

Resistance of Gram-positive bacteria to nisin is not determined by Lipid II levels

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Abstract

Lipid II is essential for nisin-mediated pore formation at nano-molar concentrations. We tested whether nisin resistance could result from different Lipid II levels, by comparing the maximal Lipid II pool in *Micrococcus flavus* (sensitive) and *Listeria monocytogenes* (relatively insensitive) and their nisin-resistant variants, with a newly developed method. No correlation was observed between the maximal Lipid II pool and nisin sensitivity, as was further corroborated by using spheroplasts of nisin-resistant and wild-type strains of *M. flavus*, which were equally sensitive to nisin.

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1. Introduction

The development of resistance of bacteria towards antibiotics is a global problem that calls for new approaches to kill harmful microorganisms. A promising alternative for antibiotics is nisin, an antimicrobial peptide that displays a broad spectrum of activity against Gram-positive bacteria and is produced by *Lactococcus lactis* [1]. The peptide is already widely used in the food industry as a safe and natural preservative [2]. Nisin kills bacteria primarily by formation of pores in the cytoplasmic membrane via binding to Lipid II [3,4].

Lipid II plays a central role in bacterial cell wall biosynthesis: it contains bactoprenyl-phosphate, which carries the cell wall building blocks UDP-GlcNAc (uridine 5'-diphospho-*N*-acetylglucosamine) and UDP-MurNAc-pp (uridine 5'-diphospho-*N*-acetylmuramoyl-pentapeptide) across the cytoplasmic membrane (Fig. 1). As a consequence of this crucial function during bacterial growth, Lipid II, and, indirectly, the bactoprenyl-phosphate pool, have become important targets for antibiotics.

Despite its prolonged use as a preservative in the food industry, no reports of emerging resistance towards nisin have yet appeared. This could be related to the double mode of action of nisin. By binding to Lipid II, it inhibits cell wall synthesis while on the other hand it forms pores in the cytoplasmic membrane. However,

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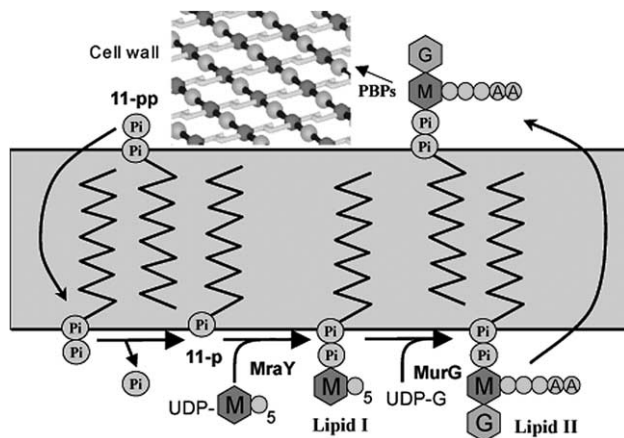


Fig. 1. *Peptidoglycan synthesis*: The transfer of the phospho-MurNAc-pentapeptide moiety of UDP-MurNAc-pentapeptide to the membrane acceptor bactoprenyl-phosphate is catalyzed by the transferase MraY and leads to synthesis of Lipid I. Addition of GlcNAc to Lipid I, by the transferase MurG, results in Lipid II, which carries the complete disaccharide peptide monomer unit: GlcNAc-MurNAc-L-Ala- γ -D-Glu-A2pm (diaminopimelic acid) (or L-Lys)-D-Ala-D-Ala. Finally, the molecule is translocated across the membrane by an unknown transport mechanism and GlcNAc-MurNAc-pentapeptide is released and integrated into peptidoglycan [26].

nisin-resistant strains can be readily obtained in laboratory settings [5–8]. The possible role of Lipid II in resistance development is still unknown.

The natural variation in the sensitivity of Gram-positive bacteria towards nisin is considerable. Even between closely related species, minimal inhibitory concentrations (MIC) range from 5 μ g/L to 5 mg/L [9]. Insights into how bacteria develop nisin resistance and the basis of the differences in nisin sensitivity are of major importance for future applications of nisin or nisin variants. Here, we investigate the possible role of Lipid II in determining the difference in sensitivity of bacteria to nisin. Two different species of Gram-positive bacteria, namely *M. flavus* and *L. monocytogenes*, and their isogenic nisin-resistant variants were selected. The four strains cover a broad range of natural variation in nisin sensitivity, varying from 20 μ g/L to 10 mg/L. Using a newly developed assay for the quantification of the maximal amount of Lipid II in bacteria, we examined whether variations in the amount of Lipid II are responsible for the differences in nisin sensitivity.

