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Biosynthesis and secretion of a precursor of nisin Z by *Lactococcus lactis*, directed by the leader peptide of the homologous lantibiotic subtilin from *Bacillus subtilis*

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The DNA sequence encoding the leader peptide of the lantibiotic subtilin from *Bacillus subtilis* was fused to the sequence encoding pronisin Z, and this hybrid gene was expressed in a *Lactococcus lactis* strain that produces nisin A. This strain simultaneously secreted nisin A and a protein of approximately 6 kDa. Amino acid sequencing of the purified 6 kDa protein and structural analysis of its main tryptic fragment by two-dimensional ^{1}H-NMR showed that it consists of the unmodified leader peptide of subtilin, without the N-terminal methionine residue, linked to a fully matured nisin Z part. The hybrid protein and its main tryptic fragment [ITPQ]-nisin Z, showed at least 200-fold lower antimicrobial activities than nisin Z against three different indicator strains.

Nisin; Subtilin; Leader peptide; Secretion; Antimicrobial activity; *Lactococcus lactis*

1. INTRODUCTION

Nisin is an antimicrobial peptide produced by certain *Lactococcus lactis* strains [1,2]. It is active against a broad range of Gram-positive bacteria, and is therefore applied in the food industry as a natural preservative [3]. Nisin is ribosomally synthesized as a precursor peptide of 57 amino acids, which is posttranslationally modified by (i) dehydration of specific serine and threonine residues, resulting in dehydroalanine (Dha) and dehydrobutyrine (Dhb) residues, respectively, (ii) formation of lanthionine and β-methyllanthionines by the specific addition of free thiol groups of cysteine residues to the double bonds of dehydroalanine and dehydrobutyrine, respectively, and (iii) cleavage of the leader peptide of 23 amino acids after secretion of the precursor molecule [4–6] (Fig. 1). Antimicrobial peptides containing lanthionines and/or β-methyllanthionines have been named lantibiotics [4] and the (bio)chemical, structural and biosynthetic aspects of more than ten lantibiotics have recently been reviewed [7]. Two natural variants, nisin A (His^{27}) [1,2] and nisin Z (Asn^{27}) [8] have been isolated, and their structural genes have been cloned and sequenced [9–13]. These two nisin species have almost identical antimicrobial properties [13,14]. The structural gene encoding the lantibiotic subtilin, produced by *Bacillus subtilis*, has also been cloned and sequenced [15]. The nisin Z leader sequence shares 57% amino acid identity with the leader sequence of subtilin, while the pro-region of nisin Z has 61% identity with the pro-region of subtilin (Fig. 1).

Lantibiotics have been shown to exert their antimicrobial activities by pore formation in membranes of susceptible Gram-positive bacteria, thereby causing leakage of cellular material and eventually cell death [16–18]. Several studies have indicated the importance of the N-terminal region of mature nisin [19–21] and subtilin [22] for activity, suggesting the involvement of this part in membrane insertion.

It has been proposed that the leader sequence, which shows some conserved regions among different lantibiotic precursors, but does not possess the characteristics of a classical signal sequence, plays a role in the modification reactions, e.g. by recognition and binding of biosynthetic enzymes or by inducing certain conformations in the pro-region of the peptide [5,7] (by definition the pro-region is the region that will constitute the mature lantibiotic after modification and processing [23]). In addition, it has been speculated that the leader peptide prevents the matured prolantibiotic from displaying antimicrobial activity intracellularly [5,7]. Precursors of the lantibiotic Pep5, either unmodified or containing a number of dehydrated residues, and in some cases (β-methyl)lanthionines in the pro-region, can be isolated from the cytoplasm of *Staphylococcus epidermidis*

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Abbreviations: Abu-S-Ala, (2*S*,3*S*,6*R*)-3-methyllanthionine, Ala-S-Ala, (2*S*,6*R*)-lanthionine; Dha, dehydroalanine; Dhb, dehydrobutyrine; NMR, nuclear magnetic resonance; NOESY, nuclear Overhauser effect spectroscopy; PCR, polymerase chain reaction; ROESY, rotating frame Overhauser effect spectroscopy; RP-HPLC, reversed-phase high-performance liquid chromatography; sl, subtilin leader; TFA, trifluoroacetic acid; TOCSY, total correlation spectroscopy.

