APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Jan. 1994, p. 1–11
0099-2240/94/$04.00+0

Vol. 60, No. 1

Growth Phase-Dependent Regulation and Membrane Localization of SpaB, a Protein Involved in Biosynthesis of the Lantibiotic Subtilin

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Received 15 July 1993/Accepted 15 September 1993

The information responsible for biosynthesis of the lantibiotic subtilin is organized in an operon-like structure that starts with the *spaB* gene. The *spaB* gene encodes an open reading frame consisting of 1,030 amino acid residues, and it was calculated that a protein having a theoretical molecular mass of 120.5 kDa could be produced from this gene. This is consistent with the apparent molecular weight for SpaB of 115,000 which was estimated after sodium dodecyl sulfate-gel electrophoresis and identification with SpaB-specific antibodies. The SpaB protein is very similar to proteins EpiB and NisB, which were identified previously as being involved in epidermin and nisin biosynthesis. Upstream from SpaB a characteristic sigma A promotor sequence was identified. An immunoblot analysis revealed that SpaB expression was strongly regulated. No SpaB protein was detected in the early logarithmic growth phase, and maximum SpaB expression was observed in the early stationary growth phase. The expression of SpaB was strongly correlated with subtilin biosynthesis. Deletion mutations in either of two recently identified regulatory genes, *spaR* and *spaK*, which act as a "two-component" regulatory system necessary for growth phase-dependent induction of subtilin biosynthesis (C. Klein, C. Kaletta, and K. D. Entian, Appl. Environ. Microbiol. 59:296–303, 1993), also resulted in failure of SpaB expression. To investigate the intracellular localization of SpaB, vesicles of *Bacillus subtilis* were prepared. The SpaB protein cosedimented with the vesicle fraction and was released only after vigorous resuspension of the vesicles. Our results suggest that SpaB is membrane associated and that subtilin biosynthesis occurs at the cytoplasmic membrane of *B. subtilis*.

Lantibiotics are peptide-derived antibiotics that exhibit high levels of antimicrobial activity against several pathogenic gram-positive bacteria, such as *Propionibacterium acnes*, staphylococci, streptococci, and clostridia. They contain the unusual amino acids *meso*-lanthionine, dehydroalanine, and dehydrobutyrine (49). Lantibiotics can be divided into two subgroups (21): (i) the linear lantibiotics, including nisin (38, 39, 42), subtilin (17), epidermin (1, 2), gallidermin (26), mersacidin (15, 33), and Pep5 (43), and (ii) the globular lantibiotics, including cinnamycin (= Ro09-0198 = lanthiopeptin) (6, 14, 27, 28, 41), duramycin (16), and ancovenin (52). The most important linear lantibiotics are nisin from *Lactococcus lactis*, subtilin from *Bacillus subtilis*, epidermin from *Staphylococcus epidermidis*, and gallidermin from *Staphylococcus gallinarum*.

Epidermin and gallidermin are used in the treatment of acne because of their high levels of activity against *P. acnes*. Subtilin and nisin are very similar to each other and have the same arrangement of lanthionine bridges. Nisin is used as a food preservative because it is very effective against certain gram-positive bacteria such as staphylococci, streptococci, and clostridia (38, 39, 42). The potent bactericidal activities of nisin and other linear lantibiotics are based on depolarization of energized bacterial cytoplasmic membranes (21).

The main difficulties in exploiting lantibiotics commercially are the low production rates of these compounds. To overcome these problems, the biosynthesis of lantibiotics has to be elucidated. Compared with other lantibiotic-producing organisms, the subtilin-producing strains of *B. subtilis* are much more suitable for genetic analysis. Therefore, we chose *B. subtilis* as a model organism to investigate lantibiotic biosynthesis.

The ribosomal origin of lantibiotics was first shown by the isolation of the epidermin structural gene, *epiA* (49). The general structure of lantibiotic genes is the same for all lantibiotics described thus far. The primary transcript of the gene for each linear lantibiotic encodes a prepeptide which consists of an N-terminal leader sequence followed by the C-terminal propeptide from which the lantibiotic matures and a characteristic proteolytic processing site with proline at position −2. This gene structure has been found for subtilin (5), nisin (8, 12, 22), gallidermin (48), Pep5 (24), and cinnamycin (23), a globular lantibiotic.

On the basis of the results of Ingram (19, 20) and the epidermin prepeptide sequence, we proposed that the leader sequence has an important function during lantibiotic maturation and that a dehydratase reaction occurs at serine and threonine residues and subsequently sulfur is added from cysteine (49). This hypothesis was supported by the isolation of prepeptides containing dehydroalanine (53).

Several genes have been identified as essential for the synthesis of the lantibiotics subtilin, epidermin, and nisin. A sequence analysis of DNA fragments adjacent to *spaS* revealed two complete open reading frames, *spaC* and *spaT*, and the C-terminal fragment of a third open reading frame, *spaB* (25, 31). Gene deletions within *spaB* and *spaC* created subtilin-negative mutants, which proved that the proteins encoded by these genes are essential for subtilin biosynthe-

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sis (25, 31). For epidermin five open reading frames, *epiB*, *epiC*, *epiD*, *epiP*, and *epiQ*, were identified adjacent to the *epiA* structural gene (46, 47). These genes supported heterologous epidermin biosynthesis in *Staphylococcus carnosus*, which proved that they are sufficient for epidermin synthesis in this host (46, 47). In addition, epidermin biosynthesis mutants could be complemented by using these genes (3, 4). For nisin biosynthesis three open reading frames, *nisB*, *nisT*, and *nisC*, were identified near the *nisA* structural gene (13). Genes *epiB* and *epiC* were homologous to *spaB* and *spaC*, respectively, and to *nisB* and *nisC*, respectively, indicating that the proteins encoded by these genes have been conserved and may have a major function in lantibiotic biosynthesis.

Isolation of the complete open reading frame of *spaB* and preliminary characterization of the protein that it encodes, SpaB, are described in this report. Our results confirm that SpaB is essential for subtilin biosynthesis and support our previous hypothesis that lantibiotic biosynthesis occurs at the cell membrane (49).

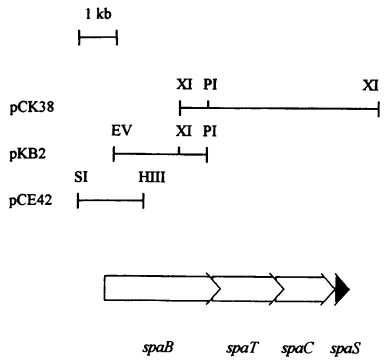

FIG. 1. Genetic analysis of *B. subtilis* DNA adjacent to *spaS*. Cloning and sequencing of plasmid pCK38 have been described recently (31). For a description of the isolation of fragments pKB2 and pCE42, see Results. Abbreviations: XI, *Xba*I; PI, *Pst*I; EV, *Eco*RV; SI, *Sac*I; HIII, *Hin*dIII.

MATERIALS AND METHODS

Strains and media. *B. subtilis* ATCC 6633 was used as the subtilin-producing strain. Recombinant plasmids were amplified in *Escherichia coli* RR1 (F⁻ *hsd520 supE44 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1*). *E. coli* strains transformed with plasmid pATH were grown in M9 medium (44) supplemented with 0.5% Casamino Acids and 40 μg of ampicillin per ml or in M9 medium supplemented with 0.5% Casamino Acids, 20 μg of tryptophan per ml, and 40 μg of ampicillin per ml.

Micrococcus luteus ATCC 9341 was used as a test strain in subtilin assays. *E. coli* and *M. luteus* were grown on Luria-Bertani medium (GIBCO, Neu-Isenburg, Germany). *B. subtilis* was grown on TY medium (0.8% tryptone, 0.5% yeast extract [Difco Laboratories, Detroit, Mich.], 0.5% NaCl). When resistance markers were used, 10 μg of kanamycin per ml and 5 μg chloramphenicol per ml were added to the medium.

