Cell wall changes in nisin-resistant variants of *Listeria innocua* grown in the presence of high nisin concentrations

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**Abstract**

Two nisin-resistant variants of a strain of *Listeria innocua* were isolated after growth in the presence of 500 and 4000 IU ml⁻¹ of nisin A showed increased cell wall hydrophobicity, resistance to phage attack and three different cell wall-acting antibiotics, as well as to the peptidoglycan hydrolytic enzymes lysozyme and mutanolysin, as compared to the parental strain. Transmission electron microscopy revealed marked thickening of the wall of nisin-resistant cells with an irregular surface. Differences in thickness were lost after cell wall purification and no significant difference in gross wall composition was observed between the parental and resistant variants. Cell wall changes in nisin-resistant *listeriae* are attributed to abnormal cell wall synthesis and autolysin inhibition, the latter possibly associated with subtle changes in cell wall structures and function.

**Keywords:** *Listeria*; Nisin resistance; Cell wall composition; Transmission electron microscopy; Lytic enzyme resistance; Cell wall antibiotic

1. Introduction

Nisin is a cationic peptide which affects the integrity of the cytoplasmic membrane of sensitive bacteria by formation of pores. It results in a rapid efflux of small metabolites [1,2]. Loss of viability generally follows with a more or less rapid cell lysis, depending on the bacterial strain or species [3,4].

Several authors [5–7] have observed nisin-resistant cells at frequencies of 10⁻⁶–10⁻⁸ in populations of different susceptible strains of *L. monocytogenes*. Although many bacterial genera have been shown to be ‘trained’ to resist high nisin concentrations, the properties of the resistant cultures have not been investigated, except for studies concerning cross-resistance with other antibiotics, or stability of nisin resistance [3].

Since colonization of the environment or of food manufacturing facilities by strains resistant to nisin may threaten the routine use of this bacteriocin as a food biopreservative, we investigated some properties of nisin-resistant cultures of *listeriae*. We describe here some cell changes induced in two nisin-resistant variants of *L. innocua* after growth in the presence of high concentrations of this inhibitor, as compared to the parental strain cultivated in the absence of nisin. We also suggest possible mechanisms of cell wall changes.

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2. Materials and methods

2.1. Organisms and culture conditions

A nisin-sensitive strain of *Listeria innocua* (serotype 6b, cheese origin from Pasteur Institute, Paris, France), hereafter referred to as *L. innocua* Lin11, was used, plus two variants of this organism which were able to grow in the presence of 500 and 4000 IU ml\(^{-1}\). The cultures were maintained at 4°C in TSBYE (tryptic soy broth, Difco, supplemented with 0.6% yeast extract, BioMérieux) containing nisin, and transferred monthly. Unless otherwise stated, prior to experimental work all bacteria were subcultured twice into fresh medium of the same composition as for storage and cultivated at 30°C to the stationary phase.

2.2. Nisin preparation

A nisin stock solution (10\(^5\) IU ml\(^{-1}\)) was prepared by dissolving purified nisin (Aplin and Barrett Ltd., Trowbridge, UK) in 0.02 N HCl and sterilized using a 0.22 μm low-protein binding filter (Millex GV, Millipore SA). Aliquots were kept frozen at −20°C and used within one month.

2.3. Determination of the MIC

The minimal inhibitory concentration (MIC) of nisin for *L. innocua* Lin11 was determined by use of serial 1:2 dilutions of the bacteriocin in TSBYE (pH 7.0). Each tube containing 10 ml of broth was inoculated with 10\(^6\) cells ml\(^{-1}\) and incubated for 24 h at 30°C. The lowest nisin concentration that prevented growth was defined as the MIC.

2.4. Selection of nisin-resistant cultures

Nisin-resistant variants were obtained by culturing *L. innocua* Lin11 into TSBYE which contained increasing concentrations of nisin. A culture able to grow in the presence of 500 IU ml\(^{-1}\) of nisin (corresponding to the MIC of *L. innocua* Lin11) and hereafter named medium resistant variant (MRV) was selected along with a highly resistant variant (HRV) which resisted 4000 IU ml\(^{-1}\) (i.e. 8 times the MIC). The identity of the derivatives and the parental strain was confirmed by biochemical tests (API-Listeria, BioMérieux, France) and restriction DNA analysis (Dr. Rocourt, Pasteur Institute, Paris, France).

