APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Apr. 2004, p. 2349–2353 Vol. 70, No. 4
0099-2240/04/$08.00+0 DOI: 10.1128/AEM.70.4.2349–2353.2004


# Subtilosin Production by Two *Bacillus subtilis* Subspecies and Variance of the *sbo-alb* Cluster

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Received 27 August 2003/Accepted 6 January 2004

**Eight different *Bacillus subtilis* strains and *Bacillus atrophaeus* were found to produce the bacteriocin subtilosin A. On the basis of the subtilosin gene (*sbo*) sequences two distinct classes of *B. subtilis* strains were distinguished, and they fell into the two *B. subtilis* subspecies (*B. subtilis* subsp. *subtilis* and *B. subtilis* subsp. *spizizenii*). The entire sequence of the subtilosin gene cluster of a *B. subtilis* subsp. *spizizenii* strain, *B. subtilis* ATCC 6633, was determined. This sequence exhibited a high level of homology to the sequence of the *sbo-alb* gene locus of *B. subtilis* 168. By using primer extension analysis the transcriptional start sites of *sbo* in *B. subtilis* strains ATCC 6633 and 168 were found to be 47 and 45 bp upstream of the *sbo* start codon, respectively. Our results provide insight into the incipient evolutionary divergence of the two *B. subtilis* subspecies.**

Almost 4% of the 4.2-Mbp *Bacillus subtilis* 168 genome codes for proteins similar to the proteins involved in the biosynthesis of antimicrobial metabolites (17). However, *B. subtilis* 168 produces only a few antibiotics because several of the biosynthetic pathways are not functional, most likely because of the X-ray mutation of the original Marburg strain (6). In contrast, various other *B. subtilis* wild-type strains produce characteristic cocktails of numerous peptide antibiotics (1, 18). For example, a well-established bioindicator strain for sterilization control, ATCC 6633 (11), was investigated with respect to biosynthesis of the lantibiotic subtilin (4, 8, 16, 27) and its regulation (26, 28). In a series of *B. subtilis* strains production of the nonribosomally synthesized cyclic lipopeptides surfactin, fengycin, and the iturins, including mycosubtilin, with different compositions has been observed (9, 18, 20, 31).

Subtilosin is a macrocyclic bacteriocin with three intramolecular bridges (14, 19). An acidic isoelectric point differentiates subtilosin from the basic lantibiotics (13, 24). Subtilosin transcription is increased under oxygen-limited and anaerobic conditions (22; T. Stein, S. Düsterhus, A. Stroh, and K.-D. Entian, 10th Int. Conf. Bacilli, abstr. P103, p. 65, 1999). The production of mature subtilosin is based on the expression of the *sbo-alb* gene cluster encompassing the subtilosin structural gene *sbo* and genes involved in posttranslational modification and processing of presubtilosin and in immunity (34, 35).

Here we describe subtilosin production by eight different *B. subtilis* wild-type strains and *Bacillus atrophaeus*. The *sbo* genes of these organisms, as well as the entire subtilosin gene cluster of *B. subtilis* ATCC 6633, were sequenced in order to analyze the genetic variation between *B. subtilis* wild-type strains.

## MATERIALS AND METHODS

**Strains and plasmids.** Strains used in this work are listed in Table 1. Recombinant plasmids were amplified in *Escherichia coli* DH5α or TG1 grown in Luria-Bertani medium (GIBCO, Neu-Isenburg, Germany). *B. subtilis* was grown either on TY (0.8% tryptone, 0.5% yeast extract [Difco, Detroit, Mich.], 0.5% NaCl) or on Landy medium supplemented with 0.1% yeast extract (33). Antibiotics were used at the following concentrations: 100 μg of ampicillin per ml and 20 μg of chloramphenicol per ml for *E. coli* and 5 μg of chloramphenicol per ml, 10 μg of kanamycin per ml, and 100 μg of spectinomycin per ml for *B. subtilis.*

