RESEARCH LETTER

Functional differences in *Leuconostoc* sensitive and resistant strains to mesenterocin 52A, a class IIa bacteriocin

Jordane Jasniewski[1], Catherine Cailliez-Grimal[1], Mohamed Younsi[2], Jean-Bernard Millière[1] & Anne-Marie Revol-Junelles[1]

[1]Laboratoire de Science et Génie Alimentaires, Institut National Polytechnique de Lorraine, Nancy-Université, Vandoeuvre-lès-Nancy, France; and [2]Laboratoire de Nutrition et Maladies Métaboliques-EA 3446, Faculté de Médecine, Nancy-Université, Vandoeuvre-lès-Nancy, France

Correspondence: Anne-Marie Revol-Junelles, Laboratoire de Science et Génie Alimentaires, Institut National Polytechnique de Lorraine, Nancy-Université, 2 avenue de la Forêt de Haye, BP 172, 54505 Vandoeuvre-lès-Nancy, France. Tel.: +33 0 3 83 59 58 84; fax: +33 0 3 83 59 57 71; e-mail: anne-marie.revol@ensaia.inpl-nancy.fr

Received 21 July 2008; accepted 19 September 2008.
First published online November 2008.

DOI:10.1111/j.1574-6968.2008.01381.x

Editor: Andre Klier

Keywords
bacteriocin; anisotropy; antibacterial peptide.

Abstract

Mesenterocin 52A (Mes 52A) is a class IIa bacteriocin produced by *Leuconostoc mesenteroides* ssp. *mesenteroides* FR52. The interaction of Mes 52A with bacterial membranes of sensitive, resistant and insensitive *Leuconostoc* strains has been investigated. The degree of insertion of Mes 52A on the phospholipid bilayer was studied by fluorescence anisotropy measurements using two probes, 1-(4-trimethylammonium)-6-phenyl-1,3,5-hexatriene (TMA-DPH) and DPH, located at different positions in the membrane, and the consequence for K^+ efflux and proton motive force was analyzed. Mes 52A caused an increase in the fluorescence of TMA-DPH and DPH in the membrane of the sensitive strain *L. mesenteroides* ssp. *mesenteroides* LMA 7, indicating that Mes 52A inserts into the cytoplasmic membrane of this sensitive strain. This insertion leads to K^+ efflux, without perturbation of ΔpH and a weak modification of $\Delta\Psi$, and is consistent with pore formation. With the high-level resistant strain *L. mesenteroides* ssp. *mesenteroides* LMA 7AR, or with the insensitive strain *Leuconostoc citreum* CIP 103405, no modification of TMA-DPH or DPH anisotropy occurred, even in the presence of high Mes 52A levels. The membrane potential was not modified and no K^+ efflux was detected. There is a clear correlation between the physico-chemical characteristics of the membrane, the degree of Mes 52A penetration, the mechanism of action and the resistance or insensitivity characteristic of the target strains.

Introduction

Bacteriocins of lactic acid bacteria have potential for interesting industrial applications. Four classes of bacteriocins have been defined on the basis of their genetic and biochemical characteristics (Klaenhammer, 1993). Among them, class I (lanthibiotics) and class II are the most studied.

Leuconostoc is an important producer synthesizing several bacteriocins of class II. *Leuconostoc mesenteroides* ssp. *mesenteroides* FR52 produced two bacteriocins: mesenterocin 52A (Mes 52A) and mesenterocin 52B (Mes 52B) (Revol-Junelles *et al.*, 1996). Mes 52B is a 32-amino-acid class IIc peptide, identical to mesenterocin B105 produced by *L. mesenteroides* Y105 (Hechard *et al.*, 1999), which displays a narrow spectrum of activity limited to the genera *Leuconostoc* and *Weissella*. Mes 52A is a class IIa bacteriocin identical to mesentericin Y105 (MesY105). These two bacteriocins are highly similar to leucocin A, with two amino acid residues difference between their sequence (Hechard *et al.*, 1992; Revol-Junelles *et al.*, 1996). Circular dichroism data of MesY105 in a buffer solution indicated that the N-terminal domain is capable of involvement in interaction with a putative receptor while the α-helix from residues 17 to 31 allows lipid binding to the membrane (Fleury *et al.*, 1996).

Some susceptible strains are known to develop bacteriocin resistance. The resistant mechanism appears to be complex and might be a critical problem when bacteriocins are used as biopreservatives. The nisin resistance of *Listeria* strains is well documented. In 1998, Crandall & Montville (1998) put forward a model for this resistance that included three factors: (1) variation of peptidoglycan composition