2. Materials and methods

2.1. Chemicals and materials

All chemicals were of analytical grade. UDP-GlcNAc was from Sigma–Aldrich Chemie (Steinheim, Germany), [14 C]-UDP-GlcNAc from NEN Life Sciences, Inc. (Boston, MA, USA).

Table 1
Bacterial strains and plasmids used in this study

Strains or plasmid	Source
<i>Micrococcus flavus</i> NIZO B423	NIZO ^a Food Research
<i>Micrococcus flavus</i> NIZO B423 Nis ^R	This work
<i>Listeria monocytogenes</i> NIZO B1242	NIZO ^a Food Research
<i>Listeria monocytogenes</i> NIZO B1242 Nis ^R	This work
<i>Escherichia coli</i> BL21 DE3	NCCB ^b
plysS; PET 21b+ <i>murG</i>	S. Walker, Princeton

^a Dutch Institute of Dairy Research.

^b The Netherlands Culture Collection of Bacteria.

2.2. Bacterial strains, cloning and culture conditions

Bacterial strains and plasmids used in this study are listed in Table 1. *M. flavus* was grown at 30 °C with shaking at 200 rpm, in Trypton Soy Broth (Oxoid, Basingstoke, Hampshire, UK). *L. monocytogenes* was grown at 37 °C in Brain Heart Infusion (Oxoid). *Escherichia coli* was grown in TY broth at 37 °C while shaking at 200 rpm. *E. coli* BL21 (DE3) (plysS; pET21b+), was used for production and isolation of MurG, carrying a N-terminal His₆-tag. Media contained ampicillin at 100 mg/L or chloramphenicol at 25 mg/L, when appropriate [10].

2.3. Generation of nisin-resistant strains

A nisin stock solution was derived from nisaplin, (2.5% nisin, Aplin and Barrett, Danisco, Copenhagen, Denmark), as described earlier [11], and was used to obtain nisin-resistant isogenic variants of *M. flavus* and *L. monocytogenes* via the following procedure; the strains were grown in broth with 10 μ g/L nisin for *M. flavus* and 900 μ g/L nisin for *L. monocytogenes*, which is just below the respective MIC values. Subsequently, the strains were repeatedly inoculated in media with increasing concentrations of nisin. After every 20th generation, nisin-resistant cultures were plated on nisin-containing plates and single colonies were picked for further enrichment. Nisin-resistant single colonies were picked, and grown in the presence of the appropriate nisin concentration and stored at –80 °C. Nisin was always added to the growth media to maintain resistance.

2.4. Antimicrobial activity determinations

M. flavus and *L. monocytogenes* were grown overnight. The Nis^R variants were grown in the presence of nisin (2.5 mg/L for *M. flavus* Nis^R and 10 mg/L for *L. monocytogenes* Nis^R). Hereafter the MIC values were essentially obtained as described before [12].

2.5. Determination of the maximal Lipid II content (Lipid II max.) in bacterial membranes

Bacterial cells were grown until mid-exponential growth phase (OD_{600} of 0.5–0.8). Membranes were isolated from lysozyme-treated cells by centrifugation at 40,000g for 45 min. They were washed twice in 50 mM Tris–HCl, 10 mM $MgCl_2$, pH 7.5 or 100 mM potassium phosphate, pH 7.5, for *M. flavus* and *L. monocytogenes*, respectively, and stored in liquid nitrogen. Membranes were thawed and incubated in the presence of sufficient precursor sugars and Triton X-100 to ensure total conversion of endogenous Lipid I, undecaprenyl di-phosphate (11-pp), and, undecaprenyl mono-phosphate (11-p pools) to Lipid II. In short: membranes (2 mM of Lipid-phosphate (Pi)) were gently mixed with a solution containing Tris–HCl (pH 8, 100 mM), $MgCl_2$ (6 mM), UDP-MurNAc-pentapeptide (45 μ M), [^{14}C]-UDP-GlcNAc (45 μ M) with a specific activity of 0.45 GBq/mmol, 1% TX-100 and an excess of H_6 -MurG (for *L. monocytogenes*) and incubated at RT for 1 h. H_6 -MurG was isolated from *E. coli* BL21 (DE3) (plysS, pET21b+ *murG*) as described previously [10]. Subsequently, 7.5 μ l of this mixture was spotted on cellulose membranes (Merck), which were developed in isobutyric acid: ammonia (7:2). The radioactive spots were visualized and quantified using a phosphor-imager (Molecular Dynamics, Sunnyvale, CA) and the Image Quant 1.2 program (Molecular Dynamics). The number of cells in 1 μ l of culture was determined using light microscopy. The amount of phospholipids per μ l of culture was determined by extracting them according to Bligh and Dyer [13]. The amount of organic phosphate in the sample was determined according to Rouser [14]. This allowed to determine the amount of Lipid II_{max} per cell, expressed as the ratio between Lipid II_{max} and phospholipids.