[5,24], confirming the proposed order of events during maturation. Moreover, we have recently shown that the last step in the biosynthesis of nisin is the cleavage of the leader peptide by the extracellular membrane-bound serine protease NisP, which is encoded in the nisin operon [6].

To investigate the role of the leader sequence in biosynthesis, activity and secretion in more detail, and to see whether it is possible to functionally exchange leader peptides of different lantibiotics, we fused a DNA fragment, which encodes the 24 amino acids of the leader sequence of subtilin, directly to the sequence encoding the 34 residues of pronisin Z. The resulting gene fusion was expressed in an *L. lactis* strain that produces nisin A, resulting in the secretion of an additional 6 kDa protein, which was purified and shown to consist of the expected hybrid pre-lantibiotic of 57 amino acid residues, lacking the N-terminal methionine residue.

2. MATERIALS AND METHODS

2.1. *Bacterial strains, plasmids, media and growth conditions*

Lactococcus lactis NZ9700 [6], a plasmid-free transconjugant of MG1614 harboring Tn*5276* [12] that produces nisin A, was used in this study for expression of the fusion gene. Cells were grown overnight without aeration at 30°C in a medium containing 1% sucrose, 1% pepton (Difco), 1% yeast extract (Difco), 0.2% NaCl, 0.002% $MgSO_4.7H_2O$ and 1% KH_2PO_4, pH 7.0, as described before [21]. During 10 liter fermentations a pH of 6.1 was maintained and sucrose was supplemented to 3%. Strain *Bacillus subtilis* ATCC6633 was used as a source of DNA encoding the leader sequence of subtilin [15]. Cells were grown at 30°C under aeration in the same medium as was used for *L. lactis*, but now supplemented with 0.5% of glucose. Plasmid pNZ9013 was used as a cloning vector for expression of the hybrid gene [21]. For cloning in *E. coli* strain MC1061 [25] was used.

2.2. *DNA manipulations, PCR, and DNA sequence analysis*

All DNA manipulations were carried out according to established methods [26]. Primers used for PCR were:

1. 5′-GGAG*CTGCAG*GTGTAGGCTTAGG-3′ (*Pst*I, subtilin leader),
2. 5′-CCCTAAA*AAGCTT*ATAAAAATAGG-3′ (*Hin*dIII, nisin Z),
3. 5′-GAG*ACCTGC*TCCATTGCGGAGTGATTTTTGAG-3′ (*Bsp*MI, subtilin leader),
4. 5′-GAG*ACCTGC*ATGAGCAAATTACAAGTATTTCGCTATGTAC-3′ (*Bsp*MI, nisin Z).

Restriction enzyme cleavage sites used in cloning are indicated in italics and between brackets. Sites of mutations to create restriction sites outside the structural gene are underlined. PCR was performed as described previously [27]. The PCR fragment encoding pronisin Z was obtained using primers **2** and **4** with plasmid pNZ9013 DNA as a template. DNA encoding the subtilin leader sequence and a 131-bp upstream region was amplified by PCR, using *B. subtilis* ATCC6633 total DNA as a template and primers **1** and **3**, which were based on the sequence of the structural gene encoding subtilin [15]. PCR fragments were digested with the enzymes indicated, and simultaneously ligated in vector pNZ9013, which had been digested with *Pst*I and *Hin*dIII. The advantage of the use of the restriction enzyme *Bsp*MI, which cuts outside its recognition sequence, has been described before [28]. The ligation mixture was used to transform *E. coli* MC1061 [24]. The relevant sequences of the recombinant plasmids were sequenced by the dideoxy chain termination method [29]. The desired plasmid pNZ9007 was isolated and used to transform *L. lactis* NZ9700. Transformants were analyzed again for sequence integrity of the hybrid gene.