Molecular biology techniques. We used previously described protocols for molecular biology techniques (44). DNA was cleaved by using the conditions recommended by the supplier of the restriction enzymes (Boehringer GmbH, Mannheim, Germany). Restriction endonuclease-digested DNA was eluted from 0.7% agarose gels by the freeze-squeeze method (50).

Plasmid isolation. The procedure of Birnboim and Doly (7) was used to isolate the plasmids of *E. coli*. When necessary, the plasmids were purified by centrifugation for 12 h at 80,000 rpm with a Beckman model TL 100 ultramicrocentrifuge and a model TLA 100.2 rotor.

For Southern hybridization double-stranded DNA fragments were labeled by nick translation by using [α-^{32}P]ATP and DNA polymerase I (Boehringer), as described by Sambrook et al. (44). For DNA sequencing, both strands of DNA were sequenced by the radioactive chain termination method by using T7 DNA polymerase (Pharmacia, Freiburg, Germany) and appropriate primers (45).

Subtilin bioassay. Test strain *M. luteus* ATCC 9341 was grown to an A_{578} of 0.8, and 0.3 ml of this culture was added to 500 ml of molten Luria-Bertani agar; this preparation was mixed, and 10-ml portions were poured into petri dishes. *B. subtilis* was spread onto each agar surface, and the diameter of *M. luteus* growth inhibition around each colony was determined.

Isolation of *B. subtilis* membrane vesicles. *B. subtilis* membrane vesicles were prepared by using a modification of the method previously described by Konings et al. (34). Cells (4 g, wet weight) were suspended in 1 liter of 0.05 M potassium phosphate (pH 8.0) containing 2 mM phenylmethylsulfonyl fluoride. Incubation was stopped by centrifuging the preparation at 48,000 × *g* for 30 min at 4°C, and the membranes were suspended by using a Potter-Elvehjem homogenizer in 0.1 M potassium phosphate buffer (pH 6.6) containing 10 mM sodium EDTA. After further centrifugation at 48,000 × *g* for 30 min at 4°C, the pellet containing the purified membrane vesicles was resuspended in 0.1 M potassium phosphate buffer (pH 6.6) containing 10 mM sodium EDTA, and aliquots (0.5 ml) were rapidly frozen and stored in liquid nitrogen. Protein was determined by the microbiuret method (54).

To test the integrity of the isolated membrane vesicles, the same procedure was performed in the presence of 100 mM 8-hydoxy-1,3,6-pyrenetrisulfonate (pyranine), a dye which has been proven not to interact with membranes. After the final centrifugation step the membranes were carefully suspended by using a Potter-Elvehjem homogenizer in 0.1 M potassium phosphate buffer (pH 6.6) containing 10 mM sodium EDTA. The vesicles containing pyranine were separated from the external pyranine on a Dowex 1x8 column (50/100 mesh; Serva, Heidelberg, Germany) (11). A 100-μl solution containing vesicles was diluted with 900 μl of 0.1 M potassium phosphate buffer (pH 10) containing 1% Triton X-100 to make the vesicles leaky, and fluorescence was measured at 450 nm.

TrpE fusion protein and antibody isolation. The 1.4-kB *Eco*RV-*Hin*dIII fragment (Fig. 1) corresponding to the C-terminal fragment (425 amino acids) of SpaB was cloned into vector pATH2 (32). For expression of the fusion protein, the plasmid was transformed into *E. coli* RR1. Cells containing the pATH vector for expression of TrpE fusion proteins were grown overnight in M9 medium containing 0.5% Casamino Acids, 20 μg of tryptophan per ml, and 40 μg of ampicillin per ml, diluted 1:10 in M9 medium containing 0.5% Casamino Acids and 40 μg of ampicillin per ml, and grown for 2 h at 30°C. Indoleacrylic acid was added to a final concentration of 2 μg/ml, and the culture was grown at 30°C for 4 h. The cells were harvested by centrifugation and lysed by adding sodium dodecyl sulfate (SDS) sample buffer (100 mM $NaPO_4$ [pH 7.0], 5% β-mercaptoethanol, 4% SDS, 8 M

urea, 0.02% bromphenol blue). The fusion protein was purified by loading 10 to 20 mg of protein onto a preparative SDS-polyacrylamide gel electrophoresis (PAGE) gel.

To prepare antibodies, bands corresponding to the fusion protein were cut from SDS-polyacrylamide gels and isolated by electroelution. The purified fusion protein was suspended in an equal volume of ABMI or ABMIII adjuvant (Sebak, Aidenbach, Germany). The mixture was injected subcutaneously into rabbits. The initial injections contained 250 to 350 μg of the fusion protein. Secondary injections (after 4 weeks) and later booster injections (every 2 weeks) contained approximately 250 μg of the fusion proteins. Serum was collected and tested 2 weeks after each booster injection. The final antisera were collected after two booster injections. The specificities of the antisera were determined by a Western blot (immunoblot) analysis by comparison with preimmunization sera.

SDS-gel electrophoresis and Western blot analysis. SDS-PAGE was performed with a Mini-PROTEIN II dual slab cell apparatus by using the discontinuous system and 7.5% acrylamide separation gels (37). Proteins were electrotransferred onto nitrocellulose paper (pore size, 0.45 μm; Schleicher & Schuell, Dassel, Germany) by using a discontinuous buffer system (model LKB 2117 Multiphor II electrophoresis system; Pharmacia-LKB, Freiburg, Germany) as recommended by the supplier. Protein was estimated by the microbiuret method (54).

After protein adsorption the nitrocellulose membranes were stained with India ink (Pelikan AG, Hannover, Germany) (18). The stain was prepared as a 0.1% (vol/vol) solution in phosphate-buffered saline (PBS) (pH 7.4) containing 1% acetic acid and 0.05% Tween 20. The nitrocellulose sheets were stained for 15 min at room temperature with shaking on a rotary shaker and then incubated in PBS containing 20% fetal calf serum (GG-free; GIBCO BRL, Berlin, Germany) for 10 min. Antibody-containing sera were diluted 1:1,000 with 20% fetal calf serum. The nitrocellulose membranes were exposed to diluted antibody solutions for 6 h or overnight on a rotary shaker, and washed three times at room temperature for 10 min with TBST (10 mM Tris, 150 mM NaCl, 0.05% Triton X-100; pH 8.0), and then incubated with an appropriate solution containing anti-rabbit immunoglobulin G (alkaline phosphatase conjugate; Sigma, Munich, Germany) in TBST for 2 h. Finally, the membranes were washed three times with TBST.

Processed nitrocellulose membranes were washed with AP buffer (100 mM Tris, 100 mM NaCl, 5 mM $MgCl_2$; pH 9.5) and exposed to AP buffer containing nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate. Bands that reacted positively became visible as brown bands within 5 to 10 min. Finally, the membranes were thoroughly rinsed with H_2O.

Nucleotide sequence accession numbers. The data bank nucleotide sequence accession numbers for proteins NisB and EpiB are X68307 and X62386, respectively. The sequence data determined in this study have been deposited in the GenBank data base under accession number L24075.