2.6. Membrane potential measurements on whole cells and spheroplasts

Cells of 10 ml overnight culture of *M. flavus* were washed in 3 ml of 10 mM potassium phosphate buffer (pH 7.5), 5 mM $MgSO_4$ and resuspended in 3 ml of the same buffer. A parallel sample was resuspended in a solution containing 1 M sucrose, 100 mM NaCl and lysozyme (1 μ g/ml) and incubated at 30 °C for 30 min to obtain spheroplasts. Cells or spheroplasts were diluted to an OD_{600} of 0.075 in the same buffer containing a final concentration of 2 μ M 3,3-dipropylthiacarbocyanine iodide (DiSC₃(5)) (Molecular Probes, Leiden, the Netherlands). The membrane potential ($\Delta\psi$) was monitored with the DiSC₃(5) probe (excitation wavelength, 651 nm; emission wavelength, 675 nm) using a Perkin–Elmer model 650-10S fluorescence spectrophotometer (Perkin–Elmer Corp., Oosterhout, the Netherlands), at

25 °C. At maximal dye incorporation, purified nisin was added (concentrations ranging from 10 nM to 1.8 μ M) to the suspension. To set the arbitrary level at 100% dissipation, valinomycin (1 μ M end concentration) was added to the suspension to completely dissipate the membrane potential. The ethanol level in the suspension never exceeded 0.5%.

3. Results and discussion

3.1. Isolation of *Nis*^R variants of *M. flavus* and *L. monocytogenes*

Nisin-resistant variants were obtained as described in Section 2. A *Nis*^R variant of *L. monocytogenes* was obtained that could grow in the presence of 10 mg/L, which is 10 times the MIC value of the *L. monocytogenes*-parent strain. Interestingly, an identical final MIC value was found earlier with different *Listeria* strains [15], suggesting that about 10 mg/L is the limit to which these bacteria can become resistant. A nisin-resistant *M. flavus* strain could grow in the presence of 2.5 mg/L, which is 125 times the MIC value of the wild-type strain (Table 2). The MICs for both parent strains are within the concentration range where nisin activity in model systems is dependent on the presence of Lipid II [16], implying that the differences in sensitivity could be due to differences in Lipid II content. The MIC value of *L. monocytogenes Nis*^R is in the concentration range where nisin can be active independent of Lipid II, but still depends on the presence of anionic phospholipids [16,17]. In this case the acquired nisin-resistance, could result in part from Lipid II independent mechanisms.

3.2. The maximal Lipid II pool in the cytoplasmic membrane is not correlated to nisin sensitivity

To test whether the Lipid II content in the bacterial membrane contributed to the difference in sensitivity of bacteria to nisin, we determined the maximal Lipid II content in the different strains described above. However, it is difficult to determine the Lipid II content directly, because of the very short half-life of Lipid II in the living cell [18], resulting in hardly detectable endogenous Lipid II in

Table 2
Minimal inhibitory concentration for nisin and bactoprenyl-phosphate content in the cytoplasmic membrane of *L. monocytogenes* and *M. flavus* strains

Strain	MIC (μ g/L)	11-p/Pi ^{a,b}	11-p/cell ($\times 10^5$)
<i>M. flavus</i>	20	1:190 \pm 85	1.9
<i>M. flavus Nis</i> ^R	2500	1:172 \pm 41	1.8
<i>L. monocytogenes</i>	1000	1:108 \pm 22	2.4
<i>L. monocytogenes Nis</i> ^R	10,000	1:119 \pm 28	2.2

^a 11-p = Bactoprenyl-phosphate and Pi = phospholipids.