(Maisnier-Patin & Richard, 1996); (2) modification of the electric charge of the membrane by phospholipid content changes, thereby preventing pore formation (Ming & Daeschel, 1993; Verheul *et al.*, 1997; Crandall & Montville, 1998); and (3) increase in membrane rigidity, preventing peptide insertion and association (Ming & Daeschel, 1993). More recently, it has been shown that four major mechanisms are implicated in *Lactococcus lactis* resistance to nisin (Kramer *et al.*, 2006). This includes (1) preventing nisin from reaching the cytoplasmic membrane, (2) reducing the acidity of the extracellular medium, thereby stimulating the binding of nisin to the cell wall, (3) preventing the insertion of nisin into the membrane and (4) possibly transporting nisin across the membrane or extruding nisin out of the membrane. Similar results were obtained with some class IIa bacteriocins. The changes in membrane components in two *Enterococcus faecium* mutants resistant to mundticin KS were similar to those in nisin-resistant strains and involved fatty acid and phospholipid modifications leading to a more rigid and less anionic membrane (Sakayori *et al.*, 2003). The resistance of *Listeria monocytogenes* LSD 530 to divergicin M35 seems to be due to modification in the fatty acid composition enhancing membrane rigidity, rather than protein modification (Naghmouchi *et al.*, 2006). In contrast to these findings, *L. monocytogenes* B73- and 412-resistant strains to leucocin A have a more fluid membrane (Vadyvaloo *et al.*, 2002). Some protein modifications, revealed using two-dimensional gel electrophoresis of whole cells, were observed in a *L. monocytogenes* P strain resistant to divercin V41 (Duffes *et al.*, 2000). Interruption of different genes encoding σ^{54}, or a σ^{54}-associated activator or two subunits of EII_t^{Man}, a phosphotransferase system (PTS) permease of the mannose family, led to resistance of *L. monocytogenes* to Mes Y105 (Dalet *et al.*, 2001; Gravesen *et al.*, 2002). The σ^{54} factor (Dalet *et al.*, 2000) or EII_t^{Man} (Hechard *et al.*, 2001) were also implicated in Mes Y105 resistance in *Enterococcus faecalis* JH2-2. The intermediate resistance of *E. faecalis* JH2-2 to divercin V41 was associated with three genes encoding σ^{54} factor, a glycerophosphoryl diester phosphodiesterase and a protein with a putative phosphodiesterase function (Calvez *et al.*, 2007).

In previous studies, we have investigated the changes in the membrane composition and in the cell wall of *Leuconostoc* strains associated with insensitivity and resistance to Mes 52A and Mes 52B. The model was made with the two bacteriocins and eight target strains from the genera *Leuconostoc* and *Weissella*, which were insensitive or had been made resistant to one mesenterocin. We observed that Mes 52A and Mes 52B resistance is unlikely to be due to changes in membrane fatty acid composition (Limonet *et al.*, 2002). Resistant strains displayed some cell morphology modifications, a large degree of increase in antibiotic resistance and modifications in lysozyme susceptibility. Moreover, Mes 52A-resistant strains displayed modifications in their membrane phospholipids, leading to a less anionic membrane (Limonet *et al.*, 2002, 2004). It was concluded that a sum of minor physiological characteristics of the membrane and/or the cell wall was probably involved in insensitivity/resistance mechanisms limiting bacteriocin diffusion through the cell wall and interaction with and penetration of the cell membrane.

In this study, we characterize the resistance or insensitivity of some *Leuconostoc* strains to extend the knowledge on resistance mechanisms developed by *Leuconostoc* strains. The model was simplified and limited to one bacteriocin, i.e. Mes 52A, only two wild-type strains from *Leuconostoc genus* chosen for their sensitivity or insensitivity to Mes 52A and one high-level resistant strain to Mes 52A. The interaction and penetration of the peptide with the viable cell membranes of these *Leuconostoc* strains were studied by fluorescence anisotropy using two probes located at different positions in the membrane: 1-(4-trimethylammonium)-6-phenyl-1,3,5-hexatriene (TMA-DPH) and DPH. Subsequently, the influence of Mes 52A on potassium efflux, on $\Delta\Psi$ and ΔpH was determined.

Materials and methods

Bacterial strains and culture conditions

Leuconostoc citreum CIP 103405 was obtained from the public collection [Collection de l'Institut Pasteur (CIP), Paris, France] and *L. mesenteroides* ssp. *mesenteroides* LMA 7 from our own collection [Laboratoire de Microbiologie Alimentaire (LMA) ENSAIA-INPL, Nancy, France]. The resistant strain, *L. mesenteroides* ssp. *mesenteroides* LMA 7AR, was obtained in a previous work (Limonet *et al.*, 2002). These strains were stored at $-24\,^{\circ}C$ without prior growth. Before use, strains were cultured twice in Man-Rogosa-Sharp (MRS) broth (Biokar, Beauvais, France) at $30\,^{\circ}C$ for 16 h. Agar medium was prepared by addition of $15\,g\,L^{-1}$ of bacteriological agar type A (Biokar).

Chemicals, bacteriocin and fluorescent probes

Mes 52A was obtained by chemical synthesis (Synt:em, Nimes, France) at 90–95% of purity according to the published sequence. This bacteriocin ($220\,mg\,L^{-1}$) was prepared in a phosphate buffer (5 mM, pH 6.5). The protein concentration was checked using the Bradford method (Bradford, 1976; Revol-Junelles *et al.*, 1996).

The fluorescent probe TMA-DPH (Sigma-Aldrich, St. Louis, MO) was prepared in *N*,*N*-dimethylformamide to a final concentration of 2 mM. The DPH probe [DPH (Sigma-Aldrich) was prepared in tetrahydrofurane] to a final concentration of 2 mM.

Nigericin and valinomycin (Sigma-Aldrich) were prepared in chloroform or in dimethylsulfoxide, respectively, to a final concentration of 10 mM. The $\Delta\Psi$ of the two strains was measured qualitatively with the fluorescent probe 3,3′-dipropylthiadicarbocyanine iodide [$DiSC_3(5)$] (Sigma-Aldrich). The ΔpH was measured with the fluorescent probe 2′,7′-bis-(2-carboxyethyl)-5[and 6]-carboxyfluorescein acetoxymethyl ester (BCECF AM) (Sigma-Aldrich).

Determination of antibacterial activity

The minimal inhibitory concentration (MIC) was determined by the critical dilution method in 96-well plates (Nunc, Roskilde, Denmark) (Verheul *et al.*, 1997). Mes 52A was prepared in the phosphate buffer. The target strains were prepared in MRS agar medium to a final $OD_{660\,nm}$ of 0.01. The plates were shacked (Titramax 100, Bioblock Fisher Scientific, Illkirch, France), and the initial $OD_{660\,nm}$ was determined with a Titertek Multiscan MCC/340 (Huntsville, AL). The plates were incubated for 24 h at 30 °C. The MIC value ($mg\,L^{-1}$) is the inverse of the highest dilution where no growth is detected.