RESULTS

Isolation and nucleotide sequence of *spaB*. Previously, we described the isolation of a 4.9-kb *Xba*I fragment (pCK38) that contained the subtilin structural gene (*spaS*), genes *spaC*, *spaB*, and *spaT*, and the 5′ part of the *spaB* gene. All five genes were involved in subtilin biosynthesis, as shown by the failure of gene disruption mutants to synthesize subtilin (31). By using the 4.9-kb *Xba*I fragment, adjacent restriction sites were determined by a Southern blot analysis, and a 2.7-kb *Eco*RV-*Pst*I fragment that overlapped the 4.9-kb *Xba*I fragment was isolated by hybridization with the labeled *Xba*I fragment (plasmid pKB2). To obtain the complete *spaB* gene, an additional overlapping 1.8-kb *Sac*I-*Hin*dIII fragment was cloned by the same procedure by using the labeled 2.7-kb fragment as a hybridization fragment, yielding plasmid pCE42 (Fig. 1).

A protein containing 1,030 amino acids was derived from the *spaB* gene; this protein had a predicted molecular mass of 1,205 kDa (Fig. 2). The *spaB* gene is preceded by a Shine-Dalgarno sequence at an appropriate distance, and its putative promoter is similar to vegetative sigma A promoters of *B. subtilis* (40).

Homology of SpaB to NisB and EpiB. SpaB was found to be homologous to proteins NisB (13) and EpiB (47) which are encoded by genes located adjacent to the structural genes of the lantibiotics nisin and epidermin. SpaB was 28.1% homologous to NisB over the entire protein and 24.5% homologous to EpiB (Fig. 3).

No additional homologies with previously described proteins were found for SpaB, NisB, and EpiB in data bases (the GenBank, EMBL, Protein Identification Resource, and Swiss-Prot data bases were examined in August 1992, September 1992, June 1992, and August 1992, respectively). According to our genetic data proteins SpaB (as well as NisB and EpiB) and SpaC (as well as NisC and EpiC) should be involved in lanthionine formation. We hypothesize that these proteins may correspond to dehydratases responsible for dehydration of serine and threonine residues and to lanthionine synthases that catalyze the addition of cysteine sulfur to the double bonds of dehydroalanine and dehydrobutyrine. Within the N-terminal parts of SpaB, NisB, and EpiB an experiment to determine levels of similarity between SpaB, NisB, or EpiB and threonine dehydatases IlvA (37) and Tdc (29) from *E. coli* revealed that there was 31% similarity over a 60-amino-acid residue region for NisB (amino acids 203 to 263 of NisB compared with amino acids 248 to 306 of *E. coli* IlvA) and 18.3% similarity over a 126-amino-acid residue region for SpaB (amino acids 172 to 294 of SpaB compared with amino acids 206 to 329 of *E. coli* IlvA/Tdc).

Within the C-terminal parts of SpaB, NisB, and EpiB there was 21% similarity for NisB over a 157-amino-acid residue region (amino acids 720 to 867 of NisB compared with amino acids 305 to 462 of *E. coli* IlvA) and 22% similarity over a 100-amino-acid residue region for EpiB (amino acids 828 to 928 of EpiB compared with amino acids 346 to 437 of *E. coli* Tdc/IlvA). Although these levels of similarity are low, they suggest that SpaB, NisB, and EpiB play a role in dehydration during lantibiotic biosynthesis.

In addition to sequence homology levels, hydrophobicity plots also revealed high levels of similarity among the three proteins because a characteristic change from a slightly hydrophobic peak to a strongly hydrophilic peak was observed (Fig. 4). However, we predicted that there should be a hydrophobic area in the C-terminal region. A computer analysis in which the method of Chou and Fasman (9) was used revealed that all three proteins may have similar secondary structures. A comparison of the predictions for α-helices and β-sheets revealed 14 α-helices and 20 β-sheets that were common to all three proteins (Fig. 3).

Identification of the *spaB* gene product. The *spaB* gene product has been implicated in posttranslational modification of the subtilin prepeptide. In order to identify and

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SacI    10        20        30        40        50        60        70        80        90       100
GAGCTCCCGCTCGCGCTTTCTGTCATTATGGCAGGCTTGCGCACGGCTCTCGTCATTGCCATCGGAATTACAGCAATCGGCACATTTGTCGGCGCCGGGG
       110       120       130       140       150       160       170       180       190       200
GGCTCGGGGATATGATTGTCCGCGGCTCAAACGCGACAAACGGAACTGCGATTATTCTCGCAGGCGCGACCCCGACTGCGGTAATGGCCATAGGAGCAGA
       210       220       230       240       250       260       270       280       290       300
TTTGATAATGGCCTGGATTGAAAGGTTCTTGAATCCGGTGAAACAAAAAAGCAGAAGAAAGGTAATAAGTGTATAGAGTTAAGCAAAAAGGATTCTTTTC
       310       320       330       340    "-35 Box"    360          "-10 Box"    390       400
