Membrane-associated proteins encoded by the nisin gene cluster may function as a receptor for the lantibiotic carnocin UI49

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Carnocin UI49, a lantibiotic produced by *Carnobacterium piscicola* shows bactericidal activity against many lactic acid bacteria. This paper describes the results of a study on the mode of action of carnocin UI49. It has previously been observed that nisin-producing *Lactococcus lactis* subsp. *lactis* strains are at least 10-fold more sensitive to carnocin UI49 relative to other lactic acid bacteria. Addition of carnocin UI49 to cells of *L. lactis* subsp. *lactis* NZ9700 resulted in a dissipation of the membrane potential and a rapid hydrolysis of internal ATP. These results suggest that carnocin UI49 may act at the cytoplasmic membrane. The correlation between production of and/or immunity to nisin and sensitivity to carnocin of *L. lactis* subsp. *lactis* strains was further investigated. Several transformed *L. lactis* subsp. *lactis* strains carrying a fragment of the nisin gene cluster were tested for their sensitivity to carnocin UI49 and their immunity to nisin. The results suggest that NisP, which is one of the membrane-associated proteins involved in the production of nisin acts as receptor for carnocin UI49. This may facilitate the binding and/or insertion of carnocin UI49 into the cytoplasmic membrane thus increasing its bactericidal activity. Lantibiotic-producing and non-producing mutants of *Staphylococcus epidermidis* and *Lactobacillus sake* did not show a difference in sensitivity to carnocin UI49. The proposed receptor-mediated action of carnocin UI49 at the cytoplasmic membrane therefore seems to be specific for the nisin-producing strains.

Keywords: *Carnobacterium piscicola*, lactic acid bacteria, lantibiotic, carnocin UI49, receptor

INTRODUCTION

The ability of lactic acid bacteria (LAB) to inhibit the growth of other bacteria is well known. One factor responsible for this inhibition is the production of antimicrobial peptides, the so-called bacteriocins. Bacteriocins are produced by all genera of LAB and many bacteriocins have been isolated and characterized. Bacteriocins produced by LAB have recently been reviewed (Hoover & Steenson, 1993; Klaenhammer, 1993).

A small number of bacteriocins have been the subject of extensive research due to their potential use as antimicrobial agents in food. In this context, one class of bacteriocins, the lantibiotics, is of special interest, because

these exert a broader spectrum of antimicrobial activity than many other bacteriocins. Lantibiotics consist of a polypeptide chain that has been post-translationally modified. These bacteriocins contain the dehydrated amino acids dehydroalanine (Dha) and dehydrobutyrine (Dhb) as well as lanthionine and β -methyl-lanthionine which are formed after S-ring formation of cysteine with Dha and Dhb respectively (Jung, 1991). These unusual amino acids may play an important role in the bactericidal activity of these peptides (Kuipers *et al.*, 1993).

Nisin was the first bacteriocin permitted as a food additive and is now used as such in at least 46 countries. It has been shown to inhibit *Clostridium* species in processed cheeses, dairy products and canned foods (Anon., 1989; Delves-Broughton, 1990). Nisin is active and stable under acidic conditions. It is therefore not the preservative of choice for meat and fish because these are not acid environments.

Abbreviations: LAB, lactic acid bacteria; TPP⁺, tetraphenylphosphonium cation.

Table 1. Characteristics of the indicator strains

Indicator strain	Relevant characteristics	Extracellular product	Reference
<i>L. lactis</i> subsp. <i>lactis</i>			
NZ9700	Tn5276	Nisin A	Kuipers <i>et al.</i> (1993)
R520	Mutated Tn5276	—	Rauch <i>et al.</i> (1991)
MG1614(pNZ9111)	<i>nisABTCIR</i>	Precursor nisin A	Kuipers <i>et al.</i> (1993)
NZ9800	Tn5276 Δ nisA	—	Kuipers <i>et al.</i> (1993)
MG1614	—	—	Gasson (1983)
<i>Lb. sake</i> L45			
LMG2340	pCIM1	Lactocin S	Mortvedt <i>et al.</i> (1991)
LMG2346	—	—	
<i>S. epidermidis</i>			
5	pED503	Pep5	Reis & Sahl (1991)
5Pep5 ⁻	Lacks pED503	—	
5Pep5 ⁻ (pMR2)	Contains fragment of pED503	prePep5	

Therefore, there is a need for novel lantibiotics with unique properties. Three lantibiotics have recently been isolated: lactocin S (Mortvedt *et al.*, 1991), lactacin 481 (Piard *et al.*, 1992) and carnocin UI49 (Stoffels *et al.*, 1992a). In order to exploit the potential of these lantibiotics, research is required on their structure, mode of action and genetics.

