Identification and Characterization of the Structural and Transporter Genes for, and the Chemical and Biological Properties of, Sublancin 168, a Novel Lantibiotic Produced by Bacillus subtilis 168*

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An antimicrobial peptide produced by Bacillus subtilis 168 was isolated and characterized. It was named sublancin 168, and its behavior during Edman sequence analysis and its NMR spectrum suggested that sublancin is a dehydroalanine-containing lantibiotic. A hybridization probe based on the peptide sequence was used to clone the presublancin gene, which encoded a 56-residue polypeptide consisting of a 19-residue leader segment and a 37-residue mature segment. The mature segment contained one serine, one threonine, and five cysteine residues. Alkylation of mature sublancin showed no free sulfhydryl groups, suggesting that one sulfydryl had formed a β-methylanthionine bridge with a dehydrobutyrine derived by posttranslational modification of threonine; with the other four cysteines forming two disulfide bridges. It is unprecedented for a lantibiotic to contain a disulfide bridge. The sublancin leader was similar to known type AII lantibiotics, containing a double-glycine motif that is typically recognized by dual-function transporters. A protein encoded immediately downstream from the sublancin gene possessed features of a dual-function ABC transporter with a proteolytic domain and an ATP-binding domain. The antimicrobial activity spectrum of sublancin was like other lantibiotics, inhibiting Gram-positive bacteria but not Gram-negative bacteria; and like the lantibiotics nisin and subtilin in its ability to inhibit both bacterial spore outgrowth and vegetative growth. Sublancin is an extraordinarily stable lantibiotic, showing no degradation or inactivation after being stored in aqueous solution at room temperature for 2 years. The fact that sublancin is a natural product of B. subtilis 168, for which a complete sequence of its genome, suggests that sublancin will be an especially good model for studying the potential of lantibiotics as sources of novel biomaterials.

Lantibiotics are bacterially produced antimicrobial peptides that possess unique chemical and biological properties owing to their containing a variety of unusual amino acid residues. Lantibiotics are defined as such by the presence of lanthionine or β-methyllanthionine, which are introduced by a posttranslational process in which serine or threonine is dehydrated to the corresponding dehydro residue, which then reacts in a Michael-type addition of a cysteine sulfhydryl group to the double bond of the dehydro residue to form a thioether link (reviewed in Refs. 1–6). Mature lantibiotics typically contain one or more dehydro residues that do not participate in lanthionine bridges. The unique properties that are conferred by these unusual residues may result in their being useful components in the design of novel biomolecules (1, 2, 7, 8).

One of the attractive features of lantibiotics is that they are comprised of gene-encoded polypeptide sequences, so their structures can be manipulated by protein engineering. Whereas this is simple in concept, putting it into practice requires the utilization of many different genetic and recombinant DNA techniques, including the removal and replacement of chromosomal segments with their genetically engineered counterparts. Ideally, these manipulations need to be done in such a way that the engineered lantibiotic analog be efficiently produced so that useful amounts of the analog are available for experimentation, which implies a need to engineer regulatory elements. Only a few bacterial strains have been sufficiently characterized to permit these manipulations to be performed in a convenient and facile manner. One such well characterized bacterial strain is Bacillus subtilis, which is second only to Escherichia coli in the extent to which tools of genetic and protein engineering have been developed, which has contributed to the extensive use of B. subtilis 168 for the industrial production of bio-engineered materials. The advantage of B. subtilis 168 over other bacterial strains has recently been increased even more by the availability of the complete sequence of the B. subtilis 168 genome (9).

It is in this context that we report the discovery of a new lantibiotic, which we have named sublancin 168, that is a natural product produced by B. subtilis 168. Although approximately 20 lantibiotics are already known, the fact that this new lantibiotic is endogenous to B. subtilis 168, and thus can be studied and manipulated using the powerful methods that are available in this strain, suggests that progress in our understanding of lantibiotics will be accelerated by our ability to study and manipulate sublancin and the genes associated with its production in its natural B. subtilis 168 host. In addition to this practical aspect of the discovery, sublancin 168 has structural features and physical properties, such as the presence of disulfide bridges and extraordinary stability, that are unprecedented among the known lantibiotics.

MATERIALS AND METHODS

Bacterial Strains, Cloning Vectors, and Culture Conditions—Sublancin was isolated from B. subtilis BR151, which is B. subtilis 168 lys-3 metB10 tryC2), obtained from the Bacillus Genetics Stock Center, Ohio
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Sublancin was produced by inoculating 1 liter of medium A with 10 ml of BR151 cultured for 16 h at 37 °C with vigorous aeration. Medium A is as described previously (10, 11), except it contained 2% sucrose instead of 5%. The culture was agitated at 200 rpm and harvested by using a 10-fold dilution of an exponential culture of cells in tubes containing growth medium together with different concentrations (5, 10, 25, 50, or 100 µg/ml) of sublancin, which were incubated with shaking at 37 °C for 18–30 h, until the respective control cultures without sublancin reached saturation. The MIC was that concentration of sublancin that completely inhibited the growth of the cells.