The minimal bactericidal concentration (MBC) was determined by subculturing each of the no-growth plates in the above MIC test to a solid medium that did not contain Mes 52A. The MBC is identified as the smallest concentration of peptide that prevents any growth of the test bacterium.

Evaluation of bacterial surface hydrophobicity and Lewis acid–base character

The microbial adhesion to solvents (MATS) method was used for the evaluation of the hydrophobic/hydrophilic cell surface properties of the strains and their Lewis acid–base characteristics. The polar solvent could be an electron acceptor or an electron donor, and was compared with a nonpolar solvent that exhibited similar van der Waals surface tension components (Bellon-Fontaine *et al.*, 1996). The following pairs of solvents were selected: chloroform, an electron acceptor solvent, and hexadecane, a nonpolar solvent; ethyl acetate, an electron donor solvent, and decane, a nonpolar solvent; and diethyl ether, an electron donor, and hexadecane, a nonpolar solvent.

A microbial suspension containing $10^8\,CFU\,mL^{-1}$ in 2.4 mL of 1.5×10^{-1} M NaCl was vortexed for 90 s with 0.4 mL of a solvent. The mixture was allowed to stand for 15 min to ensure that the two phases were separated completely before a sample (1 mL) was removed carefully from the aqueous phase, and the $OD_{660\,nm}$ was determined. The percentage of cells present in each solvent was subsequently calculated using the equation: $\%Affinity = (100 \times A)/A_0$, where A_0 is the $OD_{660\,nm}$ of the bacterial suspension before mixing and A is the OD after mixing. Each experiment was performed in triplicate using three independently prepared cultures.

Measurement of the anisotropy of fluorescence

The strains were cultivated in 50 mL of MRS medium for 16 h at 30 °C. The bacterial cells were harvested by centrifugation (10 000 ***g*** at 4 °C for 15 min) and washed three times with the anisotropy buffer [Na_2HPO_4, $3\,g\,L^{-1}$; KH_2PO_4, $6\,g\,L^{-1}$; NaCl, $2\,g\,L^{-1}$; and $(NH_4)_2SO_4$, $8\,g\,L^{-1}$, pH 7.6]. Cell pellets, suspended in the same buffer to obtain a final concentration of around $2 \times 10^8\,CFU\,mL^{-1}$, were stored at −30 °C.

The experiments were carried out in the dark to preserve fluorescent probes. The fluorescence anisotropy measurements of cells labelled with DPH or TMA-DPH were carried out using a T-format continuous excitation device (Fluofluidimeter, Regulest, Florange, France). The intensities were also measured on blank samples (unlabelled cells) for correction (Kuhry *et al.*, 1983, 1985). The samples were preincubated at 30 °C (Thermoblock, FALC, Treviglio, Italy). A diluted cell suspension (3 mL) in a quartz cuvette was labelled with 3 μL of TMA-DPH or DPH solution for 7 or 30 min, respectively (Bokas *et al.*, 2007). Mes 52A was added to a final concentration equivalent to 1/2 MIC ($19\,mg\,L^{-1}$ for $2 \times 10^8\,CFU\,mL^{-1}$) and the suspensions were incubated for 0, 5, 15, 30, 45, 60, 90 or 120 min. The influence of the concentration of Mes 52A was determined after 60 min of contact with a final concentration of peptide equal to 0, 5, 10, 15, 20, 30, 40 and $80\,mg\,L^{-1}$. The excitation and emission wavelengths were 365 and 425 nm, respectively.

During all the determinations of the fluorescence anisotropy, the cytoplasmic membrane of the studied strain was saturated with the probe; the intensity of fluorescence was constant. A control of the fluorescence anisotropy without bacteriocin was realized during the experiment; the initial fluorescence anisotropy was constant during 2 h. Without cells, in the polar solvent used, no photon emission by the probes was detected.

The temperature of the labelled and blank samples was controlled rigorously during the measurement, and the anisotropy was measured at the culture temperature (30 °C). Five measurements were performed for each sample with five repetitions. The fluorescence anisotropy $\langle r \rangle$ was calculated as described (Kuhry *et al.*, 1983, 1985).

To evaluate the stability of the cells during the anisotropy experiments, anisotropy buffer was inoculated with a 16-h subculture of the different strains in order to reach $10^4\,CFU\,mL^{-1}$. Mes 52A was added (1*MIC final concentration) to the cultures, and enumerations were performed after 0, 20 and 60 min of incubation at 30 °C, using a spiral

plating (Whitley Automatic Spiral Plater, WASP 2, AES Laboratoire, Combourg, France).

Measurement of ΔpH and ΔΨ

Cells were harvested in the log growth phase ($OD_{660\,nm} = 0.6$), washed twice with ice-cold 50 mM HEPES buffer containing 0.6 mM of KCl, glucose 0.2%, adjusted at pH 7.0 with KOH 40%, resuspended in the same buffer to 1/100 of their initial volume and stored on ice.

The ΔΨ was measured qualitatively with the fluorescent probe $DiSC_3(5)$ (Sigma-Aldrich). Glucose-energized cells (to a final population of 2.5×10^8 CFU mL^{-1}) were added to a quartz cuvette containing HEPES buffer and $DiSC_3(5)$ (5 μM). Next, after 3 min of incubation with nigericin (1.5 nM), which dissipates the pH gradient (ΔpH), Mes 52A (1*MIC) or valinomycin (1.5 nM) was added. Fluorescence measurements were performed using a spectrofluorometer FLX (Safas-Monaco, Monaco) at 622 and 670 nm for excitation and emission, respectively (Herranz *et al.*, 2001a, b).