This paper presents results on the mode of action of carnocin UI49, a polypeptide containing a large number of hydrophobic amino acid residues (Stoffels *et al.*, 1992a) as well as a cationic C-terminal tail (unpublished results). The amphiphilic character of this bacteriocin suggests that the primary target may be the cytoplasmic membrane. Therefore, the effect of carnocin UI49 on the permeability of the cytoplasmic membrane of sensitive cells was studied. In these experiments, the indicator organism was a nisin-producing *Lactococcus lactis* subsp. *lactis* strain. It had previously been observed that these strains are at least 10-fold more sensitive to carnocin UI49 than other LAB. Thus, the activity of carnocin UI49 was tested against various transformed *L. lactis* subsp. *lactis* strains carrying a fragment of the nisin gene cluster. A mechanism of action for carnocin UI49 is proposed in this paper based on the genetic characteristics and the phenotype of these strains and their sensitivity to carnocin UI49.

METHODS

Bacterial cultures and media. *Carnobacterium piscicola* UI49, the carnocin UI49 producer strain was isolated from fish (Stoffels *et al.*, 1992a). *Lactococcus lactis* subsp. *lactis* SIK-83, the nisin Z producer strain (De Vos *et al.*, 1993) was isolated from lactic acid fermented carrots (Andersson, 1986) and kindly provided by Rolf Andersson, Swedish Food Institute, Gothenburg, Sweden.

L. lactis subsp. *lactis* strains were kindly supplied by Jeroen Hugenholtz and Oscar Kuipers, Department of Microbiology, Netherlands Institute for Dairy Research (NIZO), Ede, The Netherlands. The lactocin-S-producing *Lactobacillus sake* strain

and its mutant were obtained from Ingolf Nes, Laboratory of Microbial Gene Technology, Ås, Norway. The Pep5-producing *Staphylococcus epidermidis* strain and its mutants were obtained from Hans-Georg Sahl, Institute for Medical Microbiology and Immunology, University of Bonn, Germany. The characteristics of the strains are presented in Table 1.

Cultures were maintained as frozen stocks in 20% (v/v) glycerol at -75°C . *C. piscicola* UI49 and the lactococci were propagated in BHI supplemented with 0.5% (w/v) glucose (GBHI). *L. lactis* subsp. *lactis* MG1614(pNZ9111) was grown in GBHI in the presence of 10 μg erythromycin l^{-1} (Sigma). *Lb. sake* strains were propagated in MRS. All media were obtained from Difco.

Purification of carnocin UI49 and nisin Z. Carnocin UI49 from *C. piscicola* UI49 and nisin Z from *L. lactis* subsp. *lactis* SIK-83 were purified from 1 l cultures in GM17 as described previously (Stoffels *et al.*, 1993). Protein concentrations were estimated by comparison with a Coomassie Blue binding assay (Zaman & Verwilghen, 1979) using a mixture of human albumin and globulin (5:3) (Sigma) as a standard.

Bacteriocin assay. The sensitivity of the indicator strains to carnocin UI49 and nisin Z was measured with the microtitre plate assay system as described previously (Mortvedt *et al.*, 1991; Stoffels *et al.*, 1992a, b). The nisin-producing strain *L. lactis* subsp. *lactis* NZ9700 was sensitive to minimal 0.09 mg carnocin UI49 l^{-1} and immune to maximal 3.3 mg nisin Z l^{-1} . These values were used as references for the other *L. lactis* subsp. *lactis* strains.

The well diffusion assay as described by Stoffels *et al.* (1992b) was used to measure the activity of carnocin UI49 at 12 $^{\circ}\text{C}$ and 30 $^{\circ}\text{C}$. Purified carnocin UI49 (0.19 mg ml^{-1}) was added to 5 mm diameter wells in BHI agar plates. Carnocin UI49 was allowed to diffuse into the agar at room temperature (3–4 h). *L. lactis* subsp. *lactis* NZ9700, the indicator strain, was preincubated twice at 30 $^{\circ}\text{C}$ or 12 $^{\circ}\text{C}$. BHI soft agar (0.7%, w/v, agar) was seeded with *L. lactis* subsp. *lactis* NZ9700 and poured over the surface of the agar well plates. After incubation of 24 h at 30 $^{\circ}\text{C}$ or 48 h at 12 $^{\circ}\text{C}$ the diameters of the inhibition zones were measured.