Amino Acid Sequence and Composition Analysis—Purified sublancin was sequenced from its N-terminal end using Edman degradation, using an Applied Biosystems (Foster City, CA) model 477A peptide sequencer with an on-line HPLC analyzer in the University of Maryland Core Facility (Baltimore, MD). Amino acid composition analysis was performed on HCl hydrolysates by Commonwealth Biotechnologies, Inc. (Richmond, VA). Sublancin was treated with ethanethiol in order to sequence through any dehydro residues, which are otherwise blocked, using the method of Meyer et al. (13). The modification mixture consisted of 280 µl of ethanol, 200 µl of sterile deionized water, 65 µl of NaOH, and 60 µl of ethanethiol. 150 µl of this modification mixture was added to 50 µl of freeze-dried sublancin and incubated under nitrogen for 1 h at 50 °C. The pH was lowered by addition of 5 µl of glacial acetic acid, and the product purified by HPLC as described above for sublancin.

NMR and Mass Spectral Analysis—One-dimensional NMR spectroscopy was performed with a Bruker AMX-500 NMR spectrometer interfaced to an Aspect 3000 computer using UXNMR software. Lyophilized sublancin was dissolved in 99.96% atom% D_2O to exchange protons and lyophilized (done twice) and dissolved in D_2O to a final concentration of 10 mg/ml. The proton spectra were recorded at constant 295 K in D_2O with and without the suppression of the water solvent resonance. Mass spectral analysis was performed by PeptidoGenic Research & Co (Livemore, CA) on a Sciex API I Electrospray mass spectrometer. The reported masses are those calculated as the most probable values based on the different m/z forms.

Cloning of the Sublancin Gene—A B. subtilis 168 genomic library was constructed in bacteriophage λ using total chromosomal DNA from strain BR151 grown in 50 ml of Penassay broth. Cells were lysed with a mixture of lysozyme, sodium dodecyl sulfate, and protease K; the DNA was recovered and deproteinized with phenol-chloroform as described previously (11). The genomic DNA was partially digested with HindIII and then sheared using a Gene Splicer (Bio-Rad). The DNA was cloned into LambdaGEM-12 partially filled-in vector using the protocol provided by the manufacturer. The library was screened for the sublancin gene using synthetic DNA oligomers whose sequences were chosen using the strategy of Lathe (14), based on the 16-residue N-terminal sequence of sublancin. Three single-sequence probes were designed, each one with randomly chosen degenerate bases, and the synthesis was performed by Ransom Hill Bioscience (Ramon, CA). For those amino acid residues that appeared as unidentifiable blanks in the sequence, inosines were placed in the corresponding codons in the probes. The three probes were: probe 1, GGGTTGGAATACCCAAGTTTCTACAGTTTACAGAGCTTTCC, probe 2, GGGTTGGAATACCCAAGTTTCTACAGTTTACAGAGCTTTCC, probe 3, GGGTTGGAATACCCAAGTTTCTACAGTTTACAGAGCTTTCC.

The probes were radiolabeled with ³²P at their 5’ ends using T4 polynucleotide kinase and hybridized to Southern blots of restriction digests of BR151 genomic DNA under a variety of temperature and ionic strength conditions in order to optimize the signal strength and specificity. Probe 1 gave a good signal when hybridized at 45 °C in 6x SSC and washed at 37 °C in 2x SSC, whereas probe 3 gave a good signal when hybridized at 45 °C in 6x SSC and washed at 45 °C in 2x SSC. A good signal for probe 2 could not be obtained, so its use was abandoned. The bacteriophage λ library was plated and transferred to duplicate nitrocellulose filters using standard procedures (15). One of the duplicate filters was exposed to probe 1, and the other to probe 3. The only plaques selected for further study were those that hybridized to both probes. Several such dual-hybridizing plaques were picked, and their inserts were subcloned into pT7 plasmids and screened again with probes 1 and 3. Positive inserts were cloned into M13 and subjected to dideoxy sequence analysis. The DNA sequences were conceptually translated into six reading frames, which were searched for the N-terminal amino acid sequence of sublancin. When the sublancin se
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Isolation of Sublancin 168—Our first observation of sublancin was when it appeared as a contaminant in acetone-butanol extracts of culture supernatants of B. subtilis LH45, which is a strain of B. subtilis 168 that has been genetically engineered to produce subtilin (16). When the acetone precipitate was dissolved in water and analyzed by reversed-phase HPLC, a peak that contained antimicrobial activity that emerged earlier than subtilin was observed. Its early emergence indicated that it is more hydrophilic than subtilin, but its appearance in the acetone precipitate suggested that it had subtilin-like physical properties. Several characteristics of the wild-type B. subtilis 168 strain from which LH45 had been derived suggested that wild-type B. subtilis 168 harbors an endogenous lantibiotic. 2 The possibility that this contaminating activity might be a new lantibiotic prompted its isolation and characterization.

