Nisin induction without nisin secretion

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Nisin Z, a post-translationally modified antimicrobial peptide of Lactococcus lactis, is positively autoregulated by extracellular nisin via the two-component regulatory proteins NisRK. A mutation in the nisin NisT transporter rendered L. lactis incapable of nisin secretion, and nisin accumulated inside the cells. Normally nisin is activated after secretion by the serine protease NisP in the cell wall. This study showed that when secretion of nisin was blocked, intracellular proteolytic activity could cleave the N-terminal leader peptide of nisin precursor, resulting in active nisin. The isolated cytoplasm of a non-nisin producer could also cleave the leader from the nisin precursor, showing that the cytoplasm of L. lactis cells does contain proteolytic activity capable of cleaving the leader from fully modified nisin precursor. Nisin could not be detected in the growth supernatant of the NisT mutant strain with a nisin-sensing strain (sensitivity 10 pg ml\(^{-1}\)), which has a green fluorescent protein gene connected to the nisin-inducible nisA promoter and a functional nisin signal transduction circuit. Northern analysis of the NisT mutant cells revealed that even though the cells could not secrete nisin, the nisin-inducible promoter P\(_{\text{nizA}}\) was active. In a nisB or nisC background, where nisin could not be fully modified due to the mutations in the nisin modification machinery, the unmodified or partly modified nisin precursor accumulated in the cytoplasm. This immature nisin could not induce the P\(_{\text{nizA}}\) promoter. The results suggest that when active nisin is accumulated in the cytoplasm, it can insert into the membrane and from there extrude parts of the molecule into the pseudoperiplasmic space to interact with the signal-recognition domain of the histidine kinase NisK. Potentially, signal presentation via the membrane represents a general pathway for amphiphilic signals to interact with their sensors for signal transduction.

INTRODUCTION

In bacteria as well as in some plants, fungi, protozoa and archaea, an adaptive response to environmental changes is regulated by two-component signal transduction (TCST) pathways (Koretke et al., 2000). These systems have in common a phosphoryl transfer between a sensor histidine protein kinase (HPK) and an effector response regulator (RR) (Kramer & Weiss, 1999; Fabret et al., 1999). A typical HPK is a transmembrane receptor with an amino-terminal extracellular sensing domain and a carboxy-terminal cytosolic signalling domain. The corresponding cytoplasmic RR mediates an adaptive response to this environmental signal, in many cases a change in gene expression (Dutta et al., 1999; Grebe & Stock, 1999; Stock et al., 2000). The HPK-mediated TCST circuits may sense nutrients, chemoattractants or osmotic conditions, and be involved in stress-induced sporulation, host recognition for pathogen invasion, hyphal development and ethylene response (Loomis et al., 1997; Korette et al., 2000; Wolanin et al., 2002). Recently, several chemical classes of microbially derived signalling molecules have been identified. They can be split into amino acids and short peptides utilized by Gram-positive bacteria, and fatty acid derivatives utilized by Gram-negative bacteria (Whitehead et al., 2001). The bacterial TCST autoinducer(s) signals are classified into three classes: acylhomoserine lactones used by Gram-negative bacteria, oligopeptides used by Gram-positive bacteria and 3(2H)-furanones used by Vibrio harveyi (Bassler et al., 1993; Schauder & Bassler, 2001; Pappas et al., 2004; Ahmer, 2004; Daniels et al., 2004).