Measurement of the membrane potential. Cells of the indicator strain *L. lactis* subsp. *lactis* NZ9700, grown overnight at 30 $^{\circ}\text{C}$ in GBHI, were diluted 10-fold in fresh medium and

grown to an optical density at 620 nm of 0.9. Cells were harvested by centrifugation, washed once in 50 mM potassium phosphate buffer, pH 6.0, and resuspended in the same buffer to an optical density at 620 nm of approximately 20. Cells were stored on ice and used within 3 h. The experiment was performed at 30 °C. The incubation mixture was composed of 0.1 ml of cell suspension, 1.0 ml 50 mM potassium phosphate buffer, pH 6.0, containing glucose (0.18%) and the lipophilic cation tetraphenylphosphonium (TPP⁺; 3.6 µM). By addition of the K⁺/H⁺ exchanger nigericin (0.36 µM, final concentration), the pH gradient (alkaline inside) was dissipated such that the protonmotive force was composed of the membrane potential only (Driessen *et al.*, 1987). The change in membrane potential was monitored by the distribution of TPP⁺ using a TPP⁺-selective electrode (Shinbo *et al.*, 1978).

Measurement of hydrolysis and/or efflux of ATP. The ATP content of cell suspensions was determined by the luciferase-luciferin procedure with an ATP bioluminescence assay kit (Lumac). A cell suspension of *L. lactis* subsp. *lactis* NZ9700 was prepared as described above. The experiment was performed at 30 °C. The incubation mixture was composed of 0.2 ml of cell suspension and 2.8 ml 50 mM potassium phosphate buffer, pH 6.0, containing 0.5% glucose. Aliquots from the mixture with or without carnocin UI49 (6.3 mg l⁻¹) were taken at regular time intervals for determination of ATP. For determination of the total (internal and external) amount of ATP, 20 µl of the cell suspension was rapidly lysed by the addition of 80 µl dimethylsulfoxide. The suspensions were diluted in 5.0 ml sterile water. An aliquot of 0.2 ml of the suspension or a dilution thereof was mixed with the luciferin-luciferase reagent and ATP determined with the Lumac luminometer. To determine the extracellular ATP concentration, 20 µl was diluted with 5.0 ml 50 mM potassium phosphate buffer, pH 6.0, and 0.1 ml was analysed as described above. The assays were calibrated using ATP solutions of known concentrations.

RESULTS AND DISCUSSION

Effect of carnocin UI49 on the permeability of the cytoplasmic membrane of the nisin-producing *L. lactis* subsp. *lactis* NZ9700

The wide spectrum of activity as well as the amphiphilic character of carnocin UI49 suggest that it acts on the cytoplasmic membrane in a similar fashion to nisin. Nisin acts at the cytoplasmic membrane by forming pores in the membrane. This pore formation is described in the so-called barrel-stave mechanism. The water soluble monomeric bacteriocin molecules bind and subsequently insert into the membrane. Aggregation of the molecules like barrel staves results in pore formation. The hydrophobic side of the protein is exposed to the membrane acyl chains and the hydrophilic sides line the pore. As a result, stable transmembrane pores are formed that allow the passive flux of ions and small molecules across the bilayer thereby killing the cell (Ruhr & Sahl, 1985; Abee *et al.*, 1991; Benz *et al.*, 1991; Ojcius & Young, 1991; Garcera *et al.*, 1993; Venema *et al.*, 1993). The broad spectrum of activity of nisin suggests that it does not require a specific integral membrane receptor for insertion (Ray, 1992). This was confirmed by the observation that nisin also acts on liposomes (Benz *et al.*, 1991; Gao *et al.*, 1991). The nisin activity is influenced by the phospholipid composition of the liposomal membrane, which offers an explanation for

the differences in sensitivity of bacterial species to nisin (Gao *et al.*, 1991; Garcera *et al.*, 1993).

