Entianin, a Novel Subtilin-Like Lantibiotic from *Bacillus subtilis* subsp. *spizizenii* DSM 15029^T with High Antimicrobial Activity^{∇†}

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Lantibiotics, such as nisin and subtilin, are lanthionine-containing peptides that exhibit antimicrobial as well as pheromone-like autoinducing activity. Autoinduction is specific for each lantibiotic, and reporter systems for nisin and subtilin autoinduction are available. In this report, we used the previously reported subtilin autoinduction bioassay in combination with mass spectrometric analyses to identify the novel subtilin-like lantibiotic entianin from *Bacillus subtilis* subsp. *spizizenii* DSM 15029^T. Linearization of entianin using Raney nickel-catalyzed reductive cleavage enabled, for the first time, the use of tandem mass spectrometry for the fast and efficient determination of an entire lantibiotic primary structure, including posttranslational modifications. The amino acid sequence determined was verified by DNA sequencing of the *etnS* structural gene, which confirmed that entianin differs from subtilin at 3 amino acid positions. In contrast to *B. subtilis* ATCC 6633, which produces only small amounts of unsuccinylated subtilin, *B. subtilis* DSM 15029^T secretes considerable amounts of unsuccinylated entianin. Entianin was very active against several Gram-positive pathogens, such as *Staphylococcus aureus* and *Enterococcus faecalis*. The growth-inhibiting activity of succinylated entianin (S-entianin) was much lower than that of unsuccinylated entianin: a 40-fold higher concentration was required for inhibition. For succinylated subtilin (S-subtilin), a concentration 100-fold higher than that of unsuccinylated entianin was required to inhibit the growth of a *B. subtilis* test strain. This finding was in accordance with a strongly reduced sensing of cellular envelope stress provided by S-entianin relative to that of entianin. Remarkably, S-entianin and S-subtilin showed considerable autoinduction activity, clearly demonstrating that autoinduction and antibiotic activity underlie different molecular mechanisms.

Lantibiotics are ribosomally synthesized and posttranslationally modified peptide antibiotics that contain the nonproteinogenic amino acids lanthionine and 3-methyllanthionine, as well as dihydroamino acids (38). So far as is known, lantibiotics are produced exclusively by Gram-positive bacteria (1, 9, 44). Lantibiotics have attracted much attention due to the success of the well-characterized lantibiotic nisin as a food preservative (16).

The lanthionine and 3-methyllanthionine residues exhibit the characteristic intramolecular thioether bridges of lantibiotics. Ring formation goes along with restricted proteolytic cleavability as well as inhibited fragmentation efficiency because of the necessity of two bond breakages within the cyclic parts of the sequence. Consequently, tandem mass spectrometric (MS-MS) analyses could not be used for the determination

of the primary structures and modifications of native lantibiotics (10, 33).

The N-terminal lanthionine-bridged rings A and B allow for specific binding at the pyrophosphate moiety of lipid II, the essential precursor of cell wall synthesis (2, 21). As a consequence of the formation of this lantibiotic–lipid II complex, bacterial cell wall synthesis and lipid II biosynthesis are impeded (4, 19). However, for the most intensively studied lantibiotic, nisin, the primary antibiotic activity is based on the formation of pores in the cytoplasmic membranes of Gram-positive target cells subsequent to the generation of the complex with lipid II, leading to degradation of the membrane potential (3, 18).

Lantibiotic biosynthesis receives much attention, since isolation and sequencing of the *epiA* structural gene proved that the lantibiotic epidermin from *Staphylococcus epidermidis* is ribosomally synthesized and posttranslationally modified (38). In all lantibiotics, serine and threonine residues are dehydrated to yield 2,3-didehydroalanine (ΔA) and 2,3-didehydrobutyrine (ΔB), respectively. Subsequently, cysteine sulfhydryl side chains can be subject to stereospecific intramolecular Michael addition to a 2,3-didehydroalanine or -butyrine double bond, leading to the formation of the intramolecular lanthionine or 3-methyllanthionine ring structure (for a review, see reference 44). As shown first for the lantibiotic subtilin, these posttranslational modifications are made at a multimeric protein complex consisting of dimers of three proteins. The sub-

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tilin biosynthetic proteins SpaB (and the corresponding lantibiotic protein LanB) and SpaC (LanC) are needed for dehydration and cyclization, respectively, whereas the SpaT (LanT) ABC transporter attaches the modification machinery to the cytoplasmic membrane (25, 26).

As shown first for nisin from *Lactococcus lactis*, lantibiotics have quorum-sensing activity (29). Subtilin expression in *Bacillus subtilis* is even more complex and is regulated in a dual manner. First, sigma factor H, the gene for which is controlled by the general *abrB* repressor, allows for low-level expression of the two-component regulatory system SpaRK (20, 27, 40). Second, subtilin autoinduces histidine kinase SpaK, which in turn phosphorylates the response regulator SpaR and upregulates the transcription of subtilin biosynthesis and immunity genes (40).

After the fusion of the promoter of the subtilin structural gene *spaS* with the β -galactosidase reporter gene, it was possible to establish a microtiter plate-based autoinduction bioassay for the detection of subtilin (6). This autoinduction bioassay allowed for the identification of the new subtilin-like lantibiotic entianin. According to our observation, all other subtilin-like lantibiotics known to date are produced predominantly as N-terminally succinylated forms exhibiting only low antimicrobial potential (8). Remarkably, considerable amounts of unsuccinylated entianin with high antimicrobial activity are produced by *Bacillus subtilis* subsp. *spizizenii* DSM 15029^T. As reported previously for subtilin (8), the high antimicrobial potential of entianin against several Gram-positive bacteria is strongly reduced in its N-terminally succinylated form.

MATERIALS AND METHODS

Strains used in this study. All strains used in this study are listed in Table 1.

Chromogenic subtilin autoinduction plate assay for identification of lantibiotic producer strains. For the identification of lantibiotic-producing strains, the reporter strain *B. subtilis* ATCC 6633 Δ *spaS* carrying *P_{spaS}-lacZ* was streaked out onto a TY (0.8% tryptone and 0.5% yeast extract) agar plate supplemented with 0.3 M NaCl, 1.5% (wt/vol) agar, and 40 μ g/ml X-gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside) in such a way that the plate was bisected into identical parts (6). The *B. subtilis* strains to be investigated were streaked out perpendicularly to the reporter strain, starting from the side of the plate and ending inside the reporter strain growth zone. Production of autoinductively active compounds was observable by blue coloration at the interface of the reporter strain and the strain investigated after cultivation for 48 h at room temperature.

