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Evidence for a Multimeric Subtilin Synthetase Complex

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Subtilin is a lanthionine-containing peptide antibiotic (lantibiotic) produced by *Bacillus subtilis*. It is ribosomally synthesized as a prepeptide and modified posttranslationally. Three proteins of the subtilin gene cluster (SpaB, SpaC, and SpaT) which are probably involved in prepeptide modification and transport have been identified genetically (C. Klein, C. Kaletta, N. Schnell, and K.-D. Entian, Appl. Environ. Microbiol. 58: 132–142, 1992). Immunoblot analysis revealed that production of SpaC is strongly regulated (Z. Gutowski-Eckel, C. Klein, K. Siegers, K. Bohm, M. Hammelmann, and K.-D. Entian, Appl. Environ. Microbiol. 60:1–11, 1994). Transcription of the SpaC protein started in the late logarithmic growth phase, reaching a maximum in the early stationary growth phase. No SpaC was detectable in the early logarithmic growth phase. Deletions within the *spaR* and *spaK* genes, which act as a two-component regulatory system, resulted in failure to express SpaB and SpaC, indicating that these two genes are the regulatory targets. Western blot analysis of vesicle preparations of *B. subtilis* revealed that the SpaB, SpaT, and SpaC proteins are membrane bound, although some of the protein was also detectable in cell extracts. By using the yeast two-hybrid analysis system for protein interactions, we showed that a complex of at least two each of SpaT, SpaB, and SpaC is most probably associated with the substrate SpaS. These results were also confirmed by coimmunoprecipitation experiments. In these cosedimentation experiments, SpaB and SpaC were coprecipitated by antisera against SpaC, SpaB, and SpaT, as well as by a monoclonal antibody against epitope-tagged SpaS, indicating that these four proteins are associated.

Lantibiotics such as subtilin, nisin, and epidermin are ribosomally synthesized peptide antibiotics. They contain the unusual amino acids *meso*-lanthionine, dehydrobutyrine, and dehydroalanine (36). The major characteristic of lantibiotics is the occurrence of the thioether amino acids *meso*-lanthionine and 3-methyllanthionine, which are generated during maturation of the lantibiotic prepeptides. Lantibiotics can be divided into two subgroups (19): (i) linear lantibiotics, including subtilin (16), nisin (28, 32), epidermin (1, 2), gallidermin (20), and Pep5 (33), and (ii) globular lantibiotics, including cinnamycin (RoO9-0198 or lanthiopeptin) (6, 21, 30), duramycin (15), and ancovenin (40).

Epidermin and gallidermin are potentially applicable in the treatment of acne disease because of their high activity against *Propionibacterium acnes*. Subtilin is very similar to nisin, which is the most important member of the group of linear lantibiotics. Since the realization of the mutagenic effect of nitrite, which is used against clostridia in canned food, there has been an increasing interest in the use of nisin as a food preservative. The main difficulty in exploiting lantibiotics commercially is the low production rates. To overcome this problem, the biosynthesis of lantibiotics has to be elucidated.

The structural genes of linear lantibiotics encode prepeptides which consist of an N-terminal leader sequence followed by the C-terminal propeptide from which the lantibiotics mature (36). Several genes essential for the synthesis of the lantibiotics subtilin (5, 23, 24, 25), epidermin (4, 35), and nisin (10, 38) have been identified. Sequence analysis of the DNA regions adjacent to the subtilin structural gene *spaS* revealed eight open reading frames (13). The genes *spaB*, *spaT*, and *spaC* are located upstream of *spaS*. Downstream to the *spaS* gene, *spaI*, *spaF*, *spaG*, *spaR*, and *spaK* were identified. All of these genes have been shown to be similar to respective genes of the epidermin (1, 4, 35) and nisin (9) gene clusters. Experiments with deletion of the genes *spaB*, *spaT*, *spaC*, *spaR*, and *spaK* in *Bacillus subtilis* showed that these genes are essential for subtilin biosynthesis (25). SpaIFG seems to be involved in the self-protection of the producer strain of *B. subtilis* (23). SpaR and SpaK, whose genes are located downstream of *spaIFG*, correspond to a two-component system which regulates subtilin biosynthesis (24). Similar results have also been found for the respective genes *nisIFEG* (37) and *nisRK* (12) of the nisin-producing strain *Lactococcus lactis* KS 100.

Most of the lantibiotic-producing strains investigated so far have the genes *lanB* (*epiB*, *spaB*, and *nisB*), *lanC* (*epiC*, *spaC*, and *nisC*), and *lanT* (*epiT*, *spaT*, and *nisT*) in common (13). The *lanT*-encoded proteins share strong similarities to the hemolysin transporter HlyB and are probably responsible for transport of the respective lantibiotic over the cytoplasmic membrane. Proteins encoded by the genes *lanB* and *lanC* share no similarities to previously described proteins in databases. Gene disruption experiments have revealed that all three genes are essential for biosynthesis of subtilin (25) and nisin (38), respectively. As the functions of all of the other genes involved in lantibiotic biosynthesis became obvious by their similarities to previously described proteins and by biochemical experiments, *lanB* and *lanC* are the genes which most likely encode the proteins catalyzing the modification reactions of the lantibiotic prepeptides.

