Changes in cell surface and metabolism associated with strains of *Listeria monocytogenes* displaying different sensitivities to class IIa bacteriocins

by

Viveka Vadyvaloo

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Supervisors:

Dr Marina Rautenbach

Professor John. W. Hastings

Department of Biochemistry

University of Stellenbosch

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Declaration

I, the undersigned, hereby declare that the work contained in this dissertation is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

Viveka Vadyvaloo Date

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Summary

The possible use of the bacterially produced antimicrobial peptides, and in particular class IIa bacteriocins as food preservatives is a motivating factor in studies on resistance to them by food-borne pathogens like *Listeria monocytogenes*. The high frequencies of resistance to class IIa bacteriocins have however sparked concern regarding their adequacy as potential bio-preservatives. Activity of these cationic peptides was reported to occur by membrane permeabilisation due to pore formation, which results in the leakage of the intracellular contents followed by cell death. The cell envelope (cell wall and cell membrane) is therefore envisaged as a key site of modification in susceptibility of bacteria to class IIa bacteriocins.

Mutants of the *L. monocytogenes* B73 isolate, resistant to the class IIa bacteriocin, leucocin A, were generated at the start of the study to complement the existing array of *L. monocytogenes* wild-type and resistant isolates obtained from other sources. The fifty percent inhibitory concentrations using a highly sensitive and reproducible bioassay were determined. This allowed categorisation of the mutants into intermediate and highly resistant phenotypes. Analysis of the growth patterns of all these strains showed decreased growth rates and higher growth yields for all the resistant strains in general. This provided evidence for possible effects of membrane adaptation and metabolic changes in the resistant strains and prompted further investigation. The major focus of the study on the class IIa resistant mutants were: (1) analysis of membrane compositional changes and factors influencing cell surface charge; (2) assessment of physical changes in the membrane and bacteriocin itself using circular dichroism and fourier transform infrared spectroscopy; (3) and, determination of changes in glucose metabolism.

Electrospray mass spectrometry analysis of the major listerial phospholipid, phosphatidylglycerol, revealed that membranes of resistant strains had increased levels of unsaturated and short-acyl-chain phosphatidylglycerol molecular species, indicating more fluid membranes. In addition, treatment with a desaturase inhibitor resulted in increased sensitivity of only the intermediate resistant strains to the class IIa bacteriocin, leucocin A. This indicated the influence of membrane adaptation in only lower levels of resistance. It is conceivable that more fluid membranes could also impact on decreased stability of pore formation by the bacteriocin.

Complementary biophysical studies using fourier transform infrared spectroscopy indicated the possible occurrence of greater membrane fluidity of resistant cells, by the notable shift in

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the antisymmetric CH_2 stretching vibration from 2921 cm^{-1} to 2922 cm^{-1}. Additionally, circular dichroism revealed a decreased α-helical and increased random structure of leucocin A in the presence of listerial liposomes derived from highly resistant cell membrane extracts. It is possible that this may result in reduced activity of the bacteriocin in resistant cell membranes as α-helical stucture is a critical feature for membrane insertion of cationic antimicrobial peptides.

Cell surface charge was determined by quantification of alanine and lysine esterification of the anionic cell surface polymer, teichoic acid, and membrane phospholipids respectively. Increased D-alanine, which causes neutralisation of the cell surface, was observed in all resistant cells. A tendency for greater lysine content in membrane phospholipids, which also impacts on neutralisation of the anionic phospholipid of listerial membranes, was observed in highly resistant strains only. This neutralisation of the negative charge of the cell surface may interfere with initial electrostatic interaction of bacteriocin with the cell, and subsequent interactions required for permeabilisation of the cell membrane. These differences in alanine and lysine esterification were not the result of increased expression of certain associated genes (*dltA* and *lmo1695*) and may be the result of post-transcriptional regulation. It was, however, found that all resistant *L. monocytogenes* strains, including the intermediate resistant strains, exhibited decreased expression of a putative docking molecule, the mannose-specific phosphotransferase system EIIAB subunit ($EIIAB^{Man}$). A clear correlation existed between the levels of resistance and $EIIAB^{Man}$ down-regulation.