Growth conditions and isolation of entianin isoforms and S-subtilin. For the enrichment of the different lantibiotics, 5 ml of modified Landy medium (30) was inoculated with *B. subtilis* DSM 15029^T or *B. subtilis* ATCC 6633 colonies, which were incubated under permanent shaking at 155 rpm overnight at 37°C. Fifty milliliters of the same medium was inoculated with 1 ml of preculture in a 500-ml flask and was grown under the same conditions for 30 h. The Landy medium contained 0.5% (wt/vol) glutamic acid, 0.1% (wt/vol) yeast extract, 0.01% (wt/vol) MgSO₄, 0.05% (wt/vol) KCl, and 0.004% (wt/vol) FeSO₄ and was autoclaved after adjustment to pH 7.5 with KOH. Fifty milliliters of an autoclaved solution of 40% (wt/vol) glucose and 5 ml of 20% (wt/vol) KH₂PO₄ were added to 1 liter of Landy medium. After centrifugation at room temperature for 20 min at 10,000 rpm, the culture supernatants were diluted with equal amounts of methanol and were loaded onto StrataX solid-phase extraction (SPE) cartridges (particle size, 33 μ m; Phenomenex, Aschaffenburg, Germany). After a wash with 70% methanol–0.1% trifluoroacetic acid (TFA), which led to elution of the truncated S-entianin fragment comprising amino acids 1 to 19 (fragment 1-19), intact entianin, succinylated entianin (S-entianin), and succinylated subtilin (S-subtilin) were eluted with 99.9% methanol–0.1% TFA, dried, and stored at –20°C.

Unsuccinylated and succinylated entianin and S-subtilin resulting from the crude SPE extracts were purified by semipreparative high-performance liquid chromatography (HPLC) using a System Gold solvent module (model 125) and

TABLE 1. Strains used in this study

Strain	Description ^a	Source or reference ^b
<i>B. subtilis</i>		
ATCC 6633	Wild type (Sub ⁺)	ATCC
ATCC 6633 Δ <i>spaS</i>	<i>ΔspaS amyE::P_{spaS}-lacZ</i>	6
<i>P_{spaS}-lacZ</i>	(Spec ^r Cm ^r Sub [–])	
168	Wild type (Sub [–])	DSMZ (DSM 402)
DSM 15029 ^T	Wild type (Sub ⁺)	DSMZ
BSF 2470	CU1065 <i>liaI::P_{liaR}-lacZ</i>	31
	(<i>liaI::pMUTIN</i>) (Sub [–])	
BSF 2470pX	CU1065 <i>liaI::P_{liaR}-lacZ</i>	This work
	<i>amyE::Cm^r</i>	
<i>S. aureus</i>		
ATCC 29213	Methicillin sensitive	ATCC
ATCC 43300 (MRSA)	Methicillin-resistant clinical isolate	ATCC
<i>E. faecalis</i>		
ATCC 29212	Vancomycin sensitive	ATCC
ATCC 51299 (VRE)	Vancomycin resistant	ATCC
<i>Micrococcus luteus</i>		ATCC
ATCC 9341		

^a Sub⁺, subtilin producer; Sub[–], non-subtilin producer; Spec^r, spectinomycin resistant; Cm^r, chloramphenicol resistant.

^b ATCC, American Type Culture Collection; DSMZ, German Resource Centre for Biological Material.

a System Gold detector module (model 166) (both from Beckman, Krefeld, Germany) set at 214 nm. A Gemini-NX column (height, 250 nm; diameter, 10 mm; particle size, 10 μ m; Phenomenex) was used for chromatographic separation. Eluent A was 20% acetonitrile (ACN) and 0.1% TFA, and 99.9% ACN–0.1% TFA was chosen as eluent B. The lantibiotic-containing fractions were eluted with a gradient of 24% to 29% eluent B within 25 min and were stored after drying at –20°C. The purity of the isolated isoforms was verified by analytical-scale HPLC and matrix-assisted laser desorption–time of flight mass spectrometry (MALDI-TOF MS).

Linearization of succinylated entianin. For thorough sequencing of S-entianin, including its cyclic thioether sections, by tandem mass spectrometry, previous reductive linearization of the lantibiotic was necessary. Twenty milligrams of the dried crude SPE extract containing both entianin isoforms from *B. subtilis* DSM 15029^T was diluted in 700 μ l of a solution comprising 8 M guanidinium hydrochloride, 20 mM EDTA, and 200 mM Tris-HCl. After the addition of 400 mg Raney nickel, followed by shaking for 15 h at 55°C and subsequent centrifugation for 2 min at 5,000 rpm, the supernatant was removed and desalted using C₁₈ ZipTips (Millipore, Schwalbach am Taunus, Germany) according to the manufacturer's protocol. Mass spectrometric analysis revealed quantitative reductive linearization of S-entianin.

Proteolytic digestion of entianin, S-entianin, and their linearized counterparts. For determination of the primary structure of entianin, dried HPLC-purified entianin and S-entianin were dissolved in 25 mM Tris-HCl (pH 7.4) and were separately digested in-solution with trypsin or chymotrypsin. Modified chymotrypsin (bovine; emp Biotech, Berlin, Germany) and trypsin (Sigma-Aldrich, Taufkirchen, Germany) were dissolved to final concentrations of 50 ng/ μ l and 120 ng/ μ l, respectively, in a 25 mM NH₄HCO₃–500 μ M CaCl₂ solution. After 30 μ l S-entianin solution was mixed with 1 μ l trypsin solution and 10 μ l entianin solution was mixed with 0.8 μ l chymotrypsin solution, the mixtures were incubated for 30 h at room temperature. Furthermore, a linearized mixture of SPE-purified entianin and S-entianin was dried after desalting using ZipTip purification, redissolved in 6 μ l 25 mM Tris · HCl (pH 7.4), and digested with chymotrypsin only. A 0.3- μ l chymotrypsin solution was mixed with a 6- μ l linearized-lantibiotic solution, and the mixture was incubated for 30 h at 30°C. After digestion, each mixture was desalted by C₁₈ ZipTips. Cleavage of chymotrypsin was considered after the P1 residues Y, F, W, M, L, V, Q, and A, and not before P (17). For trypsin, R and K were considered potential cleavage sites, and cleavage before P was not considered (17).

Mass spectrometric analysis. For all mass spectrometric analyses, a Voyager-DE STR mass spectrometer (Applied Biosystems, Darmstadt, Germany) and a MALDI linear trap quadrupole (LTQ) Orbitrap XL mass spectrometer (Thermo Fisher Scientific, Inc., Waltham, MA) were used. Both mass spectrometers work with nitrogen lasers emitting at 337 nm. The following parameters refer to the Voyager DE-STR mass spectrometer. Each mass spectrum resulted from the accumulation of 500 single spectra. MALDI-TOF parameters were as follows: polarity, positive; operation mode, reflector; accelerating voltage, 20 kV; grid voltage, 68.5%; delay time, 150 ns; mass range, 500 to 4,000 Da; low-mass gate, 450 Da; bin size, 0.5 ns. Data Explorer, version 4.9 (Applied Biosystems, Darmstadt, Germany), was used for analysis of the MS spectra using the following parameters: baseline correction; Gaussian smooth, 5; minimum signal/noise (S/N) ratio, 10; mass peak filter, monoisotopic peaks.