The ΔpH was measured using an acid shock (Molenaar *et al.*, 1991). Glucose-energized, BCECF-AM-loaded cells (final population of 2.5×10^8 CFU mL^{-1}) were added to a stirred cuvette containing 50 mM phosphate buffer, pH 6.0. Then, after 3 min of incubation with valinomycin (1.5 nM), which dissipates the ΔΨ, Mes 52A (1*MIC) or nigericin (1.5 nM) was added. Fluorescence was measured at 482 and 528 nm for excitation and emission, respectively. All measures were determined in triplicate.

Effect of the Mes 52A on potassium efflux

Stationary phase cells were harvested by centrifugation (10 000 ***g*** at 4 °C for 15 min). The pellets were washed three times with tryptone-salt (TS) (0.9%) supplemented with glucose (1%). Cells were resuspended in TS–glucose to a final population of 2.5×10^8 CFU mL^{-1}. Mes 52A or the same volume of buffer without the peptide was added to a final concentration corresponding to 1/2 and 1 MIC. Cells were incubated with Mes 52A for 60 min at 30 °C. The cells were harvested by centrifugation (10 000 ***g*** at 4 °C for 15 min), and the concentration of the potassium of the supernatant was determined using the atomic absorption spectrophotometer 1100 (Perkin-Elmer, Courtaboeuf, France).

Statistical analysis

All results were analyzed using ANOVA (Excel, Microsoft) with a model including one effect (time of contact, concentration of Mes 52A).

Results

The activity of Mes 52A was quantified in terms of MICs and MBCs. *Leuconostoc mesenteroides* LMA 7 exhibited a MIC value of 0.48 mg L^{-1} (124 nM), with an MBC value higher than 500 mg L^{-1}. The MIC of *L. mesenteroides* LMA 7AR and *L. citreun* CIP 103405 were higher than 2000 mg L^{-1} (516 μM). These two strains were qualified as resistant and insensitive to Mes 52A, respectively.

Impact of Mes 52A on the fluidity of the cytoplasmic membrane

The effect of Mes 52A on the mobility of the phospholipids was studied by steady fluorescence anisotropy, using two types of probes (TMA-DPH and DPH) located at different positions in the membrane. TMA-DPH is anchored at the water/lipid interface, because of its additional charged trimethylammonium group. It reflects the packaging of the polar head group and, thus, the fluidity at the membrane surface. DPH, a hydrophobic molecule, is oriented predominantly parallel to the fatty acid chains. Its fluorescence reflects the acyl chain order in lipid bilayers, and the fluidity of the core of the cytoplasmic membrane (Kuhry *et al.*, 1983, 1985).

The fluorescence anisotropy of TMA-DPH or DPH in the three target strains in the absence of peptide was not varied during the experiments (data not shown). The initial value of TMA-DPH anisotropy was higher for *L. mesenteroides* LMA 7AR (0.2001 ± 0.0005) compared with the sensitive strain *L. mesenteroides* LMA 7 (0.1785 ± 0.0022), indicating that the membrane surface of the latter was more rigid (Fig. 1a). The initial value of DPH anisotropy was similar for the three strains, indicating the same fluidity in the core of the membrane for all the strains tested (Fig. 2b). The addition of Mes 52A led to a rapid increase of the anisotropy of TMA-DPH and DPH in the membrane of *L. mesenteroides* LMA 7, maximal in the presence of 10 mg L^{-1} Mes 52A (Figs 1b and 2b), revealing an augmented lipid acyl-chain order, and in keeping with intercalation of part of the peptide into the hydrocarbon region of the bilayer. These results suggest that the Mes 52A inserts deeply into the cytoplasmic membrane of this sensitive strain. The fluorescence anisotropy of TMA-DPH and DPH in the membrane of the resistant and insensitive strains remained constant, whatever the concentration of Mes 52A added, indicating that the antimicrobial peptide did not penetrate into the first layer and into the hydrophobic core of the membrane.

Impact of Mes 52A on potassium efflux, ΔpH and ΔΨ

Mes 52A induced a significant potassium efflux from the sensitive strain, at 1/2*MIC or at 1*MIC (Fig. 3). However,

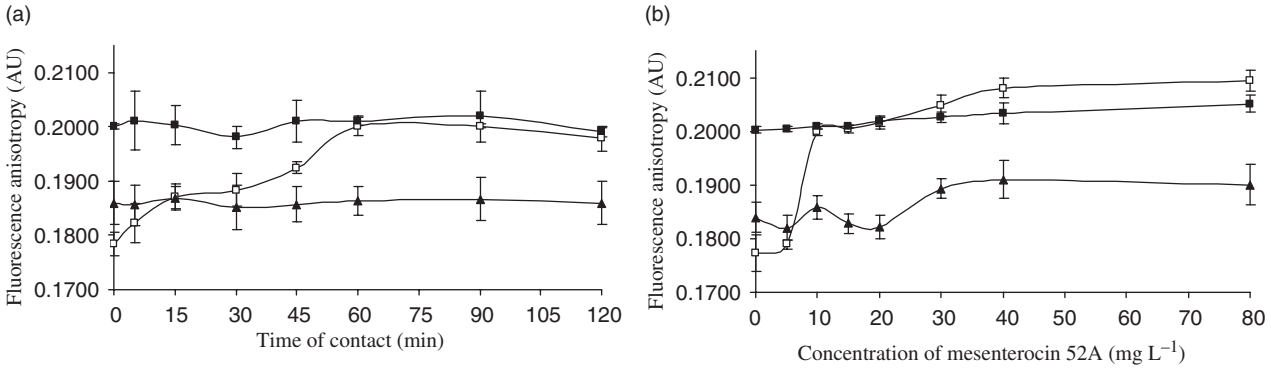

Fig. 1. Study of the fluorescence anisotropy of TMA-DPH at 30 °C (in arbitrary unit, AU), in (□): *Leuconostoc mesenteroides* ssp. *mesenteroides* LMA 7; (■): *L. mesenteroides* ssp. *mesenteroides* LMA 7AR or in (▲): *Leuconostoc citreum* CIP 103405 to a final population of 2.10^8 CFU mL^{-1}; (a): as a function of time of contact (in min) with mesenterocin 52A (1/2*MIC = 19 mg L^{-1} for an $OD_{660\,nm} = 0.8$); (b): as a function of the concentration of mesenterocin 52A (in mg L^{-1}) after 60 min of incubation with the peptide. The factor of uncertainty is $< 5\%$.

