Structure−Function Relationships of the Lanthionine Cyclase SpaC Involved in Biosynthesis of the *Bacillus subtilis* Peptide Antibiotic Subtilin

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ABSTRACT: Biosynthesis of the lantibiotic subtilin in *Bacillus subtilis* is accomplished by a synthetase complex consisting of the dehydratase SpaB, cyclase SpaC, and transporter SpaT. Genetically engineered subtilin cyclases SpaC and related NisC and EriC proteins involved in biosynthesis of the lantibiotics nisin and ericin A/S, respectively, were analyzed to functionally substitute native SpaC in vivo. We could show for the first time posttranslational modification of a lantibiotic precursor peptide (subtilin) by a hybrid lantibiotic synthetase (SpaBT/EriC). Genetically engineered SpaC alanine replacement mutants revealed the essentiality of residues His^{231}, Trp^{302}, Cys^{303}, Tyr^{304}, Gly^{305}, Cys^{349}, and His^{350}, as well as the conserved C-terminal motif Lys^{437}-Ala^{438}-Leu^{439}-Leu^{440}-Ile^{441} for subtilin biosynthesis. Assignment of these strictly conserved lantibiotic cyclase residues to the NisC structure [Li, B., Yu, J. B., Brunzelle, J. S., Moll, G. N., van der Donk, W. A., and Nair, S. K. (2006) *Science*, *311*, 1464−1467] revealed the first experimental evidence for structure−function relationships in catalytic centers of lantibiotic cyclases. SpaC residues His^{231}, Cys^{303}, and Cys^{349} are involved in coordination of the central zinc ion. The pair His^{231}/Tyr^{304} is discussed to act as general acid/base catalysts in lanthionine formation. Furthermore, pull-down experiments revealed that functional inactive SpaC mutants were still able to interact with the hexahistidine-tagged subtilin precursor peptide in vitro. Our results suggest that Trp^{302} and the C-terminal residues of SpaC are constituents of a hydrophobic cluster which is involved in stabilization of the catalytic center and binding of the subtilin precursor peptide.

Lanthionine-containing antibiotics, the lantibiotics (*1*), are ribosomal synthesized peptides with unusual structural elements such as lanthionine rings and dehydrated amino acids (*2*). The Gram-positive endospore-forming soil bacterium *Bacillus subtilis* produces more than 2 dozen antibiotics (*3*) including the lantibiotics subtilin of *B. subtilis* ATCC 6633 (*4*, *5*) and ericin A/S of the A1/3 strain (*6*) and subtilosin A, a macrocyclic peptide with three unusual intramolecular sulfide bonds (*7*) produced by *B. subtilis* strains and *B. athrophaeus* (*8*), as well as sublancin, a disulfide-containing lantibiotic (*9*, *10*). Lantibiotics like subtilin or the structurally closely related nisin of *Lactococcus lactis* exhibit nanomolar potency against a wide range of Gram-positive bacteria, including various human pathogens. The antimicrobial activity of nisin is based on pore formation in the cytoplasmic membrane (for review see ref *11*). Lipid II, the hydrophobic carrier molecule for peptidoglycan monomers, appears to be both a docking molecule that assists membrane targeting of nisin (*12*, *13*) and an integral constituent of the lethal pore associated with nisin (*14*, *15*). The subtilin and nisin producer escape the toxic effect of their products by the action of specific self-protection (immunity) systems (*16*, *17*).

Subtilin is ribosomally synthesized as a precursor peptide (*5*) and posttranslationally modified to its biologically active form (*18*−*21*) (for a structure representation see Figure 1).

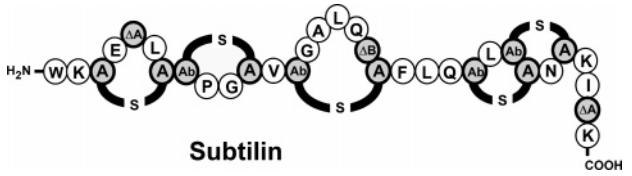

FIGURE 1: Structure of the lantibiotic subtilin produced by *B. subtilis* ATCC 6633. Amino acids (encircled) are given in one-letter code. Posttranslationally modified residues are as follows: A-S-A, *meso*-lanthionine; Ab-S-A, (2*S*,3*S*,6*R*)-3-methyllanthionine (the abbreviation Ab refers to aminobutyric acid); Δ*A*, 2,3-didehydroalanine; Δ*B*, (*Z*)-2,3-didehydobutyrine.

Its five sulfide rings are generated by the addition of cysteine thiols to didehydroalanine and didehydrobutyrine residues that originate from serine and threonine, respectively. The posttranslational modification reactions are catalyzed by the Ser/Thr dehydratase SpaB and the lanthionine cyclase SpaC; both enzymes are associated to the transmembrane lantibiotic transporter SpaT and form a macromolecular complex, the subtilin synthetase (*19*). The lantibiotic cyclases SpaC and NisC of *B. subtilis* and *L. lactis*, respectively, contain zinc ions (*22*), and the structure of the nisin cyclase NisC very recently solved by X-ray diffraction suggested that the metal center is tetrahedrically coordinated by two cysteines, one histidine, and a molecule of water (*23*). The overall structure of NisC reveals similarities in fold and substrate activation with mammalian farnesyl transferases (*23*).

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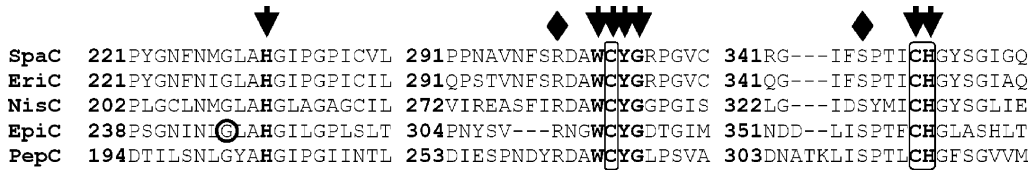

FIGURE 2: Catalytic cores of lantibiotic cyclase LanC proteins. Alignment of amino acid sequences of highly conserved regions of lantibiotic LanC proteins for the cyclization of subtilin [SpaC, Swiss-Prot accession number P33115 (*18*)], ericin A/S [EriC, AAL15566 (*21*)], nisin [NisC, CAA48383 (*46*)], epidermin [EpiC, CAA44254 (*47*)], and pep5 [PepC, CAA90026 (*48*)]. The boxed residues NisC-Cys^{284}, Cys^{330}, and His^{331} coordinate the central zinc ion in the crystal structure of NisC (*23*). Arrows indicate residues essential for the catalytic activity of SpaC (this work); diamonds symbolize positions not affected after alanine introduction (this work). The conserved residue Gly^{245} of EpiC essential for epidermin biosynthesis (*38*, *39*) is encircled (note: originally mutagenized residues G^{29}/G^{205} of EpiC (*39*) correspond to G^{107}/G^{245} after update of the *epi* gene sequences).