To achieve the highest sensitivities, 4-chloro- α -cyanocinnamic acid (CICCA) (22, 23) was used as the matrix for all MALDI-TOF MS and MS-MS experiments as a 20 mM solution in 70% ACN. CICCA was synthesized by means of a Knoevenagel condensation reaction according to the instructions given in the literature (22). The laser fluence was optimized to achieve the highest analyte S/N ratios. Internal calibration was performed by addition of the Sequazyme mass standard kit (calibration mixtures 1 and 2 additionally diluted 1:20 with 30% ACN–0.1% TFA), obtained from Applied Biosystems. A mixture of 1 μ l analyte solution or 0.1 μ l rough growth supernatants, respectively, 1 μ l matrix solution, and 1 μ l calibration mixture was spotted without premixing onto a polished steel target and was air dried. After internal calibration, the most intense analyte m/z ratios were used for internal calibration of identical samples recorded with no added calibration mixture.

For determination of the N-terminal succinylation of S-entianin, highly accurate entianin and S-entianin mass spectra were recorded using the MALDI LTQ Orbitrap XL mass spectrometer in Fourier transform mass spectrometry mode. One microliter of HPLC-purified entianin, S-entianin, and an internal calibration peptide mixture were mixed with 0.7 μ l CICCA solution on a polished steel plate, and the mixture was air dried. The parameters given under “MS-MS analysis” below, with a resolution of 100,000 and internal lock-mass calibration by means of the known m/z ratios of the added calibration mixture peptides, were used for recording the spectra.

MS-MS analysis. For sequence determination of linearized S-entianin, MS-MS spectra were recorded using the ion trap of the MALDI LTQ Orbitrap XL mass spectrometer as the mass analyzer. A mixture of 1 μ l CICCA solution (20 mM in 70% ACN) and 1 μ l ZipTip-purified SPE extract containing linearized entianin as well as linearized S-entianin dissolved in 99.9% ACN–0.1% TFA was mixed onto a polished steel plate and was air dried. The following parameters were selected: precursor selector, 3,304 Da, precursor width range, ± 5 Da; scan type, full; scan rate, normal; WideBand activation, on; microscans per step, 2; plate motion, crystal positioning system; laser energy, 12 μ J; automatic gain control, on. Qual Browser, version 2.0.7 (Thermo Fisher Scientific, Inc., Bremen, Germany), was used for peak analysis.

In-solution quantification of lantibiotics. For comparison of the specific autoinduction activities, the different HPLC-purified lantibiotics were diluted in 20 mM Tris · HCl–5% ACN (pH 7.2) in such a way that analytical-scale HPLC analyses yielded nearly identical peak integrals for separated solutions of S-subtilin and S-entianin as well as for S-entianin and entianin (see Fig. S1 in the supplemental material). The protein concentrations of these stock solutions were determined by the micro-bicinchoninic acid (micro-BCA) method (35) using a bovine albumin standard of known concentration for quantification. Additionally, solutions of S-entianin and S-entianin_{1–19} were quantified without previous dilution to identical HPLC peak integrals. The concentrations in the stock solutions were determined to be 3.7 μ M S-subtilin and 3.2 μ M S-entianin; 3.4 μ M S-entianin and 3.5 μ M entianin; and 2.9 μ M S-entianin and 15.3 μ M S-entianin_{1–19}.

Quantification of the specific autoinduction activity. The specific autoinduction of entianin and S-entianin was determined by means of a quantitative chromogenic microtiter plate bioassay developed by Burkard et al. (6). The reporter strain ATCC 6633 $\Delta spaS$ P_{*spaS*}-lacZ was cultivated overnight in 5 ml modified TY medium containing 0.3 M NaCl and was subsequently diluted with the same modified medium to an optical density at 600 nm (OD₆₀₀) of 0.1. The HPLC-purified lantibiotic stock solutions were diluted stepwise by factors of 1.3, 3, 4, 6, 12, 24, 48, and 96 with 20 mM Tris · HCl–5% ACN for the comparison between S-entianin and entianin.

Aliquots (180 μ l) of the diluted reporter strain culture were transferred to 96-well microtiter plate wells and were thoroughly mixed with 20 μ l of the lantibiotic dilution series. Every culture was tested for autoinduction activity together with a blank sample in triplicate. The combined reporter strain-lantibiotic cultures were incubated for 6 h at 37°C with 10 min of shaking at 800 rpm

FIG. 1. Analyses of subtilin autoinduction in *B. subtilis* ATCC 6633 and DSM 15029^T. (Left) Subtilin autoinduction color assay for the identification of *B. subtilis* strains producing subtilin-like lantibiotics. The β -galactosidase expression of reporter strain *B. subtilis* ATCC 6633 $\Delta spaS$ P_{*spaS*}-lacZ, streaked in a vertical direction, depends on the autoinduction of subtilin-like lantibiotics. Test strains *B. subtilis* ATCC 6633 (subtilin producer) (top), *B. subtilis* DSM 15029^T (center), and *B. subtilis* 168 (non-subtilin producer) (bottom) were streaked perpendicularly to the reporter strain and were cultivated for 24 h at 30°C. Blue indicates autoinduction due to the production of subtilin-like lantibiotics. (Right) UV chromatogram, recorded at 214 nm, of an analytical HPLC separation of the *B. subtilis* DSM 15029^T supernatant obtained after cultivation for 30 h in 50 ml Landy medium and centrifugation for 20 min at 10,000 rpm. According to mass spectrometric analyses, the marked peaks correspond to entianin_{20–32} (δ), S-entianin_{1–19} (γ), entianin (β), and S-entianin (α).

every hour. The β -galactosidase activity was determined according to the work of Burkard et al. (6). To avoid growth differences in the reporter strain, identical overnight cultures were used for each comparison.

Determination of the entianin MIC. The MIC of purified entianin was tested according to the DIN-58940-8 microdilution method (34a).

Quantification of cell wall stress induced by entianin and S-entianin. *B. subtilis* strain BSF 2470pX was used to report stress interfering with the lipid II cycle (31) as described previously (7). An overnight culture of strain BSF 2470pX was diluted in TY–0.3 M NaCl to a final OD₆₀₀ of 0.1. Aliquots (200 μ l) were separated into 96-well plates and were incubated for 3 h at 37°C. After the addition of different amounts of lantibiotics, the samples were further incubated for 1 h at 37°C. Enzyme activities were determined as described previously (40). Standard deviations were calculated by three independent approaches.

DNA sequencing. DNA sequencing was performed by Scientific Research and Development GmbH, Oberursel, Germany.

Nucleotide sequence accession number. The DNA sequence of the entianin gene cluster of *B. subtilis* strain DSM 15029^T has been deposited in GenBank under accession number HQ871873 (see Fig. S4 in the supplemental material).