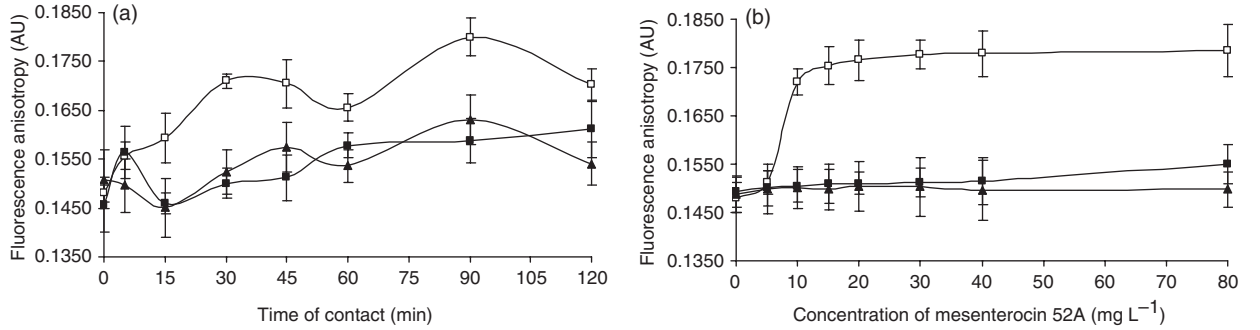

Fig. 2. Study of the fluorescence anisotropy of DPH at 30 °C (in arbitrary unit, AU), in (□): *Leuconostoc mesenteroides* ssp. *mesenteroides* LMA 7; (■): *L. mesenteroides* ssp. *mesenteroides* LMA 7AR or in (▲): *Leuconostoc citreum* CIP 103405 to a final population of 2×10^8 CFU mL^{-1}; (a): as a function of time of contact (in min) with mesenterocin 52A (1/2*MIC = 19 mg L^{-1} for an $OD_{660\,nm} = 0.8$); (b): as a function of the concentration of mesenterocin 52A (in mg L^{-1}) after 60 min of incubation with the peptide. The factor of uncertainty is $< 5\%$.

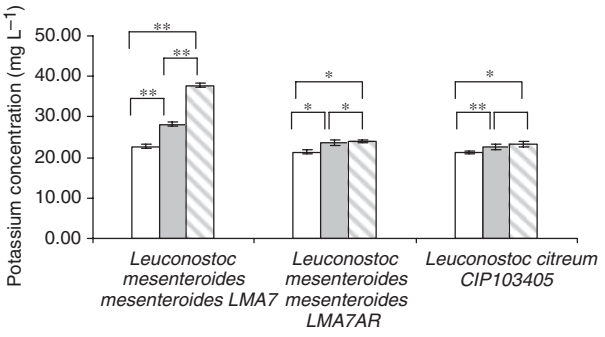

Fig. 3. Determination of the potassium concentration of the culture supernatant using atomic absorption spectrophotometry. □, without Mes 52A; ▨, with Mes 52A at 1/2*MIC; ▩, with Mes 52A at the MIC. ANOVA: *, no significant difference; **, significant difference.

the addition of Mes 52A did not allow to observe any potassium efflux with the two resistant strains.

The impact of Mes 52A on ΔpH and ΔΨ was determined (Table 1). Valinomycin and nigericin were used as positive references. After the addition of the bacteriocin, the BCECF-AM fluorescence emission remained constant, indicating that Mes 52A did not perturb the ΔpH of sensitive or resistant strains. Mes 52A disrupted the ΔΨ of the sensitive strain, observed by an increase of the fluorescence emission, whereas no change occurred in the two resistant strains.

Physico-chemical characterization of cell surfaces

The MATS test (Bellon-Fontaine *et al.*, 1996) is a simple and rapid method to study the physico-chemical properties of microorganisms including their hydrophobicity and Lewis-acid/base or electron acceptor/electron donor character. For all the strains tested, the percentage of adhesion to the apolar solvent, hexane, decane and hexadecane, is almost 0, indicating that the surfaces are rather hydrophilic (Fig. 4). The two resistant strains used exhibited a significant percent

Table 1. Increase of fluorescence emission of $DiSC_3(5)$ or of BCECF-AM expressed in percentage of the initial fluorescence normalized at 100%

	Membrane potential ($\Delta\Psi$) with $DiSC_3(5)$			pH gradient (ΔpH) with BCECF-AM		
		Mes 52A			Mes 52A	
Strains	Valinomycin	1/2*MIC	MIC	Nigericin	1/2*MIC	MIC
L. mesenteroides LMA 7	127 ± 6 (A)	104 ± 4 (B)	121 ± 3 (A)	75 ± 11 (A)	100 ± 1 (B)	100 ± 4 (B)
L. mesenteroides. LMA 7AR	118 ± 4 (A)	100 ± 2 (B)	103 ± 3 (B)	77 ± 9 (A)	100 ± 1 (B)	100 ± 3 (B)
L. citreum CIP 103405	116 ± 5 (A)	100 ± 1 (B)	100 ± 2 (B)	64 ± 13 (A)	100 ± 2 (B)	100 ± 4 (B)

(A), significant; (B), not significant.