2.3. *SDS-PAGE, purification of the 6 kDa protein and determination of antimicrobial activities*

Nisin species were detected by SDS-PAGE by use of established methods [30] and proteins were detected by silver staining. Molecular weight markers from BRL were used as size markers. Lantibiotics were purified from 10 liter of culture supernatant by hydrophobic interaction chromatography and subsequent reversed-phase HPLC, essentially as described before [21]. Preparative RP-HPLC was performed at 30°C using a 21.5 × 250 mm Hi-Pore RP-318 column (Bio-Rad). Nisin components were eluted by a linear gradient from 23% to 28% buffer B (90% CH_3CN/10% H_2O, 0.07% TFA) in buffer A (10% CH_3CN/90% H_2O, 0.1% TFA) during 50 min at a flow rate of 10 ml/min. The absorbance of the effluent was monitored at 220 nm. Minimal inhibitory concentration (MIC) values towards three indicator strains were determined as described previously [21].

2.4. *Tryptic digestion of the 6 kDa protein*

Ten milligrams of purified 6 kDa protein were incubated for 30 min at 37°C in 2 ml of a 50 mM sodium acetate buffer, pH 6.0, containing 1 mg/ml bovine pancreatic TCPK-trypsin (Sigma). Under these conditions nisin A and nisin Z are not susceptible to tryptic digestion. For this reason it was anticipated that part of the subtilin leader sequence would be cleaved off due to the presence of four lysyl residues (Fig. 1). The main tryptic product was purified by RP-HPLC and further characterized by amino acid sequencing and by ^{1}H-NMR spectroscopy.

2.5. *Amino acid sequencing*

Partial N-terminal sequencing of the 6 kDa protein and its tryptic fragment was performed by use of an automated gas-phase sequenator (Applied Biosystems) at the SON facility (Dr. R. Amons, Department of Medical Biochemistry, Sylvius Laboratory, Leiden, The Netherlands).

2.6. *NMR spectroscopy*

^{1}H-NMR spectra were taken on a Bruker AM 400 spectrometer operating at 400.13 MHz. [ITPQ]-nisin Z was dissolved in 10% D_2O/90% H_2O at a concentration of 2.5 mM. The spectra were taken at pH 3.5 and 25°C.

A complete sequential assignment of this nisin species was based on its TOCSY, NOESY, and ROESY spectra. The assignment procedure was essentially as described for other nisin species [20,31,32]; the ROESY spectrum yielded additional sequential constraints in the C-terminal part of the molecule and some long-range constraints, which were not observed in the NOESY spectra. TOCSY spectra were recorded using 20 ms and 70 ms MLEV-17 mixing sequences. NOESY spectra were taken using mixing times of 400 and 800 ms. The ROESY spectrum was recorded using a 200 ms spin-lock pulse. All 2D-spectra were recorded in the phase-sensitive mode. The water resonance was suppressed by low-power preirradiation. In the NOESY experiments the solvent resonance was also irradiated during the mixing periods. All spectra were referenced to external 3-(trimethylsilyl)-propionic 2,2,3,3-d_4 acid.

3. RESULTS

3.1. *Production of the 6-kDa hybrid protein*

Strain *L. lactis* NZ9700 harboring plasmid pNZ9007 was found to produce and secrete nisin A and a 6 kDa protein simultaneously, as determined by SDS-PAGE. The latter protein was purified from 10 liter of culture

supernatant by hydrophobic interaction chromatography followed by preparative RP-HPLC. The HPLC elution pattern is shown in Fig. 2A. The total yield of the purified protein was 43 mg.

3.2. *Amino acid sequencing of the 6-kDa hybrid protein and its main tryptic fragment*

Amino acid sequencing of the 6 kDa protein yielded the sequence SKFDDFDLDVVKVSKQDSKITPQI after which Edman degradation stopped, indicating the presence of a modified amino acid at this position. In mature nisin residue Dhb[2] is present at this position. The determined sequence clearly shows the presence of the leader peptide of subtilin, without the N-terminal methionine residue, attached to Ile[1] of (pro)nisin. The main tryptic fragment of the 6 kDa protein was also purified by RP-HPLC (Fig. 2B). N-terminal amino acid sequencing yielded the sequence ITPQI, after which the Edman degradation terminated, as expected. This fragment originates from cleavage behind the last lysyl residue in the leader peptide of subtilin (Fig. 1).