Finally, analysis of the glucose metabolism in highly resistant and wild-type strains, indicated a more efficient metabolism with regards to higher growth yields and ATP yield, in contrast to a lower specific growth rate in a spontaneous and genetically defined (*$EIIAB^{Man}$* inactivated) highly resistant mutant. The switch in metabolic end-product observed, was attributed to the loss of the glucose transporter, $EIIAB^{Man}$, and may cast doubts on the feasibility of the use of class IIa bacteriocins as food preservatives in light of a stable and efficient resistant phenotype.

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Opsomming

Die moontlike gebruik van antimikrobiese peptiede van bakteriële oorsprong, veral klas IIa bacteriocins, in voedsel preservering noodsaak die ondersoek van bakteriële weerstandigheid van organismes soos *Listeria monocytogenes* teen die peptiede. Hoë frekwensie van bateriële weerstandigheid teen die klas antimikrobiese peptied het gelei tot groot kommer aangande die effektiewieteit van die molekules as moontlike bio-voedsel preserveerders. Verslae dui daarop dat die kationies peptiede se meganisme van aksie berus op membraan permilisasie deur middle van porie vorming wat lei tot die lekasie van sellulêre inhoud en sel dood. Beide die selwand en –membraan is om die rede geïdentifiseer as teiken areas vir modifikasie in vatbaarheid vir bakterieë teen klas IIa bacteriocins

Mutante variëteite van *L. monocytogenes* B73 isolaat wat weerstandig is teen die klass IIa bacteriocin, leucocin A, is aan die begin van die studie gegenereer om die bestaande versameling van *L. monocytogenes* wildetipe en weerstandige isolate van ander bronne aan te vul. Deur gebruik te maak van 'n hoogs sensitiewe en herhaalbare biologiese toets stelsel is 50% inhibisie waardes vasgestel wat dit moontlik gemaak het om mutante varieteite te klasifiseer in intermidiêre en hoogs weerstandige fenotiepes. Analise van groei patrone en van al die variëteite het aan getoon dat laer groei tempo's en hoer groei oprengs in die algemeen aangeteken is vir al die weerstandige variëteite. Die waarnemings het voldoende bewys gelewer van membraan aanpassings en metabolise veranderings in die weerstandige variëteite om verdere navorsing te ondersteun. Die focus van die studie aangaande die klas IIa bacteriocin weerstandige bakterië het geval op (1) analise van verandering selmembraan samestelling asook faktore wat membraan oppervlak lading beïnvloed; (2) evalueering van fisiese membraan en bacteriocin verandering deur gebruik te maak van sirkulêre dichroïsme en Fourier transformasie infrarooi spektroskopie; en (3) die verandering in glucose metabolisme.

Elektrosproei massa spetrometrie analise van die hoof fosfolipied van listeria sp., fosfatidielgliserol, het aangetoon dat selmembrane van weerstandige variëteite verhooged vlakke van onversadigte en kort ketting alkiel fosfatidielgliserol molekulêre spesies wat op 'n meer vloeïede membraan dui. Verder, het behandeling met 'n desaturase inhibitor slegs in intermediêre weerstandige variëteite 'n verhoging in sensitiwiteit ten op sigte van die

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bacteriocin klass IIa, leucocin A tot gevolg gehad. Dit dui daarop dat membraan verandering 'n invloed het op laer vlakke van weerstandigheid. Dit is denkbaar dat verhoogde membraan vloeïditeit 'n negatiewe impak kan hê op die stabilliteit van porie forming deur die bacteriocin.

Komplimentêre studies met Fourier transformasie infrarooi spektroskopie het die moontlikheid verhoogde membraan vloeïditeit aangetoon deur 'n merkbare skuif in die asimetriese CH_2 strek vibrasie van 2921 cm^{-1} na 2922 cm^{-1}. Aanvulende sirkulêre dichroïsme het aangetoon dat leucicon A 'n verminderde α-heliks stuktuur en 'n verhoogde ongeordende struktuur aaneem in die teenwoordigheid van liposome afkomstig van hoog weerstandige selmembraan ekstrakte. Dit is moontlik dat die verandering in struktuur lei tot verminderede aktiewiteit van die bacteriocin in weerstandige membrane aangesien die α-heliks struktuur van kationies antimikribiese noodsaaklik is vir membraan interaksie.