RESULTS

Entianin, a new lantibiotic from *B. subtilis* DSM 15029^T. *Bacillus subtilis* subsp. *spizizenii* DSM 15029^T efficiently induced subtilin production in our previously described, highly sensitive test system (Fig. 1, left) using reporter strain *B. subtilis* ATCC 6633 $\Delta spaS$ P_{*spaS*}-lacZ (6). For further analyses, the active substances from the supernatants of *B. subtilis* DSM 15029^T and the known subtilin producer *B. subtilis* ATCC 6633 were purified by reversed-phase solid-phase extraction (SPE) and were fractionated by HPLC separation (Fig. 1, right [*B. subtilis* DSM 15029^T]). The subtilin autoinduction activities of the UV-active HPLC fractions were tested in a microtiter plate-based test system and were additionally analyzed by matrix-assisted laser desorption/ionization–time of flight mass

FIG. 2. Positive-ion mode MALDI-TOF mass spectra of 5.7 pmol (each) of HPLC-purified *B. subtilis* ATCC 6633 S-subtilin (top), *B. subtilis* DSM 15029^T S-entianin (center), and *B. subtilis* DSM 15029^T entianin (bottom). The dominant peaks correspond to the monoisotopic protonated ion species $[A + H]^+$; the corresponding oxidized species are additionally annotated in the insets. For the succinylated lantibiotics, the arrows indicate the calculated m/z ratios of unsuccinylated subtilin and entianin, which were separated from their succinylated counterparts by HPLC.

spectrometry (MALDI-TOF MS). The HPLC-purified autoinducing fraction of *B. subtilis* strain ATCC 6633 contained exclusively S-subtilin, which could be unambiguously identified within a 5-ppm error tolerance by means of the known subtilin mass/charge (m/z) ratio ($[A + H]^+ = 3,419.6$) (for the elemental formula, see reference 42) (Fig. 2). For the autoinducing strain of *B. subtilis* subsp. *spizizenii*, *B. subtilis* DSM 15029^T (also known as NRRL B-23049^T [34]), two autoinducing fractions, which contained compounds with m/z ratios of 3,347.6 and 3,447.6, could be separated (Fig. 2). These compounds exceed the mass of subtilin and that of the 100.0-Da-larger isoform S-subtilin, respectively, by 28 Da each. Additional signals shifted by +16 Da each correlate with partial oxidation apparently occurring during the purification process, since no oxidation by-products were detected by whole-cell measurements of subtilin producers by use of MALDI-TOF MS (42).

The high specificity of the autoinduction bioassay suggested

that the autoinducing compounds of *B. subtilis* DSM 15029^T correspond to a novel subtilin-like lantibiotic with unsuccinylated and succinylated isoforms, which was named entianin. Integration of the corresponding chromatogram peaks of Fig. 1 indicated a 1:3.7 ratio for the production of entianin versus S-entianin. Whereas *B. subtilis* DSM 15029^T produced a considerable amount of free entianin, the *B. subtilis* laboratory strain ATCC 6633 produced succinylated subtilin almost exclusively (data not shown). In addition to entianin and S-entianin, two peptide fragments without antibiotic activity, corresponding to the N-terminal fragment entianin₁₋₁₉ and the C-terminal fragment entianin₂₀₋₃₂, were eluted as distinct peaks from the C₁₈ column (Fig. 1, right).

The concentration of an HPLC-purified S-subtilin solution was determined according to the micro-BCA method of Olson and Markwell (35). The determination of the analytical HPLC peak areas of this stock solution and its dilutions allowed for a correlation between S-subtilin concentrations and chromato-

FIG. 3. (a) Inhibition of the growth of test strain *B. subtilis* BSF 2470pX by addition of different concentrations of entianin (left) or S-entianin (right). After 3 h of growth in microtiter plates and the subsequent addition of entianin or S-entianin at the final concentrations given in the graph, optical densities at 620 nm were determined (filled bars). After incubation for 1 h, optical densities were again determined (shaded bars) for entianin and S-entianin. Note that the S-entianin concentrations applied were much higher than those of entianin. (b) Cell wall stress induction by different concentrations of entianin (left) and S-entianin (right) using the LiaRS system for detection in *B. subtilis* BSF 2470pX ($P_{liaR-lacZ}$) (31). β -Galactosidase activity was determined by means of the absorption of generated *o*-nitrophenol.

graphic peak areas and was used for quantification of the closely related lantibiotics produced by *B. subtilis* DSM 15029^T. The entianin derivatives generated could be quantified to 16.6 $\mu\text{g/ml}$ S-entianin₁₋₁₉, 25.9 $\mu\text{g/ml}$ entianin, and 96.9 $\mu\text{g/ml}$ S-entianin.

Antibiotic activities of entianin and S-entianin. After purification by SPE and HPLC, S-subtilin and the various forms of entianin were quantified in solution and were diluted to the same concentrations (see Fig. S1 in the supplemental material). The antibiotic and autoinducing activities of these equal concentrated stock solutions and their subsequent dilutions were analyzed. Whereas entianin completely inhibited the growth of the non-subtilin-producing strain *B. subtilis* 2470pX at concentrations less than 0.6 $\mu\text{g/ml}$ (Fig. 3a), 25 $\mu\text{g/ml}$ of S-entianin (Fig. 3a) and more than 60 $\mu\text{g/ml}$ of S-subtilin (data not shown) were needed to inhibit the growth of the test strain. These results are in accordance with the strongly reduced induction of cellular envelope stress by S-entianin compared to entianin (Fig. 3b). This suggests that succinylation dramatically lowers the antibiotic activity of entianin through reduced lipid II affinity or less-efficient pore formation.

For further analysis of its antibiotic potential, the antibiotic activities of unsuccinylated entianin were analyzed in detail by the microdilution method (DIN-58940-8) against several bacterial strains, including *Staphylococcus aureus* ATCC 43300 (a methicillin-resistant *S. aureus* [MRSA] strain) and *Enterococcus faecalis* ATCC 51299 (a vancomycin-resistant enterococcus [VRE]). The MICs of entianin were in the range of 4 to 16 $\mu\text{g/ml}$ for all strains tested (Table 2) and were 8 $\mu\text{g/ml}$ for MRSA and 8 to 16 $\mu\text{g/ml}$ for VRE. This is in the same range as the known MICs of nisin A against different *S. aureus* (1 to >16 $\mu\text{g/ml}$) and *E. faecalis* (1.5 to >16 $\mu\text{g/ml}$) strains (5, 11, 32, 37).

Autoinduction activities of entianin and S-entianin. Although lantibiotic succinylation caused strongly reduced antibiotic activity, S-entianin still provided strong autoinduction of the subtilin-sensing system with similar kinetics (Fig. 4). In the case of entianin, it was possible to analyze the effect of succinylation on autoinduction also; this showed that S-entianin was less efficient than its unsuccinylated counterpart (Fig. 4). At higher concentrations, entianin causes lysis of the test strain, which explains the decrease in autoinduction.

TABLE 2. MICs of unsuccinylated entianin for different bacterial strains^a

Strain ^b	Entianin MIC (μg/ml)	No. of expts
<i>Staphylococcus aureus</i>		
ATCC 29213	4–8	3
ATCC 43300 (MRSA)	8	1
<i>Enterococcus faecalis</i>		
ATCC 29212	16	3
ATCC 51299 (VRE)	8–16	3
<i>Micrococcus luteus</i> ATCC 9341	4–8	3

^a Strains were tested for the antibiotic activity of unsuccinylated entianin by using the microdilution method.

^b MRSA, methicillin-resistant *S. aureus*; VRE, vancomycin-resistant enterococcus.