3.3. *Structural and functional characterization of the hybrid proteins*

To characterize the pronisin part of the 6 kDa protein, the [ITPQ]-nisin Z fragment was examined by 2D-NMR techniques. The results of the sequential assignment of the ^{1}H-NMR spectrum of [ITPQ]-nisin Z show that this protein consists of fully matured, intact nisin Z covalently linked to the four C-terminal residues of the subtilin leader peptide. A comparison of the chemical shifts of the resonances of [ITPQ]-nisin Z with those of nisin Z [21] shows that the major chemical shift changes induced by the additional four residues occur in the first lanthionine ring of the nisin Z part (Table I). This observation indicates that in [ITPQ]-nisin Z the first lanthionine ring has a conformation different from that in native nisin Z, which is supported by the occurrence of long-range NOEs in the N-terminal part of the hybrid molecule. The 6 kDa protein will further be referred to as sl-nisin Z (derived from 'subtilin-leader/nisin Z hybrid protein'). The deduced primary structure

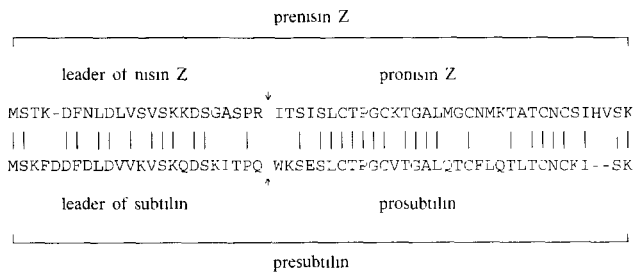

Fig. 1. Comparison of the amino acid sequences of prenisin Z and of presubtilin. Vertical lines between amino acid residues of prenisin and presubtilin indicate identical residues. Cleavage sites are indicated by arrows.

Table I

Comparison of the assignments of the ^{1}H-NMR spectra of [ITPQ]-nisin Z and nisin Z [21] recorded at 400.13 MHz in 10% D_2O/90% H_2O at pH 3.5 and 25°C

Residue	Chemical shift, ppm			
	$N^{\alpha}H$	$C^{\alpha}H$	$C^{\beta}H$	Other
Ile^{-4}	–	3.98	1.98	$C^{\gamma}H_2$ 1.24,1.52 $C^{\gamma'}H_3$ 0.99 $C^{\delta}H_3$ 0.99
Thr^{-3}	8.59	4.71	4.19	$C^{\gamma}H_3$ 1.30
Pro^{-2}	–	4.43	1.82,2.33	$C^{\gamma}H$ 2.05 $C^{\delta}H$ 3.79,3.93
Gln^{-1}	8.44	4.37	2.02,2.0	$C^{\gamma}H_2$ 2.40 $N^{\gamma}H_2$ 6.87,7.55
Ile^{1}	8.23[a]	**4.29**	**1.98**	$C^{\gamma}H_2$ 1.28,1.52 $C^{\gamma'}H_3$ **1.03** $C^{\delta}H_3$ **0.92**
Dhb^{2}	9.53[a]	–	6.66	$C^{\gamma}H_3$ 1.79
Ala^{*3}	**7.98**	4.60	3.14,3.27	
Ile^{4}	**7.69**	4.33	2.08	$C^{\gamma}H_2$ 1.15,1.38 $C^{\gamma'}H3$ 0.95 $C^{\delta}H_3$ 0.86
Dha^{5}	**10.01**	–	5.43,5.56	
Leu^{6}	8.92	4.44	1.74	$C^{\gamma}H$ **1.74** $C^{\delta}H_3$,$C^{\delta'}H_3$ 0.91,0.95
Ala^{*7}	8.22	4.51	2.96,3.07	
Abu^{*8}	8.85	5.11	3.45	$C^{\gamma}H_3$ 1.32
Pro^{9}	–	4.45	1.80,2.48	$C^{\delta}H$ 1.96,2.20 $C^{\delta}H$ 3.46
Gly^{10}	8.62	3.54,4.46		
Ala^{*11}	7.88	4.02	3.03,3.66	
Abu^{*13}	**8.39**	4.64	3.63	$C^{\gamma}H_3$ 1.34
Ile^{30}	8.07	4.18	1.84	$C^{\gamma}H_2$ 1.15,1.35 $C^{\gamma'}H_3$ 0.85 $C^{\delta}H_3$ 0.85
Lys^{34}	8.20	4.31	1.81,1.91	$C^{\gamma}H_2$ 1.46 $C^{\delta}H_2$ 1.71 $C^{\varepsilon}H_2$ 3.02 $N^{\zeta}H_3^+$ 7.56

[a] Resonance not assigned in nisin Z spectrum.