2.5. Action of cell wall antibiotics and lytic enzymes

The optical density of the cultures of *L. innocua* Lin11, MRV and HRV into fresh TSBYE (10%, v/v) was monitored using a spectrophotometer (Spectronic 20 D, Milton Roy, USA). At mid-exponential phase of growth (OD = 0.5–0.6 at 620 nm), carbenicillin (100 μg ml\(^{-1}\), Serva), D-cycloserine and vancomycin (500 μg ml\(^{-1}\) each, Sigma) were added to the cultures and OD was recorded every 30 min for 5 h.

In a separate experiment, cells in exponential phase of growth were harvested by centrifugation at 6000 × g for 10 min, washed in 10 mM Tris·HCl (pH 7.4) and resuspended in 10 mM Tris·HCl (pH 7.4) containing 10 mM MgCl\(_2\), 0.5 M of sucrose and either 5 mg ml\(^{-1}\) of chicken egg white lysozyme or 5 U ml\(^{-1}\) of mutanolysin (both from Sigma). After 1 and 3 h of incubation at 37°C, the cell suspensions were observed under a phase contrast microscope to observe protoplast formation. Samples were diluted in peptone water (0.1%, w/v, Difco) and plated onto TSBYE plates. The colonies were counted after 48 h of incubation at 30°C.

2.6. Hydrophobicity test

Cell surface hydrophobicity was determined with the hydrocarbon/buffer two phase system of Rosenberg et al. [8]. Briefly, a 1.2 ml portion of bacterial suspension (OD = 0.5 at 400 nm) in distilled water was mixed with 1.0 ml of n-hexadecane (Sigma) and shaken vigorously. After the phases were separated, the aqueous phase was carefully removed and its optical density was measured at 400 nm. The decrease (expressed in %) in optical density of the aqueous phase was used as a measure of cell surface hydrophobicity.

2.7. Lysotyping, phage sensitivity and adsorption tests

Lysotyping of *L. innocua* Lin11 and its nisin-resistant variants was performed in Dr. Rocourt’s labo-
ratory (Pasteur Institute, Paris, France). Phages 4211, 1090, 4276, 4277, 4292 were selected for their ability to cause lysis of *L. innocua* [9]. For the adsorption tests, $10^8$ cells ml$^{-1}$ of *L. innocua* Lin11, MRV and HRV grown to the stationary phase in TSBYE of appropriate nisin concentrations were mixed with 0.1 ml of a phage suspension ($10^7$ PFU ml$^{-1}$) in tryptose phosphate broth (Difco). The mixture was incubated for 1 h at 30°C and filtered with a 0.45 μm filter (Millipore). Free phage particles were enumerated in the supernatant using *L. innocua* strain PS4202 as indicator. The results were compared with the titer of a blank without bacteria to determine the percentage of phage adsorption.

2.8. Cell wall analysis

2.8.1. Isolation of cell walls

Cells from one liter of cultures in stationary phase of *L. innocua* Lin11, MRV and HRV were harvested by centrifugation at 2000 × g for 10 min. After having been washed twice with distilled water, they were disintegrated with 0.1 μm glass beads (Sigma) in a cooled cell grinder. After separation of the beads from the suspension, cells and debris were removed by centrifugation at 2000 × g for 10 min. The suspension was then heated to 98°C for 15 min to inactivate the hydrolytic enzymes. Cell walls were pelleted at 18000 × g for 30 min and washed 6 times alternately with distilled water and 0.9% NaCl (w/v) in distilled water. Washed cell walls were freeze-dried and stored at −20°C.

2.8.2. Chemical analysis

The protein concentration of the cell wall suspensions of *L. innocua* Lin11, MRV and HRV was determined using the Coomassie method (Pierce, Rockford, IL, USA) and bovine serum albumin as the protein standard. Samples for muramic acid analysis were prepared by hydrolysing cell wall suspensions with 5 N HCl for 16 h at 96°C in sealed glass tubes. Amino acid analysis was performed in a Beckman amino acid analyzer using a sodium citrate buffer system. Phosphorus was determined by the method of Chen et al. [10] and neutral sugars by the phenol-H$_2$SO$_4$ method of Dubois et al. [11].

2.8.3. Enzymatic digestion of cell walls and heat-inactivated whole cells

Bacterial autolysins were inactivated in whole cells by boiling in a water bath for 15 min. Cells were then harvested and resuspended in sodium phosphate buffer (50 mM, pH 7.0) to an OD = 0.4 at 620 nm. Lyophilized cell walls were resuspended in the same buffer to 1 mg ml$^{-1}$ final concentration. Reactions were initiated by adding 20 U ml$^{-1}$ of mutanolysin (Sigma). Incubation was carried out at 37°C, and whole cell or cell wall degradation was monitored using the spectrophotometer.