Determination of the primary structure of entianin from *B. subtilis* DSM 15029^T. For closer analysis of the primary structure of entianin, purified S-entianin was digested with trypsin (C-terminal cut after K and R, and not before P), which, however, resulted in only sparse generation of proteolytic fragments (data not shown). A comparison of the masses of the proteolytic fragments of S-entianin with those from *in silico* trypsin digestion of S-subtilin allowed for assignment of all the main fragments within an error range of 10 ppm by taking into account a mass shift of 28 Da. The ion of S-entianin detected at m/z 987.5 corresponds to subtilin fragments 21 to 29 (cleavages after amino acids F₂₀ and K₂₉ due to additional chymotryptic cleavage). Other identifiable S-entianin fragments exhibited a 28-Da increase in mass over the N-terminal peptide fragments of S-subtilin, thereby narrowing the possible modification site(s) to the region between amino acids 3 and 20.

For further analysis of the 28-Da difference in mass between S-entianin and S-subtilin, linearization was needed in order to perform tandem mass spectrometric analysis. Several methods for entianin linearization were tried (24, 36); of these, reduc-

FIG. 4. Autoinduction activities of S-subtilin, S-entianin, and entianin determined with reporter strain *B. subtilis* ATCC 6633 Δ*spaS* P_{*spaS*}-*lacZ*. Entianin concentrations exceeding 2.9 μg/ml were lethal to the reporter strain, whereas higher concentrations could be tested for the less-active compounds S-entianin and S-subtilin, resulting in saturation-like curves. Two separate tests were carried out in triplicate, one comparing S-subtilin with S-entianin (grey) and the other comparing S-entianin with entianin (black), each with the same overnight culture of the reporter strain.

FIG. 5. (a) Raney nickel-catalyzed reduction of lanthionine (where R₁ stands for H) and 3-methylanthionine (where R₁ stands for CH₃). (b) Raney nickel-catalyzed reduction of 2,3-didehydroalanine (where R₂ stands for H) and -butyrine (where R₂ stands for CH₃). The resulting mass differences are given.

tive cleavage using Raney nickel (43) was most successful at hydrolyzing the thioether 3-methyl lanthionine bonds. In comparison to the protonated S-entianin mass (m/z 3,447.6), the reduced counterpart was detected at m/z 3,303.9 (see Fig. S2 in the supplemental material). Assuming a pentacyclic structure of entianin similar to that of subtilin, the mass loss of 143.73 Da was attributed to the reductive cleavage of the 5 thioether bonds, resulting in a mass loss of 29.96 Da ($-S+2H$) each (Fig. 5a). The additional mass difference of 6 Da between the mass loss detected and that due to 3-methyl lanthionine desulfuration may be due to the additional reduction of three didehydro residues ($+2H$) present in subtilin (Fig. 5b). Therefore, the detected mass of reduced S-entianin not only supports complete thioether cleavage but also indicates the presence of three didehydro residues in entianin, in agreement with the structure of subtilin.

Linearization enabled fragmentation of the previous cyclic S-entianin/entianin parts upon proteolytic digestion. MALDI-TOF MS analysis of Raney nickel-reduced S-entianin subsequently digested by chymotrypsin yielded many more fragments than those obtained by the chymotrypsin digestion of untreated S-entianin (see Fig. S3 in the supplemental material). A comparatively long digestion time was selected in order to enable enzymatic digestions after less optimal amino acids. Analysis of the mass differences between dominant peaks within an m/z range of 1,200 to 2,100 resulted in the determination of a part of the entianin as well as the S-entianin sequence (see Fig. S3). All peaks assigned are within an error range of 20 ppm, but succinylated fragment \$1-19 has a slightly higher deviation because of saturated intensity. In addition to the known chymotryptic cleavage sites (17), analysis of the proteolytic fragments generated from reduced S-entianin uncovered low-intensity cleavages after butyrine (methyl-group-extended alanine generated upon reduction of a 3-methylanthionine ring) and glycine (see Fig. S3 in the supplemental material). Comparison of this part of the sequence with the known subtilin sequence enabled assignment of the fragment ions within the entianin sequence and furthermore revealed

FIG. 6. CID fragment ions of linearized S-entianin ($[A + H]^+ = 3,303.9$ Da) detected with the linear ion trap of a MALDI LTQ Orbitrap XL mass spectrometer. For better representation of less-intense signals, the lower m/z range of the spectrum is limited to 53% of the y_{32} ion intensity. b ions are annotated with straight vertical lines and y ions with dotted vertical lines. "Bty" refers to reduced 2,3-didehydrobutyryne or the reductively cleaved 2,3-didehydrobutyryne residue of 3-methylanthionine.

the amino acid exchange Ala₁₅ to Leu/Ile₁₅. This exchange corresponds to a mass increase of 42.05 Da, suggesting at least one additional difference in the amino acid sequence that contributes to the detected mass shift of 28 Da. A tandem mass spectrometric approach using reductively cleaved S-entianin was used to detect these assumed further differences in composition. For this purpose, the linear ion trap of a MALDI-LTQ Orbitrap XL mass spectrometer was used in combination with collision-induced dissociation (CID) (Fig. 6). Only CID fragment ions within an error tolerance of ± 0.3 Da were taken into account (see Tables S1 and S2 in the supplemental material). The fragment ion spectrum enabled complete sequencing of S-entianin except for N-terminal fragment b₃₂ and allowed for identification of the Leu₆-to-Val₆ exchange, with a mass loss of 16 Da by means of the respective y ions y_{26} and y_{27} . The C-terminal amino acid lysine could also be identified by the mass difference between fragment b₃₁ and the known mass of the precursor or those of C-terminal proteolytic peptides. Furthermore, y ions y_{17} and y_{18} confirmed the previously detected amino acid exchange of Ala₁₅ to Leu/Ile₁₅ (Fig. 6). Combining the mass shifts of both exchanges results in the detected mass difference of +28 Da for intact entianin and its succinylated counterpart versus subtilin and its succinylated form. Therefore, and in agreement with the results of MS-MS sequencing, further amino acid exchanges corresponding to mass changes can be excluded.

The nature and position of the three assumed didehydroamino acids in entianin, as well as the positions the thioether-bridged rings, could be detected as follows. Treatment of S-entianin with Raney nickel caused a mass loss due to the reductive cleavage of 5 thioether bonds and the reduction of 3 didehydroamino acids. Reduction of didehydroalanine, didehydrobutyryne, or the amino acids participating in thioether ring formation leads to the generation of alanines or butyrynes. In agreement with the conclusions from reductive linearization, 5 butyrynes and 8 alanines could be detected by CID fragmentation of reduced S-entianin, 10 of which correspond to thioether-bridged rings while 3 correspond to the didehydroamino acids of native S-entianin.

Tryptic as well as chymotryptic digestion of native S-entianin led to cleavages after amino acids F₂₀ and K₂₉ (data not shown). Therefore, no thioether bridge(s) can connect N-terminal fragment 1-20 with C-terminal fragment 21-29 or 21-32. Additionally, because of the cleavage after K₂₉ in native S-entianin, the alanine detected at position 31 close to the C terminus (Fig. 6) cannot be involved in thioether bridge formation. Because all alanines and butyrynes detected must either be involved in ring formation or be dehydrated, Ala₃₁ of reduced S-entianin must be present as 2,3-didehydroalanine in native S-entianin and entianin.