Only the assignment of those residues is shown, in which at least one of the resonances differed more than 0.02 ppm in chemical shift as compared to nisin Z [21]. The chemical shifts of the resonances differing more than 0.05 ppm are given in bold. Amino acid nomenclature is according to IUPAC-IUB recommendations (Eur. J. Biochem. 138 (1984) 9–37). Ala* and Abu* represent the alanyl- and β-methylalanyl-moieties of lanthionines. Dha and Dhb refer to dehydroalanine and dehydrobutyrine

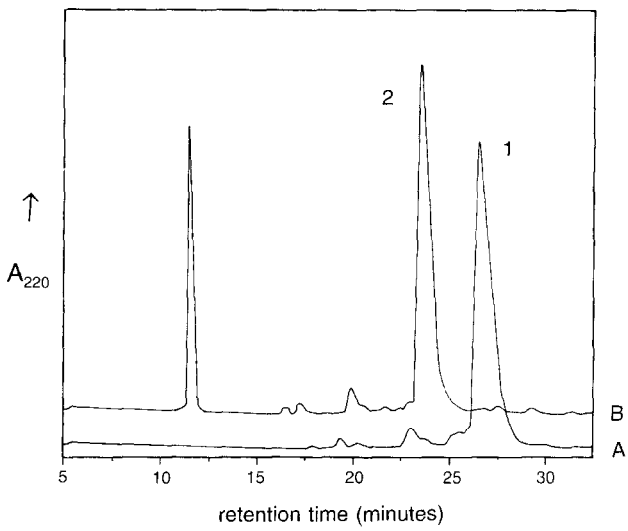

Fig 2. Analytical HPLC patterns of sl-nisin Z (A) and its tryptic digest, corrected for a trypsin blanc (B) Peak 1, sl-nisin Z; peak 2, [ITPQ]-nisin Z, the peak eluting at 12 min most probably represents a tryptic fragment of the subtilin leader peptide.

Table II

Antimicrobial activities of nisin Z, sl-nisin Z and [ITPQ]-nisin Z

Nisin	Minimal inhibitory concentration (μg/l)		
	Micrococcus flavus	*Streptococcus thermophilus*	*Bacillus cereus*
Nisin Z	11	6	130
sl-nisin Z	> 2,000	> 2,000	> 30,000
[ITPQ]-nisin Z	> 2,000	> 2,000	> 30,000

of sl-nisin Z is depicted in Fig. 3. and its calculated molecular mass is 5953 Da.

The antimicrobial activities of sl-nisin Z and of [ITPQ]-nisin Z, determined using three different indicator organisms (Table II), show a more than 200-fold decrease in bacteriocidal capacity relative to nisin Z.

4. DISCUSSION

Our results show that the leader peptide of subtilin is able to direct the complete biosynthesis and secretion of nisin Z in *L. lactis*, although it is not cleaved off after secretion. Assuming that the leader peptide is involved in the modification reactions, recognition of this sequence by putative modifying enzymes could take place via conserved residues in the leader peptide or via a specific conformation of this peptide. Synthetic leader peptides of different lantibiotics have been shown to adopt an α-helical conformation in trifluoroethanol/water (9:1) [7]. Indeed, the sequences of the leader peptides of different lantibiotics are more conserved than their pro-regions [7], suggesting a common function of these leader peptides. However, the fact that 43% of the amino acid residues in the leader peptides of nisin and subtilin are different, already indicates that absolute conservation of these residues is not necessary for its function in a heterologous host.