TGAGAGGGAAAAGAGTCCTTTTTTTATGGTATTTACTGGGTGGATCTTGATATTTTTTTGATTTTAGAATGTATAGTAAAAATAGAGTATTGTAAATAT
       410       420       430       440       450       460       470     S/D       490       500
TTTGGTTCAAATAGAATAATATGGAGGACTAAGCAGAGGGATGTAGAATTGTTTTTATTAAGAAGCGGATAAATATGGGGGGCTATAGACTAATATGAAA
                                                                                  SpaB> M   K
       510       520       530       540       550       560     EcoRI     EcoRV     590       600
TCCTTATATACACCTACAGATTATTATATGATTCGGGTTCCTTTAGTACATCAAGACTTAAAAAATGAGAATTCTCAGGATATCGATCAGTTATTACATG
S  L  Y  T  P  T  D  Y  Y  M  I  R  V  P  L  V  H  Q  D  L  K  N  E  N  S  Q  D  I  D  Q  L  L  H  D
       610       620       630       640       650       660       670       680       690       700
ACCTTTGCAACGATTCATTATTTCGGGAACAAATACTGGTATCTAGCAGGACACTATATGAAACAATACATACTTTCCTGCAAGCGCCGGATAAATTAAA
  L  C  N  D  S  L  F  R  E  Q  I  L  V  S  S  R  T  L  Y  E  T  I  H  T  F  L  Q  A  P  D  K  L  K
       710       720       730       740       750       760       770       780       790       800
AGGGAAAAAAAAAGCGCAACTTTCAACAGGCTATTTTGAAGTATGCAACAAGAAGAGCAACAAGAACCACACCTTTTGGCCTTTTTTCTTCAGTCGGTATA
 G  K  K  K  R  N  F  Q  Q  A  I  L  K  Y  A  T  R  R  A  T  R  T  T  P  F  G  L  F  S  S  V  G  I
       810       820       830       840       850       860       870       880       890       900
GGGTCGTTTTCTGATAAAAATCACTTATCTTTTAATCAACATTCATTTTACAAAAAGGCTCGTGTTGATTTGGAGTGGCTCTACCAATTAATTAGAAAAT
G  S  F  S  D  K  N  H  L  S  F  N  Q  H  S  F  Y  K  K  A  R  V  D  L  E  W  L  Y  Q  L  I  R  K  L
       910       920       930       940       950       960       970       980       990      1000
TGGAAAACGAATACACCGACCGACTTTCTTTTACATTAAATTCCGCTTGTTATATTAAGGGTGACCGGGCTTACTTGTTGTACAGCACAGATGGAAAATC
  E  N  E  Y  T  D  R  L  S  F  T  L  N  S  A  C  Y  I  K  G  D  R  A  Y  L  L  Y  S  T  D  G  K  S
      1010      1020      1030      1040      1050      1060      1070      1080      1090      1100
TGAAGAAGTTAGTGTTCGTGCGACATCTGTTTTCTATTTGATAAATGAACTGTGTGGTGAATCTGCTGCATATCAAGATATAATCCGTTGTTTGATAGAT
 E  E  V  S  V  R  A  T  S  V  F  Y  L  I  N  E  L  C  G  E  S  A  A  Y  Q  D  I  I  R  C  L  I  D
      1110      1120      1130      1140      1150      1160      1170      1180      1190      1200
AACTATCCAAATACTCCAATAAATAAAATTAATCAGTACGTAGCAGACCTTATTGACAAAGAGTTCCTTATATCAAACTTACGGCCGCCGATGACTGTTT
N  Y  P  N  T  P  I  N  K  I  N  Q  Y  V  A  D  L  I  D  K  E  F  L  I  S  N  L  R  P  P  M  T  V  S
      1210      1220      1230      1240      1250      1260      1270      1280      1290      1300
CAGATCAATTTCAGTATTTAATTGATCAAGCGGAAAGCCGCCATATTCCAAATGAACTCATTCAGGCTTGTAAAGACATTCAATATCAGATAGATGCATA
  D  Q  F  Q  Y  L  I  D  Q  A  E  S  R  H  I  P  N  E  L  I  Q  A  C  K  D  I  Q  Y  Q  I  D  A  Y
      1310      1320      1330      1340      1350      1360      1370      1380      1390      1400
TAATCGGATCACTATCGGAGAGGGAGAGCATCAGTATTTAAATCTGATTGAAACAATGAATAAACTCATAAAGGCATCATCTCCTCTGCAAGTAGACGCT
 N  R  I  T  I  G  E  G  E  H  Q  Y  L  N  L  I  E  T  M  N  K  L  I  K  A  S  S  P  L  Q  V  D  A
      1410      1420      1430      1440      1450      1460      1470      1480      1490      1500
GGGCTGGCAGACTCCTCCATTCAATTAGATAATGAAACATCTCTCGCCATAAGTGAATTGGCAAGCATGTTTACTTATATGGCTTCTCCCTCTGCCAATA
G  L  A  D  S  S  I  Q  L  D  N  E  T  S  L  A  I  S  E  L  A  S  M  F  T  Y  M  A  S  P  S  A  N  T
      1510      1520      1530      1540      1550      1560      1570      1580      1590      1600
CATTAGACCACTTGGAAAAATACCACAATGTATTTTTGGAACGCTATGGATATGAAAGAGAAGTTCCTCTCTTAGAAATGCTATGTTCCAGCACTGGCAT
  L  D  H  L  E  K  Y  H  N  V  F  L  E  R  Y  G  Y  E  R  E  V  P  L  L  E  M  L  C  S  S  T  G  I
      1610      1620      1630    EcoRI       1650      1660      1670      1680      1690      1700
TGGTGCTCCTGCTACGTACACGAATCCTGCTAATGAATTCTTTGAAGAAACATCATTTGGGGAGCAATTTTCACCGGAAATGAAACAATTTTTCATGAGA
 G  A  P  A  T  Y  T  N  P  A  N  E  F  F  E  E  T  S  F  G  E  Q  F  S  P  E  M  K  Q  F  F  M  R
      1710      1720      1730      1740      1750      1760      1770      1780      1790      1800
AAGTATTTTGAATCAGTTAGAAAGAAAGCTCCTATTCAATTAGACGACGAAACATTCCATAGAATTTGCAACTCTGAAATTGCTGATGAGGAAATTCCAT
K  Y  F  E  S  V  R  K  K  A  P  I  Q  L  D  D  E  T  F  H  R  I  C  N  S  E  I  A  D  E  E  I  P  L
      1810      1820      1830      1840    HindIII 1860      1870      1880      1890      1900
TATCATTTGAACTCAATTTCTTTGTTAAATTACGAAATGGGAGAGTTAAGCTTTATTTAGGCCCCAACGTCGGATCTACCCGCGCAGGGAAAACATTTGG
 S  F  E  L  N  F  F  V  K  L  R  N  G  R  V  K  L  Y  L  G  P  N  V  G  S  T  R  A  G  K  T  F  G
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FIG. 2. Nucleotide sequence of the subcloned fragments of pCE42, pKB2, and pCK38 containing the *spaB* reading frame and its promoter region. The putative promoter site and the Shine-Dalgarno sequence (S/D) are underlined.