LanC proteins share only low sequence similarity (20−30%), and only very few amino acids are strictly conserved (*2*). Because the zinc-complexing amino acids and their close neighborhood are among these conserved residues (Figure 2), their essential role in lantibiotic cyclization was postulated (*23*). However, no experimental evidence has been provided for the essentiality of the conserved residues for lanthionine formation. Here we describe the development of an in vivo test system (complementation of the subtilin$^-$ phenotype of Δ*spaC* cells) to investigate the capability of LanC proteins or genetically engineered SpaC proteins to substitute the native subtilin cyclase SpaC. Furthermore, an in vitro substrate binding assay was used to study the interaction of engineered SpaC variants with the subtilin precursor peptide. The relevance of highly conserved amino acid positions among lantibiotic cyclases LanC and the C-terminus of SpaC for catalytic activity and/or binding of the precursor peptide substrate was investigated.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Growth Conditions, and Plasmids. B. subtilis cells were grown in Landy medium (*24*) complemented with 0.5% yeast extract. Liquid cultures were grown under vigorous shaking at 37 °C. The *B. subtilis* strains used in this study are listed in Table 1. *L. lactis* 6F3 was grown in M17 medium. *Escherichia coli* strains were grown in Luria−Bertani (LB)[1] medium (Gibco). Solid media contained TY agar (0.8% tryptone, 0.5% yeast extract, 0.5% NaCl, 1.5% agar) or LB agar (Gibco); standard incubation conditions were overnight (15−20 h) at 37 °C. In case of the use of antibiotic resistance markers, 2.5 μg/mL phleomycin and 15 μg/mL kanamycin for *B. subtilis* were used. For antibiotic selection in *E. coli* we used 100 μg/mL ampicillin and 25 μg/mL kanamycin. Recombinant plasmids were amplified in *E. coli* DH5α and TG1. *E. coli* M15/pREP4 (Qiagen) was used for protein overproduction. For construction of the plasmid pHE38 used for in vitro mutagenesis, the P_{spaB} promoter was PCR-amplified with primers promB1 and promB2 (see Table S1 of the Supporting Information) and chromosomal *B. subtilis* ATCC 6633 DNA. The product was digested with *Spe*I and *Nde*I and ligated with the *Spe*I and *Nde*I cleaved plasmid pCE20 resulting in pHE35. The *spaC* gene was PCR-amplified with primers SpaC1 and SpaC2 (see Supporting Information, Table S1) and chromosomal *B. subtilis* ATCC 6633 DNA, digested with *Spe*I and *Xba*I, and cloned in-frame into *Spe*I/*Xba*I cleaved pHE35, resulting in plasmid pHE38 (Table 1).

Molecular Biology Techniques. Established protocols were followed for molecular biology techniques (*25*). DNA was cleaved according to the conditions recommended by the commercial supplier (Promega), plasmids were isolated due to the alkaline extraction procedure (*26*), and PCR with *Pfu* polymerase (Promega) was performed according to standard procedures (*25*) utilizing a DNA thermal cycler (Mastercycler personal, Eppendorf). DNA was eluted from agarose gels using a PCR purification kit (Qiagen).

Subtilin Induction of spa Gene Expression. B. subtilis cultures were grown overnight in Landy medium (37 °C) until an OD_{600} of 10−12. After a 2% (v/v) culture supernatant of stationary grown *B. subtilis* ATCC 6633 cells was added, the cultures were grown for a further 1.5 h.

Preparation of B. subtilis Cell Lysates, SDS−Polyacrylamide Gel Electrophoresis (SDS−PAGE), and Western Blotting. After induction of *spa* gene expression with subtilin 1 mL aliquots of the cultures were centrifuged (15000*g*, 5 min), and the cells were suspended to a final OD_{600} of 1.0 in 100 μL of TGD buffer [50 mM Tris-HCl, pH 7.5, 1 mM DTT, 10% (v/v) glycerol] containing 5 mg/mL lysozyme. After incubation (20 min, 37 °C) SDS−PAGE loading buffer was added and SDS−PAGE/western blotting was performed with 30 μL aliquots as described previously (*27*). Molecular weight standards were purchased from Bio-Rad.

Cell Fractionation. The cytoplasmic fraction from *B. subtilis* was prepared with a cell fractionation method according to ref *28*. Briefly, after the cells were harvested and washed (15000*g*, 5 min), the cell pellets were resuspended in 0.5 mL of TMS buffer [50 mM Tris-HCl, pH 8.0, 16 mM $MgCl_2$, 66% (w/v) sucrose] containing 1 mg/mL lysozyme. After incubation for 45 min at 37 °C the proteoplasts were pelleted (21000*g*, 15 min, room temperature) and disrupted by resuspending in 3 mL of lysis buffer (50 mM Tris-HCl, pH 8.0, 5 mM $MgSO_4$) or PBS buffer (137 mM NaCl, 4.3 mM Na_2HPO_4, 2.7 mM KCl, 1.4 mM KH_2PO_4, pH 7.3) and centrifuged for 30 min (1000*g* at 4 °C) to pellet undisrupted cells and cell debris. The supernatant was carefully removed and centrifuged for a further 20 min (50000*g*, 4 °C) to separate the cytoplasmic fraction (supernatant) from the membrane fraction (pellet). Both were immediately used for further analyses or stored at −80 °C.

In Vitro Mutagenesis of spaC. Site-directed mutagenesis followed the QuickChange protocol (Stratagene). In brief,

[1] Abbreviations: LB, Luria−Bertani; Lan, lantibiotic (lanthionine-containing antibiotic); IPTG, isopropyl β-D-thiogalactopyranoside; PCR, polymerase chain reaction; DTT, dithiothreitol; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethanesulfonyl fluoride; NTA, nitrilotriacetic acid; TFA, trifluoroacetic acid; MALDI, matrix-assisted laser desorption ionization; TOF, time of flight; DE, delayed extraction.