The lack of processing of the subtilin leader peptide in *L. lactis* is most probably caused by the altered cleavage site, i.e. Arg^{-1}/Ile^{1} in prenisin vs. Gln^{-1}/Ile^{1} in sl-nisin Z. This is in accordance with the observation that an Arg^{-1} to Gln^{-1} mutation in the sequence of the leader peptide of nisin Z gives rise to the secretion of an unprocessed and inactive but matured nisin Z, to which the nisin leader peptide is still attached (van der Meer et al., manuscript in preparation). Clearly, processing of the leader peptide is not a prerequisite for translocation. The absence of the N-terminal methionine residue in sl-nisin Z probably results from posttranslational processing, not uncommon in prokaryotes. While the nisin Z part of the hybrid protein was fully modified, no dehydration occurred of any of the serine or threonine residues in the subtilin leader peptide. This protection towards dehydration was previously observed for the leader peptides of nisin A [6] and Pep5 [5,24].

The sl-nisin Z and its tryptic fragment [ITPQ]-nisin

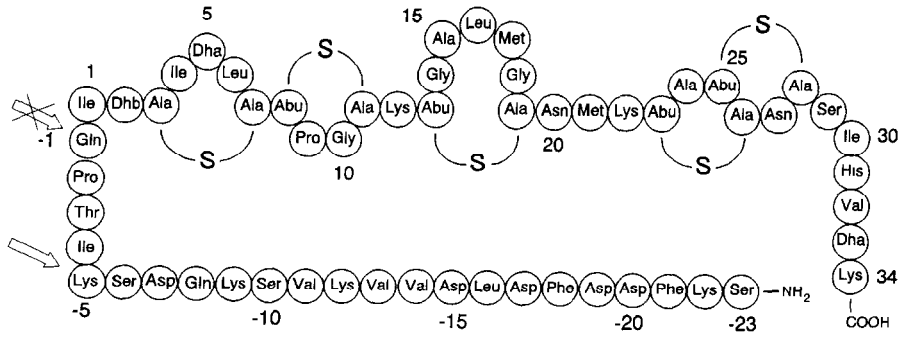

Fig 3. Primary structure of sl-nisin Z. The arrow with a cross indicates the normal processing site, which is not used when the subtilin leader is present. The other arrow indicates the most C-terminal trypsin cleavage site in the leader.

Z exhibit at least 200-fold lower antimicrobial activity than nisin Z. These data suggest that the leader sequence has a function in keeping the fully matured lantibiotic part inactive prior to secretion, as has been postulated before [7]. Possibly, no stable membrane insertion, resulting in pore-formation, can be achieved when the leader peptide or even a 4-residue rudiment of the leader peptide is still attached to the matured nisin Z part. It is noteworthy that the NMR data show that the covalent binding of the [ITPQ] sequence to nisin Z induces considerable changes in the chemical shifts of amide- and C_α-proton resonances of residues in the N-terminal part of (pro)nisin Z, suggesting a conformational change in this part of the molecule. Since the first lanthionine ring is considered to be of importance for the biological activity of nisin [20,21,31], such a conformational change could very well be responsible for the strong decrease in biological activity of the subtilin leader/nisin Z hybrids. A more detailed investigation of the 3D-structure of the nisin Z hybrids is currently in progress.

