APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Aug. 1996, p. 2966–2969 Vol. 62, No. 8
0099-2240/96/$04.00+0

Structure-Activity Relationships in the Peptide Antibiotic Nisin: Role of Dehydroalanine 5

W. C. CHAN,[1] H. M. DODD,[2] N. HORN,[2] K. MACLEAN,[1] L.-Y. LIAN,[3] B. W. BYCROFT,[1] M. J. GASSON,[2] AND G. C. K. ROBERTS[3]*

Department of Pharmaceutical Sciences, University of Nottingham, Nottingham,[1] Institute of Food Research, Norwich Research Park, Colney, Norwich,[2] and Department of Biochemistry, and Biological NMR Centre, University of Leicester, Leicester,[3] United Kingdom

Received 15 December 1995/Accepted 28 May 1996

A mutant of the peptide antibiotic nisin in which the dehydroalanine residue at position 5 has been replaced by an alanine has been produced and structurally characterized. It is shown to have activity very similar to that of wild-type nisin in inhibiting growth of *Lactococcus lactis* and *Micrococcus luteus* but is very much less active than nisin as an inhibitor of the outgrowth of spores of *Bacillus subtilis*. These observations, which parallel those of W. Liu and J. N. Hansen (Appl. Environ. Microbiol. 59:648–651, 1993) on the corresponding mutant of the related antibiotic subtilin, are discussed in terms of the mechanism(s) of action of these antibiotics.

The lantibiotics are a growing group of posttranslationally modified peptide antibiotics that characteristically have cyclic structures formed by lanthionine or 3-methyl-lanthionine residues and often also have dehydroalanine (Dha) and/or dehydrobutyrine residues (14). These natural products are currently attracting considerable interest, in terms both of their biosynthesis and of their wide range of biological activities. Nisin, produced by strains of *Lactococcus lactis* that carry a transposon containing genes coding for the nisin precursor and for proteins involved in nisin biosynthesis and resistance (1, 7, 15, 24), was the first member of this group of antibiotics to be characterized in detail. It has been quite widely used as a food preservative, notably in cheese and other dairy products and in canned vegetables, for some 30 years (13, 25). Nisin inhibits the growth of a wide range of gram-positive organisms and also inhibits the germination and/or outgrowth of spores of *Bacillus* and *Clostridium* species (13). It has been proposed to act by forming voltage-dependent pores in biological membranes (27, 28), but the details of its mechanism of action remain incompletely understood (20).

The structure of nisin, initially determined by chemical degradation (10) and subsequently confirmed by nuclear magnetic resonance (NMR) spectroscopy (6), is shown in Fig. 1; it can be seen to contain five rings formed by lanthionine or methyllanthionine residues, together with three dehydro amino acid residues: dehydrobutyrine 2, Dha5, and Dha33. Something is known of the importance of the two Dha residues for the growth-inhibitory effects of nisin from studies of breakdown products which have undergone hydrolytic cleavage at Dha5 and/or Dha33. Cleavage at Dha33 to yield nisin$^{1-32}$ has very little effect on biological activity, whereas the additional cleavage at Dha5, to yield [*des*-Dha5]-nisin$^{1-32}$, leads to a substantial loss of activity (3). It has not, however, been established whether this loss of activity is due to the loss of Dha5 per se or to the change in conformation consequent of the opening of ring A (16, 17).

* Corresponding author. Mailing address: Biological NMR Centre, University of Leicester, Medical Sciences Building, P.O. Box 138, University Rd., Leicester LE1 9HN, United Kingdom. Phone: 44-(0)116-252-5534. Fax: 44-(0)116-252-5526. Electronic mail address: gcr @le.ac.uk.

Liu and Hansen (18, 19) have studied the role of Dha5 in the activity of the closely related lantibiotic subtilin. They showed that replacement of Dha5 by alanine in the Glu-4-to-Ile mutant of subtilin had no effect on its action against vegetative cells of *Bacillus cereus* but abolished its inhibition of spore outgrowth. The observation of such different effects of this single substitution in the two assays clearly suggested that different mechanisms of action underlie these two effects of subtilin. In order to establish whether the existence of these dual mechanisms is a general feature of lantibiotics, we now report the isolation and characterization of a mutant of nisin in which the Dha5 residue has been replaced by an alanine (by means of a serine-to-alanine substitution in the precursor peptide).