Sel oppervlak lading is vasgestel deur die kwantifisering van alanien en lisien esterifisering van die anioniese cell oppervlak polimeer, teigoënsuur en membraan fosfolipiede onderskeidelik. Verhoogde D-alanien vlakke, wat neutraliserign van die buite sel oppervlak veroorsak, is waargeneem in alle weerstandige sell waargeneem. 'n Neiging tot verhoogde lisien inhoud in membraan fosfolipiede, wat ook 'n invloed het op die neutralisering van listeria anioniese fosfolipiede is waargeneem slegs in hoogs weerstandige selle. Die neutralisering van die negatiewe sel oppervlak lading kan imeng met aanvanklike elektrostaties interaksie tussen bacteriocin en selmembraan en daarop volgende interaksies wat benodig word vir membraan permialisasie. Die verskil in alanien en lisien esterifikasie is nie die gevolg van was nie die resultaat van verhoogde geen ekspressie van sekere geasosieerde gene (*dlt* en *lmo1695*) maar mag wel die resultaat wees van post-translasionele regulasie. Daar is egter gevind dat alle weerstandige *L. monocytogenes* variëteite, insluitend die intermidiêre weerstandige variëteite, 'n afname in die ekspressie van vermeende reseptor-tipe molekuul, die manose spesifieke fosfotransferase stelsel EIIAB subeenheid ($EIIAB^{Man}$). 'n Duidelike korrelasie is gevind tussen weerstandigheidsvlakke en $EIIAB^{Man}$ af-reguleering.

Laastens, analise van die glukose metabolisme van hoog weerstandig en wildetipe het aangetoon dat 'n meer effektiewe metabolisme met betrekking tot hoër groei en ATP opbrengs in teenstelling met 'n laer spesifieke groeikoers in 'n geneties gedefiniëerde ($EIIAB^{Man}$ geïnaktiveerde) hoog weerstandige mutant. Die waargenome verandering in

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metabolies eind produk, was toegeskryf aan die verlies van die glukose transporter $EIIAB^{Man}$. Dit mag twyfel laat onstaan aangande die geskiktheid van klass IIa bacteriocin as voedsel preserveerder in die lig van ‘n stabiele, weerstandige fenotiepe

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List of Abbreviations and Acronyms

A_{600}	absorbance at wavelength of 600 nm
α-helix	alpha-helix
β-sheet	beta-sheet
β-turn	beta-turn
CaF_2	calcium fluoride
CD	circular dichroism
D_2O	deuterium oxide
DMPG	dimyristoyl-L-α-phosphatidylglycerol
DPPG	dipalmitoyl-L-α-phosphatidylglycerol
DOPG	dioleoyl-L-α-phosphatidylglycerol
DSPG	distearoyl-L-α-phosphatidylglycerol
ESMS	electrospray ionization mass spectrometry
$EIIAB^{Man}$	mannose phosphotransferase system enzyme IIAB subunit
Fmoc	N^9-fluorenylmethyloxycarbonyl
FTIR	fourier transform infrared spectroscopy
H_2SO_4	sulphuric acid
HPLC	high performance liquid chromatography
IC_{50}	50 % inhibitory concentration
IC_{min}	mimimal inhibitory concentration
IC_{max}	maximum inhibitory concentration
IR	infrared
leucocin A	leuA
LTA	lipo-teichoic acid
mM	milli molar
[M]	molecular ion
M	molar
m/z	mass over charge ratio
Na-MES	sodium 2-N-morpholino-ethanesulfonic acid
NMR	nuclear magnetic resonance
OD	optical density
PG	phosphatidylglycerol

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PEP	phosphoenolpyruvate
PTS	phosphotransferase system
SDS	sodium dodecyl sulphate
SME	sterculic acid methyl ester
TA	teichoic acid
TFA	trifluoroacetic acid
TFE	trifluoroethanol
UV	ultraviolet

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Chapter 1

Introduction to class IIa bacteriocins and resistance to these antimicrobial peptides

General introduction

Food safety and preservation has become an increasing concern globally, due to frequent occurrences of food poisoning by food pathogens. One of the major food pathogens is *Listeria monocytogenes*, and it causes the major food-borne illness, listeriosis [ohio.osu.edu/hyg-fact/5000/5562.html]. *L. monocytogenes* generally affects pregnant women and immuno-compromised individuals, including people being treated for AIDS, cancer, and diabetes. Listeriosis is characterised by flu-like symptoms such as fever, headache, nausea, vomiting and diarrhoea, and may spread to the nervous system, resulting in more serious illnesses like meningitis and septicaemia.