To obtain this putative lantibiotic, a strain of B. subtilis 168 was cultured as described under “Materials and Methods,” and the active material was recovered from the supernatant using a hydrophobic interaction column and purified to near-homogeneity on a reversed-phase HPLC column, as shown in Fig. 1. The active peak showed absorbances at 214, 254, and 280 nm; when the active peak was treated with ninhydrin, it gave the purple color that is characteristic of proteins and peptides. The antimicrobial substance was named sublancin 168, to connote its being an antimicrobial peptide that is produced by B. subtilis 168.

Structure Analysis of Sublancin 168—The sublancin peptide was subjected to N-terminal sequence analysis using Edman degradation. It gave a sequence of Gly-Leu-Gly-Lys-Ala-Gln—sequence found, the actual DNA sequence that encoded the sublancin gene could be identified, which provided the sequence information needed to synthesize probes that were exactly homologous to the sublancin gene. These were used to identify library clones that contained sequences that surrounded the subtilin gene, which were then also subcloned and sequenced.

RESULTS

The putative functions of the upstream and downstream ORFs were explored by searching the GenBank/EMBL nucleotide data bases for homologies to proteins with known functions. The 332-residue upstream ORF (denoted wrxX) showed extensive homologies to proteins involved in repair of uv damage to DNA, so a role in the biosynthetic pathway of sublancin seems unlikely. The 560-residue segment of the downstream ORF showed homologies to known ABC transporter proteins including PepT, which is the transporter that is responsible for secretion of PepG during its biosynthesis (19). The gene for this downstream ORF (denoted sunT) is therefore a strong candidate as the corresponding transporter that participates in the secretion of sublancin. Fig. 3 shows the segment of the DNA sequence that contains the sublancin gene (sunA), and the 5-prime end of sunT, together with their conceptual translations (SunA and the N-terminal portion of SunT), the putative promoter region of the sun operon, and the ribosome binding site of the SunA mRNA. The complete sequences and their conceptual translations are available as accession number AF069294 in GenBank.

If sublancin 168 is a lantibiotic, then SunA is presublancin, and accordingly should contain structural features that are similar to known prelantibiotics. Fig. 4 compares presublancin typically found in lantibiotics. For example, lanthionine residues do not produce peaks that are identifiable as normal amino acids, and the dehydro residues block the sequence analysis because they spontaneously lose their N-terminal amino group and are therefore unable to react with the Edman reagent (17), thus bringing the sequence analysis to a halt. This dehydro-residue block can be alleviated by reacting the peptide with ethanethiol, which adds across the double bond, thus preventing loss of the N-terminal amino group (13). Sublancin was accordingly derivatized with ethanethiol, whereupon it was possible to sequence past the apparent block at position 16, and to obtain Gly both at positions 17 and 18; however, a blank was then encountered at position 19. The fact that ethanethiol derivatization alleviated the block at position 16 is strong evidence that residue 16 is a dehydro residue.

Cloning and Sequence Analysis of the Sublancin Gene—Since lantibiotics are biosynthesized from gene-encoded precursors, one approach to determine if sublancin is a lantibiotic is to see if it is gene-encoded, and if it is, to examine the gene and the operon in which it is found to see if they possess features that are characteristic of lantibiotics. To determine whether sublancin is a gene-encoded peptide, the N-terminal sequence was used to design a hybridization probe, which was then used to screen a B. subtilis 168 genomic library that had been constructed in bacteriophage λ. Clones containing positive signals were subjected to DNA sequence analysis. The probe design, screening, and sequencing are described under “Materials and Methods.” Nearly 5 kb of sequence was obtained, which we published in a public data base as soon as it was complete (GenBank accession no. AF014938 (1997)). This sequence was searched for open reading frames (ORFs), which were in turn searched for the N-terminal amino acid sequence of sublancin 168. A 56-residue ORF, shown in Fig. 2, that contained a perfect match to the N-terminal sequence of the sublancin peptide was found near the center of the 5-kb sequence. In addition, a 332-residue ORF was found upstream from the sublancin gene, and about 560 residues of a partially complete ORF was found downstream from the sublancin gene. The locations of these three ORFs within the 5-kb sequence are shown in Fig. 2. Several months after our sequence was published in GenBank, the Bacillus Genome Project published the complete B. subtilis 168 genome (9), which mapped these genes at a position of 193.8° on the B. subtilis 168 chromosome.

2 A. Chakicherla and J. N. Hansen, unpublished observations.
with the type A lantibiotics, which are divided into two subtypes, AI and AII. The type A lantibiotics include those that are the most thoroughly studied, such as nisin A, subtilin, epidermin, and Pep5. Type A lantibiotics are characterized by being elongated and cationic with molecular masses ranging from 2151 to 4635 Da (1). The mature region of the sublancin peptide is cationic, and its predicted molecular mass is approximately 3900 Da (depending on what posttranslational modifications have occurred), and thus possesses characteristics of a type A lantibiotic. Type AI and type AII lantibiotics differ in their leader segments, with the AII leaders containing a GA/GS/GG (“double-glycine”) sequence motif immediately preceding the.