2.9. Transmission electron microscopy

Mid-exponential cultures (OD = 0.5) of *L. innocua* Lin11, MRV and HRV in TSBYE were prefixed with 2% (v/v) glutaraldehyde (Tabb Laborato-

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![Fig. 1](https://academic.oup.com/femsle/article/140/1/29/542056)

Fig. 1. Action of cell wall-acting antibiotics at 30°C in TSBYE on cultures of *Listeria innocua*, strain Lin11: (a) parental susceptible strain, (b) medium nisin-resistant variant, (c) highly nisin-resistant variant. Control (■), carbenicillin 100 μg ml$^{-1}$ (▲), d-cycloserine 500 μg ml$^{-1}$ (●). Arrow indicates antibiotic addition.
ries Equipment Ltd.) and centrifuged at 6000 × g for 10 min at 4°C. Thin sections were prepared and stained using the method previously described [12]. Cell walls were prepared in a similar way except that fixation in glutaraldehyde and osmium tetroxide was performed at room temperature for 2 h. All preparations were observed with a Zeiss EM10 electron microscope (Oberkochen, Germany).

3. Results

3.1. Action of cell wall-acting antibiotics and muramidases

Fig. 1 illustrates the effect of cell wall antibiotics on log phase of *L. innocua* Lin11 and nisin-resistant variants. Growth inhibition of the three cultures was observed but marked bacteriolysis occurred only with *L. innocua* Lin11. It is worth noting that before adding the lytic enzymes, the resistant cultures were growing at a lower rate than the sensitive ones. Similar results were observed with 500 μg ml⁻¹ of vancomycin (data not shown). In addition, HRV cells were much more resistant to both lytic enzymes than *L. innocua* Lin11 as shown by viability loss (Table 1).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Loss of viability (log CFU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysozyme, 1 h</td>
<td>LIN11 2.5 HRV 0.6</td>
</tr>
<tr>
<td>Lysozyme, 3 h</td>
<td>LIN11 3.1 HRV 1.2</td>
</tr>
<tr>
<td>Mutanolysin, 3 h</td>
<td>LIN11 3.1 HRV 0.4</td>
</tr>
</tbody>
</table>

* Difference as compared to a control without lysozyme after 1 or 3 h of incubation at 37°C.
* LIN11: parental sensitive strain; HRV: highly resistant variant.

3.2. Hydrophobicity and phage sensitivity

Both *L. innocua* MRV and HRV showed less affinity towards n-hexadecane than *L. innocua* Lin11 and were considered to possess a less hydrophobic surface (Table 2). Moreover, the nisin-resistant variants were also phage-resistant, although they did not differ from the sensitive strain in particle adsorption.

3.3. Transmission electron microscopy of whole cells and cell walls

Light microscopy revealed that cultures of *L. innocua* MRV and HRV, unlike those of *L. innocua* Lin11, were composed of short chains and clumps

![Fig. 2. Transmission electron microscopy of ultra-thin sections of *Listeria innocua*, strain Lin11, grown in TSBYE at 30°C: (a) parental susceptible strain and (b) medium nisin resistant variant showing irregularly formed cells. Bars represent 120 nm.](https://academic.oup.com/femsle/article/140/1/29/542056 by guest on 03 October 2023)
Table 2
Cell wall hydrophobicity and phage sensitivity of *Listeria innocua* strain LIN1 as a function of resistance to nisin

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Sensitive cells</th>
<th>Resistant cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LIN1</td>
<td>MRV</td>
</tr>
<tr>
<td>Hydrophobicity (%)</td>
<td>32 (0.4)</td>
<td>20 (1.6)</td>
</tr>
<tr>
<td>Phage sensitivity</td>
<td>5/5</td>
<td>0/5</td>
</tr>
<tr>
<td>Phage adsorption (%)</td>
<td>99.9</td>
<td>99.5</td>
</tr>
</tbody>
</table>

* a Mean result of two independent experiments (SD).
* b Number of phages capable of lysis/number of tested phages.
* c Ratio of number of phages adsorbed on cells after 1 h at 30°C to initial number of phages in solution.

LIN1: parental sensitive strain; MRV: medium resistant variant; HRV: highly resistant variant.

which resisted vigorous mechanical shaking. As shown in Fig. 2, growth in the presence of high nisin concentrations resulted in irregularly shaped cells with localized thickening of the walls. No difference between the two nisin-resistant variants was noticed. Resistant cells also showed uneven surface and cytoplasmic abnormalities such as lamellae. However, the difference in wall thickness between sensitive and resistant cells was almost completely lost in washed cell walls (data not shown).