Table 1: Strains and Plasmids

	description	ref
B. subtilis strain		
ATCC 6633	wild type, subtilin producer (Sub^+)	ATCC
Δ*spaB*	*spaB::cat* (Cm^r), subtilin nonproducer (Sub^-)	*18*
Δ*spaT*	*spaT::kan* (Kan^r), Sub^-	*18*
Δ*spaC*	*spaC:::cat* (Cm^r), Sub^-	*18*
Δ*spaS*	*spaS::spec* ($Spec^r$), Sub^-	*8*
Δ*spaC amyE::spaS*	Neo^r, Cm^r; Sub^-	this work
Δ*spaC amyE::spaC*	Neo^r, Cm^r; Sub^+	this work
Δ*spaS amyE::*P_{spaS}–	Δ*spaS* cells transformed with pSB5 (*27*)	Burkard, unpublished
lacZ	Cm^r, $Spec^r$; Sub^-	
A1/3	wild type, producer of ericin A and ericin S	*6*
plasmid		
pCE20	*B. subtilis*/*E. coli* shuttle vector (Amp^r, Kan^r, $Phleo^r$)	*18*
pQE60	*E. coli* expression vector specifying His_6 tag, T5 promoter (Ap^r)	Qiagen
pQE60-HA	pQE60 derivative specifying a C-terminal $HAHis_6$ tag (Ap^r)	this work
pSD27	pMLK83 derivative (*51*) (*gus* gene was cut out with *Eco*RI) integrative vector for *B. subtilis amyE* locus (Ap^r, Neo^r)	Düsterhus, unpublished
pCE34	PCE20 (*18*) carrying *spaS* (Kan^r, $Phleo^r$)	Klein, unpublished
pMB32	*spaS* gene with native promoter in pSD27 (Ap^r, Neo^r)	Burkard, unpublished
pHE6	*spaS* 3′ fused to HA and His_6 codons, *Nco*I/*Bam*HI in pQE60 (Ap^r)	this work
pCE20SubHAHis_6	*spaS* 3′ fused to HA and His_6 codons, *spo*-promoter, *Pst*I/*Xba*I in pCE20 (Kan^r, $Phleo^r$)	this work
pHE34	*spaC* 3′ fused to His_6 codons, overexpression in *E. coli* M15/pREP4; *Nco*I/*Bgl*II in pQE60 (Ap^r)	this work
pHE35	*SpaB* promoter region, *Nde*I/*Spe*I in pCE20 (Kan^r, $Phleo^r$)	this work
pHE36	*spaC* 3′ fused to His_6 codons, *Spe*I/*Xba*I in pHE35 (Kan^r, $Phleo^r$)	this work
pHE38	*SpaC* gene; wild type, *Spe*I/*Xba*I in pHE35 (Kan^r, $Phleo^r$)	this work
pHE38H231A	pHE38 derivative, SpaC H231A mutation (Kan^r, $Phleo^r$)	this work
pHE38W302A	pHE38 derivative, SpaC W302A mutation (Kan^r, $Phleo^r$)	this work
pHE38C303A	pHE38 derivative, SpaC C303A mutation (Kan^r, $Phleo^r$)	this work
pHE38Y304A	pHE38 derivative, SpaC Y304A mutation (Kan^r, $Phleo^r$)	this work
pHE38ACF	pHE38 derivative, SpaC W302A and Y304F mutation (Kan^r, $Phleo^r$)	this work
pHE38C349A	pHE38 derivative, SpaC C349A mutation (Kan^r, $Phleo^r$)	this work
pHE38H350A	pHE38 derivative, SpaC H350A mutation (Kan^r, $Phleo^r$)	this work
pHE44	*spaC* with P_{spaB} promoter, *Eco*RI in pSD27 (Ap^r, Neo^r)	this work
pHE45	*spaC* 3′ 15 bp truncated (SpaC-Δ5), *Spe*I/*Xba*I in pHE35 (Kan^r, $Phleo^r$)	this work
pHE46	*spaC* 3′ 30 bp truncated (SpaC-Δ10), *Spe*I/*Xba*I in pHE35 (Kan^r, $Phleo^r$)	this work
pHE47	*spaC* 3′ 75 bp truncated (SpaC-Δ25), *Spe*I/*Xba*I in pHE35 (Kan^r, $Phleo^r$)	this work
pHE48	*eriC*, *Spe*I/*Sac*I in pHE35 (Kan^r, $Phleo^r$)	this work
pHE49	*nisC*, *Spe*I/*Xba*I in pHE35 (Kan^r, $Phleo^r$)	this work

entire plasmids were PCR-amplified in 50 μL of *Pfu* polymerase buffer (2 pmol of complement oligonucleotides; see Supporting Information, Table S1), 1.5 units of *Pfu* polymerase, 300 μM dNTP mix), and 30−50 ng of template DNA pHE38 (2 min at 95 °C, 35 cycles of 30 s at 94 °C, 1 min at 40 °C, and 20 min at 72 °C). Parental methylated plasmid DNA was digested with 10 units of *Dpn*I (Promega), and after purification of the remaining unmethylated plasmids by the PCR purification kit (Qiagen) they were transformed into *E. coli* TG1. Plasmids were sequenced by SRD (Oberursel, Germany) using oligonucleotides SpaC_SEQ1 5′-GGCGAATTTCCTACTGG-3′ for alanine replacements of Trp^{302}, Cys^{303}, Tyr^{304}, Cys^{349A}, and His^{350} and SpaC_SEQ2 5′-GACATTATCGTGGATGG-3′ for His^{231}.

Subtilin Monitoring. Subtilin was detected by the subtilin reporter strain *B. subtilis* 6633 ATCC Δ*spaS amyE::*P_{spaS}-*lacZ* containing a subtilin-inducible β-galactosidase specifying gene (*lacZ*). The reporter strain was grown overnight in TY medium containing 0.3 M NaCl (37 °C). After 1:50 inocula of the cells were grown for a further 4 h in fresh TY/0.3 M NaCl, culture supernatants to be tested for subtilin content were added, and cultures were grown for an additional 2 h. After OD_{600} determination 10 μL aliquots were added to 990 μL of Z-buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 50 mM β-mercaptoethanol, pH 7.0), and cells were permeabilized by adding 20 μL of toluene. After toluene removal (37 °C for 45 min) the β-galactosidase reaction was started by adding of 200 μL of *o*-nitrophenyl β-D-galactoside (ONPG, 4 mg/mL in Z-buffer) at 28 °C and stopped with 500 μL of 1 M Na_2CO_3.

Enrichment of Heterologously Produced C-Terminal Double-Tagged Subtilin Prepropeptide. Double-tagged subtilin prepropeptide was overproduced in *E. coli* strain M15/pREP4, under the control of a T5 promoter, using a modified pQE60 (Qiagen) vector with an additional HA-specifying sequence in front of the His_6 sequence. Protein expression was induced with 100 μM isopropyl β-D-thiogalactopyranoside (IPTG) at an OD_{600} of 0.8 and continued for 4 h. Cells were resuspended in PBS (137 mM NaCl, 4.3 mM Na_2HPO_4, 2.7 mM KCl, 1.4 mM KH_2PO_4, pH 7.3) containing 1 mM PMSF and 0.1 mg/mL DNase I and disrupted by sonication. Double-tagged subtilin could be enriched from inclusion bodies obtained after centrifugation (30 min, 30000*g*, 4 °C). The pellet was resuspended in PBS containing 1 mM PMSF, 0.1 mg/mL DNase I,and 1% Triton X-100, centrifuged for 30 min (30000*g*, 4 °C), and washed twice with PBS containing 1 mM PMSF (30 min, 30000*g*, 4 °C). The remaining pellet was resuspended in PBS containing 8 M urea, 1 mM PMSF, and 5 mM DTT. After solubilization

the sample was further centrifuged for 20 min (50000*g*, 4 °C); the supernatant was carefully removed and used for further experiments or stored at −20 °C.

Isolation of a Double-Tagged Subtilin Prepropeptide. For heterologous production of SubHAHis$_6$ the *spaS* gene was cloned in-frame into the modified pQE60 vector which encodes an additional C-terminal HA sequence. The SubHAHis$_6$ peptide was isolated from *E. coli* M15/pREP4 inclusion bodies after treatment with 8 M urea containing buffer and nickel-NTA affinity purification.