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REFERENCES

[1] Gross, E. and Morell, J.L. (1971) J. Am. Chem. Soc. 93, 4634–4635
[2] Hurst A. (1981) in: Advances in Applied Microbiology (Perlman, D. and Laskin, A.I., Eds.) Vol. 27, pp. 85–123, Academic Press, New York
[3] Delves-Broughton, J. (1990) Food Technol. 44, 100–117.
[4] Schnell, N., Entian, K.-D., Schneider, U., Gotz, F., Zahner, H., Kellner, R. and Jung, G. (1988) Nature 333, 276–278
[5] Weil, H.-P., Beck-Sickinger, A.G., Metzger, J., Stefanovic, S., Jung, G., Josten, M. and Sahl, H.-G. (1990) Eur. J. Biochem. 194, 217–223.
[6] van der Meer, J.R., Polman, J., Beerthuyzen, M.M., Siezen, R.J., Kuipers, O.P. and de Vos, W.M. (1993) J. Bacteriol. 175, 2578–2588.
[7] Jung, G. (1991) in: Nisin and Novel Lantibiotics (Jung, G., and Sahl, H.-G., Eds.) pp. 1–31, ESCOM, Leiden.
[8] Mulders, J.W.M., Boerrigter, I.J., Rollema, H.S., Siezen, R.J. and de Vos, W.M. (1991) Eur. J. Biochem. 201, 581–584.
[9] Buchman, G.W., Banerjee, S. and Hansen, J.N. (1988) J. Biol. Chem. 263, 16260–16266.
[10] Kaletta, C. and Entian, K.-D. (1989) J. Bacteriol. 171, 1597–1601.
[11] Dodd, H.M., Horn, N. and Gasson, M.J. (1990) J. Gen. Microbiol. 136, 555–566
[12] Rauch, P.J.G. and de Vos, W.M. (1992) J. Bacteriol. 174, 1280–1287.
[13] Kuipers, O.P., Yap, W.M.G.J., Rollema, H.S., Beerthuyzen, M.M., Siezen, R.J. and de Vos, W.M. (1991) in: Nisin and Novel Lantibiotics (Jung, G., and Sahl, H.-G., Eds.) pp. 250–259, ESCOM, Leiden.
[14] De Vos, W.M., Mulders, J.W.M., Hugenholtz, J., Siezen, R.J. and Kuipers, O.P. (1993) Appl. Environ. Microbiol. 59, 213–218
[15] Banerjee, S. and Hansen, J.N. (1988) J. Biol. Chem. 263, 9508–9514.
[16] Sahl, H.-G. (1991) in: Nisin and Novel Lantibiotics (Jung, G., and Sahl, H.-G., Eds.) pp. 347–358, ESCOM, Leiden.
[17] Benz, R., Jung, G. and Sahl, H.-G. (1991) in: Nisin and Novel Lantibiotics (Jung, G., and Sahl, H.-G., Eds.) pp. 359–372, ESCOM, Leiden.
[18] Gao, F.H., Abee, T. and Konings, W.N. (1991) Appl. Environ. Microbiol. 57, 2164–2170.
[19] Chan, W.C., Bycroft, B.W., Lian, L.-Y. and Roberts, G.C.K. (1989) FEBS Letters 252, 29–36.
[20] Rollema, H.S., Both, P. and Siezen, R.J. (1991) in: Nisin and Novel Lantibiotics (Jung, G., and Sahl, H.-G., Eds.) pp. 123–130, ESCOM, Leiden.
[21] Kuipers, O.P., Rollema, H.S., Yap, W.M.G.J., Boot, H.J., Siezen, R.J. and de Vos, W.M. (1992) J. Biol. Chem. 267, 24340–24346
[22] Liu, W. and Hansen, J.N. (1992) J. Biol. Chem. 267, 25078–25085.
[23] De Vos, W.M., Jung, G. and Sahl, H.-G. (1991) in: Nisin and Novel Lantibiotics (Jung, G., and Sahl, H.-G., Eds.) pp. 457–463, ESCOM, Leiden
[24] Sahl, H.-G., Reis, M., Eschbach, M., Szekat, C., Beck-Sickinger, A.G., Metzger, J., Stefanovic, S. and Jung, G. (1991) in: Nisin and Novel Lantibiotics (Jung, G. and Sahl, H.-G., Eds.) pp. 332–346, ESCOM, Leiden
[25] Casadaban, M.J., Chou, J. and Cohen, S.N. (1980) J. Bacteriol. 143, 971–980.
[26] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
[27] Kuipers, O.P., Boot, H.J. and de Vos, W.M. (1991) Nucleic Acids Res. 19, 4558.
[28] Tomic, M., Sunjevaric, I., Savtchenko, E.S. and Blumenberg, M. (1990) Nucleic Acids Research 18, 1656.
[29] Sanger, F., Nicklen, S. and Coulson, A.R. (1977) Proc. Natl. Acad. Sci. USA 74, 5463–5467.
[30] Schägger, H. and Jagow, G. (1987) Anal. Biochem. 166, 368–379.
[31] Chan, W.C., Lian, L.-Y., Bycroft, B.W. and Roberts, G.C.K. (1989) J. Chem. Soc. Perkin Trans. I, 2359–2367.
[32] Slijper, M., Hilbers, C.W., Konings, R.N.H. and van de Ven, F.J.M. (1989) FEBS Lett. 252, 22–28.