FIG. 2. Sequence of pre-sublancin 168. The conceptual translation of the 56-residue ORF in the middle of the 5-kb sequence is shown, cleaved into a 19-residue leader segment, and a 37-residue mature segment. The N-terminal end of the mature segment is a perfect match to the N-terminal amino acid sequence of the HPLC-purified sublancin shown in Fig. 1. Serine, threonine, and cysteine residues which are candidates for posttranslational modification during maturation are shown in an enlarged font. The wuV gene encodes an ORF with homologies to proteins involved in UV repair, and is therefore presumed to be unrelated to sublancin biosynthesis. The sunT gene encodes the pre-sublancin polypeptide as indicated, followed by a non-coding region, and then sunT gene, which is the putative sublancin transporter as described in the text and in Figs. 3 and 4. The direction of transcription of these genes is indicated by the horizontal arrows. P indicates the location of a consensus prokaryotic promoter site as described in the legend of Fig. 3.

FIG. 3. Nucleotide sequence of the sublancin gene. The sublancin gene (sunA) and the 5’-end of the sunT gene are shown, along with their conceptual translations. Homologies of the N-terminal end of SunT to PepT and LcnDR3, which are lantibiotic and dual-function transporters, respectively, are shown in Fig. 5. The TatATT (~10) and TIGAAT (~35) regions show strong homology to the consensus prokaryotic promoter (35), and is the likely promoter of the sun operon. The GGAGG identified as rbs is a standard prokaryotic consensus ribosome binding site that is appropriately located to be functional. The sunT gene encodes an unusual UUG initiation codon that can be identified by the purine-rich Shine-Dalgarno sequence (18) that is underlined. This may result in the sunT mRNA being translated less efficiently than the sunA mRNA. The complete coding sequences of the sunA and sunT genes and their conceptual translations are available as Accession Number AF069294 in GenBank.

FIG. 4. Alignment of pre-sublancin with type AI and type AII pre-lantibiotics. Figure shows conserved leader segments of AI and AII as identified by Nes and Tagg (3). Sublancin shows homologies that are characteristic of type AII lantibiotics, including the “diglycine motif” found in leaders that are normally cleaved by dual-function transporters that contain a leader peptidase function (20), as described under “Results.”
For sublancin to be a lantibiotic, it should contain at least one lanthionine residue, either Lan or MeLan; and at least one dehydro residue, either Dha formed from serine, or Dhb formed from threonine. The putative mature region of sublancin contains only one serine (residue 16), and one threonine (residue 19). For sublancin to contain at least one dehydro residue and one lanthionine residue would require that both the Ser\textsuperscript{16} and Thr\textsuperscript{19} be converted to Dha and Dhb, respectively, and for one of them to form a cross-linkage with a cysteine, and for the other to remain as a dehydro residue. These possibilities can be distinguished by NMR spectroscopy. Both Dha and Dhb contain vinyl protons, which typically give resonance peaks in the δ = 5.2–6.9 ppm region of the NMR spectrum, with Dha appearing as a doublet, and Dhb appearing as a quartet (7, 21–23). The NMR spectrum of sublancin is shown in Fig. 6. A portion of the NMR spectrum shows a doublet appearing at δ 6.2 ppm, which is in the middle of the vinyl proton region, and therefore argues that a dehydro residue is present, and its being a doublet further argues that it is a Dha. The other peaks are in the aromatic proton region (δ = 6.5–8.0), and can be attributed to the aromatic residues in sublancin. It is to be noted that the Edman degradation of native sublancin was blocked from residue 16 on, and this block was alleviated by reacting with ethanethiol, which is also consistent with residue 16 being a dehydro residue. Since the gene sequence shows a Ser at position 16, one can conclude that the Dha shown in the NMR spectrum is due to one or more additional modifications of the amino acids, due to one or more additional modifications of the amino acids, as considered under “Discussion.”

**Biochemical Properties of Sublancin**—For sublancin to be a typical lantibiotic, it should contain at least one lanthionine residue, either Lan or MeLan; and at least one dehydro residue, either Dha formed from serine, or Dhb formed from threonine. The putative mature region of sublancin contains only one serine (residue 16), and one threonine (residue 19). For sublancin to contain at least one dehydro residue and one lanthionine residue would require that both the Ser\textsuperscript{16} and Thr\textsuperscript{19} be converted to Dha and Dhb, respectively, and for one of them to form a cross-linkage with a cysteine, and for the other to remain as a dehydro residue. These possibilities can be distinguished by NMR spectroscopy. Both Dha and Dhb contain vinyl protons, which typically give resonance peaks in the δ = 5.2–6.9 ppm region of the NMR spectrum, with Dha appearing as a doublet, and Dhb appearing as a quartet (7, 21–23). The NMR spectrum of sublancin is shown in Fig. 6. A portion of the NMR spectrum shows a doublet appearing at δ 6.2 ppm, which is in the middle of the vinyl proton region, and therefore argues that a dehydro residue is present, and its being a doublet further argues that it is a Dha. The other peaks are in the aromatic proton region (δ = 6.5–8.0), and can be attributed to the aromatic residues in sublancin. It is to be noted that the Edman degradation of native sublancin was blocked from residue 16 on, and this block was alleviated by reacting with ethanethiol, which is also consistent with residue 16 being a dehydro residue. Since the gene sequence shows a Ser at position 16, one can conclude that the Dha shown in the NMR spectrum is due to one or more additional modifications of the amino acids, as considered under “Discussion.”