The membrane potential of sensitive nisin-producing lactococcal cells was measured after addition of carnocin UI49 to test the activity of carnocin UI49 on the cytoplasmic membrane. Energized cells of *L. lactis* subsp. *lactis* strain NZ9700 retained a membrane potential of -124 mV (negative inside) at pH 6 in the presence of nigericin (0.36 µM) which could be dissipated by valinomycin (0.72 µM) (Fig. 1). In a second experiment, addition of carnocin UI49 (8.5 mg l⁻¹, final concentration) also resulted in complete depolarization of the cytoplasmic membrane (Fig. 1). No further dissipation of the membrane potential was observed when valinomycin (0.72 µM) was added to the cells after treatment with carnocin UI49 (data not shown).

The increase in membrane permeability caused by carnocin UI49 was further studied by measuring the hydrolysis and/or efflux of ATP after addition of carnocin UI49. When the starved lactococcal cells were energized with glucose, the ATP concentration increased significantly (Fig. 2). Carnocin UI49 (6.3 mg l⁻¹, final concentration) was added to the cell suspension when 50% or 100% of the total ATP had been produced by the cells (Fig. 2a and b, respectively). ATP production ceased immediately after addition of carnocin UI49 to the cells (Fig. 2a). This suggests that carnocin has an immediate effect on the cells. ATP hydrolysis was observed after a short lagtime (Fig. 2a, b). Interestingly, the decrease in the pH of the cell suspension which normally drops from 6 to approximately 4.2 stopped immediately after addition of carnocin UI49 (data not shown). This is a further indication of inhibition of the sugar metabolism of the cells.

Both the membrane potential dissipation and ATP hydrolysis by cells of *L. lactis* subsp. *lactis* NZ9700 indicate that the cytoplasmic membrane is the primary target for carnocin UI49. The bacteriocin may form pores according to the barrel-stave mechanism of action as described for nisin. In this study, no efflux of ATP was observed (data not shown) which suggests that the pores are very small. However, if carnocin acts in the same fashion as nisin, the pores are formed upon oligomerization of carnocin UI49 molecules in the membrane. A higher bacteriocin concentration may therefore result in larger pore sizes resulting in the efflux of larger compounds such as ATP (Sahl *et al.*, 1987; Benz *et al.*, 1991).

The ATP hydrolysis caused by carnocin UI49 may be induced upon dissipation of the protonmotive force, i.e. increased ATP consumption due to higher activity of the H⁺-ATPase. The ATP hydrolysis may also be due to the efflux of phosphate and subsequent shift in the ATP hydrolysis equilibrium as was recently described for the action of pore-forming colicins and phage T5 against *Escherichia coli* (Guihard *et al.*, 1993).

Effect of temperature on the activity of carnocin UI49

The effect of temperature on the activity of carnocin UI49 has been studied previously (Stoffels *et al.*, 1992b). In those studies, the sensitive strain *L. lactis* subsp. *lactis*

Fig. 1

Fig. 2

Fig. 1. Membrane potential changes induced by carnocin UI49. The membrane potential was measured as described in Methods. Experiments were performed at 30 °C in 1.0 ml 50 mM phosphate buffer pH 6.0. At the points indicated by the arrows the following additions were made: (1) 0.1 ml cell suspension; (2) glucose, final concentration 0.18% (w/v); (3) nigericin, final concentration 0.36 μ M; and (4) carnocin UI49, final concentration 8.5 mg l^{-1} (—) or valinomycin, final concentration 0.72 μ M (---).

Fig. 2. Effect of carnocin UI49 on intracellular ATP levels in *L. lactis* NZ9700. The ATP content was measured as described in Methods. The assay was performed in 50 mM phosphate buffer pH 6.0. At time zero the cells were energized with glucose (final concentration 0.5%, w/v). Values are presented as a percentage of the maximum intracellular ATP concentration (100% is 10 mM). The change in the ATP level in the cells was monitored with (Δ) or without ($\circ$) addition of carnocin UI49 (final concentration 6.3 mg l^{-1}) after 10 min (a) or 25 min (b) of incubation at 30 °C (as indicated by arrows).

NCDO497 was grown overnight in GM17 at 30 °C and added to 50 mM sodium phosphate buffer, pH 6.5, with and without carnocin UI49 (1000 bacteriocin units ml^{-1}). Viable cells were determined after incubation of the samples at 4 °C, 15 °C, 22 °C, 30 °C and 34 °C. The results suggested that maximum bactericidal activity of carnocin UI49 occurred at 34 °C with a decrease in activity down to 15 °C where it was completely abolished (Stoffels *et al.*, 1992b).