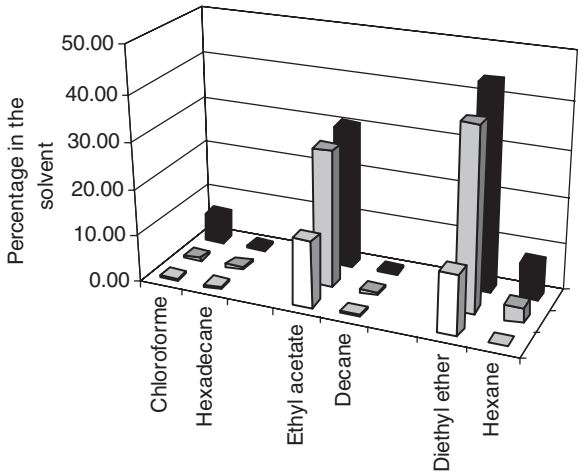

Fig. 4. Partition in organic solvents of the strains studied expressed in percentage of the initial $OD_{660\,nm}$ in the solvent. □, *Leuconostoc mesenteroides* ssp. *mesenteroides* LMA 7; ▨, *L. mesenteroides* ssp. *mesenteroides* LMA 7AR; ■, *Leuconostoc citreum* CIP 103405. SD ≤ 3%.

of adhesion to ethyl acetate and diethyl ether, indicating a Lewis-acid character.

Discussion

In previous studies, we investigated the changes in the membrane composition and in the cell wall of *Leuconostoc* strains associated with insensitivity and resistance to Mes 52A and Mes 52B. The model was made with the two bacteriocins and eight target strains from the genera *Leuconostoc* and *Weissella* that were insensitive and had been made resistant to one mesenterocin (Limonet *et al.*, 2002, 2004). Resistance to bacteriocins seems to be a complex phenotype, involving membrane and/or protein modifications depending on the bacteriocin and the target strain studied (Sakayori *et al.*, 2003; Kramer *et al.*, 2006; Naghmouchi *et al.*, 2006). To obtain more informations on *Leuconostoc* resistance, the model was simplified and limited to the study of the interaction of one bacteriocin, Mes 52A, with a membrane of the only *Leuconostoc* strain insensitive to Mes 52A, *L. citreum* CIP 103405, and of *L. mesenteroides* LMA 7 and its variant *L. mesenteroides* LMA 7AR, which is 4000-fold resistant to Mes 52A.

With the sensitive strain *L. mesenteroides* LMA 7 addition of the peptide induced a rapid increase of the fluorescence anisotropy of the two probes DPH and TMA-DPH, which proves that Mes 52A interacts both with the surface and with the deep of the phospholipidic bilayer. The presence of a potassium efflux was in accordance with the degree of penetration of Mes 52A. These results support the hypothesis that the bacteriocin could insert into the cytoplasmic membrane to form pores. Mes 52A does not disrupt the pH gradient, but has a significant impact on the membrane potential of this sensitive strain. Other described bacteriocins from the class IIa induce a potassium efflux or have an impact on $\Delta\Psi$ like enterocin P (Herranz *et al.*, 2001a, b) or piscicocin CS526 (Suzuki *et al.*, 2005).

For the resistant strain *L. mesenteroides* LMA 7AR or for the insensitive strain *L. citreum* CIP 130405, the presence of Mes 52A does not modify the proton motive force, nor potassium efflux. The resistance phenotype is not always associated with modification of such parameters. The K^+ efflux from five variants of *L. monocytogenes* LSD 530 resistant to nisin A, nisin Z, pediocin PA-1 and divergicin M35 was significantly lower than from the parent strain, but still in effect (Naghmouchi *et al.*, 2007). In some nisin *L. monocytogenes* Scott A-resistant strains, the $\Delta\Psi$ was affected by nisin concentration up to the MIC value compared with the wild-type strain (Verheul *et al.*, 1997). The fluorescence anisotropy of TMA-DPH or DPH is not modified in the presence of bacteriocin. This indicates the absence of interaction between the membrane surface and the bacteriocin, and the absence of penetration in the deep of the membrane. We suppose that the absence of Mes 52A action could be due to modifications of the membrane surface or the membrane composition. In fact, the fatty acid and phospholipid composition of the three strains revealed significant differences as described in previous studies (Limonet *et al.*, 2002, 2004). The unsaturated and cyclic fatty acid contents of the three strains are significantly different. *Leuconostoc mesenteroides* LMA 7 has the highest

content of unsaturated fatty acid, compared with *L. mesenteroides* LMA 7AR and *L. citreum* CIP 130405, leading to a less rigid membrane. This is in accordance with the penetration of Mes 52A in the deep of the membrane. Moreover, it has been shown in previous studies (Limonet *et al.*, 2002) that the presence of Mes 52A induced a significant increase in saturated fatty acid contents in the resistant strain *L. mesenteroides* LMA 7AR and a decrease in unsaturated fatty acid content in the insensitive strain *L. citreum* CIP 130405. These modifications lead to a more rigid membrane. These two facts could explain the absence of penetration of Mes 52A into the heart of the membrane of these two strains. The membrane phospholipids of the wild-type strain are mostly anionic due to the presence of phosphatidylglycerol and diphosphatidyglycerol, while the membranes of the resistant and insensitive strains were *a priori* less negative due to an increase in the phosphatidylethanolamine content and a decrease in phosphatidylglycerol ones (Limonet *et al.*, 2004). It is established that the first step of the mechanism of action of bacteriocin involved electrostatic interactions between the cationic peptide and the negatively charged cell surfaces (Chen *et al.*, 1997). Such interactions are absent in the resistant and insensitive strains due to the less negative surface of the membrane and this has resulted in the absence of TMA-DPH anisotropy modifications. Similar results were obtained with Plantaricin A (Zhao *et al.*, 2006). The addition of negatively charged phospholipid increases the DPH emission anisotropy in the liposome, indicating the penetration of the peptide. The relation between Mes Y105 insertion and the charge of lipid mono- or bilayers had been demonstrated using polarization modulation infrared reflection absorption spectroscopy (Castano *et al.*, 2005). Results indicate that Mes Y105 selectively interacts with negatively charged lipids. However, a very limited insertion of the peptide, which remains localized at the interface, was observed by *in situ* infrared data. Such results were obtained with lipid vesicles, which are different from bacterial cells (Papo & Shai, 2003).