**Plasmid isolation and PCR.** Established protocols were used for molecular biology techniques (25), and *E. coli* plasmids were isolated by the rapid alkaline extraction procedure (5). DNA amplification with Taq DNA polymerase was performed according to the instructions of the commercial supplier (Boehringer GmbH, Mannheim, Germany) by using a Hybaid R2 Combi-thermal reactor. DNA was cleaved and isolated with a QIAquick purification kit (Qiagen GmbH, Hilden, Germany). Oligonucleotides were purchased from ARK Scientific GmbH Biosystems, Darmstadt, Germany. Sequencing by primer walking was carried out by Scientific Research and Development, Oberursel/Frankfurt, Germany; nucleotide sequences were determined at least two times for each DNA strand.

***sbo* deletion in *B. subtilis* 168 and ATCC 6633.** Primers TS13C (GAATTGACACTATCTAGAGAAATGCCG) and TS14 (ATCCGGTGGTGCGGAATTCGATGA) (restriction sites are underlined) were designed by using the genome sequence of *B. subtilis* 168 (15). A 1,375-bp DNA fragment including the *sbo* gene was PCR amplified, cleaved with EcoRI and XbaI, and cloned into pUC19. To remove an NdeI restriction site, the resulting plasmid, pTS*sbo*, was cleaved with NarI and AatII, and blunt ends were generated after exonuclease treatment. The self-ligation product (pSD13) was cleaved with NdeI and the Klenow fragment, and the *sbo* gene was removed by BglII cleavage. Into the resulting blunt and BglII sites, a Sau3A/HincII site-containing *cat* gene obtained from pCE26 (16) was cloned. The plasmid constructed, pSD15, was linearized and used for transformation of competent *B. subtilis* 168 cells as described previously (2), with slight modifications as described by Klein et al. (16). An *sbo* deletion in *B. subtilis* ATCC 6633 was obtained after transformation with chromosomal DNA obtained from the corresponding *B. subtilis* 168 strain. For construction of the *B. subtilis* Δ*spaS*/Δ*sbo* strain ATCC 6633, the chloramphenicol resistance cassette of the Δ*spa*S strain (16) was replaced by a gene conferring resistance to spectinomycin (pJL62) by in vivo recombination (30) prior to deletion of *sbo*.

**Primer extension.** Total RNA was prepared from overnight cultures grown in Landy medium by using an RNeasy mini-kit (Qiagen) and was treated with 20 U of high-performance liquid chromatography (HPLC)-grade DNase. (Amersham Biosciences, Freiburg, Germany) in 40 mM Tris-HCl (pH 7.5)–60 mM $MgCl_2$ in a 50-μl (final volume) mixture for 30 min at 37°C. RNA was isolated, precipitated with ethanol, and dissolved in 20 μl of $H_2O$. Primer extension analyses with primer AS26 (CCCCATAGACCGAATAGACCTG) were performed as previously described (26).

**Reversed-phase HPLC and mass spectrometry.** Culture supernatants of *B. subtilis* strains were separated by reversed-phase HPLC by using $C_{18}$-Hypersil (particle size, 5 μm; precolumn dimensions, 4 by 10 mm; main column dimensions, 2 by 100 mm; Maisch, Ammerbuch, Germany). Eluents A and B were composed of 0.1% (vol/vol) trifluoroacetic acid and 20% (vol/vol) acetonitrile in water and 0.1% (vol/vol) trifluoroacetic acid in acetonitrile, respectively. A sample was applied with 100% eluent A and eluted with segmented gradients of