In this study, *L. lactis* subsp. *lactis* NZ9700 was cultured twice at 12 °C and 30 °C before assaying its sensitivity to carnocin UI49 at high and low temperatures. The diameters of the inhibition zones when assayed at 12 °C and 30 °C were 33 and 27 mm, respectively. These results clearly demonstrate that carnocin UI49 is indeed also active at low temperatures. Growth of the indicator strain at low temperatures is apparently accompanied by the adaption of the cytoplasmic membrane such that membrane fluidity is maintained allowing insertion of and channel formation by carnocin UI49 resulting in growth inhibition at low temperature.

Effect of carnocin UI49 on the permeability of the cytoplasmic membrane of non-nisin-producing *L. lactis* R520

Thus far, all experiments with carnocin UI49 were performed with nisin-producing *L. lactis* indicator strains. These strains have been the indicator strains of choice,

Table 2. Strains of *L. lactis* subsp. *lactis* tested for sensitivity to carnocin UI49 and immunity to nisin Z

Values represent the means of five measurements.

Indicator strain	Immunity to nisin Z* (%)	Sensitivity to carnocin UI49† (%)
<i>L. lactis</i> subsp. <i>lactis</i>		
NZ9700	100	100
R520	6	6
MG1614(pNZ9111)	3	1
NZ9800	10	11
MG1614	0.09	1.5

* 100% immunity of *L. lactis* subsp. *lactis* NZ9700 to nisin Z: 3.3 mg l^{-1} .

† 100% sensitivity of *L. lactis* subsp. *lactis* NZ9700 to carnocin UI49: 0.09 mg l^{-1} .

because they are at least 10-fold more sensitive to carnocin UI49 than the non-nisin-producing LAB (Stoffels *et al.*, 1993). Dissipation of the membrane potential was not observed for the less sensitive cells of *L. lactis* subsp. *lactis* R520 (Table 2) after addition of carnocin UI49 to a final concentration of 34 mg l^{-1} (data not shown). Due to the

Fig. 3. Simplified scheme of membrane-associated proteins encoded within the nisin gene cluster in *L. lactis* NZ9700 and possible mechanisms of action of carnocin UI49 (1, 2, 3). See text for details. I, NisI; P, NisP; T, NisT; CM, cytoplasmic membrane; $\blacksquare$, carnocin UI49; $\square$, nisin.

high salt concentration of the bacteriocin samples (0.2 M NaCl), it was not possible to add more carnocin UI49 to the cell solution without changing the experimental conditions. Unfortunately, the sample could not be desalted without a reduction in bacteriocin activity of at least 90 %. This could be due to a decrease in the solubility of carnocin UI49. The loss in activity may also be due to the aggregation of carnocin UI49 molecules as was observed for nisin (Liu & Hansen, 1990).

Possible role of membrane-associated proteins in the sensitivity of nisin-producing lactococcal cells to carnocin UI49

The reason for the high sensitivity of the nisin-producing *L. lactis* subsp. *lactis* strains to carnocin UI49 was studied further. The results of this study indicate that carnocin UI49 acts at the cytoplasmic membrane and the high sensitivity of the nisin-producing cells may be due to proteins encoded within the nisin gene cluster. Three membrane-associated proteins have been described that are involved in nisin biosynthesis and immunity to nisin. NisI is a lipid-modified extracellular membrane-anchored protein of 32 kDa that is involved in the immunity of the nisin producer strain to nisin (Kuipers *et al.*, 1993). NisP is a subtilisin-like serine protease of 54 kDa that cleaves off the leader sequence from fully matured precursor nisin. The protease is attached to the cellular membrane via a C-terminal anchor sequence (Van der Meer *et al.*, 1993). NisT is a 69 kDa ATP-dependent membrane translocator which is involved in nisin transport (Blight & Holland, 1990; Engelke *et al.*, 1992). These proteins are encoded on a lactococcal gene cluster involved in nisin biosynthesis, immunity and genetic mobility (Klaenhammer, 1993). This gene cluster is located on the conjugative transposon Tn5276. This element also encodes the genes involved in sucrose utilization (Rauch & De Vos, 1992). The fermentation of sucrose depends initially upon the translocation and phosphorylation of the sugar mediated by the membrane-associated phosphoenolpyruvate-dependent sucrose:phosphotransferase system (sucrose-PTS) (Thompson *et al.*, 1991). The expression of the *suc* genes may be under glucose (catabolite) repression control in a manner similar to the *lac* genes (Poolman, 1993). Therefore, in order to repress

the expression of the sucrose-PTS, all *L. lactis* subsp. *lactis* strains were grown in BHI supplemented with glucose.