In the present report, we describe the results of several experiments giving strong evidence for a membrane-associated subtilin synthetase complex consisting of proteins SpaB, SpaC, and SpaT, catalyzing modification and transport of the SpaS

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TABLE 1. Plasmids used in this study

Plasmid	Description	PCR primers used for amplification[a]
pPKi40	160-bp PCR fragment containing *spaS* gene cloned into pGAD424 *Eco*RI-*Bam*HI site	5′ GGAGGTGACCGAATTCTCAAAGTTCG 3′ 5′ CTTATTTAGGGATCCTGCAGTTACAAG 3′
pPKi41	160-bp PCR fragment containing *spaS* gene cloned into pGBT9 *Eco*RI-*Bam*HI site	5′ GGAGGTGACCGAATTCTCAAAGTTCG 3′ 5′ CTTATTTAGGGATCCTGCAGTTACAAG 3′
pPKi42	1,310-bp PCR fragment containing *spaC* gene cloned into pGAD424 *Eco*RI-*Pst*I site	5′ GCAGTAAATGGAATTCGGCACTGTATC 3′ 5′ TTAAATTAATACTGCAGTTGTCCAATCTG 3′
pPKi43	1,310-bp PCR fragment containing *spaC* gene cloned into pGBT9 *Eco*RI-*Pst*I site	5′ GCAGTAAATGGAATTCGGCACTGTATC 3′ 5′ TTAAATTAATACTGCAGTTGTCCAATCTG 3′
pPKi48	1,253-bp PCR fragment containing *spaT* gene (aa 200–614) cloned into pGAD424 *Eco*RI-*Pst*I site	5′ GACAGGAAGAATTCTTCATACAC 3′ 5′ CAATTTCAACTTCTCTGCAGGATACAGTGCC 3′
pPKi49	1,253-bp PCR fragment containing *spaT* gene (aa 200–614) cloned into pGBT9 *Eco*RI-*Pst*I site	5′ GACAGGAAGAATTCTTCATACAC 3′ 5′ CAATTTCAACTTCTCTGCAGGATACAGTGCC 3′
pPKi50	808-bp PCR fragment containing *spaT* gene (aa 345–614) cloned into pGAD424 *Sma*I-*Pst*I site	5′ GTCACAAGAACCCGGGTGTTGAGC 3′ 5′ CAATTTCAACTTCTCTGCAGGATACAGTGCC 3′
pPKi51	808-bp PCR fragment containing *spaT* gene (aa 345–614) cloned into pGBT9 *Sma*I-*Pst*I site	5′ GTCACAAGAACCCGGGTGTTGAGC 3′ 5′ CAATTTCAACTTCTCTGCAGGATACAGTGCC 3′
pPKi54	1,530-bp PCR fragment containing 5′ end of *spaB* cloned into pGAD424 *Sma*I-*Bam*HI site	5′ GATTATTATATGATCCCGGGTCCTTTAG 3′ 5′ CGTAAAAAGAGGGATCCCTTTTTCTCG 3′
pPKi55	1,530-bp PCR fragment containing 5′ end of *spaB* cloned into pGBT9 *Sma*I-*Bam*HI site	5′ GATTATTATATGATCCCGGGTCCTTTAG 3′ 5′ CGTAAAAAGAGGGATCCCTTTTTCTCG 3′
pPKi56	1,686-bp PCR fragment containing 3′ end of *spaB* cloned into pGAD424 *Sma*I-*Sal*I site	5′ GGATCTACCCCGGGAGGGAAAAC 3′ 5′ CTTTACTTCCATGCTAGTCGACCATTC 3′
pPKi57	1,686-bp PCR fragment containing 3′ end of *spaB* cloned into pGBT9 *Sma*I-*Sal*I site	5′ GGATCTACCCCGGGAGGGAAAAC 3′ 5′ CTTTACTTCCATGCTAGTCGACCATTC 3′
pSW6	546-bp PCR fragment encoding AU1 epitope fused to N terminus of SpaS cloned into pCE20 (27) *Bst*EII-*Xba*I site	5′ GAAAGGAGGTGACCAATATGGATACCTATAGATAC ATTTCCAAGTTCGATGATTTCG 3′ 5′ TTACTTATCTAGATTAAATGTATCTATAGGTA TCTTTAGAGATTTTGCAGTTACAGG 3′

[a] Restriction sites within the primers are underlined.

prepeptide. These results, which are in good agreement with findings concerning nisin synthesis (38), were obtained by two independent experimental approaches. To detect weak and transient interactions of the proteins, we used the yeast two-hybrid system (8, 14). This system is based on the functional reconstitution of Gal4p, a transcriptional activator from yeast. The interaction of two hybrid proteins, one fused to the Gal4p activation domain and the other fused to the Gal4p DNA-binding domain, restores a functional activator, which binds to the GAL4-binding sites activating the transcription of a β-galactosidase gene. Interactions identified by the two-hybrid approach were verified by coimmunoprecipitation experiments. Our results strongly support the notion that a lanthionine synthetase complex, consisting of two or more SpaB, SpaC, and SpaT subunits, is needed for modification of the subtilin prepeptide.