**Comparison of SunT to other bacteriocin ABC-transporter proteins.** The N-terminal end of SunT is compared with the N-terminal end of LcnDR3, the lactococcin DR transporter (20), and the N-terminal end of PepT, the ABC-transporter that exports the lantibiotic Pep5 during biosynthesis (19). Pep5 is a type AI lantibiotic (non-diglycine leader), and its transporter does not possess a N-terminal proteolytic domain. However, the C-terminal ATP-binding domain of PepT shows strong homology to SunT (not shown). LcnDR3 does possess an N-terminal proteolytic domain, and a homologous counterpart appears in SunT, including the conserved histidine and cysteine regions (identified by enclosing boxes and by stars) that are part of the active site of the proteolytic domain (20). The GenBank accession numbers of SunT, LcnDR3 (also called LctT), and PepT are AF069294, U91581, and Z49865, respectively.

**Analysis of Disulfide Cross-linkages in Sublancin**—The sublancin prepeptide contains five cysteine residues, which is the same number of cysteines as are present in the prepeptides of nisin and subtilin. However, in nisin, subtilin, and all other...
known lantibiotics, all of the cysteine residues are converted to unusual residues such as the five Lan and MeLan in nisin (24) and subtilin (11), or the aminovinylcysteine in epidermin (25). For a natural lantibiotic to contain unmodified cysteines or disulfide cross-linkages is unprecedented, so the cysteine residues in sublancin were examined to see if any possessed the characteristics of either free sulfhydryl groups or disulfide bridges. The amino acid analysis that was employed cannot detect free cysteine residues, but can detect them as carboxymethyl-cysteine if they are alkylated prior to acid hydrolysis. Alkylation of native sublancin followed by amino acid analysis gave no detectable carboxymethyl-cysteine, which rules out the presence of free sulfhydryl groups (data not shown). Reduction of sublancin with dithiothreitol followed by alkylation gave 3.3 (suggesting a real value of 4, since the 3.3 is likely a minimum value, and the nearest integer value larger than 3.3 is 4) carboxymethyl-cysteines/mol of sublancin. SDS-PAGE and ion-spray mass spectroscopy results described above established that sublancin exists exclusively as a monomer, so there cannot be any intermolecular disulfide bridges. These observations are all consistent with four of the cysteines of sublancin participating in two disulfide bridges, with the fifth cysteine having been converted to a MeLan residue by reacting with a Dhb residue (derived from post-translational dehydration of Thr19), leaving the unreacted Dha16 that is revealed in the NMR spectrum.

The number and location of disulfide bridges was further explored by analysis with proteolytic enzymes. The native form of sublancin, and the denatured form, and the denatured-reduced form of sublancin were all resistant to trypsin, despite the presence of a Lys at position 4 and an Arg at position 33. When the denatured and reduced sublancin was alkylated, trypsin cleavage gave detectable amounts of fragments of 3200 and 1581 Da, neither of which is an expected product. Sublancin was more sensitive to chymotrypsin, with even the native molecule being substantially degraded, to give products of 1392 and 1823 Da. The first is consistent with a polypeptide consisting of residues 1–11 being cross-linked by a disulfide bridge to a peptide consisting of residues 36 and 37 (G1–W11-S-S-C36–R37, with an expected value of 1392 Da), and the second is consistent with a polypeptide consisting of residues 1–11 cross-linked by a disulfide bridge to a peptide consisting of residues 33–37 (G1–W11-S-S-R33–R37, with an expected value of 1,823 Da); with chymotrypsin having cleaved at typical major cleavage sites (Try11, Tyr12, Phe36). From this, we can conclude that native sublancin has a disulfide bridge between Cys7 and Cys36. To decide upon the location of the second disulfide bridge, we compare sublancin to other type A lantibiotics, and note that formation of a thioether link between Cys22 and Dhb19, to give a Aba19-Ala22 MeLan-type cross-linkage would put a two-residue Gly20-Gly21 sequence in the ring enclosed by the MeLan cross-link, which is similar to the two-residue Pro9-Gly10 sequence enclosed by the Aba8-Ala11 MeLan cross-link in both nisin and subtilin. Moreover, formation of this particular MeLan bond is consistent with the observation that the Cys-dehydro partner selection in lantibiotics consistently involves a dehydro residue that is on the N-terminal side of the Cys residue. Assuming that the MeLan that actually forms conforms to these standard patterns, then Cys22 will react with Dhb19 which would require the second disulfide bridge to form between Cys14 and Cys29, as shown in Fig. 7.

FIG. 6. Proton NMR spectrum of sublancin 168. The part of the spectrum that encompasses portions of the aromatic and vinyl proton regions is shown. The doublet resonance centered at δ = 6.2 is identified as Dha16 for reasons described under “Results.” There were no other peaks in the vinyl proton region. The peaks in the aromatic region are assumed to represent the aromatic residues in sublancin.