MATERIALS AND METHODS

Production and purification of nisin Dha5A. Nisin Dha5A (with an alanine residue at position 5) was produced by means of an expression system in *L. lactis* FI7332, whose construction is described elsewhere (8). *L. lactis* FI7332 was grown to stationary phase in 500 ml of MCM medium. Nisin Dha5A was isolated from the culture supernatant by hydrophobic interaction chromatography on Merck Fractogel TSK 650S (22) and purified by reverse-phase high-pressure liquid chromatography (HPLC) on a Hypersil Pep100 $5C_{18}$ column (7 by 150 mm). The solvents used for elution from the reverse-phase HPLC column were 0.06% aqueous trifluoroacetic acid (solvent A) and 0.06% trifluoroacetic acid acetonitrile-water (9:1) (solvent B); elution was isocratic 75% A–25% B for 5 min, then a linear gradient to 35% B over 9 min, and finally a linear gradient to 37% B over 18 min. Nisin was purified by HPLC from a commercial preparation as previously described (6).

Characterization of nisin Dha5A. Amino acid analysis was performed by a pre-column derivatization procedure on an automated analyzer (model 420H; Applied Biosystems). Plasma desorption mass spectrometry was performed on a Bioson 20 spectrometer. One- and two-dimensional ^1H NMR spectra were obtained at 500 and 600 MHz with Bruker AM500 and AMX600 spectrometers (2, 3, 6, 16, 17); samples contained nisin or nisin Dha5A in 0.4 ml of ^2H_2O containing 0.1 M phosphate, pH 2.25.

Assays of biological activity. Assays of inhibition of growth of *L. lactis* MG1614 and of *Micrococcus luteus* NCDO8166 were carried out either by agar diffusion or by monitoring growth in suspension, which both gave similar results; the MICs quoted (means of duplicates) are those for inhibition of growth in suspension determined by a microtiter plate assay as recently described in detail (9). For determination of the effects on spore germination and outgrowth, strains of *B. subtilis* containing the *luxAB* genes under the control of the SASP2 promoter (12) were kindly provided by G. Stewart and P. Hill (Department of Applied Biochemistry and Food Science, University of Nottingham, Sutton Bonington, United Kingdom). Spores were prepared (23), and germination and outgrowth were initiated by heat shock, after which phase-contrast microscopy was performed, A_{600} was determined, and a luciferase assay was performed. The luciferase bioluminescence assay was carried out as described previously (12).

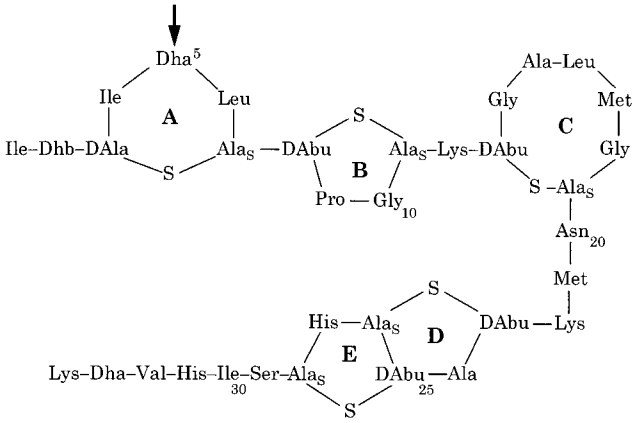

FIG. 1. Structure of nisin. Dha5 (Dha^5) is indicated in ring A. Dehydrobutyrine is abbreviated Dhb. *meso*-Lanthionine and 3-methyl-lanthionine residues are indicated by DAla—S—Ala$_s$ and DAbu—S—Ala$_s$, respectively (in which the amino-terminal moieties have the D configuration). Every fifth residue is numbered.