MATERIALS AND METHODS

Strains and media. *B. subtilis* ATCC 6633 was used as the subtilin-producing strain. As negative controls in coimmunoprecipitation assays, *spaS*, *spaB*, *spaC*, and *spaT* deletion strains were used (25). Recombinant plasmids were amplified in *Escherichia coli* RR1 (F$^-$ *hsd-520 supE44 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1*). In the two-hybrid assay, yeast strain *Saccharomyces cerevisiae* SFY-542 (MAT**a** *ura3-52 his3-200 ade2-101 lys2-801 trp1-901 leu2-3,112* can^r *gal80-538* URA3::GAL1-*lacZ*) was used to monitor protein-protein interactions. *B. subtilis* was grown on TY medium (0.8% tryptone, 0.5% yeast extract [Difco, Detroit, Mich.], 0.5% NaCl), and *E. coli* strains were grown in Luria-Bertani medium (GIBCO, Neu-Isenburg, Germany) or on M9 medium (34) at 37°C. The following concentrations of antibiotics were used: ampicillin at 40 μg/ml for *E. coli* and chloramphenicol at 5 μg/ml and kanamycin at 10 μg/ml for *B. subtilis*. Yeast cells were grown at 30°C in YEPD liquid medium (2% [wt/vol] Bacto Peptone, 1% [wt/vol] yeast extract, and 4% [wt/vol] glucose). Transformants were selected on synthetic complete medium (0.67% yeast nitrogen base [Difco], 0.5% ammonium sulfate, 4% glucose or galactose) supplemented with the required amino acids.

Plasmids. For detection of the SpaS prepeptide by Western blot analysis, shuttle plasmid pSW6 was used. The plasmid codes for a six-amino-acid (DTYRYI) AU1 epitope (HISS Diagnostics GmbH) fused to the N terminus of *spaS* (Table 1). The epitope is derived from the L1 capsid protein of bovine papillomavirus. In *S. cerevisiae*, plasmids pGBT9 and pGAD424 (8, 14) were used to express fusion proteins with the Gal4p-binding domain and the Gal4p activation domain (Table 1). For expression of the TrpE-SpaC fusion protein, the vector pATH1 (26) was used.

Transformation of *B. subtilis*. *B. subtilis* was transformed by the competence method (3), with slight modifications (25).

Molecular biology techniques. Established protocols for molecular biology techniques were followed (34). DNA was cleaved as recommended by the commercial 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 (39).

Plasmid isolation and PCR. The procedure of Birnboim and Doly (7) was followed to isolate plasmids of *E. coli*. When necessary, these were purified by