FIG. 7. The locations of thioether and disulfide bridges in sublancin. The position of the lanthionine residue and the pattern of disulfide bridge formation were inferred from the NMR spectrum, N-terminal amino acid sequence analysis, amino acid composition analysis, reaction with sulfhydryl-directed agents, proteolytic digestions, and conserved features among lantibiotics as described under “Results.” The representation of the sublancin structure as three open circles and a salt-bridge between the N-terminal amino group and the C-terminal carboxyl group is arbitrary, since no information about the secondary structure of sublancin is available.

Spectrum of Antimicrobial Activity of Sublancin 168—The lantibiotic family of antimicrobial peptides shows broad spectrum activity against Gram-positive bacteria, and very little activity against Gram-negative bacteria (1, 2). To see if sublan-
Antimicrobial peptides that are chemically stable are better suited for practical applications than are unstable ones. The chemical stability of sublancin was therefore assessed when it was an unpurified component of the

### Table I

Spectrum of activity of sublancin against exponentially growing Gram-positive and Gram-negative strains of bacteria

| Strains                      | Sensitivity to sublancin | MIC (µg/ml) |
|------------------------------|--------------------------|-------------|
| **Gram-positive strains**    |                          |             |
| Bacillus cereus T            | +                        | >100        |
| Bacillus megaterium (14581)  | ++                       | 5           |
| Bacillus subtilis (6633)     | +++                      | 5           |
| Enterococcus fecalis (19433) | -                        |             |
| Lactococcus lactis (11454)   | -                        |             |
| Listeria monocytogenes (15313)| -                       |             |
| Staphylococcus aureus (12600)| +                        | >100        |
| Staphylococcus epidermidis (12228)| +                    | 100         |
| Streptococcus pyogenes (49399)| +                       |             |
| **Gram-negative strains**    |                          |             |
| Bordetella bronchiseptica (10580)| -                    |             |
| Escherichia coli JM101       | -                        |             |
| Pseudomonas aeruginosa (10145)| -                       |             |
| Yersinia enterocolitica (23715)| -                     |             |

**Discussion:** The concentration of sublancin used to treat the spores prior to washing had no effect on the outcome of the recovery experiment, with the 0.1 µg/ml treatment showing the same effect as the 100 µg/ml treatment. This shows that the spore sites to which the sublancin become associated are saturated at very low levels of sublancin.
culture supernatant, and after it had been purified by HPLC chromatography. Activity was assessed using the agar-plate halo assay against bacterial spores. Culture supernatant stored at room temperature showed little change in halo size during the first 4 days, but showed significant loss after 1 week. Culture supernatants stored at either 4 °C or −20 °C showed no change in halo size after 6 months. HPLC-purified sublancin was remarkably stable, and one sample was stored as a 10 mg/ml solution of sublancin in sterile D₂O, pH 6.5, in an NMR tube for 2 years (protected from light), after which its activity remained undiminished and its NMR profile unchanged (data not shown). Sublancin was stable to a wide range of pH values when either phosphoric acid or ammonium acetate buffers were used to adjust the pH of culture supernatants over a range of 1.5–9.5. The samples were assayed after incubating them for 2 h at 4 °C. The pH 9.5 halo was diminished slightly, but the halos produced by the lower pH samples were unchanged. Finally, a sample of the culture supernatant that was autoclaved for 3 min at 121 °C showed undiminished activity. These stability characteristics resemble those of nisin, which is very stable at low pH and can survive autoclaving at pH 2.5 without damage, but is fairly unstable above pH 7 (31). However, the ability of sublancin to survive in aqueous solution, at a pH that is nearly neutral, for 2 years without any apparent chemical or biological degradation shows that it is a peptide whose intrinsic stability is extremely high. This extraordinary stability may prove to be a useful characteristic, perhaps enhancing the utility of sublancin in practical applications, or as a model compound whose study may inspire strategies for enhancing the stabilities of non-sublancin antimicrobial peptides.