L. monocytogenes is associated with foods such as milk, soft-cheeses, raw vegetables, all types of raw meats, cooked poultry and smoked fish. The public is now becoming increasingly aware of the advantages of natural preservation methods and minimally processed foods for maintenance of a healthy lifestyle. There is thus a demand for more natural bio-preservative value food additives, rather than the use of chemical preservation. This has led to great interest in the antimicrobial peptides or bacteriocins produced by bacterial flora that are normally found in foods.

1. A general classification of bacteriocins

Bacteriocins are formally defined as ribosomally synthesized antibacterial proteins produced by bacteria that kill or inhibit the growth of closely related species of bacteria [40]. They are divided as follows into 3 or 4 groups [40, 49]: (1) Class I, also known as the lantibiotics because of their unusual amino acids, e.g. lanthionine, dehydrobutyrine, and dehydroalanine; (2) Class II are divided into 2 sub-classes, class

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IIa and Class IIb and consists of small heat stable peptides; and (3) Class III consists of large heat labile proteins [49].

2. Significance and nature of class IIa bacteriocins

The class IIa bacteriocins have been extensively studied because of their ability to strongly inhibit *L. monocytogenes*. This antilisterial nature of the class IIa bacteriocins has also gained them much attention as potential food bio-preservatives, since they are mostly produced by food-related strains of lactic acid bacteria, and are non-toxic [19, 12, 40, 1]. These peptides are also generally cationic in nature. Besides their high homology to each other, another important feature of class IIa bacteriocins is the presence of an N-terminal YGNGV motif [19, 12, 1]. A list of some class IIa bacteriocins and their amino acid sequences appear in Fig. 1.

```
Pediocin PA-1:             KYYGNGVTCGKHSCSVDWGKATTCIINNGAMAWATGGHQGNHKC
Coagulin:                  KYYGNGVTCGKHSCSVDWGKATTCIINNGAMAWATGGHQGTHKC
Sakacin P:                 KYYGNGVHCGKHSCTVDWGTAIGNIGNNAAANWATGGNAGWNK
Piscicolin:                KYYGNGVSCNKNGCTVDWSKAIGIIGNNAAANLTTGGAAGWNKG
Mundticin:                 KYYGNGVSCNKKGCSVDWGKAIGIIGNNSAANLATGGAAGWSK
Sakacin 5X:                KYYGNGLSCNKSGCSVDWSKAISIIGNNAVANLTTGGAAGWKS
Leucocin C:                KNYGNGVHCTKKGCSVDWGYAWTNIANNSVMNGLTGGNAGWHN
Bacteriocin31:            ATYYGNGLYCNKQKCWVDWNKASREIGKIIVNGWVQHGPWAPR
Curvacin A:               ARSYGNGVYCNNKKCWVNRGEATQSIIGGMISGWASGLAGM
Carnobacteriocin BM1:AISYGNGVYCNKEKCWVNKAENKQAITGIVIGGWASSLAGMGH
Enterocin P:             ATRSYGNGVYCNNSKCWVNWGEAKENIAGIVISGWASGLAGMGH
Enterocin A:          TTHSGKYYGNGVYCTKNKCTVDWAKATTCIAGMSIGGFLGGAIPGKC
Divergicin V41:           TKYYGNGVYCNSKKCWVDWGQASGCIGQTVVGGWLGGAIPGKC
Carnobacteriocin B2:       VNYGNGVSCSKTKCSVNWGQAFQERYTAGINSFVSGVASGAGSIGRRP
Leucocin A:                KYYGNGVHCTKSGCSVNWGEAFSAGVHRLANGGNGFW
Mesentericin Y105:         KYYGNGVHCTKSGCSVNWGEAASAGIHRLANGGNGFW
Plantaricin C19:           KYYGNGLSCSKKGCTVNWGQAFSCGVNRVATAGHGK...
```

Fig. 1. Amino acid sequences of 17 class IIa bacteriocins (after Kazazic et al. 2002 [39]).