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TABLE 1. Strains used

| Strain | Other designations, description, and/or relevant genotype[a] | Reference |
|---|---|---|
| *Bacillus subtilis* strains | | |
| ATCC 6633 | CCM 1999, DSM 347, IAM 1069, NCIB 8054, NCTC 10400 | 6 |
| 168 | Used for genome sequencing; DSM 402, NCIB 10106, *B. subtilis* subsp. *subtilis* | 21 |
| 168 Δ*sbo* | *sbo*::*cm* ($Cm^r$) | This study |
| DSM 618 | Test strain for detection of antibiotics in meat | |
| DSM 1088 | IFO 13169, *Bacillus natto* | |
| DSM 2109 | ATCC 11774, NCTC 8236 | |
| DSM 2277 | ATCC 51189, CIP 103406, IAM 1633, NCIB 8649, NCTC 10073, *B. atrophaeus* | 11 |
| DSM 6405 | Mutant of *B. subtilis* W23, *B. subtilis* subsp. *spizizenii* | 21 |
| 60015 | Marburg strain | 10 |
| $10^T$ | Type strain; ATCC $6051^T$, CCM $2216^T$, IAM $12118^T$, IFO $13719^T$, NCIB $3610^T$, NCTC $3610^T$ | |
| ATCC 6633 | | 16 |
| Δ*spaS* | *spaS*::*cm* ($Cm^r$) | |
| Δ*spaS* | *spaS*::*cm*::*spec* ($Spec^r$) | This study |
| Δ*sbo* | *sbo*::*cm* ($Cm^r$) | This study |
| Δ*spaS* Δ*sbo* | *spaS*::*cm*::*spec* ($Spec^r$), *sbo*::*cm* ($Cm^r$) | This study |
| *Micrococcus luteus* ATCC 9341 | Test strain for antimicrobial activity | |
| *Escherichia coli* DH5α | *supE44 lacU169* [Φ80*lacZ*M15] *hsdR17 recA1 endA1 gyrA96 thi-1 relA1* | 23 |

[a] ATCC, American Type Culture Collection; CCM, Czech Collection of Microorganisms; CIP, Collection de l'Institut Pasteur; DSM, German Collection of Microorganisms (http://www.dsmz.de); IAM, Institute of Applied Microbiology; IFO, Institute for Fermentation; NCIB, National Collection of Industrial Bacteria; NCTC, National Collection of Type Cultures.

acetonitrile (20% eluent B for 5 min, 20 to 40% eluent B for 20 min, and 40 to 100% eluent B for 5 min) (29). Delayed-extraction matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectra were recorded with a Voyager-RP-DE instrument (PerSeptive, Framingham, Mass.) by using a 337-nm nitrogen laser for desorption and ionization (19). The total acceleration voltage was 20 kV; 11.6 kV was used for the first grid. Reversed-phase HPLC fractions (0.7 μl) were mixed with 0.7 μl of matrix solution (20 μg of α-cyano-β-hydroxycinnamic acid [Sigma] $\mu l^{-1}$ in eluent A) on the sample target and dried in the ambient air. Between 128 and 256 single scans were accumulated for each mass spectrum. The delay time was 375 ns.

**Nucleotide sequence accession number.** The nucleotide sequence reported here has been deposited in the EMBL nucleotide sequence database under accession number AJ430547.

## RESULTS AND DISCUSSION

Antibiotic production by different *B. subtilis* strains was investigated by performing antimicrobial activity tests, reversed-phase HPLC separation of culture supernatants, and MALDI-TOF mass spectrometry (Fig. 1). For strain ATCC 6633, a representative strain, subtilin and its isoform [*N*-alpha-succinyl-Trp1]-subtilin are responsible for the main anti-*Micrococcus* activity. Consequently, the Δ*spaS* subtilin deletion strain exhibited no subtilin production. For the previously unidentified active antimicrobial compound in peak II (Fig. 1B) an *m*/*z* value of 3400.7 was determined (Fig. 1C). Both the *m*/*z* value and the elution position are consistent with the properties of authentic subtilosin produced by *B. subtilis* 168 (3, 19). In addition, peak II (subtilosin) was not observed in the supernatant of the Δ*spaS*/Δ*sbo* double mutant (Fig. 1B), clearly demonstrating that the newly identified ATCC 6633 bacteriocin is based on *sbo* gene expression and demonstrating that it is identical to subtilosin. In fractions eluting around 20 min (Fig. 1B) the lipopeptides surfactin and mycosubtilin were observed. The production of these compounds was not affected in the gene deletion mutants (data not shown). Surprisingly, all eight *B. subtilis* wild-type strains investigated, including *B. atrophaeus* (black-pigmented *B. subtilis*; formerly *B. subtilis* DSM 2207 or ATCC 51189) (11), that are listed in Table 1 have been