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      1910       1920       1930       1940       1950       1960       1970       1980       1990       2000
GAGATTTTCTCATATGTCTGATTCAATCAGTGAAATCATAAAGACCTTACATAACAAGGAGAAAGAGTTAACAGAGTGTAATACGAAAGTTTGTGAACTG
 R  F  S  H  M  S  D  S  I  S  E  I  I  K  T  L  H  N  K  E  K  E  L  T  E  C  N  T  K  V  C  E  L
      2010       2020       2030       2040       2050       2060       2070       2080       2090       2100
AGTATTGTGCCTAATCAAACTAGGTCTGGAAATGTAACAAGAAATGTAAGCTACCGAGAAAAAAGAGATGTCTCTTTTTACGAACAGTGCTCTGCATCTCA
 S  I  V  P  N  Q  T  R  S  G  N  V  T  R  N  V  S  Y  R  E  K  E  M  S  L  F  T  N  S  A  L  H  L  N
      2110       2120       2130       2140       2150       2160       2170       2180       2190       2200
ATGATTCCGTCAAAGCTGAAGATATTCTAATTGGAATCAATAAAGACCATAACTTTTATGCTAGACATAAAACAACTGGCGAAATTCTGTCTTTTGAGTC
   D  S  V  K  A  E  D  I  L  I  G  I  N  K  D  H  N  F  Y  A  R  H  K  T  T  G  E  I  L  S  F  E  S
      2210       2220       2230       2240       2250       2260       2270       2280       2290       2300
AAATCATATGTTTAATCCTTTATTAATGACCAACGCTGTACGGTTTTTATTAGAGATTTCAAGAGATGGTAAAAGAAAATGGAATGATTTCCCGTGGTTT
 N  H  M  F  N  P  L  L  M  T  N  A  V  R  F  L  L  E  I  S  R  D  G  K  R  K  W  N  D  F  P  W  F
      2310       2320       2330       2340       2350       2360       2370       2380       2390       2400
AGTATCTATAGTGATTTCAAGTATATTCCTGAAATCAAATATAAAGAGATAACCTTATCTTGTGAACAATGGCTGATATACAAAAATGATTTAAGCATGC
 S  I  Y  S  D  F  K  Y  I  P  E  I  K  Y  K  E  I  T  L  S  C  E  Q  W  L  I  Y  K  N  D  L  S  M  H
      2410  XbaI       2430       2440       2450       2460       2470       2480       2490       2500
ACTCGAATGCATCTCTAGAAGAGATAAAATCTCCTTTTTTTTGAATTTCATCGTACTTATGAACTGCCGCAAACATTTTATATCGTTAACGCAGACAATCG
   S  N  A  S  L  E  E  I  K  S  P  F  F  E  F  H  R  T  Y  E  L  P  Q  T  F  Y  I  V  N  A  D  N  R
      2510       2520       2530       2540       2550       2560       2570       2580       2590       2600
ATTATTGATTGATATAGAGAATGATTGTACTTTGGATGTTTTTTTCTGGGAATTGAAAAAAACGAACCATAACCAGCCATTACAACTTGTGGCTGTTGAG
 L  L  I  D  I  E  N  D  C  T  L  D  V  F  F  W  E  L  K  K  T  N  H  N  Q  P  L  Q  L  V  A  V  E  H
      2610       2620       2630       2640       2650       2660       2670       2680       2690       2700
CATGATGCAGATGCGTTAATGGATAGAAACCAAAATGACTATTCGGGAGAAATCGTCGTTCCGCTGCTTAGAAAACAACCAGAAAAACCATTGTATTTAC
   D  A  D  A  L  M  D  R  N  Q  N  D  Y  S  G  E  I  V  V  P  L  L  R  K  Q  P  E  K  P  L  Y  L  P
      2710       2720       2730       2740       2750       2760  HindIII    2790       2800
CGGTTCTTAACGCAATAGAGGGAAGTGGTTCCGACAGAATAAAAATGCCATTTGAAGACTGGCTGTTTATTAAGCTTTATTGTAAACAAACAAGAGAAGA
   V  L  N  A  I  E  G  S  G  S  D  R  I  K  M  P  F  E  D  W  L  F  I  K  L  Y  C  K  Q  T  R  E  E
      2810       2820       2830       2840       2850       2860       2870       2880       2890       2900
AGAGCTAATTGCTTTTGAAATAGCGGATTTTTATAACCAGATTTCTGATCAATATCCAGTCAGACATTTCTTTATGAGGTATCGGGATCCAAAGCCTCAT
 E  L  I  A  F  E  I  A  D  F  Y  N  Q  I  S  D  Q  Y  P  V  R  H  F  F  M  R  Y  R  D  P  K  P  H
      2910       2920       2930       2940       2950       2960       2970       2980       2990       3000
ATAAGACTTAGATTTAATGGAAAAGCCGAAGTGCTGTACAGCTTGTTTCCCCAATTATTGAATTGGCTGAAAAGCTTAAGAGAAAAAGGACTGGTTTCAG
 I  R  L  R  F  N  G  K  A  E  V  L  Y  S  L  F  P  Q  L  L  N  W  L  K  S  L  R  E  K  G  L  V  S  E
      3010       3020       3030       3040       3050       3060       3070       3080       3090       3100
AGTCTGTTATCACTCAATACGAGCGGGAGATAGAACGATATGGCGGGCTAAGCCTTATGGAGGCTGCAGAACAGCTTTTCTGTGAAGACAGCAAAGTTGT
   S  V  I  T  Q  Y  E  R  E  I  E  R  Y  G  G  L  S  L  M  E  A  A  E  Q  L  F  C  E  D  S  K  V  V
      3110       3120       3130       3140       3150       3160       3170       3180       3190       3200
TGAAATGATGATTAGAATGCACCGGATGAAAGATATTACGATAAGCAAGGAAATTGCAGGCATGGTTTCGGTTATACAGTTTTTAGAACAGTTCGAGCTA
 E  M  M  I  R  M  H  R  M  K  D  I  T  I  S  K  E  I  A  G  M  V  S  V  I  Q  F  L  E  Q  F  E  L
      3210       3220       3230       3240       3250       3260       3270       3280       3290       3300
ACGTTTGAAGAACAGTTAACTTTTTTAGAGAGAAATTCCTTACAGAATGAGTATCGTACTGAATTTAAAAAGGATAGAGAAATGTATATTGAAATATGCA
 T  F  E  E  Q  L  T  F  L  E  R  N  S  L  Q  N  E  Y  R  T  E  F  K  K  D  R  E  M  Y  I  E  I  C  N