The mass of fragment 21-29, generated upon digestion of intact S-entianin, indicates the presence of two thioether bonds. Sequencing of the reduced counterpart reveals butyrynes at positions 23 and 25 as well as alanines at positions 26 and 28. DNA sequencing of the entianin structural gene (Fig. 7) shows that both alanines originate from the reduction of cysteines. For steric reasons, Cys₂₆ cannot be connected to position 25. Consequently, ring formation must occur between amino acids 23 and 26 as well as between amino acids 25 and 28 (Fig. 7).

For the N-terminal part of entianin, two rings must be gen-

FIG. 7. Comparison of the primary structures of subtilin (12–14) and entianin by schematic representation. Amino acids (circled) are given in one-letter code. Posttranslational modifications are abbreviated as follows: A-S-A, *meso*-lanthionine; Ab-S-A, 3-methylanthionine ("Ab" refers to α -aminobutyric acid); Δ A, 2,3-didehydroalanine; Δ B, 2,3-didehydrobutyryne. Arrows indicate amino acid residues in entianin that differ from those in subtilin.

erated, one between amino acids 3 and 7 (ring A) and one between amino acids 8 and 11 (ring B), for the generation of the typical lipid II binding motif; otherwise, no antibiotic activity would be detectable. In agreement with this, DNA sequencing discloses cysteines at positions 7 and 11.

It is very unlikely that reduced Ala₅ is involved in the generation of the last missing thioether bond, since this would lead to the generation of an additional outer ring, leading to structural interference with the binding motif. Therefore, native entianin most probably exhibits a 2,3-didehydroalanine at position 5.

The three remaining reduced amino acids comprise butyrynes at positions 13 and 18 and Ala₁₉. DNA analysis reveals that Ala₁₉ originates from the reduction of cysteine. The thiol group of Cys₁₉ cannot be connected to position 18 for steric reasons. Therefore, the amino acids at positions 13 and 19 must be involved in ring formation, and butyryne at position 18 originates from the reduction of didehydrobutyryne.

In summary, all didehydroamino acids and positions of the thioether bridges could be identified and coincide perfectly with the corresponding modifications in subtilin and its succinylated counterpart (Fig. 7).

Differences between isobaric Leu-to-Ile exchanges and vice versa in subtilin versus entianin are difficult to detect by conventional mass spectrometric techniques. For this purpose, additional sequencing of the *etnS* structural gene of entianin was performed (see Fig. S4 in the supplemental material). Sequencing of the *etnS* gene of *B. subtilis* DSM 15029^T, which codes for the unmodified prepropeptide, including the N-terminal leader sequence, confirmed the detected exchange Leu₆ → Val₆, specified the exchange Ala₁₅ → Leu₁₅, and furthermore revealed the isobaric exchange Leu₂₄ → Ile₂₄ (Fig. 7).

Determination of N-terminal succinylation. Accurate mass determination of entianin and its supposed succinylated counterpart was performed with the MALDI LTQ Orbitrap mass spectrometer. The signal of protonated entianin was detected at *m/z* 3,347.60716 (calculated, 3,347.60185 Da; Δ = 1.6 ppm) and that of protonated S-entianin at *m/z* 3,447.61798 (calculated, 3,447.6179 Da; Δ = 0.2 ppm). Taking into account the known composition of neutral entianin (C₁₅₀H₂₃₁N₃₉O₃₈S₅) as well as an error tolerance of ± 0.5 ppm, the only possible chemical composition of the S-entianin mass increase with regard to an even electron ion was calculated to be C₄H₄O₃. N-terminal localization of the derivatization could be verified by means of a mass shift of about 100 Da of all N-terminal b ions but not for any C-terminal y ions (Fig. 6).

In view of the known N-terminal succinylation of S-subtilin (8) as well as the determination of the N-terminal derivatization of S-entianin and of C₄H₄O₃ as the only possible elemental composition of the derivatization, it seems obvious that, like S-subtilin, S-entianin also exhibits N-terminal succinylation.

Homology between the subtilin- and entianin-related gene clusters. Although the operon structure of the genes for the biosynthesis, immunity, and regulation of *B. subtilis* DSM 15029^T entianin was similar to that of the subtilin gene cluster of *B. subtilis* ATCC 6633, with a nucleotide identity of 93%, striking differences at the 5' ends of *etnI*, *etnG*, and *etnK* were found: three N-terminal elongations of 26 (SpaI), 33 (SpaG), and 6 (SpaK) amino acid residues were detected for the proteins responsible for self-immunity (SpaI and SpaG) and the

quorum-sensing histidine kinase (SpaK) of *B. subtilis* DSM 15029^T relative to these proteins in *B. subtilis* ATCC 6633. Since the SpaI immunity protein is posttranslationally lipid acylated (15), the question of whether the 26-amino-acid extension might interfere with posttranslational modification arose. However, Western blot analysis clearly showed that after lipid acylation and N-terminal processing, EtnI and SpaI are similar in size (data not shown). High levels of sequence identity were detected for the proteins responsible for the posttranslational modification of subtilin and entianin: 94% for EtnB, 96% for EtnT, and 92% for EtnC. This homology suggests and supports the hypothesis that 3-methyl lanthionine, 2,3-didehydroalanine, and 2,3-didehydrobutyryne are formed and localized in the same manner in entianin as in subtilin (12–14).

DISCUSSION

In this work, we identified *B. subtilis* DSM 15029^T as the producer of a novel subtilin-like lantibiotic, termed entianin, by using our recently developed subtilin autoinduction bioassay in combination with MALDI-TOF MS analysis of the purified culture supernatants and DNA sequencing of the corresponding gene clusters. *B. subtilis* DSM 15029 is the type strain of *B. subtilis* subsp. *spizizenii*, also referred to as the W23 group. This confirms our previous findings that all *B. subtilis* subsp. *spizizenii* strains produce subtilin-like lantibiotics, whereas *B. subtilis* subsp. *subtilis* strains are not lantibiotic producers (6). Therefore, the subtilin autoinduction bioassay provides a fast and easy tool for differentiating between strains of these two *B. subtilis* subspecies.

By use of Raney nickel-catalyzed reductive linearization combined with tandem mass spectrometric analysis, we developed an approach that, for the first time, allowed for thorough lantibiotic sequencing, including the characterization of difficult-to-access intramolecular ring structures. This strategy will be highly advantageous for elucidating the structure of novel lantibiotics, where linearization will facilitate great progress in the elucidation of ring formations and posttranslational modifications. By the combination of this strategy with DNA sequencing, all posttranslational modifications will become easily accessible.