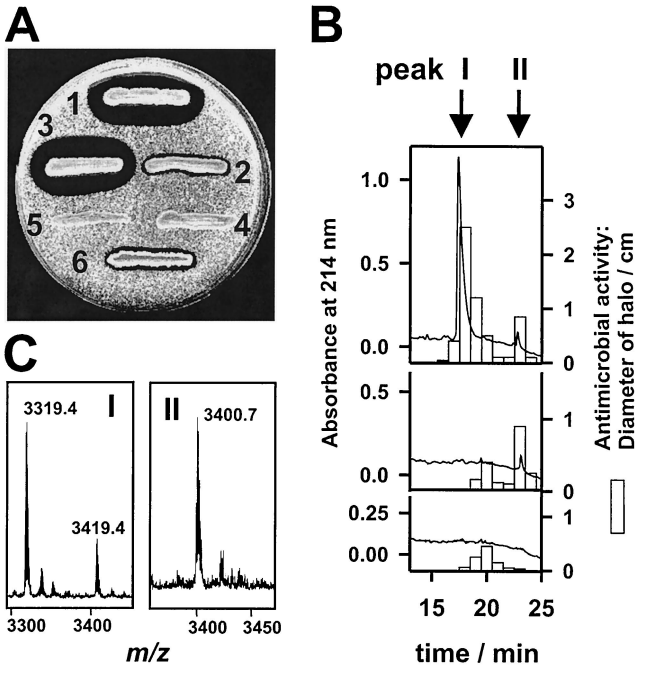


FIG. 1. Analysis of *B. subtilis* peptide antibiotics. (A) *Micrococcus luteus* growth inhibition assay. Streak 1, *B. subtilis* wild-type strain ATCC 6633; streak 2, strain ATCC 6633 Δ*spa*S deletion mutant; streak 3, strain ATCC 6633 Δ*sbo* deletion mutant; streak 4, strain ATCC 6633 Δ*spa*S/Δ*sbo* deletion mutant; streak 5, strain 168 Δ*sbo* deletion mutant; streak 6, *B. subtilis* wild-type strain 168. (B) Reversed-phase HPLC separation of *B. subtilis* ATCC 6633 culture supernatants. The flow rate was 0.4 ml/min, and 400-μl fractions were collected. Aliquots (20 μl) of the wild type (top panel), the Δ*spa*S mutant (middle panel), and the Δ*spa*S/Δ*sbo* double mutant (bottom panel) were used to inhibit the growth of *M. luteus*. (C) Detail of MALDI-TOF mass spectra of peak I (*m*/*z* 3319.4 and 3419.4 correspond to subtilin and succinylated subtilin, respectively) and peak II (*m*/*z* 3400.7 corresponds to subtilosin A). The variance of the *m*/*z* measurements was ±0.2 Da.