RESULTS

Production and structural characterization of nisin Dha5A. We have earlier described the construction and expression of the gene for the nisin mutant having an alanine residue at position 5 (nisin Dha5A) in *L. lactis* FI7332 (8). The peptide was isolated and purified from the culture supernatant of this strain by HPLC and was shown to be >98% pure by analytical HPLC. In view of the extensive posttranslational modifications involved in nisin biosynthesis, it was important to carry out a detailed chemical characterization of the isolated peptide to confirm its structural identity. Comparison of the results of amino acid analysis of nisin Dha5A (data not shown) with those for nisin demonstrated the presence of an additional alanine residue in nisin Dha5A; the results of the analysis were in satisfactory agreement with expected values for nisin Dha5A for all residues. Mass spectroscopic analysis (Fig. 2A) yields a mass corresponding closely to that expected for nisin Dha5A (MH^+ required, 3356.13; MH^+ found, 3356.3). The ^1H NMR spectrum (Fig. 2B) showed the absence of the characteristic olefinic proton resonance of Dha5, while the resonances of Dhb3 and Dha33 remained. The spectrum was analyzed in detail by two-dimensional experiments; a new methyl resonance (from Ala-5) was observed at 1.5 ppm, and the remainder of the spectrum was closely similar to that of nisin. Taken together, these data confirm that the isolated nisin Dha5A is pure and has the anticipated structure, that is, the posttranslational modifications have been carried out and it differs from nisin only in the substitution of alanine for Dha at position 5 (i.e., in having two additional hydrogen atoms).

Biological activity of nisin Dha5A. Two different biological activities of nisin Dha5A were evaluated. First, its ability to inhibit bacterial growth was assayed against a sensitive strain of *L. lactis* and against *M. luteus*. For *L. lactis*, the MIC of nisin Dha5A was 0.25 μg/ml, compared with a nisin value of 0.13 μg/ml, while for *M. luteus*, the MIC of both nisin and nisin Dha5A was 0.25 μg/ml (see also reference 9). Thus, the mutant is only slightly less active against *L. lactis* and is just as active as nisin in inhibiting the growth of *M. luteus*. It is clear that Dha5 is not essential for inhibition of vegetative bacterial growth by nisin.

As noted above, nisin is also active against spores of *Bacillus* spp. and *Clostridium* spp., although much less is known about this activity than about its bacteriocidal activity. We have studied the effects of nisin and nisin Dha5A on the germination and outgrowth of spores of *B. subtilis* using two simple assays which appear to be sensitive to two different stages of the process. A simple measurement of the sample turbidity can be used to monitor spore germination and outgrowth, as shown in Fig. 3A; in the control sample, germination is associated with a decrease in turbidity (over the first 30 to 50 min) while the later stages of outgrowth and the onset of cell division are associated with a marked increase in optical density. In the presence of 0.5 μg of nisin per ml, the turbidity initially decreases and then remains more or less constant until after ~150 min. Microscopy showed that in the presence of this concentration of nisin, there was little effect on germination but that the spores remained at the swollen, phase dark stage of early outgrowth. There was no loss in viability of the spore suspensions until after ~180 min. By contrast, the same concentration of nisin Dha5A had no effect on the time course of the changes in turbidity when compared with the control (Fig. 3A). In order to obtain information on the earlier stages of the germination-outgrowth process, we employed a system (12) in which luciferase is used as a reporter, the expression of the *luxAB* genes being under the control of the sporulation-specific SASP2 promoter. As shown in Fig. 3B, in this system the light output, reflecting luciferase activity, increases rapidly over the first 20 min, during the germination phase. Addition of 0.5 μg of nisin per ml had little effect on this initial increase, but while the level of light output remained constant in the control sample, it dropped rapidly in the presence of nisin. As seen in the turbidity assay, addition of 0.5 μg of nisin Dha5A per ml gave results identical to those with the control sample. In both spore assays, therefore, nisin Dha5A is inactive or at least much less active than nisin itself.