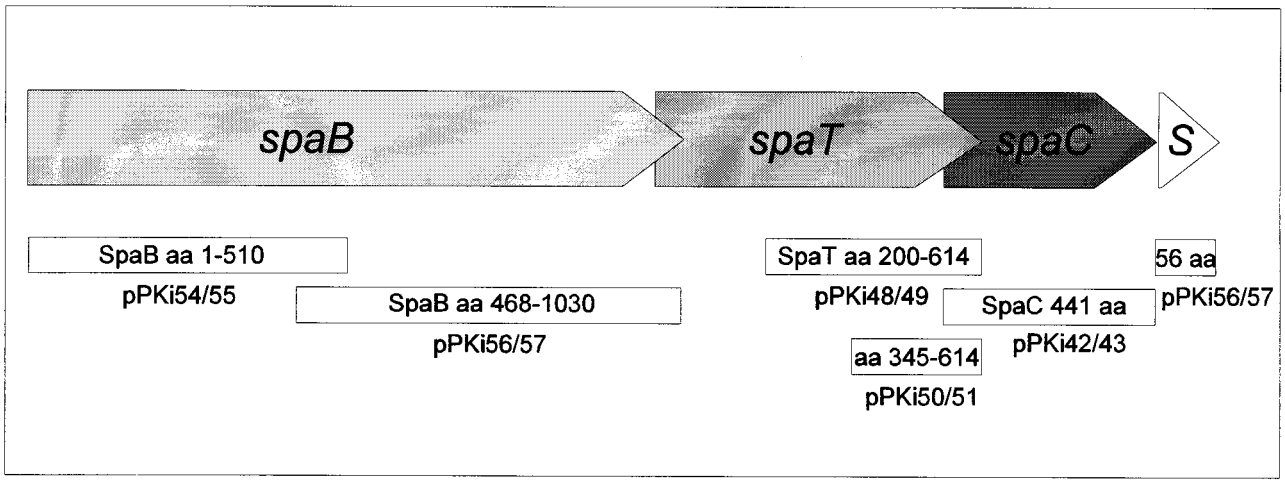

FIG. 1. Organization of the genes *spaB*, *spaT*, *spaC*, and *spaS* and corresponding protein fragments used in the two-hybrid assays. The genes are indicated by arrows. The boxes represent the lengths of the protein fragments fused to the GAL4 activation or DNA-binding domain.

ultramicrocentrifugation (Beckman TL 100, rotor TLA 100.2) at 80,000 rpm for 12 h. PCR was carried out by following standard procedures (34) with a Hybaid R2 Combi-Thermal reactor. With *Taq* DNA polymerase (Boehringer), 35 cycles of 20 s at 94°C, 20 s at 55°C, and 2.5 min at 72°C were performed.

Transformation of *S. cerevisiae*. Transformation of yeast was carried out by using the bicine method described by Klebe et al. (22).

Two-hybrid assay. To observe interactions between proteins which are involved in subtilin maturation, we used the yeast two-hybrid system (8, 14). DNA fragments used to construct fusion proteins with the DNA-binding and transcriptional activation domains of Gal4p were obtained by PCR with chromosomal DNA of *B. subtilis* ATCC 6633 as the template. Plasmids derived from pGBT9 and pGAD424 encoding the two hybrid proteins were cotransformed into a yeast strain harboring a *lacZ* reporter gene containing an upstream activation sequence (UAS_{Gal4}) and assayed for β-galactosidase activity.

β-Galactosidase assay. For qualitative studies, β-galactosidase filter assays were carried out. After 2 to 4 days of growth at 30°C, yeast transformants were transferred to sterile filters soaked in selection medium. Filters were placed on plates containing selection medium, and the colonies were allowed to grow for an additional 2 to 3 days at 30°C. The filter assay for β-galactosidase activity was carried out as follows. A filter with yeast transformants grown on top was carefully lifted off of the agar plate and transferred (colonies facing up) to liquid nitrogen. The frozen filter was removed from the nitrogen and allowed to thaw at room temperature. This filter with permeabilized cells was placed, colony side up, on another filter presoaked with a Z buffer–X-Gal solution (25 ml of Z buffer [60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, pH 7], 70 μl of β-mercaptoethanol, 420 μl of X-Gal solution [100 mg of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside in 1 ml of *N*,*N*-dimethylformamide]). The filters were incubated at 30°C and checked for the appearance of blue colonies.

Antisera. Two different approaches were used to get antisera against SpaC and SpaT. The 0.935-kb *Eco*RI-*Xba*I PCR fragment (coding for 305 amino acid residues of the C-terminal part) of *spaC* was fused to the *trpE* gene by using vector pATH1. For expression of the TrpE fusion protein, the plasmid was transformed into *E. coli* RR1. Cells containing the resulting plasmid were grown overnight in M9 with 20 μg of tryptophan per ml, diluted 1:10 in M9 without tryptophan, and further 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 an additional 4 h. Cells were harvested by centrifugation and lysed by addition of sodium dodecyl sulfate (SDS) sample buffer (100 mM $NaPO_4$ [pH 7.0], 0.5% β-mercaptoethanol, 4% SDS, 8 M urea, 0.02% bromphenol blue). Purification of the fusion protein, by subjecting 10 to 20 mg of protein to preparative SDS-polyacrylamide gel electrophoresis (PAGE), was followed by electroelution of the protein. The purified fusion protein was used for immunization of rabbits as described previously (17). The SpaC antiserum was preincubated 1:1 with a crude extract from a *spaC* deletion strain for 1 h at 37°C and centrifuged, and the supernatant was used for Western blot analysis. An 18-amino-acid peptide of the C terminus of SpaT was synthesized, coupled to keyhole limpet hemocyanin, and used for immunization (Eurogentec). Preimmune sera were taken before immunization of each rabbit started.

Preparation of membrane vesicles. Membrane vesicles of *B. subtilis* were prepared as described previously (17).

Coimmunoprecipitation. A 200-μl vesicle preparation was mixed with 200 μl of TENT buffer (25 mM Tris-HCl [pH 7.5], 5 mM EDTA, 250 mM NaCl, 0.1% Triton X-100), 50 μl of washed 10% Pansorbin (*Staphylococcus aureus* cells; Calbiochem-Novabiochem GmbH, Bad Soden, Germany) was added, and the samples were incubated for 1 h at 22°C. After centrifugation (10,000 × *g*, 1 min, 4°C), the supernatant was collected and incubated overnight at 4°C with 5 μl of the respective antisera. A 100-μl volume of prewashed 10% Pansorbin was added to the suspension, and the sample was incubated for 15 min at 22°C. After centrifugation of the StaphA (Pansorbin)-antibody-antigen complex (2 min, 10,000 × *g*, 4°C) through a sucrose cushion (1 M sucrose in TENT buffer), the supernatant was removed. The pellet was washed twice and subjected to SDS-PAGE, followed by Western blot analysis.