**DISCUSSION**

The evidence that sublancin 168 is a lantibiotic is strong. The presublancin gene sequence encodes a serine residue at position 16 of the mature region, which can serve as the precursor to dehydroalanine. Sequential Edman degradation was blocked at position 16, which is characteristic of dehydro residues. As has been demonstrated for dehydro residues in other lantibiotics, the block was alleviated by derivatization with ethanethiol. The NMR spectrum of sublancin showed a doublet in the vinyl proton region of the spectrum, which is consistent with the presence of a dehydroalanine. Sequence analysis of the sublancin gene showed a leader segment with homologies to known type II lantibiotics, including the “double-glycine” sequence motif immediately preceding the cleavage site, indicating that it is probably translocated by a dual-function ABC transporter that both translocates the peptide and proteolytically cleaves the leader segment. The gene immediately downstream from the sublancin gene confirms this, in that it encodes a protein that is homologous to known dual-function transporters, with an identifiable proteolytic domain in addition to a transporter domain. Although the presublancin gene encodes five cysteines, reaction of sublancin with an alkylating agent failed to demonstrate the presence of a free sulfhydryl group, which is consistent with at least one of the cysteines having reacted with a dehydrobutyryne residue to form a β-methyllanthionine bridge. The spectrum of activity of sublancin is similar to other lantibiotics in that it is active against a variety of Gram-positive bacteria and inactive against Gram-negative bacteria. It also showed strong inhibition of bacterial spore outgrowth in addition to inhibition of exponentially growing cells, as is seen with both nisin and subtilin. However, unlike nisin and subtilin, washing sublancin-inhibited spores could cause a small percentage (about 1%) of them to proceed through outgrowth and then grow vegetatively, suggesting that the inhibitory effect of sublancin against spores is slightly reversible. For both nisin and subtilin, it has been demonstrated that the mechanism of inhibition of spore outgrowth is different from the inhibition of vegetative growth, in that an intact dehydroalanine is required for spore outgrowth inhibition, but not for vegetative growth inhibition. The fact that sublancin contains only one dehydro residue compared with the three dehydro residues in nisin and subtilin may account for sublancin showing reversibility of inhibition of spore outgrowth, whereas nisin and subtilin do not. It has been suggested that the dehydro residue can react with a nucleophilic target (2, 27, 29), in which case the larger number of possible attachment points of nisin and subtilin could reduce the likelihood of dissociation and reversal of inhibition, although this explanation is hypothetical.

With this report of the discovery and characterization of sublancin 168, the family of known lantibiotics increases in both size and scope, and there are now over 20 known lantibiotics (2). A striking feature of lantibiotics is their diversity in terms of structure, chemical properties, and biological properties (1, 2). The defining characteristic of lantibiotics is that they contain the unusual amino acid lanthionine or β-methyllanthionine, which are formed by posttranslational dehydration of serine or threonine, respectively, followed by a Michael-type nucleophilic addition of a cysteine sulfhydryl across the double bond. Because of this mechanism, the presence of the lanthionine requires that the cell possess the machinery to dehydrate serines and/or threonines in addition to the ability to form the thioether linkage. Reflecting this, all the currently known lantibiotics possess at least one lanthionine and one dehydro residue in the mature peptide, although there is little reason to believe that exceptions to this are impossible. Especially notable is that, prior to our discovery of sublancin, all the cysteine residues in known lantibiotics had undergone posttranslational modifications, and never existed as disulfide bridges or free sulfhydryl groups. Sublancin breaks this trend in that only one of its five cysteines has been posttranscriptionally modified, and the other four cysteines instead participate in two disulfide bridges.

Lantibiotics can be considered as a subset of the prodigious number of ribosomally synthesized antimicrobial peptides that have been discovered recently, many of which are produced by eukaryotic organisms, such as the defensins and cercepins (1, 32). Mammalian and insect defensins, tachyplesins, and plant thionins all tend to be disulfide-rich, typically containing two or three disulfide bridges within a peptide consisting of 30–40 amino acid residues (33). The ubiquity and frequency of disulfide bridges argues an important role, perhaps by their ability to impose conformational constraints on the peptide and contribute to conformational and chemical stability. Because the thioether of the lanthionine bridge contains one sulfur atom instead of two, the lanthionine would be expected to be more conformationally constrained than the disulfide. Moreover, the lanthionine is insensitive to redox conditions, while the disulfide is easily broken under mild reducing conditions. In view of the apparent superiority of the lanthionine bridge in terms of conformational and chemical stability, it is somewhat surprising that sublancin contains one lanthionine and two disulfides, instead of the three lanthionines and no disulfides that are found in other lantibiotics such as subtilin, which is produced by *B. subtilis* ATCC 6633, and nisin. The fact that sublancin possesses both types of linkages suggests that having both types confers a selective advantage. It has been observed that antimicrobial peptides represent a remarkable example of convergent evolution, in which a wide variety of organism types have evolved antimicrobial peptides of common function from very different ancestral origins (33). Perhaps sublancin repre-
sents a converging evolutionary branch-point between prokary-
otic lantibiotics and eukaryotic defensins, in which sublancin
takes advantage of both types of linkages.

One aspect of the sublancin structure that this work does not
resolve is the reason why the molecular mass of sublancin, as
determined by ion-spray mass spectroscopy, is 164.48 Da
greater than expected from the amino acid composition. This is
very likely due to an unidentified posttranslational modifica-
tion. A precedent for additional posttranslations is seen in
sublancin for two reasons. One is that succinylation blocks the
N terminus against Edman degradation (8, 34), but sublancin
is not blocked. A second reason is that the succinyl group
should increase the molecular mass by only 100 Da instead of
164.48 Da. Our results that provided molecular masses of sub-
lancin fragments after chymotrypsin degradation establish
that the modification must occur at a residue that lies between
Trp11 and Arg33, because the disulfide cross-linked chymotryp-
tic fragment consisting of (G1–W11-S-S-R33–R37) has a molecu-
lar mass that is exactly that predicted from the amino acid
composition. The molecular mass discrepancy therefore must
come from the modification of one (or more) of the residues that
resides outside this fragment.