Substrate Binding Assay. Nickel-NTA (10 μL) (Qiagen, Hilden, Germany) was incubated with 50 μL of SubHAHis$_6$ dissolved in PBS buffer containing 8 M urea for 30 min at 4 °C. Nickel-NTA beads were washed once with PBS containing 8 and 4 M urea, respectively, and once with PBS buffer (5 s, 1000 rpm, 4 °C) and incubated for 1 h at 4 °C under shaking with cytoplasmatic *B. subtilis* fractions containing the subtilin synthetase subunits SpaB and SpaC. The supernatant was removed, and the nickel-NTA was washed three times with PBS buffer (5 s, 1000 rpm, 4 °C) and resuspended in 1× SDS−PAGE loading buffer.

MALDI-TOF MS. For MALDI mass spectrometric analyses dried samples were dissolved in 5 μL of 50% acetonitrile (ACN)/1% (v/v) trifluoroacetic acid (Fluka, Buchs, Switzerland). The sample (0.5 μL) was mixed with 0.5 μL of matrix [5 mg/mL α-cyano-4-hydroxycinnamic acid (Bruker, Bremen, Germany) in 50% ACN/0.5% (v/v) trifluoroacetic acid] directly on a stainless steel MALDI target [Applied Biosystems (ABI), Darmstadt, Germany] and dried under ambient conditions. Delayed extraction (DE) MALDI time-of-flight (TOF) mass spectra were recorded on a Voyager-DE STR instrument (ABI) on a nitrogen laser (λ = 336 nm, repetition rate = 20 Hz) for desorption and ionization with an acquisition mass range from *m*/*z* 600 to *m*/*z* 10000 and the low mass gate set to *m*/*z* 550. The total acceleration voltage was 20 kV with 68.5% grid voltage on the first grid, 0.02% guide wire voltage, 200 ns delay, and a mirror voltage ratio of 1.12. All experiments were carried out with the reflector positive ion mode. Between 500 and 1000 laser shots were accumulated for each mass spectrum. Spectra were externally equilibrated using the Sequazyme peptide mass standards kit (ABI).

RESULTS

LanC Complementation Studies. To establish a system for the investigation of genetically engineered subtilin cyclase SpaC proteins, the Sub$^-$ phenotype of a Δ*spaC* deletion mutant was complemented (Figure 3). As the promoter for the subtilin precursor specifying *spaS* gene is located in the 3′-region of the *spaC* gene (*27*, *29*, *30*), the Sub$^-$ phenotype of Δ*spaC* cells (*18*) could only be complemented after expression of both an additional *spaS* copy and *spaC* under control of their native promoters (*27*, *29*, *30*) P$_{spaS}$ and P$_{spaB}$ (plasmid pHE38) in *B. subtilis* Δ*spaC amyE*::P$_{spaS}$-*spaS* cells. Subtilin production was monitored by growth inhibition tests using *Micrococcus luteus* and MALDI-TOF mass spectrometry (see below). The most sensitive subtilin detection system was based on the subtilin reporter strain *B. subtilis* ATCC 6633 Δ*spaS amyE::P$_{spaS}$-lacZ* that contains a subtilin-inducible *lacZ* gene, and signal transduction is via the subtilin-specific two-component autoregulatory system SpaRK

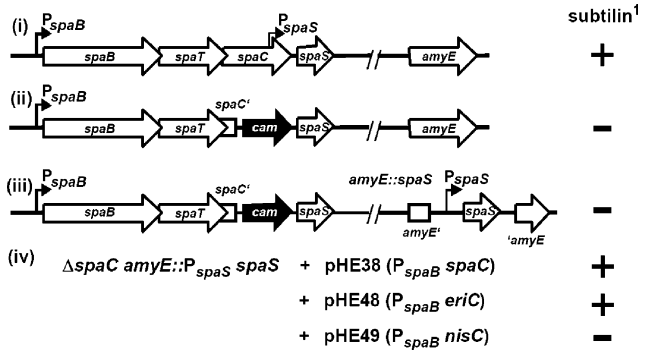

FIGURE 3: Complementation of a *spaC* deletion mutant. The subtilin gene cluster and amylase locus of the *B. subtilis* ATCC 6633 strains wild type (i), Δ*spaC* [ii, (*18*)], and (iii) Δ*spaC amyE::spaS*, respectively. (iv) The Sub$^-$ phenotype of the Δ*spaC* strain could be restored after transformation of Δ*spaC amyE::spaS* cells with plasmids pHE38 and pHE48 carrying the *spaC* and *eriC* genes under control of P$_{spaB}$, the native promoter of the *spaBTC* operon (*27*). Complementation was not successful with plasmid pHE49 carrying *nisC*. Footnote 1: The subtilin phenotype has been determined by growth inhibition tests, MALDI-TOF MS, and an autoinduction assay (Table S2 of the Supporting Information).

(*27*, *31*) (original data are summarized in Table S2, Supporting Information). Detection was about 10 ng/mL subtilin (M. Burkard, K.-D. Entian, and T. Stein, to be published elsewhere), which is 3 orders of magnitude less than the 5.2 μg/mL subtilin determined within stationary *B. subtilis* ATCC 6633 cultures (*32*). The Δ*spaC amyE*::P$_{spaS}$-spaS cells expressing the SpaC complementation plasmid pHE38 produced about 80% of the subtilin amount of the *B. subtilis* wild-type cells (see also Table S2 in the Supporting Information).

Remarkably, Δ*spaC amyE::spaS* cells produced subtilin after expression of *eriC* (pHE48), to our knowledge the first in vivo posttranslational modification of a lantibiotic precursor peptide with a hybrid lantibiotic synthetase (SpaB-EriC-SpaT). It seems that the close relationship between EriC, the cyclase for the two lantibiotic peptides ericin A/S (*6*) and SpaC (85% sequence identity), was sufficient for formation of a functional hybrid subtilin synthetase complex. In contrast, after expression of *nisC* (pHE49) in Δ*spaC amyE::spaS* cells no subtilin production was detected. A possible explanation for the dysfunction of the NisC to substitute SpaC is missing interactions with the subtilin precursor peptide and/or the subtilin dehydratase SpaB as SpaC and NisC exhibit only 32% sequence identity. SDS−PAGE and immunoblotting of whole cell extracts demonstrated homologous (SpaC) or heterologous (EriC, NisC) production of LanC proteins as well as their stability in ATCC 6633 cells (Figure 4A). Thus, the Sub$^-$ phenotype of the pHE49 (*nisC*) transformed Δ*spaC amyE::spaS* strain is not explainable by instability of the NisC protein in *B. subtilis*.