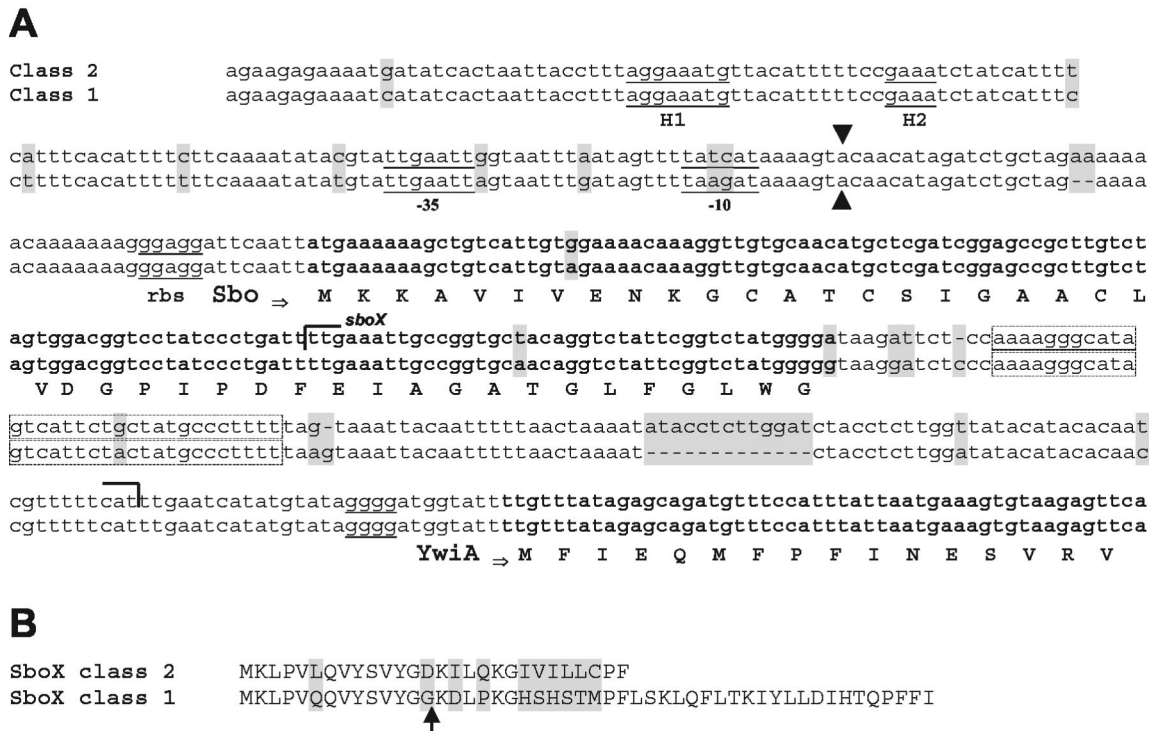


FIG. 2. Comparison of *sbo* alleles of two *B. subtilis* classes. (A) Subtilosin A-encoding gene sequence *sbo* and flanking regions and the derived amino acid sequence. rbs, standard prokaryotic ribosome binding site. Transcriptional start sites (see Fig. 3) are indicated by arrows, and the positions of derived −10 and −35 regions are indicated. H1 and H2 indicate a putative sigma factor H region located 80 to 100 bp upstream of the transcriptional start site of *sbo*. A putative termination loop is enclosed in a box. The putative open reading frame *sboX* (150 nucleotides) is indicated by brackets. (B) Alignment of the amino acid sequences of the putative SboX gene product. The arrow indicates the putative processing site (double Gly motif). Differences between the two alleles are indicated by shading.

found to produce subtilosin. This finding was unprecedented because most of the known *B. subtilis* wild-type strains produce individual antibiotic cocktails. For example, subtilin production has been described only for the ATCC 6633 strain, and distinct lipopeptides are produced only by a few individual strains. The widespread occurrence of subtilosin might reflect an important physiological role. As subtilosin is produced at the end of exponential growth, particularly under stress conditions, a specific function of subtilosin as an antibiotic, killing factor (12) or as a pheromone during anaerobic or biofilm growth of *B. subtilis* (15) has to be considered.

During cloning of *B. subtilis* ATCC 6633 DNA we observed restriction sites not present in the genome of strain 168 (15), while proposed sites were absent. We sequenced the *sbo* genes and flanking regions of all investigated *B. subtilis* wild-type strains in order to analyze the structural basis of these observations and to evaluate possible evolutionary relationships among the subtilosin producers. The main result of a comparison of the *sbo* alleles was identification of two distinct *B. subtilis* classes (Fig. 2A). Class 1 (168-like) includes strains 60015 (Marburg strain), 168, and $10^T$ (type strain), as well as DSM 1088 and DSM 2109. Class 2 (W23-like) comprises strains ATCC 6633 and DSM 618, as well as DSM 6405, a mutant of the W23 strain. This observation is in good agreement with the recent classification of strain 168 as *B. subtilis* subsp. *subtilis* and the recent classification of W23-related strains as *B. subtilis* subsp. *spizizenii* based on DNA reassociation studies (21).