SDS-PAGE and Western blot analysis. SDS-PAGE and Western blot analysis were performed as described previously (11, 12). Molecular weight standards for SDS-PAGE were obtained from Anamed (Offenbach, Germany).

RESULTS

Construction of fusion proteins for two-hybrid analysis. To study possible physical interactions of proteins encoded by the subtilin gene cluster, the yeast two-hybrid system was used. Proteins with physical interaction are able to reconstitute the Gal4p gene activation function when one protein is fused to the Gal4p activation domain and the other is fused to the Gal4p DNA-binding domain. In-frame fusions of genes under investigation with the Gal4p activation domain and the Gal4p DNA-binding domain were constructed (Table 1 and Fig. 1) and confirmed by DNA sequencing of the constructs. The results of the β-galactosidase filter assays monitoring the interactions of the fusion proteins are shown in Table 2. For control experiments, all fusions were tested with the Gal4p-binding and activation domains alone.

To identify proteins probably catalyzing the modification reactions, we first tested for possible interactions with the SpaS prepeptide. A blue coloration of the yeast cells indicating an interaction of SpaC and the SpaS prepeptide was observed. Additionally, we saw an interaction of SpaS with the C-terminal part of SpaB (amino acids [aa] 468 to 1030) but not with the N-terminal part of SpaB (aa 1 to 510). This suggested that the prepeptide is associated with the C terminus of SpaB. Second, we observed interactions between the SpaC protein and both the N- and C-terminal parts of SpaB. In addition to this, we found interactions of the N-terminal part of SpaB with the bigger part of SpaT (aa 200 to 614) missing two of the four hydrophobic domains in the N-terminal part. Additionally, interactions of the C-terminal part of SpaB with both parts of SpaT were observed.

The two-hybrid analysis system also allowed us to test for self-interactions of the proteins involved in subtilin maturation

TABLE 2. Results of β-galactosidase filter assay[a]

Activation domain	Colony color when protein was fused to following binding domain:						
	SpaS	SpaC	SpaB aa 1–510	SpaB aa 468–1030	SpaT aa 200–614	SpaT aa 345–614	Gal4p
SpaS	White	Blue	White	Blue	White	White	White
SpaC	Blue	Blue	Blue	Blue	White	White	White
SpaB aa 1–510	White	Blue	White	Blue	Blue	White	White
SpaB aa 468–1030	Blue	Blue	Blue	Blue	Blue	Blue	White
SpaT aa 200–614	White	White	Blue	Blue	Blue	White	White
SpaT aa 345–614	White	White	White	Blue	White	White	White
Gal4p	White	White	White	White	White	White	White

[a] All proteins were fused to both the Gal4p activation domain and the Gal4p-binding domain.

and transport. After cotransformation of the yeast cells with plasmids encoding fusions of SpaC with the GAL4 activation domain and DNA-binding domain, β-galactosidase activity was observed, which suggests that the lanthionine synthetase complex contains at least a SpaC dimer. Self-interactions of the C-terminal and N-terminal parts of SpaB were also observed. Furthermore, we saw self-interactions when plasmids coding for fusions of a larger part of SpaT (aa 200 to 614) were investigated. This suggested that two or more SpaB and SpaT molecules are part of a common protein complex. The finding that SpaT is at least a dimer is in accordance with the general view that bacterial ABC transporters are homodimers (18). The dimerization site of SpaT is probably located between aa residues 200 and 345. The results of the two-hybrid assays suggested that the proteins involved in subtilin biosynthesis are associated in a multimeric complex consisting of proteins SpaB, SpaC, and SpaT (Fig. 2).