DISCUSSION

It is clear from the results presented here that the replacement of Dha5 in nisin by alanine has no effect on nisin's activity as an inhibitor of bacterial growth but does drastically decrease its activity level as an inhibitor of spore outgrowth. These observations closely parallel those reported for the Dha5A mutant of the related lantibiotic subtilin (19). The assays used by Liu and Hansen (19) were quite different from those employed in the present work—in particular they measured lysis of vegetative cells rather than growth inhibition—and the structure-activity relationships of nisin and subtilin show some differences (4, 5). The present report and that of Liu and Hansen (19) together provide strong evidence that nisin and subtilin have two distinct biological effects, inhibition of bacterial growth and inhibition of spore outgrowth, that these effects are based on distinct structure-activity relationships, and that these effects must therefore be produced by two distinct molecular mechanisms.

The presence of a Dha residue at position 5 is clearly not essential for inhibition of the growth of *L. lactis* or *M. luteus*. Earlier work showed that Dha33 could be removed without loss of nisin's ability to inhibit the growth of these organisms but that additional loss (by hydrolytic cleavage) of Dha5 led to a substantial decrease in the level of antibacterial activity (3). The present work shows that this decrease is not due to the absence of a Dha residue at position 5 but is due either to the absence of a side chain at this position or, more probably, to the effect of cleavage at this position on the conformation and flexibility of ring A. Nisin ring A is conformationally well defined (16, 17, 29), but in [*des*-Dha5]-nisin$^{1-32}$ there is much more flexibility, although the native conformation of ring A is still accessible (16, 17). As described previously (3), [*des*-