      3310       3320       3330       3340       3350       3360       3370       3380       3390       3400
ATTCTGACAGAGATTGGGATAATCTCAAGAAAACAAGTGATGGCGGTATGTTATATGAAACTTTGAAAACAAGAAAAATGGCTGCAGCTCATTATGCATT
   S  D  R  D  W  D  N  L  K  K  T  S  D  G  G  M  L  Y  E  T  L  K  T  R  K  M  A  A  A  H  Y  A  F
      3410       3420       3430       3440       3450       3460       3470       3480       3490       3500
TTTAATCAAAAAGGCATTTGATAACAAAGATGAAGTTTATTCACGTATAGGAAGTATCATCCATCTGCATTGCAATCGTTTATTCGGAACCGACAGAGAA
 L  I  K  K  A  F  D  N  K  D  E  V  Y  S  R  I  G  S  I  I  H  L  H  C  N  R  L  F  G  T  D  R  E
      3510       3520       3530       3540       3550       3560       3570       3580       3590       3600
CTGGAAAATAAAATTCTCACCCTATGCAGACATTCTTTATATGCGCAACGATATCAAAAGATGAATGGTAGTTTAGCATGGAAGTAAAGGAACAACTGAA
 L  E  N  K  I  L  T  L  C  R  H  S  L  Y  A  Q  R  Y  Q  K  M  N  G  S  L  A  W  K
```

FIG. 2—*Continued.*

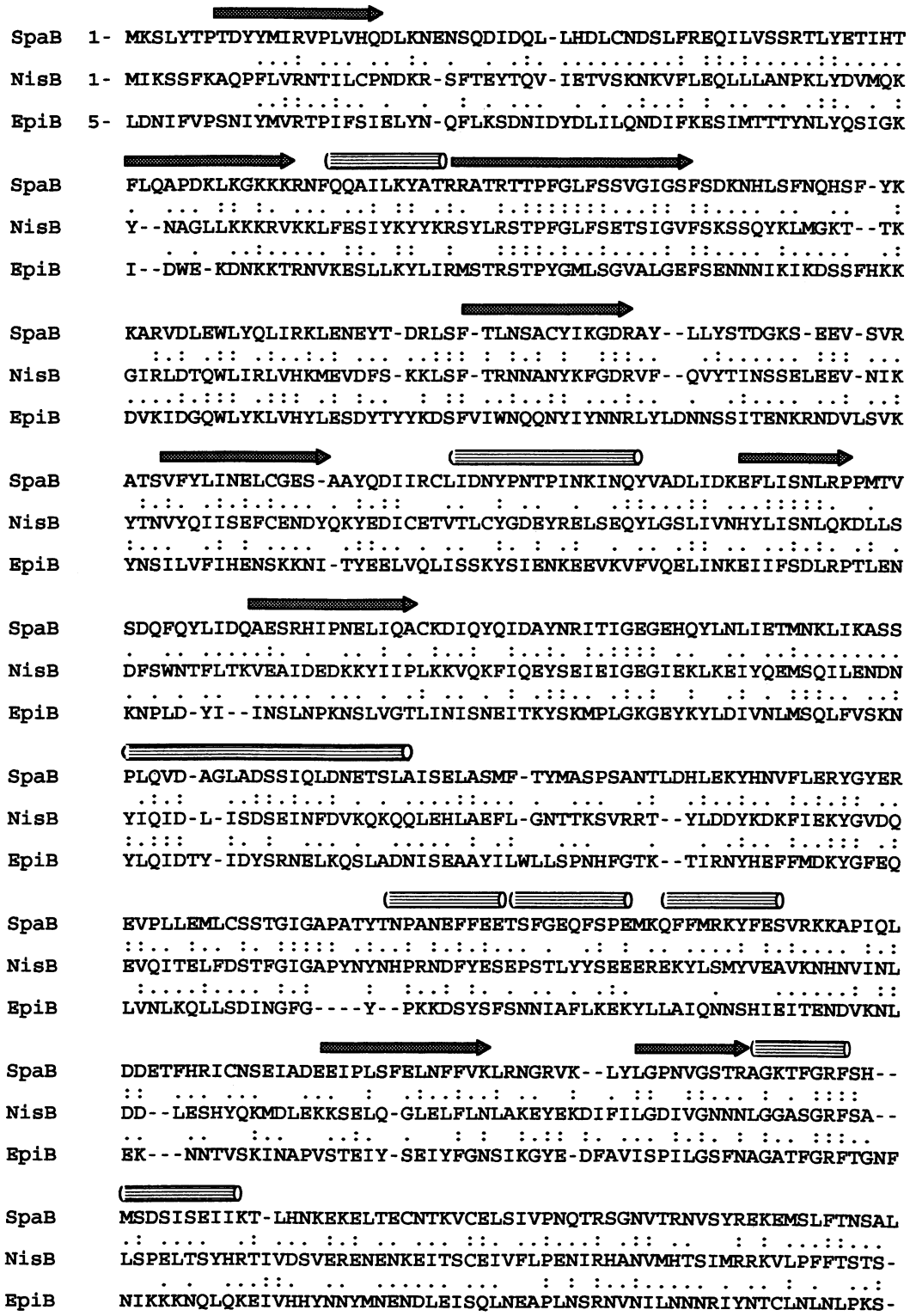

FIG. 3. Sequences and predicted secondary structures of proteins SpaB, NisB, and EpiB. Similar amino acid residues are indicated by dots, and identical amino acid residues are indicated by colons. The arrows indicate areas where β-sheets are predicted, and the rods indicate areas having an α-helical character. Predictions were made by using the method of Chou and Fasman (9) and "Prosis" (Hitachi).

characterize this protein, polyclonal antibodies were obtained by immunizing rabbits with a TrpE-SpaB fusion protein expressed in *E. coli*. The protein was manufactered by fusing the *spaB* open reading frame (425 amino acid residues of the C-terminal part) to the *trpE* open reading frame of *E. coli*. The antibodies obtained cross-reacted with a protein having a molecular mass of approximately 115 kDa after SDS-gel electrophoresis (Fig. 5).

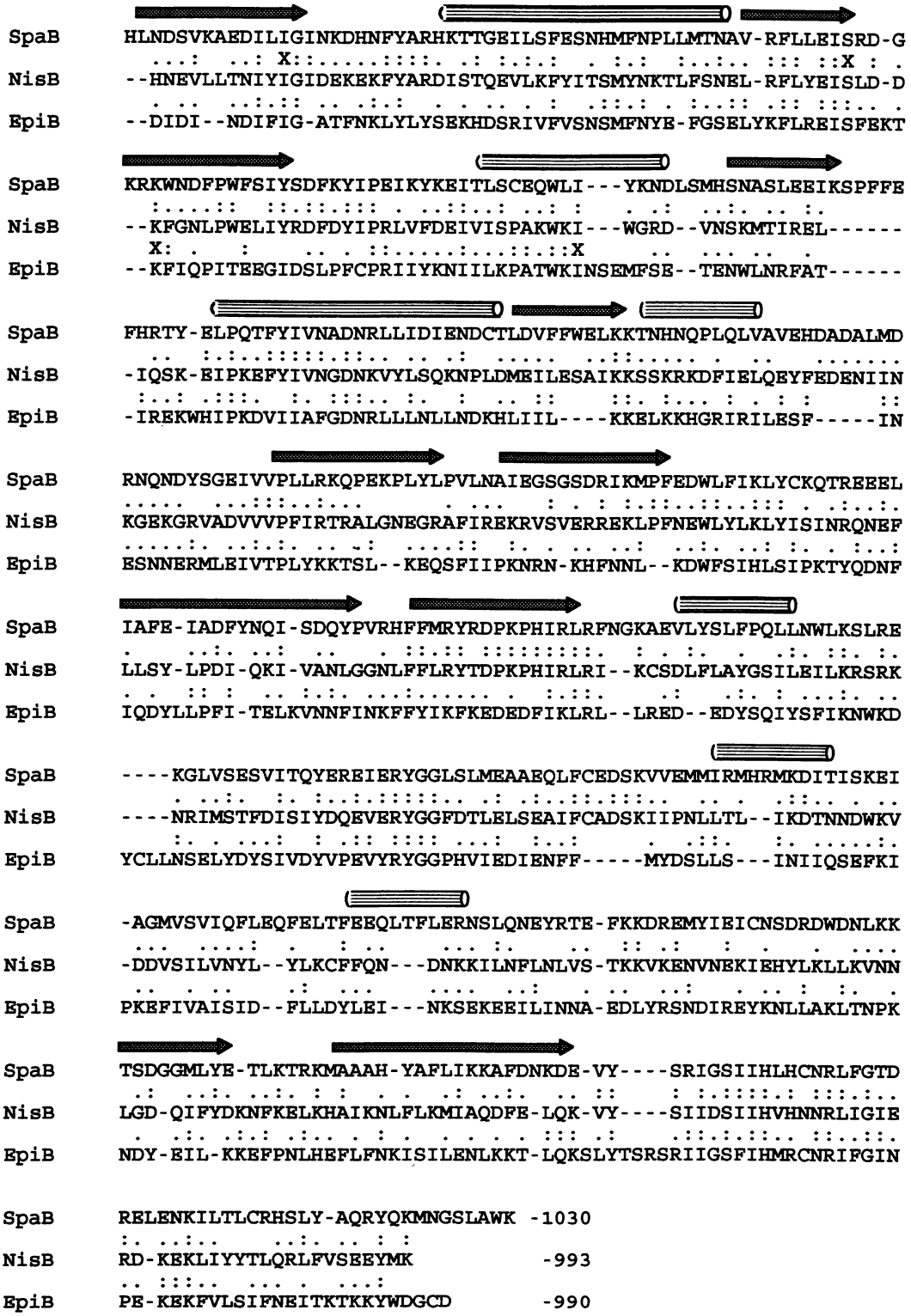

FIG. 3—*Continued.*

Neither signal was detected with the preimmune serum or with the *spaB* disruption mutant, confirming that the cross-reaction with SpaB was specific. Considering that membrane proteins (see below) migrate slightly differently on SDS-gels, the estimated protein molecular mass of 115 kDa is consistent with the molecular mass of 120.5 kDa derived from the *spaB* sequence.

The *spaR* and *spaK* disruption mutants were also tested

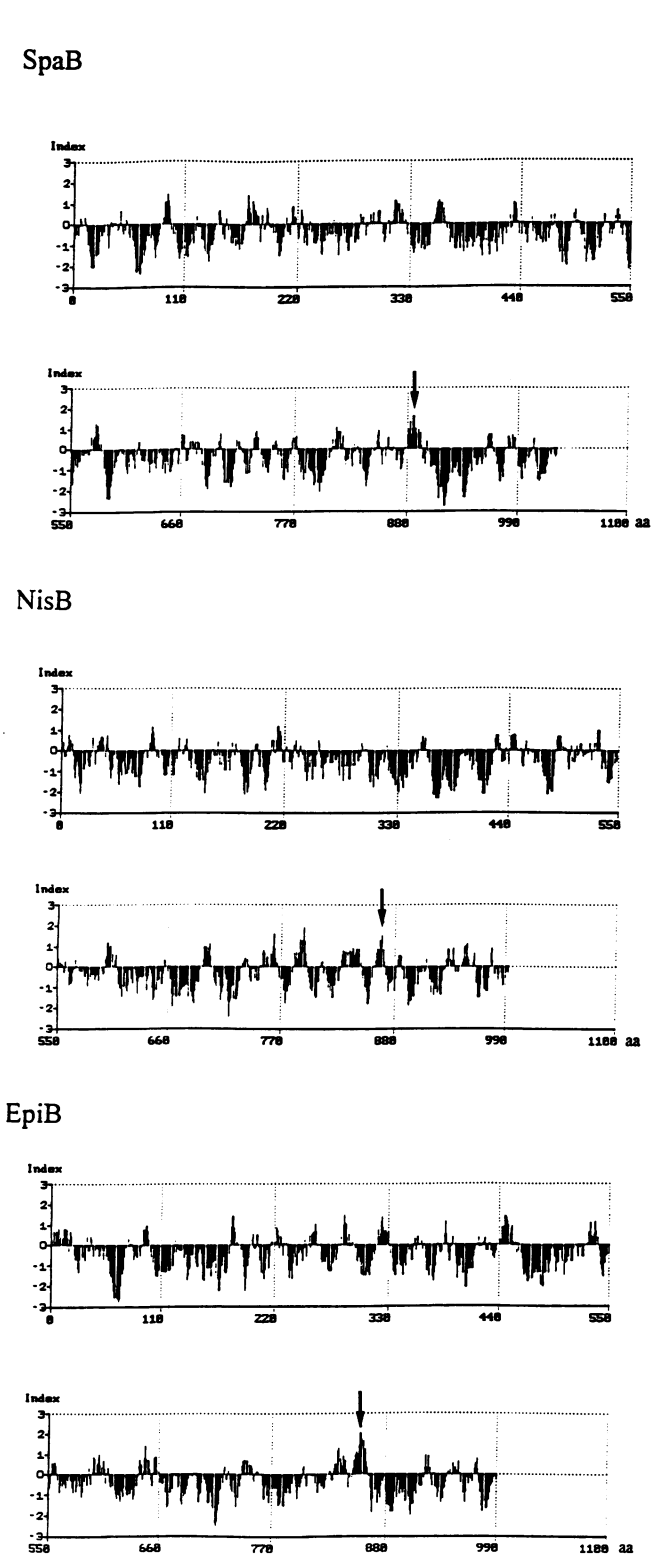

FIG. 4. Hydrophobicity plots for SpaB of *B. subtilis*, NisB of *L. lactis* 6F3, and EpiB of *S. epidermidis* Tü 3298 (= DSM 3095). Positive numbers indicate hydrophobicity. aa, amino acids. The arrows indicate the hydrophobic region present in all three proteins.

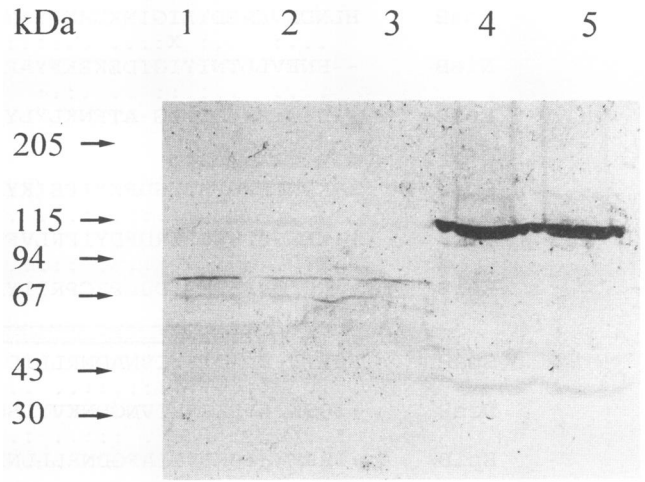

FIG. 5. Immunoblot analysis of SpaB. Lanes 1 through 3, protein extracts of *spaR*, *spaK*, and *spaB* disruption mutants, respectively; lanes 4 and 5, wild-type protein extract.

for SpaB expression by immunoblotting. SpaB protein was observed in wild-type cells, whereas no SpaB protein was detected in *spaR* and *spaK* mutants. These results show that the previously described "two-component" regulatory system for subtilin biosynthesis (30) acts via *spaB* expression. In order to analyze the regulation of SpaB, SpaB expression was monitored during growth. Wild-type cells were grown on TY media, and after vesicle preparation SpaB expression was monitored by performing a Western blot analysis. As shown in Fig. 6b, the SpaB protein was first detected after 3.5 h. The intensity of the SpaB protein signal increased until the stationary phase was reached and then decreased after overnight incubation of stationary-phase cells.

The expression of the SpaB protein correlated strongly with subtilin biosynthesis, as shown by bioassay tests performed with *M. luteus* as the test organism. Severe growth inhibition was observed with wild-type cells after 5 h, which coincided with the detection of the SpaB protein (Fig. 6c). No growth inhibition occurred with the *spaB* disruption mutant (31). These results indicated that *spaB* is involved in subtilin biosynthesis and showed that *spaB* expression and subtilin biosynthesis are strongly related.

Preparation of *B. subtilis* vesicles and membrane localization of SpaB. After centrifugation, most of the cross-reacting activity was present in the sediment, indicating that SpaB was associated with the membrane. In order to establish the membrane localization, *B. subtilis* vesicles were prepared.