Properties of Genetically Engineered SpaC Cyclases: Subtilin Production and SpaC Stability. To get deeper insights into the catalytic mechanism of the subtilin cyclase SpaC, we decided to identify amino acids involved in its catalytic activity. Sequence alignments reveal a series of amino acids highly conserved among LanC proteins [Figure 2 (*22*)]. We genetically engineered SpaC by in vitro mutagenesis of the plasmid pHE38 (Table 1) and replaced

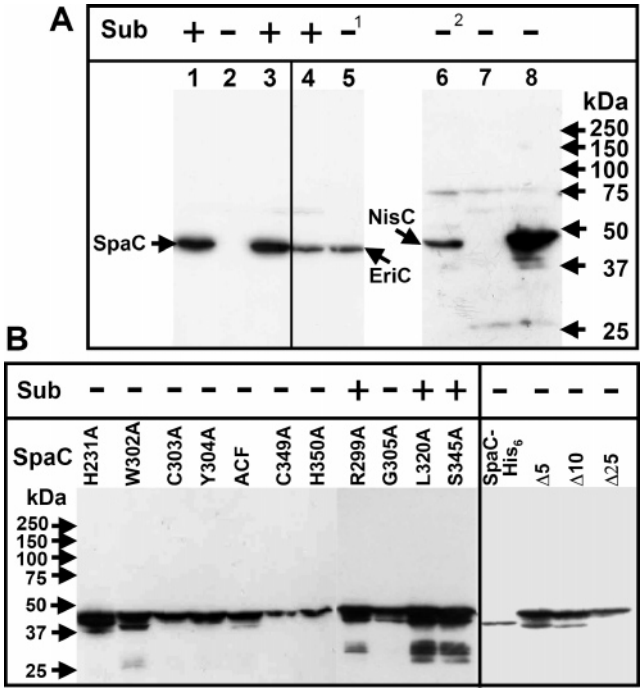

FIGURE 4: Complementation of the Sub$^-$ phenotype of *B. subtilis* Δ*spaC* with SpaC homologues and mutagenized SpaC proteins. (A) Detection of LanC proteins after immunoblotting of SDS−PAGE (10% Tris−glycine gels) separated *B. subtilis* cell lysates. Lanes 1 (wild type), 2 (Δ*spaC amyE::spaS*), 3 (Δ*spaC amyE::spaS* + SpaC), 4 (Δ*spaC amyE::spaS* + EriC), and 5 [ericin producer *B. subtilis* A1/3 (*27*)] were visualized by SpaC-directed immunoserum. Lanes 6 (nisin producer *L. lactis* 6F3), 7 (Δ*spaC amyE::spaS*), and 8 (Δ*spaC amyE::spaS* + NisC) were detected with anti-NisC antisera. The respective cells were grown in Landy (*B. subtilis*) or M17 (*L. lactis*) media, as described under Experimental Procedures, and harvested in the late exponential growth phase. (B) Immunoblots of SDS−PAGE (10% gels) separated of *B. subtilis* cell lysates incubated with anti-SpaC immunoserum. Cultures were grown in Landy medium and treated with a subtilin-containing culture supernatant to induce *spa*-promoter-dependent gene expression. Samples were taken during late exponential growth (OD_{600} ~ 12). The subtilin-producing phenotype of the respective strains (parallel analyses without subtilin induction) is indicated in the headline. Footnotes: 1, wild-type *B. subtilis* A1/3 produces the lantibiotic ericin, a close relative of subtilin (*6*); 2, *L. lactis* 6F3 produces the lantibiotic nisin.

highly conserved amino acid residues by alanine, and furthermore, we engineered SpaC protein with exchanges in two positions (ACF refers to the accidentally generated SpaC double mutant with the sequence $Ala^{302}Cys^{303}Phe^{304}$ instead of the wild-type sequence $Trp^{302}Cys^{303}Tyr^{304}$). Genetically engineered SpaC proteins were tested in vivo to substitute the native SpaC protein. The SpaC alanine substitution mutants His^{231}, Trp^{302}, Cys^{303}, Tyr^{304}, Gly^{305}, Cys^{349}, His^{350}, and ACF were deficient in subtilin production (Figure 4B). Remarkably, all engineered SpaC proteins were produced and highly stable (Figure 4B); less intensive signals at ~40 and 30 kDa are most probably due to proteolytic degradation; however, corresponding signals could also be observed for wild-type SpaC in some cases (not shown). Conclusively, the Sub$^-$ phenotype of cells expressing engineered SpaC proteins was not due to an instability of the altered proteins, as those would get rapidly degraded by the action of one or more of the 35 proteases encoded by the *B. subtilis* genome (*33*). If residues Arg^{299}, Leu^{320}, or Ser^{345} of SpaC have been replaced with alanine, the Sub$^-$ phenotype of the Δ*spaC amyE::spaS* strain could be complemented (Figure 4B). All mutants produced comparable amounts of subtilin, corresponding to 80−90% of the subtilin amount produced by Δ*spaC amyE::*P_{spaS}-spaS cells expressing the wild-type SpaC from pHE38 (see also Table S2 in the Supporting Information).

The C-terminus of SpaC was truncated by 5 (SpaC-Δ5), 10 (SpaC-Δ10), and 25 amino acids (SpaC-Δ25), and furthermore, SpaC with a C-terminal His_6 fusion was constructed. Immunoblotting demonstrated production and stability for all three truncated SpaC species (Figure 4B, last three lanes), whereas the His_6 fusion protein gets rapidly degraded. All of these C-terminally engineered proteins were not able to complement the Sub$^-$ phenotype of Δ*spaC amyE::spaS* cells, which suggests an important influence of the C-terminus of SpaC in the catalytic activity and/or stabilization of the tertiary structure.

SpaC Substrate Binding Assay. After lysis of *B. subtilis* ATCC 6633 cells with lysozyme and hypoosmotic shock (*28*) SpaB and SpaC proteins were present exclusively in the cytoplasm fraction, whereas the transmembrane protein SpaT remained in the membrane fraction (Figure 5A). This implicates that membrane association of the subtilin synthetase observed earlier (*19*, *34*) might represent only a transition state. For example, in the absence of the SpaS precursor peptide the components SpaB and SpaC might be localized in the cytosol as either monomeric proteins or loosely associated oligomers. Membrane association does not occur until formation of a subcomplex, for example, SpaBCS, that might be a prerequisite for binding to the membrane-spanning transporter SpaT, and possibly, the subtilin synthetase complex dissociates after export of the modified precursor peptide.

Substrate binding by subunits of the subtilin synthetase SpaB and/or SpaC was analyzed using in vitro pull-down experiments with the heterologously produced double-tagged subtilin precursor peptide $SubHAHis_6$ (see Figure S1 of the Supporting Information) immobilized to Ni-NTA beads. SpaC specifically interacted with immobilized $SubHAHis_6$ (Figure 5B, anti-SpaC blot, lanes wild type and Δ*spaB* + chromosomally integrated SpaC) independent of the presence of SpaB. Consistently, the SpaC$^-$ strains were avoid of a corresponding signal [Δ*spaB* with a transcriptional terminator blocking the expression of the subsequent *spaC* gene (*27*) and Δ*spaC*]. Unfortunately, SpaB interacted with Ni-NTA no matter if the subtilin prepropeptide was present or not (Figure 5B, anti-SpaB blot, lanes for wild type and Δ*spaC*). The $SubHAHis_6$-containing Ni-NTA beads bound SpaB to a higher extent compared to control reactions without immobilized $SubHAHis_6$ (the corresponding signals are 30−50% more intensive). However, we restricted further pull-down experiments on the clear and highly specific interaction between SpaC and the double-tagged subtilin precursor peptide.