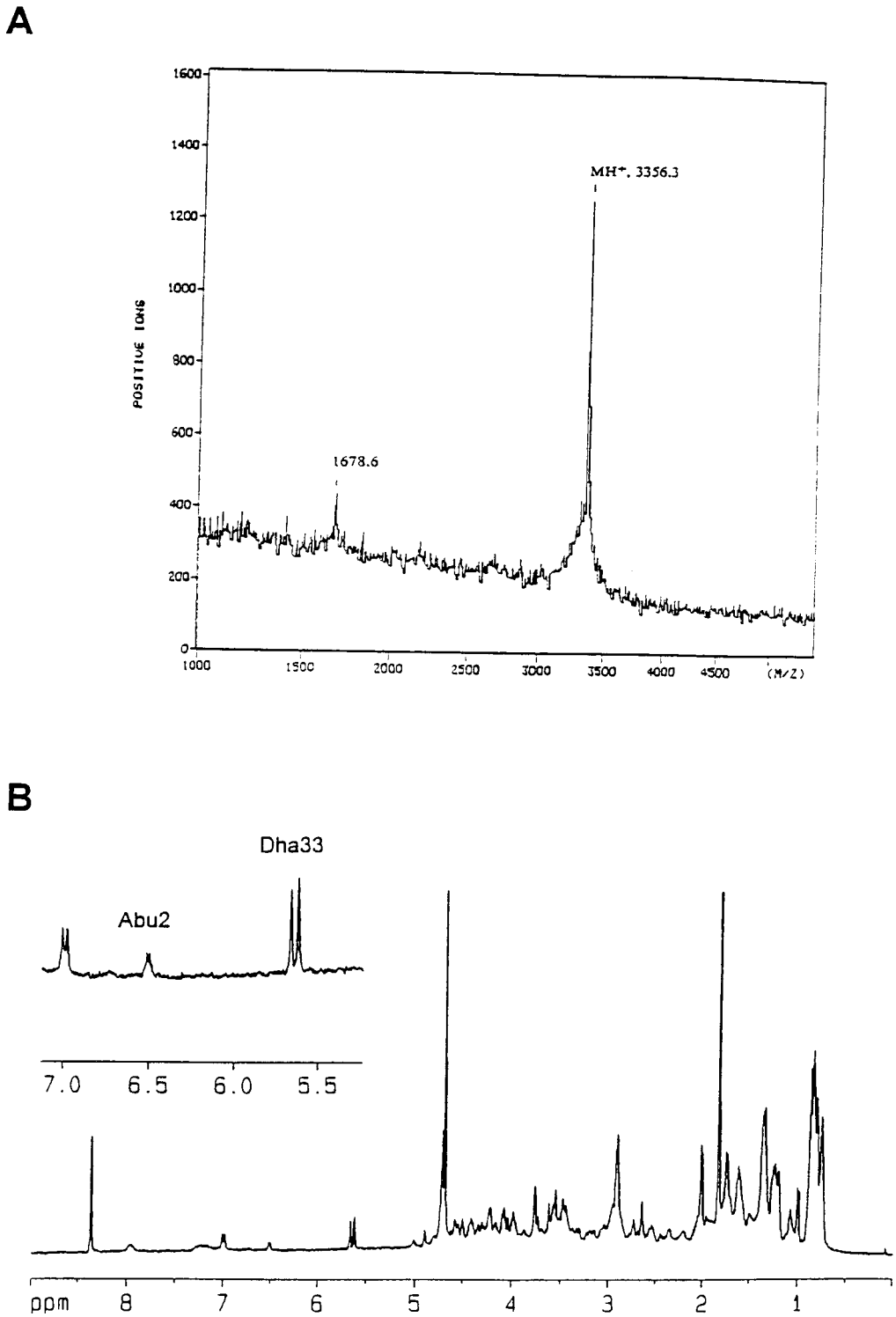

FIG. 2. Characterization of nisin Dha5A. (A) Mass spectrum of the nisin mutant Dha5A. (B) ^{1}H NMR spectrum (500 MHz) of the nisin mutant Dha5A (^2H_2O, pH ~2.5). The inset shows the olefinic proton region of the spectrum with the resonances of dehydrobutyrine 2 (Abu2) and Dha33 identified; in wild-type nisin, the olefinic proton resonances of Dha5 appear just to high field of that of Dha33 and are clearly absent in this spectrum.

Dha5]-nisin$^{1-32}$ is formed from nisin on prolonged storage or, much more rapidly, on heating in acid, and formation of this product appears to be the main reason for the loss of activity of nisin on storage. Nisin Dha5A would be expected to be much more stable on storage in terms of its bacteriocidal activity; the cleavage at Dha33 will presumably still occur, but this does not significantly change the activity.

However, nisin Dha5A has lost the ability to inhibit the

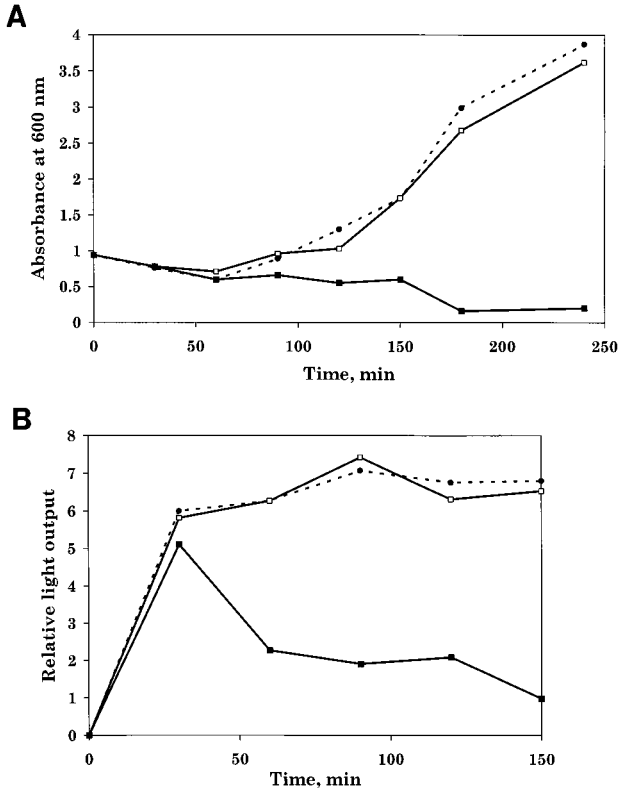

FIG. 3. Actions of nisin and nisin Dha5A on germinating spores of *B. subtilis*. (A) Turbidity changes during the germination and outgrowth of spores of *B. subtilis* alone (●---●) and in the presence of 0.5 μg of nisin (■—■) or nisin Dha5A (□—□) per ml. (B) Luciferase expression, under the control of the SASP2 promoter, during the germination and outgrowth of spores of *B. subtilis* alone (●---●) and in the presence of 0.5 μg of nisin (■—■) or nisin Dha5A (□—□) per ml.

outgrowth of bacterial spores. While it is widely accepted that the bactericidal effect of nisin is associated with the formation of pores in the cell membrane (20, 27, 28), virtually nothing is known about the mechanism of its effect (or that of subtilin [11, 19, 21]) on spore outgrowth. The existence of distinct structure-activity relationships for the two actions of nisin and subtilin will provide a valuable tool in further mechanistic investigations.