Coimmunoprecipitation of SpaB, SpaC, and SpaT. To exclude possible artifacts of the two-hybrid results, further support by alternative experiments was necessary. To prove physical interactions between SpaB and SpaC, coimmunoprecipitation experiments (Table 3) were carried out by using antisera against SpaB (17), SpaT, and SpaC. Membrane vesicles of *B. subtilis* ATCC 6633 were incubated with antisera directed against the SpaC or SpaT protein, and the protein-antibody complexes were precipitated with protein A from *S. aureus*. After SDS-PAGE and Western transfer, the precipitates were analyzed by SpaB-specific antisera. By using vesicles from the wild-type strain, it was shown that SpaB was coprecipitated by SpaC- and SpaT-specific antisera (Fig. 3). When vesicles were treated with SpaB- or SpaT-specific antisera and further analyzed with SpaC antisera (Fig. 4), SpaC was detected in vesicles derived from the wild type. Neither SpaB nor SpaC was precipitated in vesicle preparations of *spaB* or *spaC* deletion strains (Fig. 3 and 4, lanes 5 and 6). We also observed a number of unspecific bands due to protein A cross-reactions, mainly the unspecific signals of the heavy chain of rabbit immunoglobulins G (IgGs). To distinguish unspecific signals from specific ones, the experiments were repeated by using preimmune sera for precipitation. With all three preimmune sera, neither SpaB, SpaC, nor SpaT was detected (data not shown). Coimmunoprecipitation of SpaT with SpaC- or SpaB-specific antiserum could not be shown because the SpaT protein and the heavy chain of the rabbit IgGs could not be resolved by SDS-PAGE. Therefore, the unspecific IgG signals interfere with the specific signal for SpaT. The results obtained by coimmunoprecipitation of SpaB, SpaT, and SpaC biochemically confirmed the results obtained by two-hybrid experiments.

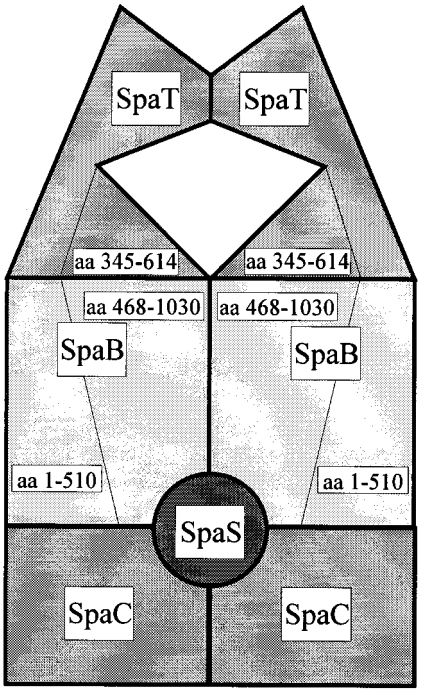

FIG. 2. Composition of the suggested lanthionine synthetase complex based on the data obtained by two-hybrid assays. The thin lines represent protein fragments. The thick-lined borders show the interactions between the protein fragments.

Coimmunoprecipitation of SpaS and SpaC or SpaB. To investigate the interaction of the subtilin prepeptide with the SpaC and SpaB proteins shown by the two-hybrid experiments, coimmunoprecipitation experiments with antisera directed against the SpaB and SpaC proteins and against a tagged subtilin prepeptide were done (Table 3). Lacking an antiserum against the subtilin prepeptide, we used a *spaS* deletion strain transformed with plasmid pSW6, which codes for the AU1 tag fused to the N terminus of SpaS (see Materials and Methods and Table 1). Cell extracts of this *B. subtilis* strain were incu-

TABLE 3. Results of coimmunoprecipitations

Second antiserum	Result obtained with following first antiserum:			
	SpaB	SpaT	SpaC	AU1
SpaB	+	+	+	+
SpaT	–	–	–	–
SpaC	+	+	+	+
AU1	–	–	–	–

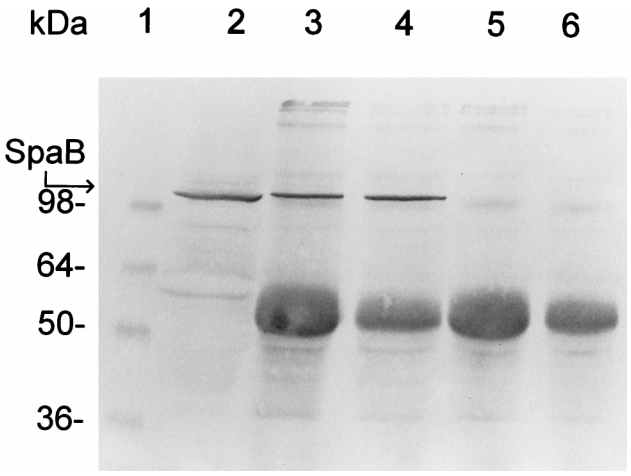

FIG. 3. Coimmunoprecipitation of SpaB. After precipitation, protein complexes were separated on an SDS–9% polyacrylamide gel, blotted, and analyzed with SpaB-specific antiserum. The SpaB-cross-reacting band is marked. Lanes: 1, molecular mass standards; 2, vesicle fraction of *B. subtilis* ATCC 6633 used as a positive control; 3, SpaC antibody-protein complex precipitated with protein A of *S. aureus*; 4, SpaT antibody-protein complex precipitated with protein A of *S. aureus*; 5 and 6, control experiments done with *B. subtilis* ATCC 6633 vesicles lacking *spaB* precipitated with primary antisera against SpaC and SpaT, respectively. Unspecific bands were caused mainly by unspecific cross-reactions between protein A and rabbit IgGs.