Combining all data from DNA and MS-MS sequencing, we find that entianin exhibits the primary sequence of subtilin except for the amino acid exchanges Leu₆ → Val₆, Ala₁₅ → Leu₁₅, and Leu₂₄ → Ile₂₄ and represents a third subtilin-like lantibiotic along with ericin S from *B. subtilis* A1/3 (41). Although the differences in primary structure among subtilin, ericin, and entianin are based on the exchange of hydrophobic amino acids, such changes can influence antibiotic activity considerably. This has been shown for ericin, where the exchange of two hydrophobic residues caused strongly increased antibiotic activities (41), and also for gallidermin, with a single amino acid exchange relative to the sequence of epidermin (39). In *B. subtilis* DSM 15029^T, the lantibiotic entianin is produced not only in its succinylated form but also as an unsuccinylated species with an antimicrobial activity much higher than those of S-entianin and S-subtilin, as determined by use of a *B. subtilis* test strain. Therefore, succinylation seems to dramatically decrease the antibiotic properties of entianin and subtilin,

perhaps through diminished lipid II-lantibiotic interaction or hampered integration of the complex into the cytoplasmic membrane.

As previously reported, N-terminally succinylated subtilin showed much lower antibiotic activity than subtilin (8). This is confirmed by the new subtilin-like lantibiotic entianin, which exhibits a 40-fold higher antibiotic activity in its unsuccinylated form. Remarkably, autoinduction is not severely affected by succinylation; S-entianin reaches 70% of the autoinduction level of unsuccinylated entianin. However, it should be taken into account that the maximal autoinduction level of entianin is difficult to calculate, because this lanthionine becomes toxic at concentrations clearly below 1 µg/ml. Nevertheless, it is obvious that S-entianin has a strong autoinduction capacity, whereas its antibiotic activity is nearly eliminated. Therefore, succinylation does not interfere with the lantibiotic sensing system to the same degree that it influences antibiotic activity, perhaps due to a lowered cell wall permeability caused by the negative charge of the N-terminal modification.

DNA sequencing of the *etn* gene cluster of *B. subtilis* DSM 15029^T, which is responsible for entianin biosynthesis, regulation, and autoimmunity, revealed a high degree of homology (93%) with the *spa* gene cluster of *B. subtilis* ATCC 6633 (responsible for subtilin biosynthesis). Still, open reading frame extensions of 26 and 33 additional amino acids relative to their subtilin immunity protein counterparts indicate N-terminally extended sequences for the entianin immunity proteins EtnI and EtnG. However, at least for EtnI, maturation and cleavage of the N-terminal extension result in the same size as that of SpaI (28).

All *B. subtilis* subsp. *spizizenii* strains investigated hitherto for lantibiotic production have been shown to be subtilin producers (such as ATCC 6633, DSM 618, DSM 1087, DSM 6395, DSM 6405, and DSM 8439 [6]); however, the type strain of *B. subtilis* subsp. *spizizenii*, DSM 15029, does not produce subtilin but the subtilin variant entianin. This is in agreement with unpublished data from the German Collection of Microorganisms and Cell Cultures (<http://www.dsmz.de/microorganisms/html/strains/strain.dsm015029.html>) which reveal that the type strain has a riboprint pattern different from those of other strains of the same subspecies. Consequently, a reevaluation of DSM 15029 as the type strain of *B. subtilis* subsp. *spizizenii* should be considered.

In summary, we report on the identification of the new subtilin-like lantibiotic entianin, which can be isolated in its unsuccinylated form and provides comparably high antibiotic activity in the same concentration range as nisin A, which is active against various Gram-positive pathogens (5, 11, 32, 37). Considering the increasing resistance to antibiotic treatment and the need for new antibiotics, entianin might be a promising candidate for potential applications against MRSA and *E. faecalis* infections. Furthermore, our finding that the less-toxic isoforms S-entianin and S-subtilin have autoinduction activity may provide a new tool for the biotechnological production of entianin.

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REFERENCES

- Bierbaum, G., and H. G. Sahl. 2009. Lantibiotics: mode of action, biosynthesis and bioengineering. *Curr. Pharm. Biotechnol.* **10**:2–18.
- Breukink, E., and B. de Kruijff. 1999. The lantibiotic nisin, a special case or not? *Biochim. Biophys. Acta* **1462**:223–234.
- Breukink, E., et al. 1999. Use of the cell wall precursor lipid II by a pore-forming peptide antibiotic. *Science* **286**:2361–2364.
- Brötz, H., et al. 1998. Role of lipid-bound peptidoglycan precursors in the formation of pores by nisin, epidermin and other lantibiotics. *Mol. Microbiol.* **30**:317–327.
- Brumfitt, W., M. R. Salton, and J. M. Hamilton-Miller. 2002. Nisin, alone and combined with peptidoglycan-modulating antibiotics: activity against methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant enterococci. *J. Antimicrob. Chemother.* **50**:731–734.
- Burkard, M., K. D. Entian, and T. Stein. 2007. Development and application of a microtiter plate-based autoinduction bioassay for detection of the lantibiotic subtilin. *J. Microbiol. Methods* **70**:179–185.
- Burkard, M., and T. Stein. 2008. Microtiter plate bioassay to monitor the interference of antibiotics with the lipid II cycle essential for peptidoglycan biosynthesis. *J. Microbiol. Methods* **75**:70–74.
- Chan, C. W., B. W. Bycroft, M. L. Leyland, L.-Y. Lian, and G. C. Roberts. 1993. A novel posttranslational modification of the peptide antibiotic subtilin: isolation and characterization of a natural variant from *Bacillus subtilis* ATCC 6633. *Biochem. J.* **291**:23–27.
- Chatterjee, C., M. Paul, L. Xie, and W. A. van der Donk. 2005. Biosynthesis and mode of action of lantibiotics. *Chem. Rev.* **105**:633–684.
- Georgalaki, M. D., et al. 2002. Macedocin, a food-grade lantibiotic produced by *Streptococcus macedonicus* ACA-DC 198. *Appl. Environ. Microbiol.* **68**:5891–5903.
- Giacometti, A., O. Cirioni, F. Barchiesi, and G. Scalise. 2000. In-vitro activity and killing effect of polycationic peptides on methicillin-resistant *Staphylococcus aureus* and interactions with clinically used antibiotics. *Diagn. Microbiol. Infect. Dis.* **38**:115–118.
- Gross, E. 1975. Subtilin and nisin: the chemistry and biology of peptides with alpha, beta-unsaturated amino acids, p. 31–42. *In* R. Walter and J. Meienhofer (ed.), *Peptides: chemistry, structure, and biology: proceedings of the fourth American Peptide Symposium*. Ann Arbor Science, Ann Arbor, MI.
- Gross, E. 1978. Polypeptide antibiotics, p. 415–462. *In* M. J. Weinstein and G. H. Wagan (ed.), *Antibiotics: isolation, separation and purification*. Elsevier, New York, NY.
- Gross, E., H. H. Kiltz, and E. Nebelin. 1973. Subtilin, VI: the structure of subtilin. *Hoppe-Seyler's Z. Physiol. Chem.* **354**:810–812. (In German.)
- Halami, P. M., T. Stein, A. Chandrasekar, and K.-D. Entian. 2010. Maturation and processing of SpaI, the lipoprotein involved in subtilin immunity in *Bacillus subtilis* ATCC 6633. *Microbiol. Res.* **165**:183–189.
- Hansen, J. N. 1994. Nisin as a model food preservative. *Crit. Rev. Food Sci. Nutr.* **34**:69–93.
- Harris, J. L., et al. 2000. Rapid and general profiling of protease specificity by using combinatorial fluorogenic substrate libraries. *Proc. Natl. Acad. Sci. U. S. A.* **97**:7754–7759.
- Hasper, H. E., B. de Kruijff, and E. Breukink. 2004. Assembly and stability of nisin-lipid II pores. *Biochemistry* **43**:11567–11575.
- Hasper, H. E., et al. 2006. An alternative bactericidal mechanism of action for lantibiotic peptides that target lipid II. *Science* **313**:1636–1637.
- Heinzmann, S., K. D. Entian, and T. Stein. 2006. Engineering *Bacillus subtilis* ATCC 6633 for improved production of the lantibiotic subtilin. *Appl. Microbiol. Biotechnol.* **69**:532–536.
- Hsu, S. T., et al. 2004. The nisin-lipid II complex reveals a pyrophosphate cage that provides a blueprint for novel antibiotics. *Nat. Struct. Mol. Biol.* **11**:963–967.
- Jaskolla, T. W., W.-D. Lehmann, and M. Karas. 2008. 4-Chloro- α -cyanocinnamic acid is an advanced, rationally designed MALDI matrix. *Proc. Natl. Acad. Sci. U. S. A.* **105**:12200–12205.
- Jaskolla, T. W., D. G. Papasotiropoulos, and M. Karas. 2009. Comparison between the matrices alpha-cyano-4-hydroxycinnamic acid and 4-chloro-alpha-cyanocinnamic acid for trypsin, chymotrypsin, and pepsin digestions by MALDI-TOF mass spectrometry. *J. Proteome Res.* **8**:3588–3597.
- Kawulka, K. E., et al. 2004. Structure of subtilisin A, a cyclic antimicrobial peptide from *Bacillus subtilis* with unusual sulfur to α -carbon cross-links: formation and reduction of α -thio- α -amino acid derivatives. *Biochemistry* **43**:3385–3395.
- Kiesau, P., et al. 1997. Evidence for a multimeric subtilin synthetase complex. *J. Bacteriol.* **179**:1475–1481.
- Klein, C., C. Kaletta, N. Schnell, and K. D. Entian. 1992. Analysis of genes involved in biosynthesis of the lantibiotic subtilin. *Appl. Environ. Microbiol.* **58**:132–142.
- Klein, C., C. Kaletta, and K. D. Entian. 1993. Biosynthesis of the lantibiotic Subtilin is regulated by a histidin kinase/response regulator system. *Appl. Environ. Microbiol.* **59**:296–303.