The production of antimicrobial peptides by Gram-positive bacteria involves a tight interplay of three genetically linked components: a secreted peptide pheromone, an HPK and an RR (Kleebezem et al., 1997). In the case of nisin, the antimicrobial peptide produced by certain strains of Lactococcus lactis, the bacteriocin itself acts as a peptide pheromone, inducing expression of genes that are necessary for its own production (Kuipers et al., 1995; Qiao et al., 1996; Kleebezem et al., 1997). Nisin is translated as a precursor with an N-terminal leader and processed post-translationally. During maturation of nisin, some of the serine and threonine residues are dehydrated by NisB (Koponen et al., 2002), followed by lanthionine ring formation by NisC (Koponen et al., 2002). Modified nisin is secreted by a dedicated ATP-binding-cassette exporter, NisT (Qiao & Saris, 1996),
and activated by cleavage of the leader by NisP (Kuipers et al., 1993; Qiao et al., 1996). After activation by the serine protease NisP the majority of nisin is found in the supernatant after the mid-exponential growth phase in pH-uncontrolled fermentations (Ra et al., 1996). From the culture supernatant nisin can insert into the cytoplasmic membrane of sensitive bacteria, resulting in voltage-dependent permeabilization of the membrane and cell death (Chatterjee et al., 2005). In nisin producers the transmembrane HPK NisK senses nisin and activates the intracellular RR NisR, which in turn activates the nisin promoters, creating an autoinduction loop (Kuipers et al., 1995; de Ruyter et al., 1996; Qiao et al., 1996). It has been shown that derivatives of nisin induce the transcription of the nisin structural gene (nisA or nisZ), while unmodified precursor peptide and several other antimicrobial peptides could not induce transcription (Kuipers et al., 1995; Qiao et al., 1996). Activation of NisR results in transcriptional activation of the nisA/ZBTSIPRK and the nisFEG operons, encoding the nisin maturation machinery, nisin immunity and regulation (Engelke et al., 1994; Immonen et al., 1995; de Vos et al., 1995; Kuipers et al., 1995; Saris et al., 1996; Immonen & Saris, 1998; Stein et al., 2003). The nisA promoter can also be induced with galactose independently of the NisRK system in some strains (Chandrapati & O'Sullivan, 1999, 2002).

In this study we show that the nisZ promoter is activated in a nisin translocator deficient strain even though no nisin could be detected in the growth supernatant with an assay having a detection limit of 10 pg ml$^{-1}$. Due to the deficiency of the nisin transport function, nisin accumulated inside the cells. Intracellular proteolytic activity cleaved the leader from the fully modified nisin precursor and active nisin could be detected in the cytoplasm of these cells. We conclude that nisin can also induce from the inside of the cell, most likely via the membrane, by presenting part of the nisin molecule to the water-soluble side in the pseudoperiplasm for interaction with the signal recognition domain of NisK. This would represent a novel way for a signal transduction pathway to recognize its signal.

**METHODS**

**Bacterial strains, plasmids, media and growth conditions.** LAC46 (AnisI; Ra et al., 1996), LAC53 (AnisIb; Qiao et al., 1996), LAC104 (AnisIc; Koponen et al., 2002) and LAC67 (AnisIz; Qiao et al., 1996) deletion strains derived from L. lactis subsp. lactis N8 (Graeffe et al., 1991) were used. L. lactis subsp. lactis N8 is a nisin Z producing strain. L. lactis MG1614 (Gasson, 1983) was used as a host strain for constructed plasmids. For isolation of fully modified nisin precursor L. lactis MG1614/pNZ29111, capable of secreting fully modified prenisin, was utilized (Kuipers et al., 1995). Lactococcal cells were cultivated without aeration at 30 °C in M17 broth (Oxoid) containing 0.5% (w/v) glucose or sucrose instead of lactose (M17GS). When needed, media were supplemented with 5 μg erythromycin ml$^{-1}$ or 10 μg chloramphenicol ml$^{-1}$. Micrococcus luteus NCIMB 86166 (National Collection of Industrial and Marine Bacteria, UK) was used as a nisin-sensitive indicator strain in nisin biosassays. The expression plasmid pLEB384 (Qiao et al., 1996) contains the constitutive P$_{as}$ promoter (Sibakov et al., 1991) followed by the P$_{nisZ}$ promoter and the nisZ gene (Graeffe et al., 1991). The L. lactis LAC240 (Reunanen & Saris, 2003) and LAC275 (Hakovirta et al., 2006) strains were used for quantification of nisin. LAC240 is a derivative of the non-nisin-producing L. lactis strain MG1614, containing the plasmid pLEB99 encoding the NisRK and GFP proteins; the gfp gene is under the control of the nisin-inducible nisF promoter. Cells of LAC275 can also sense nisin and produce GFP, but have the nisRK genes integrated into the chromosome (Kuipers et al., 1998) and the gfp$_{UV}$ gene (Clonetech) is under the control of the nisA promoter.

