subtilosin molecules escape from disulfide bond formation during their export in contrast to sublancin 168 molecules.

Strikingly, both for subtilosin and sublancin 168, molecule species were observed with increased molecular mass when *B. subtilis bdbB*-X*bdbB* was grown in the presence of 1% xylose. This unprecedented finding shows that subtilosin as well as sublancin 168 can undergo growth medium-dependent modifications. Based on the observed mass increases, it is possible that subtilosin has one xylose adduct (+132 Da), whereas sublancin 168 has either one (+132 Da) or two (+264 Da) of these adducts. Notably Paik *et al.* (9) have previously reported a modification of sublancin 168 that increases its mass with 164.48 Da. The precise nature of these modifications and their effect on bactericidal activity remain to be elucidated.

In conclusion, the present observations show for the first time an involvement of thiol-disulfide oxidoreductases in the production of a peptide antibiotic. Furthermore, our results reveal an important function of the BdbB and BdbC proteins in the *B. subtilis* cell. Thus far the BdbB protein was only known to be involved in the folding of the heterologous secretory protein PhoA, whereas BdbC was shown to be required for competence development in addition to the secretion of the heterologous protein PhoA (15, 42). Our ongoing research is focused on the identification of the determinants for the substrate specificities of the thiol-disulfide oxidoreductases of *B. subtilis*, BdbB and BdbC in particular. For this purpose, it will be of major importance to answer the question of whether BdbB is directly or indirectly involved in the formation of the disulfide bonds of sublancin 168.

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Thiol-Disulfide Oxidoreductases Are Essential for the Production of the Lantibiotic Sublancin 168

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