28. Klein, C., and K. D. Entian. 1994. Genes involved in self-protection against the lantibiotic subtilin produced by *Bacillus subtilis* ATCC 6633. *Appl. Environ. Microbiol.* **60**:2793–2801.
29. Kuipers, O. P., M. M. Beerthuyzen, P. G. de Ruyter, E. J. Luesink, and W. M. de Vos. 1995. Autoregulation of nisin biosynthesis in *Lactococcus lactis* by signal transduction. *J. Biol. Chem.* **270**:27299–27304.
30. Landy, M., G. H. Warren, S. B. Rosenman, and L. G. Colio. 1948. Bacillomycin: an antibiotic from *Bacillus subtilis* active against pathogenic fungi. *Proc. Soc. Exp. Biol. Med.* **67**:539–541.
31. Mascher, T., S. L. Zimmer, T. A. Smith, and J. D. Helmann. 2004. Antibiotic-inducible promoter regulated by the cell envelope stress-sensing two-component system LiaRS of *Bacillus subtilis*. *Antimicrob. Agents Chemother.* **48**:2888–2896.
32. Mota-Meira, M., G. Lapointe, C. Lacroix, and M. C. Lavoie. 2000. MICs of mutacin B-Ny266, nisin A, vancomycin, and oxacillin against bacterial pathogens. *Antimicrob. Agents Chemother.* **44**:24–29.
33. Müller, W. M., T. Schmiederer, P. Ensle, and R. D. Süssmuth. 2010. In vitro biosynthesis of the prepeptide of type-III lantibiotic labyrinthopeptin A2 including formation of a C-C bond as a post-translational modification. *Angew. Chem. Int. Ed. Engl.* **49**:2436–2440.
34. Nakamura, L. K., M. S. Roberts, and F. M. Cohan. 1999. Relationship of *Bacillus subtilis* clades associated with strains 168 and W23: a proposal for *Bacillus subtilis* subsp. *subtilis* subsp. nov. and *Bacillus subtilis* subsp. *spizizenii* subsp. nov. *Int. J. Syst. Bacteriol.* **49**:1211–1215.
- 34a. Normenausschuss Medizin im DIN, Deutsches Institut für Normung e.V. 1999. Microdilution methods. DIN 58940-8. Deutsches Institut für Normung e.V., Berlin, Germany.
35. Olson, B. J., and J. Markwell. 2007. Assays for determination of protein concentration. *Curr. Protoc. Protein Sci.* Chapter 3, Unit 3.4.
36. Petricci, E., M. Renzulli, M. Radi, F. Corelli, and M. Botta. 2002. Solid-phase synthesis (SPS) of substituted uracils via Oxone cleavage methodology. *Tetrahedron Lett.* **43**:9667–9670.
37. Piper, C., L. A. Draper, P. D. Cotter, R. P. Ross, and C. Hill. 2009. A comparison of the activities of lacticin 3147 and nisin against drug-resistant *Staphylococcus aureus* and *Enterococcus* species. *J. Antimicrob. Chemother.* **64**:546–551.
38. Schnell, N., et al. 1988. Prepeptide sequence of epidermin, a ribosomally synthesized antibiotic with four sulphide-rings. *Nature* **333**:276–278.
39. Schnell, N., et al. 1989. Structural gene isolation and prepeptide sequence of gallidermin, a new lanthionine containing antibiotic. *FEBS Microbiol. Lett.* **58**:263–268.
40. Stein, T., et al. 2002. Dual control of subtilin biosynthesis and immunity in *Bacillus subtilis*. *Mol. Microbiol.* **44**:403–416.
41. Stein, T., et al. 2002. Two different lantibiotic-like peptides originate from the ericin gene cluster of *Bacillus subtilis* A1/3. *J. Bacteriol.* **184**:1703–1711.
42. Stein, T. 2008. Whole-cell matrix-assisted laser desorption/ionization mass spectrometry for rapid identification of bacteriocin/lantibiotic-producing bacteria. *Rapid Commun. Mass Spectrom.* **22**:1146–1152.
43. Uchida, K., and E. R. Stadtman. 1992. Selective cleavage of thioether linkage in proteins modified with 4-hydroxynonenal. *Proc. Natl. Acad. Sci. U. S. A.* **89**:5611–5615.
44. Willey, J. M., and W. A. van der Donk. 2007. Lantibiotics: peptides of diverse structure and function. *Annu. Rev. Microbiol.* **61**:477–501.