**Northern hybridization analysis.** L. lactis strains for Northern analysis were grown to the exponential growth phase, collected by centrifugation and broken by sonication. RNA was isolated according to Ra & Saris (1995). Thirty micrograms of RNA and 3 μg RNA ladder (Gibco-BRL) were fractionated on 2% agarose gels containing formaldehyde. After electrophoresis the gel was soaked in water for 20 min and then for 1 h in transfer buffer (8 mM Na$_3$HPO$_4$, 17 mM Na$_2$PO$_4$). RNA was transferred to Hybond N membrane with a Bio-Rad Trans-Blot cell overnight at 250 mA at 4 °C. After transfer, membranes were rinsed in transfer buffer and then incubated in a vacuum oven at 80 °C for 2 h. Membranes were prehydrized for 4 h at 57 °C (5 × SSC, 5 × Denhardt’s solution, 0.5% SDS and 200 μg denatured herring sperm DNA ml$^{-1}$). After addition of the probe, hybridization was performed in the same buffer overnight at 57 °C. Membranes were washed for 10 min with 2 × SSC/0.1% SDS at 57 °C, for 20 min with 1 × SSC/0.1% SDS at 57 °C, for 20 min with 0.5 × SSC/0.1% SDS at 57 °C and for 20 min at 65 °C, and finally with 0.1 × SSC/0.1% SDS at 65 °C. Membranes were exposed to Kodak X-Omat 100 films at −70 °C overnight before developing.

**Labelling of the probes.** Labelling of 50 ng of probe was performed with the Multiprime labelling system (Amersham) according to the manufacturer’s instructions, in the presence of Amersham’s [32P]dCTP (3000 Ci mmol$^{-1}$, 111 TBq mmol$^{-1}$). Free nucleotides were separated from labelled DNA with Sephadex C-50 Nick columns (Pharmacia). The nisZ-specific DNA for the probe was made by digestion of plasmid pLEB1984 (Graeffe et al., 1991) with Sphl and SaeI, generating a 227 bp fragment (the ends of the fragment are at bp 125–352 in the nisZ sequence at EMBL accession no. Z18947), which was isolated from a 0.8% agarose gel using a Magic PCR prep kit (Promega).

**Nisin bioassay.** In order to detect the activity of nisin in the cytoplasm of lactococcal cells, L. lactis strains were grown to OD$_{600}$ 0.6. The cells were collected from 10 ml of culture by centrifugation and resuspended in 200 μl distilled water acidified to pH 2.5 with HCl. PMSF at a final concentration of 10 μg ml$^{-1}$ was used to irreversibly inhibit serine proteases NisP and trypsin. For isolation of intracellular nisin the cells were frozen by liquid nitrogen and thawed several times prior to sonication. To prevent the activity of NisP, the serine protease inhibitor PMSF was used. Cytoplasm of LAC85 cells was also isolated without addition of PMSF for comparison. After sonication using a Labsonic U (Labsystems), the particulate fraction was removed by centrifugation at 14 000 g for 10 min and the supernatant was further filtered through a membrane with a cut-off of 0.2 μm. Antibacterial activity of nisin was determined as growth inhibition zones on Luria–Bertani soft agar plates inoculated with M. luteus. The indicator strain M. luteus was grown to OD$_{600}$ 0.7 and 10 μl of the culture was added to 3 ml Luria–Bertani soft agar. On the top of the agar surface, 3 μl of the samples (both cells and supernatant for each strain) to be tested was applied as a droplet on the agar surface and allowed to dry. Plates were incubated overnight at 37 °C. The samples (both cells and supernatant) were heated at 80 °C for 10 min and stored frozen prior to the GFP bioassay. Isolation of the cytoplasm of LAC67 cells for detecting proteolytic
activity in the cytoplasm was done as above except that PMSF was not included. The isolated cytoplasm was used to digest fully modified nisin precursor prior to applying on Luria–Bertani soft agar plates inoculated with *M. luteus*.