The proteins attached to the vesicles were analyzed by performing an immunoblot analysis. The 115-kDa SpaB protein was associated with the vesicle fraction and was released only after treatment with SDS (Fig. 7). After several resuspension steps followed by repeated centrifugation, the SpaB protein was still found in the vesicle fraction, and no depletion was observed. Even after vesicle disintegration by vigorous agitation with glass beads, SpaB was still in the membrane fraction. These results strongly suggest that SpaB is membrane bound and support our hypothesis that lantibiotic biosynthesis occurs at the cell membrane.

The integrity of the prepared vesicles was tested by using pyranine, which does not significantly bind to phospholipid vesicles because of its polyanionic character (11). Control

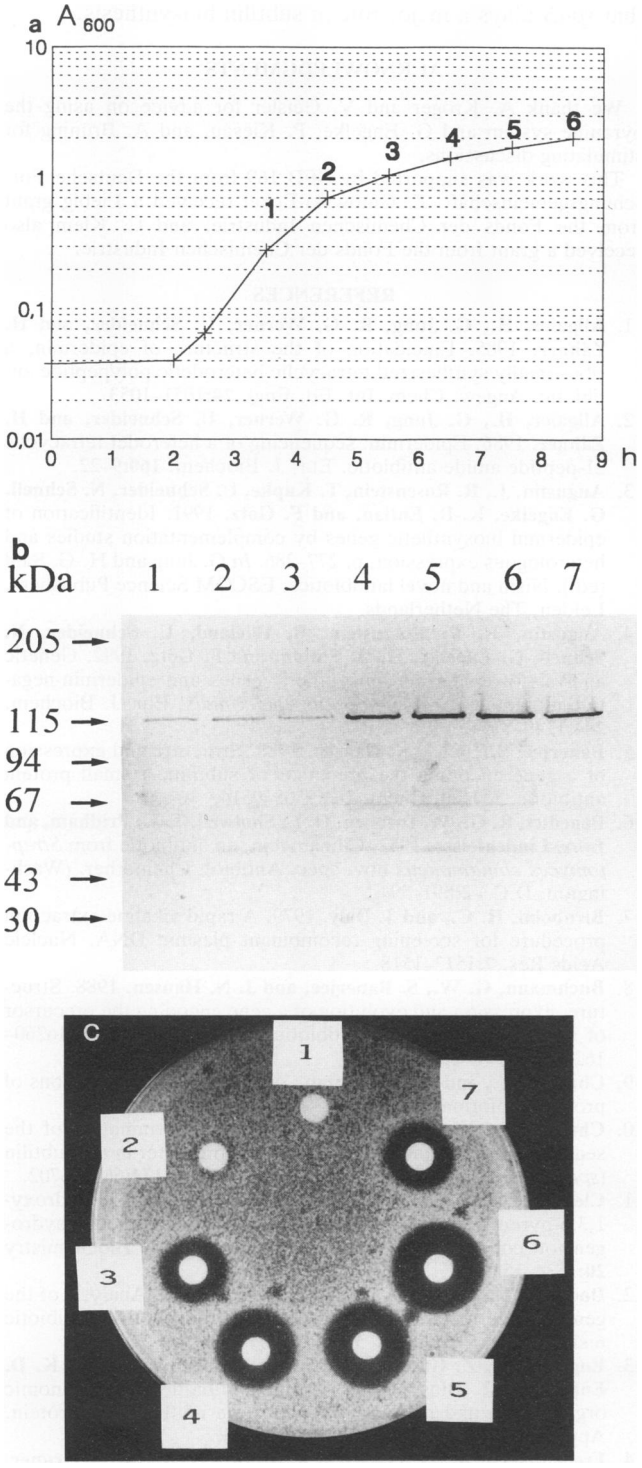

FIG. 6. Regulation of SpaB expression and subtilin biosynthesis. (a) Growth curve of *B. subtilis* ATCC 6633. The numbers next to the curve correspond to lane designations in panel b and to colony designations in panel c. (b) Immunoblot analysis of SpaB expression during growth. Lane 1, 3.5 h; lane 2, 4.5 h; lane 3, 5.5 h; lane 4, 6.5 h; lane 5, 7.5 h; lane 6, 8.5 h; lane 7, 24 h. The times are the amounts of time after batch culture inoculation. (c) Bioassay for subtilin production. The numbers 1 to 6 correspond to those in panels a and b; number 7 corresponds to supernatant from a culture that was grown overnight.

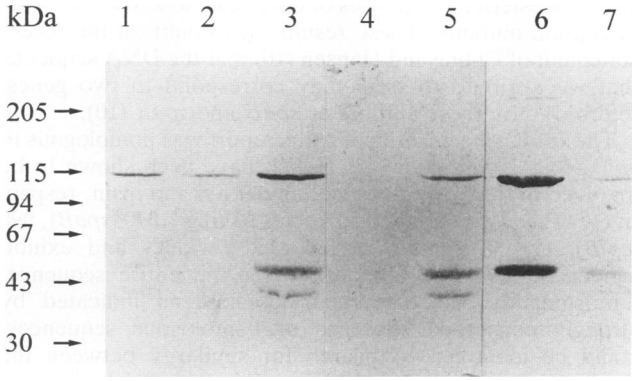

FIG. 7. Membrane localization of SpaB. Protein extracts were separated by SDS-gel electrophoresis and detected by Western blot analysis. Crude extracts (lane 1) were centrifuged at 48,000 × *g* for 30 min at 4°C (lane 2, supernatant; lane 3, pellet), and the resulting membrane fraction (pellet) was resuspended and centrifuged again under the same conditions (lane 4, supernatant; lane 5, vesicle fraction). The final vesicle fraction (pellet) was treated with SDS and analyzed after further centrifugation at 48,000 × *g* for 30 min at 4°C (lane 6, supernatant; lane 7, pellet).

vesicles were formed in the presence of pyranine. Vesicles with incorporated pyranine were separated from external pyranine by gel chromatography. The amount of pyranine incorporated into the vesicles was estimated after vesicles were lysed with Triton X-100 and fluorescence was measured at 450 nm. From these results a pyranine concentration of 2.6×10^{-8} M was calculated. The vesicles remained stable in the absence of Triton X-100.

DISCUSSION

The genes involved in subtilin biosynthesis are organized in an operon-like structure (25, 31). Genes *spaC*, *spaT*, and *spaB* are located upstream of the structural gene, *spaS*. These genes play a major role in subtilin biosynthesis as gene disruptions of *spaC* and *spaB* resulted in subtilin-negative mutants (25, 31). The SpaT protein exhibited a high level of homology with ATP-binding cassette transport proteins. The initial gene disruption experiments identified a mutant that was capable of producing subtilin but had a clumpy phenotype and became nonviable at the growth phase at which subtilin biosynthesis began (25, 31). Additional experiments, however, revealed that this phenotype occurred only when kanamycin was used as a resistance marker for gene disruption. When the *cat* gene was used as a resistance marker, the resulting mutants were subtilin negative (30). These results showed that all three genes which are upstream of *spaS* that have been identified are necessary for subtilin biosynthesis. In this study we isolated the complete *spaB* gene and deduced that the open reading frame encodes a protein having 1,030 amino acids. Upstream from *spaB* a putative promoter that has similarities to vegetative sigma A promoters of *B. subtilis* (40) was identified. No additional open reading frames upstream from *spaB* on the DNA fragment that was isolated could be identified for the following 300 bp. As deduced from the *spaB* gene, a protein with a molecular mass of 1,250 kDa was expected. By using antibodies directed against SpaB, a protein having the expected molecular mass was detected by Western blot analysis. No cross-reactions were observed with the preimmune serum and