REFERENCES
1. Hansen, J. N. (1993) Annu. Rev. Microbiol. 47, 535–564
2. Hansen, J. N. (1997) in Bio/Technology of Antibiotics (Strohl, W. R., ed) 2nd
Ed., pp. 437–470, Marcel Dekker, Inc., New York
3. Nes, I. F., and Tagg, J. R. (1996) Antonie Van Leeuwenhoek 69, 89–97
4. Sahl, H.-G. (1994) Ciba Found. Symp. 186, 27–53
5. Sahl, H. G., Jack, R. W., and Bierbaum, G. (1995) Eur. J. Biochem. 230,
827–855
6. de Vos, W. M., Kuipers, O. P., Vandermeer, J. R., and Siewen, R. J. (1995) Mol.
Microbiol. 17, 427–437
7. Liu, W., Hansen, J. N. (1992) J. Biol. Chem. 267, 25078–25085
8. Chakicherla, A., and Hansen, J. N. (1995) J. Biol. Chem. 270, 23533–23539
9. Kunst, F., Ogawara, N., Moszer, I., Albertini, A. M., Alloni, G., Azevedo, V.,
Bertero, M. G., Bessieres, P., Bolotin, A., Borchert, S., Borris, R., Boursier,
L., Brans, A., Braun, M., Brignell, S. C., Bron, S., Broutillet, S., Bruschi,
C. V., Caldwell, B., Capuano, V., Carter, N. M., Choi, S. K., Codani, J. J.,
Connerton, I. F., Danchin, A., et al. (1997) Nature 390, 249–256
10. Feeney, R. E., Garibaldi, J. A., and Humphreys, E. M. (1948) Arch. Biochim.
Biophys. 17, 435–445
11. Banerjee, S., and Hansen, J. N. (1988) J. Biol. Chem. 263, 9508–9514
12. Vary, J. C., and Halvorson, H. O. (1965) J. Bacteriol. 89, 1340–1347
13. Meyer, H. E., Heber, M., Eisermann, B., Korte, H., Metzger, J. W., and Jung,
G. (1994) Anal. Biochem. 223, 185–190
14. Luthe, R. (1985) J. Mol. Biol. 183, 1–12
15. Maniatis, T., Fritsch, E. F., and Sambrook, J. (1982) Molecular Cloning: A
Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor,
NY
16. Liu, W., and Hansen, J. N. (1991) J. Bacteriol. 173, 7387–7390
17. Wakamiya, T., Ueki, Y., Shiba, T., Kido, Y., and Motoki, Y. (1985) Tetrahedron
Lett. 26, 665–668
18. Kozak, M. (1980) Microbiol. Rev. 47, 1–45
19. Meyer, C., Bierbaum, G., Heidrich, C., Reis, M., Suling, J., Iglesias-Wind, M. I.,
Kemper, C., Molitor, E., and Sahl, H.-G. (1995) Eur. J. Biochem. 232,
478–489
20. Havravstein, L. S., Diep, D. B., and Nes, I. F. (1995) Mol. Microbiol. 16, 229–240
21. Fisk, C. L. (1975) A Nuclear Magnetic Resonance Study of the Polypeptide,
Nisin, Its Fragments and α, β-Unsaturated Amino Acid Constituents. Ph.D.
thesis, Georgetown University, Washington, D. C.
22. Jones, A. J., Helmerhorst, E., and Stokes, G. B. (1983) Biochem. J. 211,
499–502
23. Asquith, R. S., and Carthew, P. (1972) Tetrahedron 28, 4769–4773
24. Buchman, G. W., Banerjee, S., and Hansen, J. N. (1988) J. Biol. Chem. 263,
16260–16266
25. Kukpe, T., Stevanovic, S., Sahl, H. G., and Gotz, F. (1992) J. Bacteriol. 174,
5354–5361
26. Cleeland, R., and Squires, E. (1991) in Antibiotics in Laboratory Medicine
(Lorian, V., ed) pp. 739–786, Williams & Wilkins, Baltimore, MD
27. Liu, W., and Hansen, J. N. (1993) Appl. Environ. Microbiol. 59, 648–651
28. Chan, W. C., Dodd, H. M., Horn, N., Maclean, K., Lian, L. Y., Bycroft, B. W.,
Gasson, M. J., and Roberts, G. C. (1996) Appl. Environ. Microbiol. 62,
2966–2969
29. Morris, S. L., Walsh, R. C., and Hansen, J. N. (1984) J. Biol. Chem. 259,
13590–13594
30. Buchman, G. W. (1988) Ph.D. thesis, University of Maryland, College Park,
MD
31. Liu, W., and Hansen, J. N. (1990) Appl. Environ. Microbiol. 56, 2551–2558
32. Hancock, R. (1997) Lancet 349, 418–422
33. Hancock, R., Falla, T. J. (1997) in Bio/Technology of Antibiotics (Strohl, W. R.,
ed) 2nd Ed., pp. 471–496, Marcel Dekker, Inc., New York
34. Chan, W. C., Bycroft, B. W., Leyland, M. L., Lian, L. Y., and Roberts, G. C.
(1993) Biochem. J. 291, 23–27
35. Rosenberg, M., and Court, D. (1979) Annu. Rev. Genet. 13, 319–353