**GFP-based nisin bioassay.** The nisin bioassay was performed as described by Reunanen & Saris (2003). When LAC275 was used in the GFP bioassay the freezing step was omitted, chloramphenicol (10 µg ml⁻¹) was added to the growth medium instead of erythromycin, less nisin was used for the standard curve, and the excitation and emission filters were different. Cells of the two indicator strains were inoculated 1 : 100 in M17 (Oxoid) containing 0·5 % (w/v) glucose, 0·1 % (w/v) Tween 80; when needed, 5 µg ml⁻¹ erythromycin or 10 µg ml⁻¹ chloramphenicol was added. The indicator strain inoculum was divided into aliquots of 175 µl on a microtitre plate, then 50 µl aliquots of the samples were applied and the nisin standards (Sigma) were prepared so that the concentration of nisin in the culture medium ranged from 2·5 to 125 ng ml⁻¹ for LAC240 and from 10 to 90 pg ml⁻¹ for LAC275. The bacterial suspensions were divided into 225 µl aliquots on a microtitre plate, and incubated overnight at 30 °C. Before the fluorescence measurements 175 µl of each supernatant was removed. The maturation of newly synthesized GFP molecules is a temperature-dependent process proceeding faster at low temperature (Lim et al., 1995). Thus, the incubated LAC240 cells were frozen for 30 min at −20 °C and thawed at room temperature prior to the detection of fluorescence. Cells of LAC275 did not require freezing for correct folding due to mutations in the gfp gene (Crameri et al., 1996). Green protein fluorescence was detected in terms of relative fluorescence units (RFU) with a Fluoroscan Ascent 374 fluorometer using the Ascent software (version 2.4.2; Labsystems). The excitation and emissions filters were 485 and 538 nm for LAC240, and 373 and 583 nm for LAC275. Growth was measured by determining the OD₆₀₀ with an UltraspecII spectrophotometer (Pharmacia LKB).

**RESULTS**

**Activation of the nisZ promoter in nisT, nisB and nisC mutant strains**

It has been shown (Qiao & Saris, 1996) that a knock-out of the NisT ABC transporter in the *L. lactis* LAC46 mutant renders the cells incapable of nisin secretion and that the cells contain nisin in their cytoplasm. Plasmid pLEB384, which has the nisin structural gene under the control of the nisin-inducible PnisZ promotor, was transformed into LAC46 in an attempt to achieve greater nisin production inside the cells. Northern analysis of these LAC46/pLEB384 cells, named LAC85, with a nisZ probe showed that the PnisZ promotor was activated without induction with extracellular nisin (Fig. 1c, lane 1). In order to evaluate if fully modified nisin was required for this activation, pLEB384 was transformed into strains LAC53 (NisB inactive) and LAC104 (NisC inactive), yielding strains LAC109 and LAC108, respectively. As the nisB and nisC mutations are in the nisin modification genes the strains do not produce mature nisin and their nisin-inducible promoters are activated only upon addition of external nisin (Koponen et al., 2002). Cells of LAC85 (active nisin in the cytoplasm), LAC109 (unmodified nisin precursor in the cytoplasm upon external nisin induction) and LAC108 (dehydrated nisin precursor in the cytoplasm upon external nisin induction) were grown with and without nisin. Total RNA of these cells was analysed by Northern blotting using a nisZ probe. The results (Fig. 1) revealed that of the cells not induced with external nisin only the NisT mutant (LAC85) showed transcripts from the nisZ promotor. Therefore, production of fully modified nisin is needed for the observed activation of the PnisZ promotor when externally added nisin is absent. The nisZ promotor region of the pLEB384 plasmid of the NisT mutant was isolated and sequenced, but no mutations were identified, indicating that the activation of the nisZ promotor in pLEB384 in the NisT mutant was not due to any mutation resulting in a constitutive promotor (results not shown). Plasmid pLEB384 from the NisT mutant was also transformed into the NisB and NisC mutants, but in these cells the nisZ promotor was again only activated upon nisin induction (results not shown). This further showed that the activation of the nisZ promotor in pLEB384 without externally added nisin occurred only in the NisT mutant, producing and containing active nisin inside the cells.