The in vitro substrate binding assay was used to study if the SpaC alterations (point mutations, truncation) had any effects on substrate binding. For wild-type as well as plasmid-encoded *spaC* (pHE38) expressing cells, the signal intensities for SpaC precipitated in the presence of Ni-NTA-immobilized $SubHAHis_6$ were more than 20-fold superior to intensities in the absence of the subtilin precursor (Figure 5C). The genetically engineered SpaC proteins with alanine

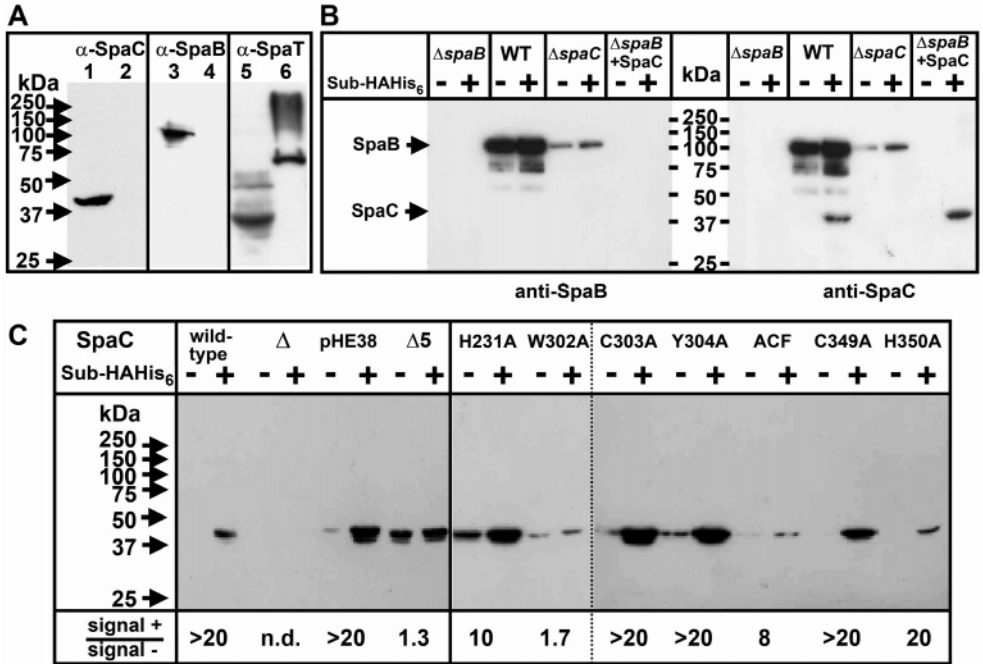

FIGURE 5: Interaction between SpaC and subtilin precursor peptide SubHAHis$_6$. (A) Localization of subtilin synthetase components. After lysis of *B. subtilis* ATCC 6633 cells the cytoplasmic (lanes 1, 3, and 5) and membrane fractions (lanes 2, 4, and 6) were separated by SDS−PAGE (10% Tris−glycine gels). Immunodetection was with the indicated immunosera for SpaC (lanes 1 and 2), SpaB (lanes 3 and 4), and SpaT (lanes 5 and 6), respectively. (B) Interaction of immobilized SubHAHis$_6$ with SpaB or SpaC (pull-down assay). The cytoplasmic fractions of *B. subtilis* cell lysates were incubated with nickel-NTA in the presence (+) or absence (−) of double-tagged subtilin prepropeptide. Strains analyzed were the wild-type *B. subtilis* (ATCC 6633) and its derivatives Δ*spaB*, Δ*spaC*, and the Δ*spaB* strain after chromosomal integration of P_{spaB}-*spaC* into the *amyE* locus (Δ*spaB* + SpaC). Immunodetection was with the indicated immunoserum for SpaB or SpaC, respectively. (C) Interaction between SubHAHis$_6$ and engineered SpaC proteins. Cytoplasmic fractions of cell lysates (500 μg of protein each) were incubated with nickel-NTA in the presence (+) or absence (−) of SubHAHis$_6$. Strains analyzed were wild-type *B. subtilis* ATCC 6633 (native SpaC), Δ*spaC* (Δ), Δ*spaC* after transformation with the *spaC* gene carrying plasmid pHE38, and Δ*spaC* producing C-terminally truncated SpaC (Δ5), as well as Δ*spaC* expressing engineered SpaC variants. Cultures were grown in Landy medium and harvested in the late exponential growth phase ($OD_{600} \sim 10-12$). The cytoplasmic fractions were isolated by cell fractionation, and samples were separated by SDS−PAGE. For western blotting a SpaC-directed antiserum was used. The numbers in the bottom line are quotients of western blot signal intensities in the presence of the subtilin precursor peptide (+) divided by the intensities in the absence of the prepeptide (−). The figure is composed of different blots separated by solid or dashed lines, and thus, absolute signal intensities are not directly comparable.

at positions His^{231}, Cys^{303}, Tyr^{304}, Cys^{349}, and His^{350} also show specific interaction with the double-tagged subtilin prepropeptide: The SpaC signal intensities were more than 10-fold stronger in the presence of SubHAHis$_6$ compared to intensities in the absence of the precursor (Figure 5C). This clearly shows that engineered SpaC variants inactive in the in vivo assay were still able to bind the subtilin precursor peptide specifically. The C-terminally truncated SpaC proteins did not specifically interact with the double-tagged subtilin prepropeptide SubHAHis$_6$ as exemplified for SpaCΔ5 (Figure 5C). This suggested that the C-terminus of SpaC plays an important role in binding of the prepropeptide. Possibly, the five C-terminal amino acids are involved in stabilization of the catalytic center of SpaC. Since the signals for the SpaC proteins engineered at positions Trp^{302} and His^{350} as well as the ACF mutant were relatively low (Figure 5C), it seems to be that the Trp^{302} mutant did not specifically interact with SubHAHis$_6$ and that at least Trp^{302} is important for the interaction of SpaC with the subtilin precursor peptide. One explanation for the low SpaC signal intensities is different subtilin production rates resulting in different induction patterns and consequently to different SpaC amounts. Such effects also have been observed if different preparations of wild-type *B. subtilis* ATCC 6633 cells were compared (not shown). Furthermore, variations in stability of the engineered SpaC variants during the time-consuming cell fractionation preparation protocol have to be taken into account.