Physico-chemical differences were also indicated by the partition profiles in organic solvents (Fig. 4). The surface of all the strains studied is hydrophilic. Both resistant strains exhibit a high affinity for Lewis-basic solvents, indicating a Lewis-acid character of their envelope. These observations suggest that the envelope of the resistant strains is less anionic than the sensitive strain (Bellon-Fontaine *et al.*, 1996). The cationic character of the cell surface could limit the crossing of the cationic bacteriocin through the envelope by electrostatic repulsion. In this case, the peptide could not reach the cytoplasmic membrane. The envelope could be a new support of resistance.

Our results were in accordance with the results obtained with mundticin KS on *E. faecium* or divergicin M35 on *L. monocytogenes* LSD 530, for which membrane modifications are directly implicated in the resistance phenotype (Sakayori *et al.*, 2003; Naghmouchi *et al.*, 2007).

In our model with a class IIa bacteriocin, Mes 52A, and insensitive or resistant strains belonging to the *Leuconostoc* genus, there is a clear correlation between the physico-chemical characteristics of the membrane, the degree of Mes 52A penetration, the mechanism of action and resistance or the insensitivity characteristic of the target strain. In the presence of a fluid membrane and a negative surface, Mes 52A penetrates deep into the membrane, leading to a K^+ efflux and modification of the $\Delta\Psi$, which induce a bacteriostatic effect. This is observed with the sensitive strain *L. mesenteroides* LMA7. In the presence of a less negative and rigid membrane, no penetration of Mes 52A occurred and no modification of $\Delta\Psi$ or ΔpH was observed. This was observed with an induced resistant strain, *L. mesenteroides* LMA7 AR, and one natural insensitive strain, *L. citreum* CIP 130405. However, the diversity of the results obtained in the literature to explain the resistance mechanism indicates that not just one universal mechanism exists.

Acknowledgements

We thank Michèle Turban and Delphine Roger for excellent technical assistance.

References

Bellon-Fontaine MN, Rault J & van Oss CJ (1996) Microbial adhesion to solvents: a novel method to determine the electron-donor/electron-acceptor or Lewis acid–base properties of microbial cells. *Colloid Surface B* **7**: 47–53.

Bokas D, Uy D, Grattepanche F, Duportail G, Guedon E, Delaunay S & Goergen JL (2007) Cell envelope fluidity modification for an effective glutamate excretion in *Corynebacterium glutamicum* 2262. **76**: 773–781.

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**: 248–254.

Calvez S, Rince A, Auffray Y, Prevost H & Drider D (2007) Identification of new genes associated with intermediate resistance of *Enterococcus faecalis* to divercin V41, a pediocin-like bacteriocin. *Microbiology* **153**: 1609–1618.

Castano S, Desbat B, Delfour A, Dumas JM, da Silva A & Dufourcq J (2005) Study of structure and orientation of mesentericin Y105, a bacteriocin from Gram-positive *Leuconostoc mesenteroides*, and its Trp-substituted analogues in phospholipid environments. *Biochim Biophy Acta* **1668**: 87–98.

Chen Y, Ludescher RD & Montville TJ (1997) Electrostatic interactions, but not the YGNGV consensus motif, govern the

binding of pediocin PA-1 and its fragments to phospholipid vesicles. *Appl Environ Microb* **63**: 4770–4777.

Crandall AD & Montville TJ (1998) Nisin resistance in *Listeria monocytogenes* ATCC 700302 is a complex phenotype. *Appl Environ Microb* **64**: 231–237.

Dalet K, Briand C, Cenatiempo Y & Hechard Y (2000) The rpoN gene of *Enterococcus faecalis* directs sensitivity to subclass IIa bacteriocins. *Curr Microbiol* **41**: 441–443.

Dalet K, Cenatiempo Y, Cossart P & Hechard Y (2001) A sigma(54)-dependent PTS permease of the mannose family is responsible for sensitivity of *Listeria monocytogenes* to mesentericin Y105. *Microbiology* **147**: 3263–3269.

Duffes F, Jenoe P & Boyaval P (2000) Use of two-dimensional electrophoresis to study differential protein expression in divercin V41-resistant and wild-type strains of *Listeria monocytogenes*. *Appl Environ Microb* **66**: 4318–4324.

Fleury Y, Dayem MA, Montagne JJ, Chaboisseau E, Le Caer JP, Nicolas P & Delfour A (1996) Covalent structure, synthesis, and structure-function studies of mesentericin Y 105(37), a defensive peptide from gram-positive bacteria *Leuconostoc mesenteroides*. *J Biol Chem* **271**: 14421–14429.

Gravesen A, Ramnath M, Rechinger KB *et al.* (2002) High-level resistance to class IIa bacteriocins is associated with one general mechanism in *Listeria monocytogenes*. *Microbiology* **148**: 2361–2369.

Hechard Y, Derijard B, Letellier F & Cenatiempo Y (1992) Characterization and purification of mesentericin Y105, an anti-*Listeria* bacteriocin from *Leuconostoc mesenteroides*. *J Gen Microbiol* **138**: 2725–2731.