**Analysis of nisin concentration in the medium and the cytoplasm of *L. lactis* cells**

To analyse if the nisZ promotor activation was due to cell lysis resulting in the release of nisin to the medium, causing induction of intact cells, the nisin content in cultures of the NisT mutant LAC85 was determined using a nisin bioassay based on nisin-induced fluorescence. In this assay, cells of
L. lactis LAC275 containing the nisin-inducible gfpUV gene were grown together with the growth supernatant of strain LAC85. The results showed that the growth supernatant of LAC85 contained less than 10 pg ml⁻¹ (the detection limit of the assay) as the supernatant was not able to induce any fluorescence in the cells of LAC275. To further ensure that the LAC85 cells do not release nisin to the growth supernatant for other cells to be induced, LAC85 cells were grown together with LAC275 cells, which are induced by nisin at levels above 10 pg ml⁻¹ to produce GFP. No GFP-related fluorescence was obtained from the cell mixtures, showing that LAC85 cells did not release detectable amounts of nisin during growth.

To verify that the LAC85 cells contained active nisin and not premature nisin with the leader still uncleaved, the cells were broken in the presence of PMSF, a serine protease inhibitor. The protease inhibitor was added to avoid the possibility that breakage of the cells would release nisin precursor with the leader to the NisP protease present on the surface of the cells, resulting in false positive results regarding nisin activity in the cytoplasm, as has been suggested by Chatterjee et al. (2005). LAC85 cells had NisP activity anchored to the cell wall and the capacity to cleave the leader from the nisin precursor, but PMSF could inhibit this activity (Fig. 2). Nisin from the cytoplasm of LAC85 isolated in the presence of PMSF was quantified using the nisin-induced green fluorescence bioassay (Reunanen & Saris, 2003) using L. lactis LAC240 cells, which, like LAC275, contain a nisin-inducible gfp gene. The results showed that the cytoplasm of LAC85 cells contained nisin at a concentration of 6 ± 1.6 ng ml⁻¹ (6 ± 1.6 RFU; Fig. 3). Nisin could also be isolated from the cytoplasm of LAC85 cells that had been grown in the presence of PMSF followed by breakage of the cells with PMSF included (results not shown). In addition, the nisin activity of the cytoplasm of LAC85 isolated with or without PMSF was analysed using nisin-sensitive M. luteus as an indicator. If the cytoplasm of LAC85 contained fully modified nisin precursor with the leader still attached, then isolation of the cytoplasm without PMSF would result in cleavage of the leader, resulting in increased inhibition compared to cytoplasm isolated with PMSF included. However, the cytoplasm of LAC85 cells isolated with PMSF contained slightly more nisin activity than the cytoplasm isolated without PMSF (Fig. 2b).

**Activation of fully modified nisin precursor by the cytoplasm of L. lactis**

The cytoplasm of LAC67 was isolated to further verify that the cytoplasm of L. lactis could cleave the leader from fully modified nisin precursor. LAC67 has the nisZ gene replaced by erm, but contains all the other nisin genes, which are not

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**Fig. 2.** Nisin bioassay using M. luteus as nisin-sensitive strain. (a) Spots: 1, cells of LAC85 mixed with fully modified prenisin; 2, cells of LAC85 mixed with fully modified prenisin and the serine protease inhibitor PSMF; 3, the fully modified prenisin pre-incubated with 5 mg trypsin ml⁻¹ at 37 °C; 4, fully modified prenisin treated with trypsin and the serine protease inhibitor (PSMF). Spots 1–4 are from the same plate. (b) Spots: 1, the cytoplasm fraction of LAC85 cells; 2, the cytoplasm fraction of LAC85 cells mixed with the serine protease inhibitor PSMF.