Fig. 3 shows how NisI, NisP and NisT may be inserted into the lactococcal cell membrane. Furthermore, the model shows possible ways in which these proteins may be involved in the bactericidal activity of carnocin UI49. The first mechanism (1) shows how one of the proteins could serve as a receptor for carnocin UI49 and facilitate the binding and/or subsequent insertion of carnocin UI49 into the membrane. This may increase the efficiency of bactericidal activity. The second mechanism shows another possibility for NisI involvement (2). NisI may neutralize the bactericidal activity of nisin by interception of the nisin molecules. This would explain the limited immunity of the wild-type nisin-producing strains to a high concentration of nisin. Carnocin UI49 could compete with the nisin molecules for the same site on NisI, thereby releasing nisin. Nisin kills cells more efficiently than carnocin UI49; the minimum inhibitory concentration of nisin against *L. lactis* subsp. *lactis* MG1614 is 0.003 mg l⁻¹ while the minimum inhibitory concentration of carnocin UI49 against *L. lactis* subsp. *lactis* NZ9700 is 0.09 mg l⁻¹ (Table 2). Therefore, killing of the lactococcal cells by this mechanism would be mainly the result of pore formation by the nisin molecules that have been released from NisI. The third mechanism (3) does not involve proteins, but is based upon differences in the phospholipid composition and/or other characteristics of the cytoplasmic membrane as compared to that of non-nisin-producing *L. lactis* subsp. *lactis* strains.

In order to elucidate which of the above is the most plausible mode of action of carnocin UI49, the latter was tested against various transformed *L. lactis* subsp. *lactis* strains. The *L. lactis* subsp. *lactis* MG1614 strain used has been transformed with the plasmid pNZ9111 which contains all the genes involved in nisin biosynthesis and immunity with the exception of *nisP* (Van der Meer *et al.*, 1993). Consequently strain *L. lactis* subsp. *lactis* MG1614(pNZ9111) produces fully matured inactive extracellular precursor nisin. The other mutant used, *L. lactis* subsp. *lactis* NZ9800, contains the complete Tn5276, but is deficient in nisin A production due to gene replacement of *nisA* by a truncated *nisA* gene (Kuipers *et al.*, 1993). The third mutant used, *L. lactis* subsp. *lactis*

Table 3. Strains of *Lb. sake* and *S. epidermidis* tested for sensitivity to carnocin UI49

Values represent the means of two measurements.

Indicator strain	Sensitivity to carnocin UI49 (mg l ⁻¹)
<i>Lb. sake</i>	
LMG2340	0.7
LMG2346	1.4
<i>S. epidermidis</i>	
5	> 50
5Pep5 ⁻	> 50
5Pep5 ⁻ (pMR2)	> 50

R520 has a mutated Tn5276, which has not been further specified (Kuipers *et al.*, 1993).

The sensitivities of the three lactococcus mutants to carnocin UI49 were reduced as compared to the wild-type nisin-producing strain (Table 2). However, the sensitivities of *L. lactis* subsp. *lactis* R520 and *L. lactis* subsp. *lactis* NZ9800 were still 4-fold and 7-fold higher, respectively, than the sensitivity of the non-nisin-producing plasmid-free wild-type strain *L. lactis* subsp. *lactis* MG1614. These results indicate that the sensitivity of the wild-type nisin-producing strains to carnocin UI49 cannot be explained by mechanism 2 (Fig. 3), since these mutants do not produce active nisin but are still more sensitive to carnocin UI49 than is *L. lactis* subsp. *lactis* MG1614.