ACKNOWLEDGMENTS

This work was supported by the Biotechnology and Biological Sciences Research Council.

We are grateful to G. Stewart and P. Hill, Department of Applied Biochemistry and Food Science, University of Nottingham, Sutton Bonington, United Kingdom, for providing *B. subtilis* strains.

REFERENCES

1. **Buchman, W. B., S. Banerjee, and J. N. Hansen.** 1988. Structure, expression and evolution of a gene encoding the precursor of nisin, a small protein antibiotic. J. Biol. Chem. **263:**16260–16266.
2. **Chan, W. C., B. W. Bycroft, M. L. Leyland, L.-Y. Lian, J. C. Yang, and G. C. K. Roberts.** 1992. Isolation and NMR studies of subtilin A and subtilin B. FEBS Lett. **300:**56–62.
3. **Chan, W. C., B. W. Bycroft, L.-Y. Lian, and G. C. K. Roberts.** 1989. Isolation and characterisation of two degradation products derived from the peptide antibiotic nisin. FEBS Lett. **252:**29–36.
4. **Chan, W. C., B. W. Bycroft, and G. C. K. Roberts.** 1993. Structure-activity relationships of nisin and subtilin, p. 770–771. *In* C. H. Schneider and A. N. Eberle (ed.), Peptides 1992. ESCOM Science Publishers, Leiden, The Netherlands.
5. **Chan, W. C., M. Leyland, J. Clark, H. M. Dodd, L.-Y. Lian, M. J. Gasson, B. W. Bycroft, and G. C. K. Roberts.** Structure-activity relationships in the peptide antibiotic nisin: antibacterial activity of fragments of nisin. FEBS Lett., in press.
6. **Chan, W. C., L.-Y. Lian, B. W. Bycroft, and G. C. K. Roberts.** 1989. Confirmation of the structure of nisin by complete ^{1}H nmr resonance assignment in aqueous and dimethyl sulfoxide solution. J. Chem. Soc. Perkin Trans. I **1989:**2359–2367.
7. **Dodd, H. M., N. Horn, and M. J. Gasson.** 1990. Analysis of the genetic determinant for production of the peptide antibiotic nisin. J. Gen. Microbiol. **136:**555–566.
8. **Dodd, H. M., N. Horn, and M. J. Gasson.** 1995. A cassette vector for protein engineering the lantibiotic nisin. Gene **162:**163–164.
9. **Dodd, H. M., N. Horn, C. J. Giffard, and M. J. Gasson.** 1996. A gene replacement strategy for engineering nisin. Microbiology **142:**47–55.
10. **Gross, E., and J. L. Morell.** 1971. The structure of nisin. J. Am. Chem. Soc. **93:**4634–4635.
11. **Hansen, J. N., Y. J. Chung, W. Liu, and M. J. Steen.** 1991. Biosynthesis and mechanism of action of nisin and subtilin, p. 287–302. *In* G. Jung and H.-G. Sahl (ed.), Nisin and novel lantibiotics. ESCOM Science Publishers, Leiden, The Netherlands.
12. **Hill, P. J., L. Hall, D. A. Vinicombe, C. J. Soper, P. Setlow, W. M. Waites, S. Denyer, and G. S. A. B. Stewart.** 1994. Bioluminescence and spores as biological indicators of inimical processes. J. Appl. Bacteriol. **76:**129S–134S.
13. **Hurst, A.** 1981. Nisin. Adv. Appl. Microbiol. **27:**85–123.
14. **Jung, G., and H.-G. Sahl. (ed.)** 1991. Nisin and novel lantibiotics. ESCOM Science Publishers, Leiden, The Netherlands.
15. **Kaletta, C., and K.-D. Entian.** 1989. Nisin, a peptide antibiotic: cloning and sequencing of the *nisA* gene and posttranslational processing of its peptide product. J. Bacteriol. **171:**1597–1601.