**Fig. 3.** GFP bioassay. Standard curve for nisin-induced fluorescence of L. lactis LAC240 cells grown in M17G, given as final assay concentration; the results shown are the mean ± SD from four parallel experiments. The R² for the linear part of the curve (6.25–25 ng nisin ml⁻¹) is 0.9942. The concentrations of nisin in the cytoplasm of LAC85 cells were extrapolated from the standard curve for nisin. Without nisin addition, the RFU value was 55 ± 1.4, and this value was subtracted from the mean of RFU values of the other strain samples. The concentration of nisin in the cytoplasm of LAC85 cells determined in this way was 6.2 ± 1.6 ng ml⁻¹ (marked with an arrow on the plot). When this experiment was done, cells of LAC85 were mixed with the protease inhibitor PSMF prior to sonication.
transcribed due to the lack of nisin induction. The isolated cytoplasm was incubated with fully modified nisin precursor. Nisin activation, reflecting removal of the leader from fully modified prenisin, was evident (Fig. 4). This showed that the cytoplasm of LAC67 cells has the capacity to activate fully modified nisin precursor into nisin, explaining why nisin could be isolated from the cytoplasm of the NisT mutant cells.

**DISCUSSION**

At subinhibitory levels, extracellular nisin induces the nisin promoters via NisK and NisR, the TCST system involved in regulation of nisin biosynthesis and immunity (Kuipers et al., 1995; Dunny & Leonard, 1997). In this study we found that the nisZ promoter was induced by intracellular nisin in the NisT mutant LAC85. Nisin has been previously detected from cytoplasmic fractions (Qiao & Saris, 1996), but induction of the nisin promoters by intracellular nisin was not analysed in that study. Further, no protease inhibitor was added when cells were broken, leading to the possibility that cell-wall-anchored NisP could cleave the leader from the nisin precursor after cell breakage. Therefore, it was not certain whether the nisin activity detected from the isolated cytoplasm was formed before or after the breakage of the cells as has been suggested by Chatterjee et al. (2005). Therefore, we added the serine protease inhibitor PMSF to the LAC85 cells before breakage of the cells. PMSF should be capable of inhibiting the NisP as it is a serine protease (van der Meer et al., 1993) anchored on the outside of the cells. We showed (Fig. 2) that PMSF could inhibit the NisP activity of intact LAC85 cells. No nisin activity was detected in LAC85 cells incubated together with PMSF and the fully modified nisin precursor still having the leader attached. The control showed that cells of LAC85 have NisP activity. In this study PMSF was added to the LAC85 cells prior to breakage of the cells so it can be concluded that the nisin activity detected in the isolated cytoplasm of LAC85 cells must have already been formed inside the cells. Cells of LAC85 were also grown in the presence of PMSF followed by breakage in the presence of PMSF. Nisin was also detected in the isolated cytoplasm of these cells. In addition, nisin activity analysis using nisin-sensitive M. luteus cells incubated with samples of the cytoplasm of LAC85 cells isolated with and without PMSF showed that the cytoplasm of LAC85 did not contain a significant amount of fully modified nisin with the leader still attached. If this had been the case, then the inhibition of the LAC85 cytoplasm isolated with PMSF should have yielded less nisin activity compared to cytoplasm isolated without PMSF. In fact, the cytoplasm isolated with PMSF yielded slightly more activity (Fig. 2b), perhaps indicating that PMSF protects nisin from degradation. In order to verify that the cytoplasm of L. lactis does contain protease activity that can cleave the leader from the fully modified nisin precursor with the leader still attached, we isolated the cytoplasm from LAC67 cells, which do not have NisP activity (Qiao et al., 1996), and incubated it with isolated fully modified nisin precursor. The fact that the cytoplasm could activate the fully modified nisin precursor provides further evidence that L. lactis does have intracellular proteolytic activity capable of degrading the leader from the fully modified nisin precursor. This explains why we could isolate nisin from the cytoplasm of LAC85 cells in the presence of PMSF: the nisin leader had been cleaved prior to the breakage of the cells. We have not identified the protease capable of nisin leader cleavage from the genome of the sequenced L. lactis subsp. lactis IL1403 genome (Bolotin et al., 2001), but the gene content of different strains may differ (Kok et al., 2005). A nisin producer contains a 70 kb transposon, which is not present in L. lactis subsp. lactis IL1403. Proteins have a certain half-life in cells and several proteolytic systems for this protein turnover are present in all living cells. The proteasome into which unfolded proteins are inserted for degradation is a potential candidate for degrading the leader from the fully modified nisin precursor. The leader has an elongated α-helical structure and could fit into the proteasome and be degraded, whereas the rest of the molecule is a more rigid and compact structure and may not fit into the proteasome (Groll & Clausen, 2003).