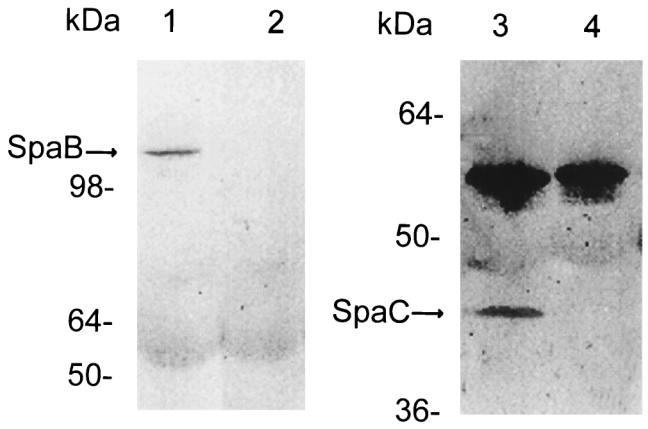

FIG. 5. Coimmunoprecipitation of SpaB and SpaC with tagged prepeptide. After precipitation, protein complexes were separated on SDS–7.5 and 9% polyacrylamide gels, respectively, blotted, and analyzed with SpaB (lanes 1 and 2)- and SpaC (lanes 3 and 4)-specific antisera. The cross-reacting bands are marked. Lanes: 1 and 3, antibody-protein complexes derived from vesicles of *B. subtilis* ATCC 6633 carrying pSW6 precipitated with protein A of *S. aureus*; 2 and 4, antibody-protein complexes derived from vesicles of *B. subtilis* ATCC producing nontagged prepeptide precipitated with protein A of *S. aureus* as a control. Unspecific bands were caused mainly by unspecific cross-reactions between protein A and rabbit IgGs.

bated with antisera directed against the AU1 epitope, and the protein-antibody complexes were precipitated with protein A of *S. aureus*. After SDS-PAGE, the proteins precipitated by monoclonal antibody AU1 were further analyzed by antiserum directed against SpaB and SpaC (Fig. 5). Cell extracts of *B. subtilis* ATCC 6633, which produces only the nontagged prepeptide, were used to prove the specificity of the signals. SpaB and SpaC were coprecipitated by specific antisera against the tagged prepeptide, whereas neither SpaB nor SpaC was precipitated in the controls (Fig. 5). These biochemical experiments support the results of the two-hybrid assay showing that the subtilin prepeptide is associated with a complex of SpaB and SpaC.

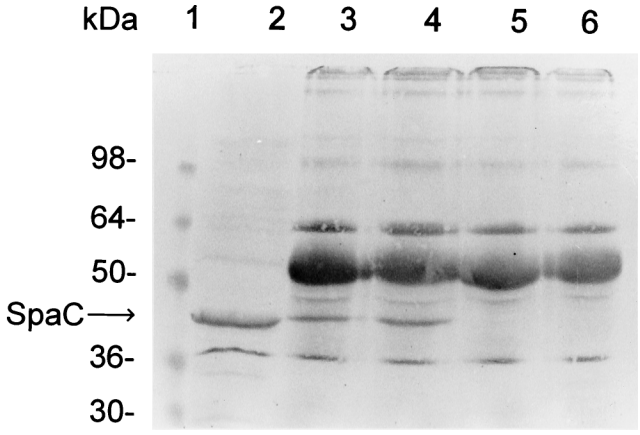

FIG. 4. Coimmunoprecipitation of SpaC. After precipitation, protein complexes were separated on an SDS–9% polyacrylamide gel, blotted, and analyzed with SpaC-specific antiserum. The SpaC-cross-reacting band is marked. Lanes: 1, molecular mass standards; 2, vesicle fraction of *B. subtilis* ATCC 6633 used as a positive control; 3, SpaB antibody-protein complex precipitated with protein A of *S. aureus*; 4, SpaT antibody-protein complex precipitated with protein A of *S. aureus*; 5 and 6, control experiments with *B. subtilis* ATCC 6633 vesicles lacking *spaC* precipitated with primary antisera against SpaB and SpaT, respectively. Unspecific bands were caused mainly by unspecific cross-reactions between protein A and rabbit IgGs.

DISCUSSION

Genes involved in subtilin biosynthesis are organized in an operonlike structure (25). Two reactions have been proposed for the maturation of subtilin, dehydration of serine and threonine residues, followed by sulfur addition from neighboring cysteine residues. The genes *spaB*, *spaT*, and *spaC*, which are located upstream of the structural gene *spaS*, are essential for subtilin biosynthesis and transport (25). For the final maturation of subtilin, a hypothetical protease of the subtilisin type should remove the leader peptide after its transport across the cytoplasmic membrane.