The Double-Tagged Subtilin Precursor Peptide SubHA-His$_6$. Expression of the C-terminally double-tagged subtilin precursor peptide SubHAHis$_6$ could complement the Sub$^-$ phenotype of Δ*spaS* cells (Figure 6A). Subtilin production was monitored by means of the autoinduction assay using the reporter strain Δ*spaS amyE::*P_{spaS}-*lacZ* (see also Table S2, Supporting Information), which was superior to *M. luteus* growth inhibition tests on agar plates. Our results clearly showed production of molecule species able to induce the subtilin-specific SpaRK two-component regulatory system. Surprisingly, the double-tagged matured subtilin (calculated *m*/*z* 5699.7) was not observed by MALDI-TOF MS in butanolic extracts of Δ*spaS* + pCE20SubHAHis$_6$ cells (Figure 6B). Instead, the mass spectra are indicative of three different degradation products, *m*/*z* 3419.7 fits with the mass of succinylated subtilin without any tag; additionally, succinylated subtilin species carrying a C-terminal extension of Gly (*m*/*z* 3476.7) and Gly-Ser (*m*/*z* 3563.8) were observed. Obviously, the C-terminal HA-His$_6$ tag was removed, and only the first amino acids Gly-Ser of the C-terminal HA-His$_6$ tag were left over. This provides experimental evidence that SubHAHis$_6$ is a substrate for the subtilin modification

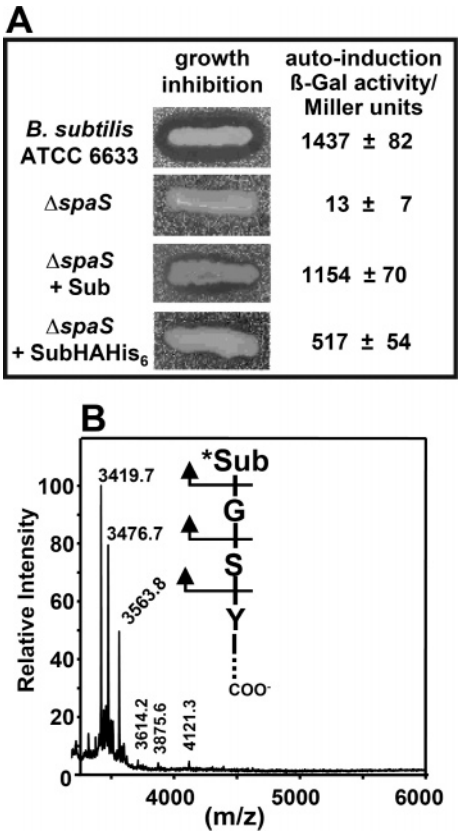

FIGURE 6: The double-tagged subtilin prepropeptide SubHAHis$_6$. (A) Subtilin production assayed with a growth inhibition test using *M. luteus* (left) and an autoinduction test (right). In the latter, the strain Δ*spaS amyE::P$_{spaS}$-lacZ* reports the presence of subtilin via the two-component system SpaRK that induces the *spaS*-promoter-controlled β-galactosidase specifying *lacZ*. Strains analyzed were wild-type *B. subtilis* (ATCC 6633) and the Δ*spaS* strain, as well as Δ*spaS* cells after transformation with plasmids pCE34 (+Sub) and pCE20SubHAHis$_6$ (+SubHAHis$_6$). Values below 15 Miller units have been interpreted as background (Sub$^-$ phenotype). (B) Part of a MALDI-TOF mass spectrum of the culture supernatant of a SubHAHis$_6$-expressing *B. subtilis* Δ*spaS* strain. Instead of a signal for the modified double-tagged subtilin the most intensive peaks at *m*/*z* 3419.7, 3476.7, and 3563.8 exactly fit with the calculated values for succinylated subtilin species without extension (*m*/*z* 3419.6) as well as species C-terminally extended by Gly (3476.7) or Gly-Ser (3563.8). The N-terminal succinylation of subtilin (+100 mass) was described previously (*21*, *32*, *49*).

proteins. C-Terminal truncation of the tagged SpaS precursor species could occur before, during, or after the secretion process. Even though the other both alternatives cannot be ruled out, the C-terminal truncation was most likely due to frequently occurring proteolytic "shaving-like" activities in the *B. subtilis* culture supernatant (*35*) that are also involved in removal of the subtilin leader sequence (*20*, *21*). Earlier observations showed that the C-terminally AspHis$_6$-tagged nisin precursor was posttranslationally modified, but in contrast to the *B. subtilis* system the tagged nisin could be isolated from the *L. lactis* culture supernatant (*36*).

DISCUSSION

The high-resolution (2.5 Å) crystal structure for nisin cyclase NisC (*23*) shows that most of the amino acids highly conserved among lantibiotic cyclases (Figure 2) are closely located to the central zinc ion. It is highly remarkable that this reaction center is conserved among LanC proteins, irrespective of the electrophiles (2,3-didehydroalanine and 2,3-didehydrobutyrine) or the size and number of lanthionine rings in lantibiotic precursor peptides (*37*). It seems that all lanthionine rings within a given lantibiotic are generated by the action of a single LanC protein with a single reaction center. A highly plausible catalytic mechanism for nisin cylization was derived from the structural data in combination with a docking experiment of the nisin B-ring structure obtained from NMR spectroscopic data with NisC (*23*), however, without corroboration by experimental data. One possible way to characterize the catalytic center of LanC proteins and to test the proposed lanthionine cyclization mechanism is functional analyses of engineered LanC proteins. The present work makes significant contributions to our understanding of LanC structure−function relationships. Our first important contribution was the development of an in vivo system to test the functionality of engineered SpaC cyclases. Using this system, we could show for the first time the production of a matured lantibiotic (subtilin) biosynthesized by a hybrid lantibiotic synthetase (SpaBT/EriC).

More importantly, we provide experimental evidence that amino acid residues of SpaC highly conserved among lantibiotic cyclases are essential for subtilin biosynthesis. Until now, only two reports on mutagenized/engineered LanC cyclases exist. From *Staphylococcus epidermidis* epidermin$^-$ mutants generated with ethyl methanesulfonate (*38*) two mutagenized EpiC proteins were found with exchanges of two glycine residues against glutamate, respectively (*39*). Furthermore, genetically engineered SpaC proteins with Ala replacements of residues Cys^{303} and Cys^{349} Ala heterologously produced in *E. coli* lack the metal cofactor or contained significantly decreased amounts of it (*22*). However, the functionality of the engineered proteins in *B. subtilis* has never been analyzed. Our results are first experimental evidence for the essentiality of residues Cys^{303}, Cys^{349}, and His^{350} in subtilin biosynthesis. Together with information from the X-ray structure of NisC that the corresponding residues of NisC Cys^{284}, Cys^{330}, and His^{331} (Figure 7) were identified to coordinate the central zinc ion (*23*), our data substantiate the function of these residues as components of the catalytic center.