As nisin can be found inside LAC85 cells, it seems unlikely that the induction of the PnisZ promoter observed in these cells could have been due to intracellular production of nisin with the leader still attached, transported by some other transport protein followed by cleavage by NisP. Compared to LAC109 (the nisB mutant) and LAC108 (the nisC mutant), which contain unmodified nisin precursor and dehydrated nisin precursor (Koponen et al., 2002), LAC85 cells were the only cells having active nisin in the cytoplasm and the PnisZ promoter activated without external nisin addition (Fig. 1c, lane 1). This suggests that nisin from

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**Fig. 4.** Nisin bioassay using M. luteus as nisin-sensitive strain. Spots: 1, the fully modified prenisin; 2, 10 mg ml⁻¹ trypsin; 3, the cytoplasm fraction of LAC76 mixed with the fully modified prenisin; 4, the fully modified prenisin pre-incubated with 10 mg ml⁻¹ trypsin at 37 °C for 2 h; 5, the cytoplasm fraction of LAC76 cells; 6, the cytoplasm fraction of LAC76 cells mixed with the fully modified prenisin and the serine protease inhibitor (PMSF). Spots 1–6 are from the same plate.
inside the cells can activate the nisin-inducible P_{nisZ} promoter. Normally this promoter is activated via the two-component regulatory proteins NisRK, but that requires external nisin (Kuipers et al., 1995).

There are several possibilities for how the intracellular nisin of the LAC85 cells, lacking the nisin-specific secretion capacity, could reach the externally located signal recognition part of the NisK histidine sensor. To exclude the possibility that some of the LAC85 cells had lysed, releasing nisin to the intact cells and resulting in induction of P_{nisZ}, we quantified nisin from the growth supernatant using the most sensitive nisin detection and quantification system presently available. Nisin could not be detected in the culture supernatant with this assay, which has a detection limit of 10 pg ml^{-1}. This sensitivity is higher than the detection limit of nisin of 0.1 ng ml^{-1} of a strain with a gusA gene connected to the nisA promoter (Kuipers et al., 1995). If the LAC85 cells released less than 10 pg nisin ml^{-1}, then this concentration would probably not have been sufficient to induce the nisZ promoter as strongly as seen in Fig. 1. Therefore, as no detectable nisin was present in the medium of the LAC85 cells it is more likely that the observed activation of the nisZ promoter in the LAC85 cells was due to the fact that NisK was capable of recognizing its signal, nisin, in a non-aqueous environment. As a result of the nisin transport deficiency the nisin-mediated quorum sensing had changed to signalling with nisin only to the cells themselves.