Our experiments revealed by two different approaches, two-hybrid assay and coimmunoprecipitation, that SpaC, SpaB, and SpaT are organized in a membrane-associated complex. In this complex, SpaB is attached to SpaT and SpaC is attached to SpaB. The two-hybrid system showed interactions between SpaT and SpaB and between SpaB and SpaC but not between SpaT and SpaC. In coimmunoprecipitation experiments, we precipitated SpaB and SpaC by using antiserum against SpaT, as well as SpaB with antiserum against SpaC and vice versa. These results indicate that the whole complex was precipitated by the SpaT antiserum. Additionally, we demonstrated with both methods that SpaC and SpaB interact with the subtilin prepeptide. Therefore, SpaB and SpaC should be the part of the protein complex which is necessary for the prepeptide modification reaction.

Furthermore, we observed in the two-hybrid assay that SpaT interacts with SpaT. This indicates that the ABC transporter SpaT is at least a dimer. The interaction occurred only when the larger part of SpaT (aa 200 to 614) was fused to the Gal4p test proteins. The smaller C-terminal fragment (aa 315 to 614), containing the ATP-binding sites, showed no dimerization, indicating that the amino acids necessary for dimerization are residues 200 to 315. Dimerizations were observed for SpaB and SpaC as well. From this, we concluded that the protein com-

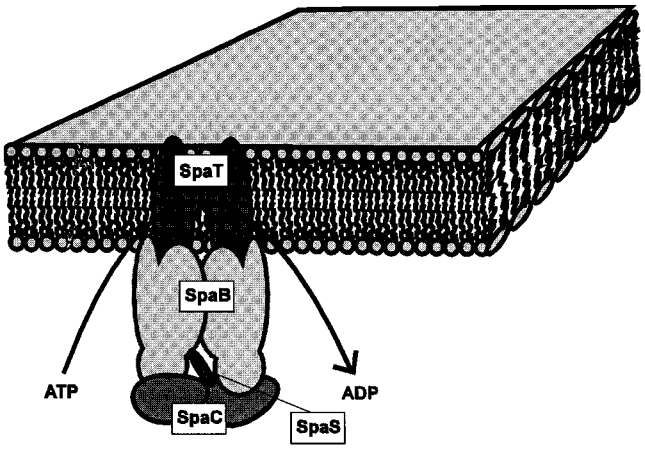

FIG. 6. Model of the minimal subtilin synthetase complex. The SpaT ABC transporter is integrated as a dimer in the cellular membrane and linked to at least two SpaC subunits via at least a SpaB dimer.

plex for subtilin modification and transport, which we named the subtilin synthetase complex, consists of at least two molecules of SpaB (120.5 kDa), two molecules of SpaC (49.3 kDa), and two molecules of SpaT (71.2 kDa) and has a total molecular mass of at least 482 kDa (Fig. 6).

The proposed model for subtilin biosynthesis is similar to the model for nisin biosynthesis (38). Both synthetase complexes are membrane associated. Whereas the subtilin complex consists of two or more subunits of each type, the nisin synthetase complex consists of two NisT and NisC subunits but only one NisB subunit. Furthermore, the interactions between the subunits differ. In the subtilin synthetase complex, SpaB is the mediator between SpaC and SpaT; in the case of nisin, NisC functions as the mediator between NisB and NisT. Whereas in the nisin synthetase complex the involvement of the transporter was elucidated only by the two-hybrid system, the involvement of SpaT in a lanthionine synthetase complex was shown here for the first time by coimmunoprecipitation, as well as by two-hybrid experiments. Furthermore, SpaB was precipitated by antiserum against the AU1-tagged prepeptide, while there was no such precipitation of NisB by antiserum against the leader peptide of nisin. A reason for this may be the use of the AU1 tag for detection of the subtilin prepeptide, which elongates the leader peptide by six amino acids. For nisin, it was proposed that NisB concealed the leader sequence, which was the epitope for the antibody. Possibly, the six amino acids of the AU1 tag were not covered by SpaB and remained detectable by the antiserum (Fig. 5).

For subtilin biosynthesis, our present data suggest that the unmodified subtilin prepeptide binds to SpaC as well as to SpaB. The prepeptide is modified by the action of SpaB and SpaC and is subsequently translocated over the cytoplasmic membrane by the SpaT ABC transporter. If SpaB is the dehydratase and SpaC is the lanthionine synthetase or vice versa is still not clear. According to a model for Pep5 biosynthesis, the catalytic domain for the dehydratization reaction could be located on the PepB protein and the thioether formation domain could be located on the PepC protein (29). For epidermin biosynthesis, Kupke and Götz showed evidence that EpiC is the thioether-forming protein and EpiB is the dehydratase (27). Additionally, Götz and coworkers did not observe any modification of the epidermin prepeptide with either purified EpiB (31) or EpiC (27). We propose that the modification of the prepeptide and the transport of the modified prepeptide require a complete and functional subtilin synthetase complex.

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