The ability of some *L. lactis* subsp. *lactis* strains to produce nisin is tightly coupled to their immunity to nisin; full immunity is observed only when matured nisin is produced (Kuipers *et al.*, 1993). The expression of all genes involved in the biosynthesis of and immunity to nisin may be linked. The immunity of the *L. lactis* subsp. *lactis* strains was measured as an indication of the expression of the nisin gene cluster and thus the presence of NisT, NisP and NisI. The results are shown in Table 2. Indeed, the decrease in immunity of *L. lactis* subsp. *lactis* R520, *L. lactis* subsp. *lactis* NZ9800 and *L. lactis* subsp. *lactis* MG1614(pNZ9111) to nisin corresponded to the decrease in sensitivity to carnocin UI49. This suggests that carnocin UI49 may act according to mechanism 1 (Fig. 3), i.e. receptor-mediated induction of carnocin UI49 activity whereby NisI, NisT and/or NisP function as a receptor.

L. lactis subsp. *lactis* MG1614(pNZ9111) showed the highest decrease in sensitivity to carnocin UI49 and immunity to nisin relative to the wild-type nisin-producing strain. Its immunity to nisin is still 30-fold higher than that of *L. lactis* subsp. *lactis* MG1614, the wild-type non-nisin-producing strain. This suggests that *L. lactis* subsp. *lactis* MG1614(pNZ9111) still harbours the immunity system against nisin. *L. lactis* subsp. *lactis* MG1614 shows similar sensitivity to carnocin UI49 as *L. lactis* subsp. *lactis* MG1614(pNZ9111), which suggests that the latter has lost the factor that induces the activity of

carnocin UI49. In contrast to the other transformed *L. lactis* subsp. *lactis* strains or the wild-type nisin-producing strain, this strain does not contain the gene encoding NisP. Therefore, if carnocin UI49 acts according to mechanism 1 (Fig. 3), NisP is the most likely candidate to function as a receptor. The last 30 C-terminal amino acid residues of NisP show homology with a number of membrane-anchoring attachment sites of proteins of Gram-positive bacteria. These membrane-spanning sequences contain a hydrophobic α -helix with the hydrophilic residues located at one site (Vos *et al.*, 1989; Van der Meer *et al.*, 1993). If the NisP attachment site is similar, one could envisage the involvement of this site in the facilitated insertion of carnocin UI49 into the membrane.

Activity of carnocin UI49 against other lantibiotic-producing strains

Table 3 shows the bactericidal activity of carnocin UI49 against two other lantibiotic-producing strains, *Lb. sake* and *S. epidermidis*. *Lb. sake* LMG2346 (Lac⁻ Imm⁻) showed similar sensitivity towards carnocin UI49 as the wild-type producer strain *Lb. sake* LMG2340 (Lac⁺ Imm⁺). *Lb. sake* LMG2346 has been cured for the plasmid containing the genes that are involved in lactocin S immunity and production. Since this drastic mutation does not affect the sensitivity towards carnocin UI49 our results suggest that lactocin S production and/or immunity is not coupled to carnocin UI49 sensitivity. Finally, *S. epidermidis*, the Pep5 producer strain and its derivatives were not sensitive to carnocin UI49.

Conclusion

This paper presents results on the mode of action of carnocin UI49. Carnocin UI49 increased the permeability of the cytoplasmic membrane of *L. lactis* subsp. *lactis* NZ9700 in a manner similar to other bacteriocins. The high sensitivity of the nisin-producing *L. lactis* subsp. *lactis* to carnocin UI49 relative to other *L. lactis* subsp. *lactis* strains was further investigated. One of the membrane-associated proteins involved in the production of nisin or the immunity to nisin seemed to be involved in the sensitivity of these cells to carnocin UI49. Furthermore, the results suggested that NisP may act as a receptor for carnocin UI49, thereby increasing carnocin UI49 activity. It is not yet known whether the phospholipid composition of the nisin-producing *L. lactis* subsp. *lactis* strains differs from that of the non-nisin producing *L. lactis* subsp. *lactis* strains. Cloning and expression of each of the genes involved in nisin production and immunity to nisin may show whether NisP is the only protein which induces carnocin UI49 activity and possibly acts as its receptor.

C. piscicola UI49, the carnocin UI49 producer strain, is as sensitive to nisin as other carnobacteria (Stoffels *et al.*, 1993). Furthermore, the lantibiotic-producing and non-producing mutants of *S. epidermidis* and *Lb. sake* did not show a difference in sensitivity to carnocin UI49. The proposed receptor-mediated action of carnocin UI49 at the cytoplasmic membrane therefore seems to be specific

for carnocin UI49 and the nisin-producing *L. lactis* subsp. *lactis* strains.

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