Nisin is an amphiphilic molecule and can interact with and insert into the membrane (Hasper et al., 2004). However, it is possible that nisin could to some extent be translocated by some transporter other than NisT to the pseudoperiplasmic space, i.e. the space between the membrane and the peptidoglycan. From there nisin could be recognized by NisK and/or adsorbed to the membrane surface or the cell wall. Potentially, nisin would never escape from the pseudoperiplasmic space or to such a low extent that it could not be detected in the growth medium. In the phosphorelay signal transduction system of Bacillus subtilis it has been postulated that the secreted signal PhrA is processed after secretion and taken back into the cells via the oligopeptide transport system (Perego, 1997). As a consequence only a little physiologically detectable peptide is found in the culture supernatant and the majority is inside the cells, where the PhrA can act as a signal. In nisin signal transduction, the signal, i.e. nisin, is needed only outside the cell and normally the majority of nisin produced is found in the culture supernatant (Ra et al., 1996). Activity of the nisin immunity transporter NisFEG should result in accumulation of nisin in the growth medium according to the results of Stein et al. (2003). They showed that NisFEG-expressing B. subtilis in a nisin-containing medium could keep more nisin in a soluble form in the growth medium compared to cells not expressing NisFEG. This suggested that the function of NisFEG is to pump nisin adsorbed on the surface or membrane of the cells into the medium. Clearly, the NisFEG of LAC85 cells could not translocate nisin from the pseudoperiplasmic space or membrane into the growth supernatant in detectable amounts, as nisin could not be detected in the growth medium of these cells.

Another explanation for why the nisZ promoter in pLEB384 was activated in the LAC85 cells is that nisin that had accumulated inside the cells inserted into the membrane, where it was recognized by NisK. It is not known which part of nisin is required for its function as a signal for NisK. The part of nisin needed for interaction with NisK may possibly stick out from the membrane into the aqueous pseudoperiplasmic space and be available for signal recognition by NisK.

It has been suggested that when nisin is inserted in the membrane the C-terminal part of the protein would not be inside the membrane and that the positive charge(s) would interact with the negatively charged phospholipids (van den Hooven et al., 1996). This part of nisin could well be involved in signalling, as the C-terminally His-tagged nisin we have previously constructed could not act as a signal for NisK (Koponen et al., 2002). In addition, changing one amino acid in the C-terminal part of nisin has been shown to reduce signalling capacity to 8% compared to nisin Z (van Kraaij et al., 1997), further showing that the C-terminal part of nisin is important for the signal function of nisin. Our results suggest that functional nisin inside the cells is capable of inducing the P_{nisZ} promoter in the cells of LAC85. This induction is likely to occur via NisK, as partly modified nisin precursors inside the cells could not induce (Koponen et al., 2002) and active nisin could not be detected from the cytoplasm of a wild-type nisin producer (Qiao & Saris, 1996). To verify that NisK is involved in the activation of the LAC85 P_{nisZ} promoter, a nisK knock-out mutation is needed. However, such a mutation abolishes transcription of the nisin-induced promoters (de Ruyter et al., 1996), making the analysis difficult, as a nisTK mutant strain would not have any nisin in the cytoplasm. Verifying the presence of nisin in the membrane of LAC85 cells is also difficult, as separation of the membrane from the cytoplasm would involve breakage of the cells and then nisin would be found in both fractions due to its amphiphilic nature. However, the inability of partially modified nisin precursors, accumulated in the cytoplasm of NisB and NisC mutants, to induce the P_{nisZ} promoter suggests that the induction of the P_{nisZ} promoter in LAC85 cells occurred via NisK. How nisin reaches NisK is unclear, but we favour the possibility that nisin inserts into the membrane and from there presents parts of the molecule into the pseudoperiplasmic space for the NisK signal recognition domain. This could mean that in wild-type nisin producers NisK recognizes nisin not from the water-soluble but rather from a non-soluble phase. This could be a general feature of amphiphilic peptides functioning as signal molecules in TCST pathways.

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