after a Western blot analysis of crude extracts from the *spaB* disruption mutants. These results cast doubt on the recent statement of Chung and Hansen (10) that the DNA sequence that we attribute to *spaB* may correspond to two genes, referred to by these authors as *spaD* and *spaE* (10).

The *spaB* gene described in this report was homologous to *epiB* (46, 47) and *nisB* (13), which have been shown to be involved in the biosynthesis of epidermin and nisin, respectively. These genes encode proteins having 1,030 (*spaB*), 990 (*epiB*), and 993 (*nisB*) amino acid residues and exhibit significant levels of homology over the entire sequence. Unfortunately, no functional domains, as indicated by strongly conserved stretches of homologous sequences, could be identified. A search for similarity between the SpaB, NisB, and EpiB proteins and threonine dehydratases revealed some similarity, which suggests that SpaB, NisB, and EpiB have a dehydratase function.

To understand the function of SpaB in subtilin biosynthesis, we used specific antibodies to investigate the cellular localization of the SpaB protein. The method of Konings et al. (34) was used for *B. subtilis* membrane preparation; vesicles were isolated, and the integrity of the isolated vesicles was proven with the dye pyranine, which does not interact with membranes (11). Our results showed that SpaB copurified with the vesicle fraction, which indicates that the SpaB protein is associated with the cellular membrane of *B. subtilis*. A similar result was found previously for NisB, which is associated with the cellular membrane of *L. lactis* (13). Surprisingly, SpaB, NisB, and EpiB are rather hydrophilic proteins. There is a hydrophobic region present in SpaB between amino acid residues 875 and 900 which may play a role in membrane integration. A similarly localized hydrophobic area is also present in EpiB between amino acids 830 and 860. For NisB a change from hydrophilic to hydrophobic character is also found between amino acid residues 825 and 850, but this change is less pronounced than the change in the other proteins. Perhaps this area is important for membrane attachment. Alternatively, if no regions for membrane integration are present on these proteins, they might attach to membranes by interactions with other proteins, such as the more hydrophobic SpaC, NisC, and EpiC proteins, indicating that there is a membrane-localized multifunctional protein complex for lantibiotic biosynthesis. This possibility is consistent with the ideas that modification reactions occur in the prepeptides and that the mature lantibiotic is released from the modified prepeptide by proteolytic cleavage during or after transport through the cytoplasmic membrane (49). Thus far, two observations confirm this hypothesis. First, a modified prepeptide was isolated with antibodies directed against the leader sequence of lantibiotic Pep5 (53), and second, a protease specific for cleavage of the nisin prepeptide was recently localized extracellularly (51).

We recently showed that subtilin biosynthesis is regulated by a so-called two-component regulatory system which is composed of a membrane-localized histidine kinase and a response regulator (30). A similar regulatory system was also identified for nisin biosynthesis by identifying the *nisR* gene encoding the putative response regulator (51). This indicates that the genes encoding the proteins involved in lantibiotic biosynthesis could be the target for this regulatory system. Indeed, we showed that *spaB* is not expressed in *spaR* and *spaK* gene disruption mutants. In addition, we monitored subtilin production and *spaB* expression in a batch culture experiment. Our results clearly revealed that *spaB* expression strongly coincided with subtilin production, indicating that *spaB* plays a major role in subtilin biosynthesis.

ACKNOWLEDGMENTS

We thank A. Kröger and V. Geisler for advice on using the pyranine system and G. Engelke, P. Kiesau, and A. Brüning for stimulating discussions.

This work was supported by SFB 169 from the Deutsche Forschungsgemeinschaft. Z. Gutowski-Eckel received a Liebig grant from the Fonds der Chemischen Industrie, and C. Klein also received a grant from the Fonds der Chemischen Industrie.

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