Remarkably, the nucleotide sequences of the *sbo* genes and flanking regions are identical in strains belonging to the same subspecies, and the sequences differ by three nucleotides in the two subspecies (Fig. 2A). However, the encoded Sbo prepeptides are identical in all cases. Primer extension analyses of *sbo* transcripts in representatives of both *B. subtilis* classes revealed transcriptional start sites that are 47 nucleotides (class 2 strain ATCC 6633) and 45 nucleotides (class 1 strain 168) upstream of the start ATG codon of *sbo* (Fig. 3). Similar 5′ transcriptional start sites are utilized by both *B. subtilis* classes. Due to a two-nucleotide insertion into class 2 sequences, the sizes of the transcripts differ by two nucleotides. The −10 and −35 regions derived from the transcriptional start sites resemble promoter regions utilized by sigma factor A (Fig. 2A). Remarkably, within the −10 region two nucleotide substitutions in both *B. subtilis* classes were observed, which suggests that there is an effect on *sbo* expression. However, a region upstream of the −35 region (positions −70 to −110) is perfectly conserved. This region represents a perfect sigma factor H binding site; however, involvement of this region in regulation of subtilosin biosynthesis has not been shown yet.

Downstream of *sbo* a gene cluster with seven open reading frames (*ywiA* and *ywhRQPOMN*) has been identified and sequenced in *B. subtilis* ATCC 6633, a representative of the *B.*

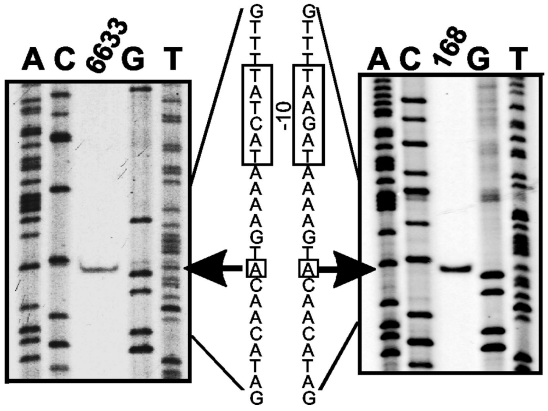


FIG. 3. Primer extension analysis of *sbo*: mapping of the transcriptional start site of *sbo* by primer extension analysis with RNA from *B. subtilis* ATCC 6633 and 168 (middle lanes). The outside lanes show the results of autoradiography of dideoxynucleotide sequencing reactions with primer AS26 complementary to the 5′ region of *sbo*. The transcriptional start sites are indicated by arrows, and the −10 regions are enclosed by boxes in the derived nucleotide sequence.

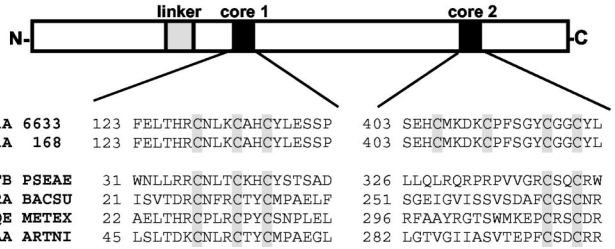


FIG. 4. Domain structure of YwiA (AlbA): schematic representation of the putative domain structure of YwiA resulting from amino acid alignment of the sequences of *B. subtilis* ATCC 6633 and 168. Highly conserved regions (cores) are indicated by solid boxes; a gray box indicates a less conserved region. The first cysteine cluster (core 1) is highly homologous to active sites of proteins belonging to the MoaA-NifB-PqqE family carrying Fe-S centers, like NifB from *Pseudomonas aeruginosa* (NIFB PSEAE), NarA from *B. subtilis* (NARA BACSU), PqqE from *Methylobacterium extorquens* (PQQE METEX), and MoaA from *Arthrobacter nicotinovorans* (MOAA ARTNI).