Furthermore, our data provide evidence for the essentiality of SpaC residues His^{231} and Tyr^{304}. Lanthionine formation involves deprotonation of substrate cysteines and protonation of enolate intermediates generated by conjugate addition to the β-carbon of the didehydroalanine or didehydrobutyrine substrates on opposite faces of the emerging lanthionine ring. Li et al. (*23*) proposed general acid/base catalyst functions for the pair Arg^{280}/His^{212} of NisC. However, the counterpart amino acid pair Arg^{299}/His^{231} represents most likely not the general acid/base catalysts of SpaC, since SpaC residue Arg^{299} is not essential for subtilin biosynthesis (Figure 4B). Presumably, SpaC residue Tyr^{304}, which has been demonstrated to be obligate for subtilin biosynthesis and which is highly conserved among LanC proteins and the only residue to be considered as an acid/base catalyst in close proximity to the catalytic center, is involved in the catalytic activity (Figure 7). The *S. epidermidis* mutant expressing epidermin cyclase EpiC carrying an exchange of the strictly conserved Gly^{245} residue against glutamate was not able to produce epidermin. The corresponding residues of SpaC Gly^{228} and

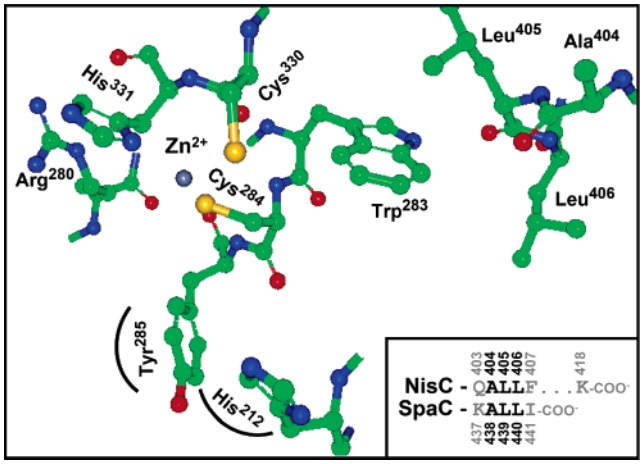

FIGURE 7: Catalytic core structure of lantibiotic cyclases. The catalytic core structure of NisC (*23*) is shown including the Zn^{2+} coordinating residues Cys^{284} (SpaC-Cys^{303}), Cys^{330} (Cys^{349}), and His^{331} (His^{350}). Additionally, the residues His^{212} (SpaC-His^{231}), Trp^{283} (Trp^{302}), and Tyr^{285} (Tyr^{304}) in close proximity to the catalytic center are indicated. The SpaC cyclase counterparts of these residues (Arg^{280} is the only exception) were identified to be essential for subtilin biosynthesis (see Figure 4B). The residues close to the C-terminus Ala^{404}, Leu^{405}, and Leu^{406} (for SpaC positions see the insert) are most likely involved in stabilization of the catalytic core structure (see the text). The counterparts of NisC residues His^{212}/Tyr^{285} to SpaC are His^{231}/Tyr^{304} that are discussed to be acid/base catalysts (see the text) are partially encircled. This figure was created with Protein Workshop (*50*) using the X-ray data for NisC (PDB code 2G02) (*23*).

NisC Gly^{205} are localized in close proximity to essential SpaC His^{231} and NisC His^{212} residues involved in Zn^{2+} complexing (*23*). Thus, the conserved Gly residue might be part of a region involved in maintenance of the catalytic core structure. When residues Arg^{299}, Leu^{320}, or Ser^{345} of SpaC were replaced with alanine, the Sub^- phenotype of Δ*spaC amyE::spaS* cells could be complemented (Figure 4B). This result demonstrates that the corresponding residues are not involved in the catalytic activity of SpaC. Most likely, their NisC counterparts (Arg^{280}, Leu^{301}, or Ser^{326}) also do not contribute to nisin cyclization.

The development of an in vitro test to investigate interactions between engineered SpaC proteins with the subtilin precursor peptide was important for a greater understanding of the function of SpaC. We showed that functional inactive SpaC alanine replacement mutants His^{231}, Cys^{303}, Tyr^{304}, Cys^{349}, and His^{350} are still able to interact with the subtilin precursor. Remarkably, substrate binding was also detected with engineered SpaC proteins unable to complement native SpaC in vivo (compare Figures 4B and 5C). The first evidence for specific interactions between lantibiotic precursor peptides and components of modification machineries was provided for nisin and subtilin synthetase with the yeast two-hybrid system and co-immunoprecipitation (*19*, *40*). LanC−LanA interactions with purified proteins were biochemically evidenced for EpiC and EpiA (*39*). The results shown herein (Figure 5) are further evidence that the subtilin prepropeptide does not have to be dehydrated to interact with SpaC. Leader peptides are essential for the substrate recognition of LanC cyclases, as for example dehydrated NisA from which the leader peptide was removed was no substrate for NisC (*23*).

When engineered SpaC proteins are investigated for their ability to interact with the subtilin precursor peptide Sub-HAHis$_6$, the C-terminal five amino acid residues as well as Trp^{302} were identified to be important (Figure 5). This residue, Trp^{302}, and Gly^{305} as well as the last five amino acid residues (Lys^{437}−Ile^{441}) of SpaC were also identified to be essential for subtilin biosynthesis (Figure 4B). As summarized in Figure 7, the last 10 amino acids of NisC are localized outside of its core structure (*23*), whereas the preceding 10 residues including Ala^{404}-Leu^{405}-Leu^{406} (counterparts to SpaC-Ala^{438}-Leu^{439}-Leu^{440}) build a hydrophobic cluster together with Trp^{283} (SpaC-Trp^{302}) and stabilize the conformation of the catalytic center. SpaC is 10 amino acids shorter than NisC and ends with the sequence motif KALLI (Figure 7, insert) highly conserved among LanC proteins (not shown). The essentiality of Trp^{302} and the last five SpaC residues for subtilin biosynthesis and precursor peptide binding implies important contributions of these elements in stabilization of the reaction center. A plausible explanation for the nonfunctionality of the C-terminal hexahistidine-tagged SpaC protein is a destabilization of the catalytic center, possibly due to interaction between the histidine residues and the catalytic zinc ion, and thus, the protein structure gets unstable, possibly the prerequisite for rapid proteolytic degradation (Figure 4B).

The huge potential of in vivo and in vitro syntheses of lantibiotics/lantibiotic derivatives inaccessible for chemical peptide synthesis (*23*, *41*−*44*) is highly stimulating for further studies in lantibiotic biosynthesis. For example, a recent study shows the mechanism of the highly processive LanM dehydration catalyzed by lacticin 481 synthetase, which uses ATP for phosphorylation of the Ser/Thr residues prior to elimination to didehydroalanine or didehydrobutytine, respectively (*45*).

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SUPPORTING INFORMATION AVAILABLE

PCR primers used in this work (Table S1), subtilin monitoring using the subtilin reporter strain *B. subtilis* ATCC 6633 Δ*spaC amyE::spaS* (Table S2), and a MALDI-TOF mass spectrum of RP-HPLC isolated SubHAHis$_6$ heterologously expressed in *E. coli* (Figure S1). This material is available free of charge via the Internet at http://pubs.acs.org.

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