### 3.4. Chemical composition of cell wall and resistance to lytic enzymes

Chemical analysis of the cell walls of the three cultures did not reveal any significant difference in the quantitative composition (Table 3). Difference in protein concentrations could be due to variations in washing efficiency between preparations. Whole cells

<table>
<thead>
<tr>
<th>Component</th>
<th>Dried cell wall (μg/mg) (SE) from LIN1</th>
<th>MRV</th>
<th>HRV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensitive cells</td>
<td>120 (25)</td>
<td>68 (5)</td>
<td>145 (22)</td>
</tr>
<tr>
<td>Resistant cells</td>
<td>15</td>
<td>25</td>
<td>13</td>
</tr>
<tr>
<td>Proteins (n = 6)</td>
<td>15</td>
<td>25</td>
<td>13</td>
</tr>
<tr>
<td>Muramic acid (n = 1)</td>
<td>29 (3)</td>
<td>36 (3)</td>
<td>28 (3)</td>
</tr>
<tr>
<td>Phosphorus (n = 2)</td>
<td>95 (22)</td>
<td>81 (11)</td>
<td>89 (17)</td>
</tr>
<tr>
<td>Neutral sugars (n = 2)</td>
<td>95 (22)</td>
<td>81 (11)</td>
<td>89 (17)</td>
</tr>
</tbody>
</table>

n: number of experiments.

LIN1: parental sensitive strain; MRV: medium resistant variant; HRV: highly resistant variant.

Fig. 3. Degradation of (a) heat-inactivated whole cells and (b) washed cell wall suspensions (1 mg dry material ml⁻¹) by adding mutanolysin (20 U ml⁻¹), a cell wall lytic enzyme, of the parental susceptible strain of *Listeria innocua*, strain Lin1 (●), the medium nisin-resistant variant (▲) and the highly nisin-resistant variant (●) in 50 mM sodium phosphate buffer, pH 6.8 at 30°C.

of *L. innocua* MRV and HRV were lysed at a slower rate than the sensitive ones (Fig. 3), with a small difference between *L. innocua* HRV and MRV cells. In contrast, purified cell walls of *L. innocua* MRV were hydrolyzed at a rate similar to that of the sensitive strain whereas the purified wall of *L. innocua* HRV were more resistant.

### 4. Discussion

Thickening of the cell wall was the most striking observation when nisin-resistant listeriae were grown in the presence of high nisin concentrations, but such a feature partially disappeared during cell wall purification. The second observation was that nisin-resistant cultures also resist cell wall-acting antibiotics and hydrolysis by murein enzymes.

Reisinger et al. [13] using an in vitro system found that nisin either inhibits or slightly activates the formation of murein, depending on the concentra-
tion. It is not known whether this phenomenon really occurs in living bacteria, but one can deduce from our study that nisin did not inhibit peptidoglycan synthesis since the cell population increased. However, cell wall synthesis was undoubtedly disturbed with apparent accumulation of material between the cytoplasmic membrane and the cell wall. The disappearance of the difference in purified cell wall thickness between susceptible and resistant cultures suggests that nascent or poorly cross-linked peptidoglycan or any other cell wall components were lost during the purification steps of cell wall preparation.

On the other hand, Bierbaum and Sahl [4,14] have shown that cationic peptides (including nisin) adsorb to the teichoic acids of the cell wall of the test organism and thereby activate the autolytic enzymes of the bacteria. As the autolysins are also cationic proteins, their activation is described as an ion exchange process where the cation nisin ‘elutes’ the autolysins. More interestingly, these authors have also noted that high concentrations of the cationic peptides inhibited the autolysin activity.

Therefore, both activation of murein synthesis and autolysin inhibition may have contributed to the cell wall thickening of the nisin variants grown in the presence of high nisin concentrations. Consistent with autolysin inhibition is the increased resistance of nisin-resistant cells to lysozyme and mutanolysin, as well as the absence of lysis in presence of the cell wall-acting antibiotics. Moreover, resistance to phage attack while adsorption occurred may be attributed to the incapacity of phage lytic enzymes to attack the peptidoglycan from inside (if we assume that phage infection and production of phage particles occurred).

Lack of lysis probably occurred because some changes in cell wall composition and structure, e.g. in the peptide network, have reduced the access of cell wall antibiotics or lytic enzymes to their targets [15]. However, since nisin-resistant cells are able to grow, although at a lower rate than the parental strain, one can conclude that autolysin activity is only partially inhibited. This partial and possibly localized inhibition could be responsible for uneven surface stress, resulting in cell deformation [16]. Similar localized wall thickening and changes in surface morphology were also observed with a mutant of *Bacillus subtilis* whose growth was inhibited due to insufficient autolysin production [17].

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**References**