*subtilis* subsp. *spizizenii* strains (accession number AJ430547). The identified gene cluster exhibits a high level of homology to the *sbo-alb* gene cluster of *B. subtilis* 168 involved in the biosynthesis of subtilosin, including the structural gene, as well as genes encoding the posttranslational modification machinery and subtilosin immunity proteins. BLAST alignments (Table 2) revealed that the first four genes are highly conserved with those of *B. subtilis* subsp. *subtilis* (96 to 100% amino acid identity), while the remaining four genes are less conserved (83 to 88% identity). These differences reveal incipient evolutionary divergence of the *B. subtilis* subspecies. This low level of conservation is unprecedented; for example, thymidylate synthases A (*thyA*) in *B. subtilis* subsp. *spizizenii* ATCC 6633 and W23 and *B. subtilis* subsp. *subtilis* (168) exhibit more than 95% amino acid identity (32). Even the average level of amino acid identity for the DNA gyrases (*gyrA*) in seven *Bacillus* type strains was 95.1% (7).

The nucleotide sequences of the closely related species and subspecies can be used for identification of genes and highly conserved regions in the gene products putatively corresponding to functional domains. For example, in strain 168 a new gene with an unknown function, *sboX*, encoding a bacteriocin-like product, was hypothesized (Fig. 2A) (34), which resides in an open reading frame overlapping the coding region of *sbo*. Notably, the expression of *sboX* would result in a 22-amino-acid truncated peptide in W23-like strains compared to the peptide produced by 168-like strains (Fig. 2B), which makes it unlikely that SboX is produced by W23-like strains.

YwiA (AlbA) is involved in subtilosin biosynthesis, most likely in the posttranslational modification of presubtilosin (34, 35), although its molecular function is unknown. The amino acid sequences of YwiA in the two *B. subtilis* subspecies (Fig. 4) were compared, and two highly conserved regions (amino acids 1 to 90 and 109 to 450) separated by a less conserved linker region (amino acids 91 to 105) were identified. The large conserved domain from amino acid 109 to amino acid 450 exhibits homology to proteins belonging to the MoaA-NifB-PqqE family, which carry Fe-S centers in their active sites. Also, this cysteine-rich cofactor binding region is conserved in YwiA (core 1), as is a second CXXC motif near the C terminus (core 2). A pattern search for core 2 of YwiA proteins (Fig. 4) revealed homology to arylsulfatases and metallothioneins. Upstream of this signature sequence an unusual sulfur-rich motif (CMXXXC) with an unknown function has been found in YwiA proteins.

As this study revealed, two distinct *B. subtilis* subspecies (*B. subtilis* subsp. *subtilis* and *B. subtilis* subsp. *spizizenii*) are distinguishable only on the basis of their *sbo* genes. Comparisons between the subtilosin gene clusters of the two subspecies led to identification of highly conserved protein domains and also provided insight into incipient evolutionary divergence.

TABLE 2. Proteins derived from the subtilosin gene cluster

| Designation | | Length (amino acids) | | % Amino acid identity |
|---|---|---|---|---|
| This study[a] | Study of Zheng et al.[b] | ATCC 6633 | 168 | |
| SboA | | 43 | 43 | 100 |
| | SboX[c] | | 50 | |
| YwiA | AlbA | 449 | 448 | 96 |
| YwhR | AlbB | 53 | 53 | 100 |
| YwhQ | AlbC | 239 | 239 | 97 |
| YwhP | AlbD | 436 | 436 | 86 |
| YwhO | AlbE | 386 | 386 | 88 |
| YwhN | AlbF | 427 | 427 | 87 |
| YwhM | AlbG | 232 | 233 | 83 |

[a] Also see reference 17.
[b] Designation used in reference 35 unless otherwise indicated.
[c] Open reading frame hypothesized for *B. subtilis* 168 (34) (see text).


## ACKNOWLEDGMENTS

We greatly acknowledge Michael Karas, University Frankfurt, for the opportunity to use his MALDI-TOF mass spectrometric equipment and J. Hofemeister for valuable discussions and for the kind gift of various *B. subtilis* wild-type strains.


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