16. **Lian, L.-Y., W. C. Chan, S. D. Morley, G. C. K. Roberts, B. W. Bycroft, and D. Jackson.** 1991. Nmr studies of the solution structure of nisin A and related peptides, p. 43–58. *In* G. Jung and H.-G. Sahl (ed.), Nisin and novel lantibiotics. ESCOM Science Publishers, Leiden, The Netherlands.
17. **Lian, L. Y., W. C. Chan, S. D. Morley, G. C. K. Roberts, B. W. Bycroft, and D. Jackson.** 1992. Solution structures of nisin A and its two major degradation products determined by NMR. Biochem. J. **283:**413–420.
18. **Liu, W., and J. N. Hansen.** 1992. Enhancement of the chemical and antimicrobial properties of subtilin by site-directed mutagenesis. J. Biol. Chem. **267:**25078–25085.
19. **Liu, W., and J. N. Hansen.** 1993. The antimicrobial effect of a structural variant of subtilin against outgrowing *Bacillus cereus* T spores and vegetative cells occurs by different mechanisms. Appl. Environ. Microbiol. **59:**648–651.
20. **Moll, G., G. C. K. Roberts, W. N. Konings, and A. J. M. Driessen.** 1995. Mechanism of lantibiotic-induced pore formation. Antonie Leeuwenhoek Int. J. Gen. Mol. Microbiol. **69:**185–191.
21. **Morris, S. L., R. C. Walsh, and J. N. Hansen.** 1984. Identification and characterization of some bacterial membrane sulfhydryl groups which are targets of bacteriostatic and antibiotic action. J. Biol. Chem. **259:**13590–13594.
22. **Mulders, J. W. M., I. J. Boerrigter, H. S. Rollema, R. J. Siezen, and W. M. de Vos.** 1991. Identification and characterisation of the lantibiotic nisin Z, a natural nisin variant. Eur. J. Biochem. **201:**581–584.
23. **Nicholson, W. L., and P. Setlow.** 1990. Sporulation, germination and outgrowth, p. 391–403. *In* C. R. Harwood and S. M. Cutting (ed.), Molecular biology methods for Bacillus. Wiley, New York.
24. **Rauch, P. J. G., M. M. Beerthuysen, and W. M. de Vos.** 1990. Nucleotide sequence of IS904 from *Lactococcus lactis* subsp. *lactis* strain NIZO R5. Nucleic Acids Res. **18:**4253–4254.
25. **Rayman, K., and A. Hurst.** 1984. Biotechnology of industrial antibiotics, p 607–628. Dekker, New York.
26. **Roberts, G. C. K., L.-Y. Lian, M. Leyland, W. C. Chan, B. W. Bycroft, H. Dodd, and M. Gasson.** 1992. Nisin and subtilin: towards the engineering of new antibiotics, p. 134–146. *In* P. Goodenough (ed.), Protein engineering. CPL Press/AFRC, London.
27. **Ruhr, E., and H.-G. Sahl.** 1985. Mode of action of the peptide antibiotic nisin and influence on the membrane potential of whole cells and on cytoplasmic and artificial membrane vesicles. Antimicrob. Agents Chemother. **27:**841–845.
28. **Sahl, H.-G.** 1991. Pore formation in bacterial membranes by cationic lantibiotics, p. 347–358. *In* G. Jung and H.-G. Sahl (ed.), Nisin and novel lantibiotics. ESCOM Science Publishers, Leiden, The Netherlands.
29. **Van de Ven, F. J. M., H. W. Van den Hooven, R. N. H. Konings, and C. W. Hilbers.** 1991. NMR studies of lantibiotics: the structure of nisin in aqueous solution. Eur. J. Biochem. **202:**1181–1188.