^b Average of five independent experiments.

isolated membranes. A direct method to determine the pools of Lipids I and II has been described for *E. coli* [19]. Unfortunately, this method is not generally applicable to other bacteria as it makes use of an *E. coli* strain with a specific mutation in the diaminopimelic acid biosynthesis route. As an alternative, we devised a method to determine the maximal amount of Lipid II that the bacterial membrane could contain, by complete conversion of the 11-p, 11-pp and Lipid I pools into Lipid II. [¹⁴C]-UDP-GlcNAc was used to quantify Lipid II as described in Section 2. Although this method does not directly measure the pool level of Lipid II, it is capable of revealing (up or down)-regulation in bactoprenyl-phosphate biosynthesis. Levels of bactoprenyl-pyrophosphate and Lipid I are presumably very low as compared to the bactoprenyl-phosphate levels [20], and therefore we assume that the amount of Lipid II in our assay is proportional to the bactoprenyl-phosphate pool of the bacteria. This assumption seems justified when the thick peptidoglycan layer of Gram-positive bacteria is considered: peptidoglycan synthesis is most likely the main pathway that uses the bactoprenyl-phosphate pool.

With this method, the ratios of Lipid II_{max} to phospholipids in the four strains used in this study were estimated to range from 1:190 to 1:108 (Table 2). Thus, between two bacterial strains with a 50-fold difference in MIC value only a 2-fold difference in maximal Lipid II content could be detected, which cannot be responsible for the large differences in MIC values. Storm and Strominger [21] also determined the total pool level of bactoprenyl-phosphate, by determining the number of bound bacitracin molecules per bacterium at saturation, concluding that the total pool level of bactoprenyl-phosphate in *M. lysodeikticus* was 2×10^5 molecules per cell, which is very similar to our result.

The maximal amount of Lipid II in the membranes of the Nis^R variants of *M. flavus* and *L. monocytogenes* and the two parental strains did not show a significant difference (Table 2). Because the availability of bactoprenyl-phosphate is rate-limiting for several aspects of cell wall synthesis in *Staphylococcus aureus* [22] and *Bacillus* sp. [23], we conclude that nisin resistance of our Nis^R strains is not caused by down-regulation of the bactoprenyl-phosphate content, which would result in lower Lipid II pool levels in the cells. Thus, the nisin resistance of the Nis^R strains is not directly related to the membrane Lipid II levels. The results do emphasize the importance of a stable Lipid II cycle in bacterial cell wall synthesis, probably because this molecule is indispensable for cell wall biosynthesis.

3.3. Removal of the cell wall dramatically changes the sensitivity to nisin

The above conclusion implies that spheroplasts of the nisin-sensitive and -resistant strains would be equally

Table 3

3,3-Dipropylthiacarbocyanine iodide (DisC₃₍₅₎) release from whole cells and protoplasts of sensitive and resistant strains of *M. flavus* after exposure to nisin

	<i>M. flavus</i> ^a (%)	<i>M. flavus</i> Nis ^{Ra} (%)
<i>Whole cells</i>		
1 nM nisin	ne	ne
10 nM nisin	17 ± 6	ne
100 nM nisin	31 ± 10	12 ± 1
500 nM nisin	58 ± 2	14 ± 2
1.8 μM nisin	nd	38 ± 5
<i>Protoplasts</i>		
10 nM nisin	58 ± 8	38 ± 5
100 nM nisin	100	70 ± 3

ne = No detectable effect.

nd = Not done.

^a Mean of three independent experiments.

sensitive to nisin. Interestingly, when the sensitivity of the spheroplasts was compared to the sensitivity of the intact parental cells, it became apparent that removal of the cell wall dramatically changed the sensitivity of both *M. flavus* strains (Table 3). Dissipation of the membrane potential was already observed after addition of 10 nM of nisin to the spheroplasts of both sensitive and resistant strains (Table 3). This suggests that the cell wall is an important factor in acquiring nisin resistance, as has been proposed before [15,24,25]. Strikingly, only a slight (1.4-fold) difference in dissipation remained between the spheroplasts of wild-type and the Nis^R variant. The 1.4-fold difference in sensitivity that remains could be due to a decrease in negatively charged phospholipids composition observed in the nisin-resistant strains (data not shown). These findings support our earlier suggestion that Lipid II levels are kept constant.

In conclusion, our results indicate that, despite the essential role of Lipid II in the mode of action of nisin, there is no direct role of Lipid II in nisin-resistance. The observed resistance must originate from other mechanisms. The results of the spheroplast experiments suggest that the cell wall has been changed such that nisin is unable to gain access to Lipid II, and hence that the cell wall is the major determinant in acquiring nisin resistance.

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