Hechard Y, Berjeaud JM & Cenatiempo Y (1999) Characterization of the mesB gene and expression of bacteriocins by *Leuconostoc mesenteroides* Y105. *Curr Microbiol* **39**: 265–269.

Hechard Y, Pelletier C, Cenatiempo Y & Frere J (2001) Analysis of sigma(54)-dependent genes in *Enterococcus faecalis*: a mannose PTS permease (EII(Man)) is involved in sensitivity to a bacteriocin, mesentericin Y105. *Microbiology* **147**: 1575–1580.

Herranz C, Chen Y, Chung HJ, Cintas LM, Hernandez PE, Montville TJ & Chikindas ML (2001a) Enterocin P selectively dissipates the membrane potential of *Enterococcus faecium* T136. *Appl Environ Microb* **67**: 1689–1692.

Herranz C, Cintas LM, Hernandez PE, Moll GN & Driessen AJ (2001b) Enterocin P causes potassium ion efflux from *Enterococcus faecium* T136 cells. *Antimicrob Agents Ch* **45**: 901–904.

Klaenhammer TR (1993) Genetics of bacteriocins produced by lactic acid bacteria. *FEMS Microbiol Rev* **12**: 39–85.

Kramer NE, van Hijum SA, Knol J, Kok J & Kuipers OP (2006) Transcriptome analysis reveals mechanisms by which *Lactococcus lactis* acquires nisin resistance. *Antimicrob Agents Ch* **50**: 1753–1761.

Kuhry JG, Fonteneau P, Duportail G, Maechling C & Laustriat G (1983) TMA-DPH: a suitable fluorescence polarization probe for specific plasma membrane fluidity studies in intact living cells. *Cell Biophys* **5**: 129–140.

Kuhry JG, Duportail G, Bronner C & Laustriat G (1985) Plasma membrane fluidity measurements on whole living cells by fluorescence anisotropy of trimethylammonium-diphenylhexatriene. *Biochim Biophys Acta* **845**: 60–67.

Limonet M, Revol-Junelles AM & Milliere JB (2002) Variations in the membrane fatty acid composition of resistant or susceptible *Leuconostoc* or *Weissella* strains in the presence or absence of Mesenterocin 52A and Mesenterocin 52B produced by *Leuconostoc mesenteroides* subsp. *mesenteroides* FR52. *Appl Environ Microb* **68**: 2910–2916.

Limonet M, Cailliez-Grimal C, Linder M, Revol-Junelles AM & Milliere JB (2004) Cell envelope analysis of insensitive, susceptible or resistant strains of *Leuconostoc* and *Weissella* genus to *Leuconostoc mesenteroides* FR 52 bacteriocins. *FEMS Microbiol Lett* **241**: 49–55.

Maisnier-Patin S & Richard J (1996) Cell wall changes in nisin-resistant variants of *Listeria innocua* grown in the presence of high nisin concentrations. *FEMS Microbiol Lett* **140**: 29–35.

Ming X & Daeschel MA (1993) Nisin resistance of foodborne bacteria and the specific resistance responses of *Listeria monocytogenes* ScottA. *J Food Protect* **56**: 944–948.

Molenaar D, Abee T & Konings WN (1991) Continuous measurement of the cytoplasmic pH in *Lactococcus lactis* with a fluorescent pH indicator. *Biochim Biophys Acta* **1115**: 75–83.

Naghmouchi K, Drider D, Kheadr E, Lacroix C, Prevost H & Fliss I (2006) Multiple characterizations of *Listeria monocytogenes* sensitive and insensitive variants to divergicin M35, a new pediocin-like bacteriocin. *J Appl Microbiol* **100**: 29–39.

Naghmouchi K, Kheadr E, Lacroix C & Fliss I (2007) Class I/Class IIa bacteriocin cross-resistance phenomenon in *Listeria monocytogenes*. *Food Microbiol* **24**: 718–727.

Papo N & Shai Y (2003) Can we predict biological activity of antimicrobial peptides from their interactions with model phospholipid membranes? *Peptides* **24**: 1693–1703.

Revol-Junelles AM, Mathis R, Krier F, Fleury Y, Delfour A & Lefebvre G (1996) *Leuconostoc mesenteroides* subsp. *mesenteroides* FR52 synthesizes two distinct bacteriocins. *Lett Appl Microbiol* **23**: 120–124.

Sakayori Y, Muramatsu M, Hanada S, Kamagata Y, Kawamoto S & Shima J (2003) Characterization of *Enterococcus faecium* mutants resistant to mundticin KS, a class IIa bacteriocin. *Microbiology* **149**: 2901–2908.

Suzuki M, Yamamoto T, Kawai Y, Inoue N & Yamazaki K (2005) Mode of action of piscicocin CS526 produced by *Carnobacterium piscicola* CS526. *J Appl Microbiol* **98**: 1146–1151.

Vadyvaloo V, Hastings JW, van der Merwe MJ & Rautenbach M (2002) Membranes of class IIa bacteriocin-resistant *Listeria monocytogenes* cells contain increased levels of desaturated and

short-acyl-chain phosphatidylglycerols. *Appl Environ Microb* **68**: 5223–5230.

Verheul A, Russell NJ, Van THR, Rombouts FM & Abee T (1997) Modifications of membrane phospholipid composition in nisin-resistant *Listeria monocytogenes* Scott A. *Appl Environ Microb* **63**: 3451–3457.

Zhao H, Sood R, Jutila A, Bose S, Fimland G, Nissen-Meyer J & Kinnunen PK (2006) Interaction of the antimicrobial peptide pheromone Plantaricin A with model membranes: implications for a novel mechanism of action. *Biochim Biophys Acta* **1758**: 1461–1474.