2.1 Predicted structures of class IIa bacteriocins

Class IIa bacteriocins show great similarity in their primary structures (Fig. 1), containing from 37 residues (e.g. leucocin A and mesentericin Y105 [30, 24, 32]) to 48 residues (e.g. carnobacteriocin B2 [51]). A consensus YGNGV sequence is present at the extreme N-terminus of the structural motif and this motif is being extended to include more common residues as even more bacteriocins are being found. The C-terminus shows more variability [21] but with the emergence of numerous bacteriocins, it is now being recognised in delineating the class IIa bacteriocins into

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subgroups based on their C-terminal sequence similarity [19]. The percentage similarity at the C-terminus varies between 34 and 80.5 % [19] except between leucocin A and mesentericin Y105, which have only two amino acid differences in their overlapping sequences [32].

Another characteristic of the primary structure of class IIa bacteriocins is the occurrence of two cysteines at positions 9 and 14, which are known to form a disulphide bridge, which stabilizes a β-hairpin loop in the YGNGV motif [2]. This N-terminal disulphide has also been shown to be essential for antilisterial activity [24]. Pediocin PA-1 was the first class IIa bacteriocin to be identified, which contained another 2 cysteines forming a disulphide bridge at positions 24 and 44 on the C-terminus, that is important for high levels of activity [18, 20]. The C-terminal disulphide bridge folds the peptide into a conformation that allows exposure of the hydrophobic residues which is crucial for antilisterial activity [2, 25], antimicrobial specificity and decreases temperature dependency of bacteriocin activity [20].

Tryptophan residues at positions 18, 33 and 41 in class IIa bacteriocins are also highly conserved within the peptide [23], and are also crucial for activity [2, 23, 24]. Tryptophan at positions 18 and 41 have been suggested to interact with the membrane-water interface and tryptophan at position 33 was suggested to insert into hydrophobic core of the target membrane along with the C-terminal α-helix [23].

2.2 Mode of action of class IIa bacteriocins

Most studies on understanding the mechanistic action of class IIa bacteriocins, suggest dissipation of the proton motive force (PMF) *via* dissipation of either or both the pH gradient (ΔpH) and transmembrane potential ($\Delta\Psi$) [38, 7, 34, 35, 31, 11], as being the common mechanism causing death of target cells. Depletion of intracellular ATP and amino acid and potassium ion efflux has also been observed for some class IIa bacteriocins [48, 31]. The mechanisms are thought to occur as a result of pore formation and permeabilisation resulting in the leakage of the intracellular contents of the target cell. Unlike the class I bacteriocin, nisin, there is no leakage of inorganic phosphate or ATP associated with the class IIa bacteriocin mechanism of action [19, 7], indicating a smaller pore or permeabilising complex for class IIa bacteriocins in comparison to class I members.

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The underlying molecular mechanism of bacteriocin action is hypothesized to occur *via* binding, insertion and pore formation and is currently the focus of bacteriocin investigators. This mechanism, which was proposed to adhere to the 'barrel-stave' model proposed for cationic antimicrobial peptide activity [50, 19, 48], excludes the possibility of receptor involvement in class IIa bacteriocin mode of action. Evidence for bacteriocin insertion and pore formation, studied by monitoring carboxyfluorescein leakage in liposomes also supports a 'no-receptor' scenario for bacteriocin activity [9, 38]. However, the bacteriocin preparations in these studies were not shown to be purified to chemical homogeneity with analytical methods such as high performance liquid chromatography and electrospray mass spectrometry and thus may have contained contaminants that influenced the bacteriocin's activity on liposomes.

Alternatively, it was suggested that for pore formation to occur a protein receptor was required in *Pediococcus acidilactici* PAC 1.0 [11]. More recent studies on leakage of radioactive Rb^{+} in wild-type and resistant *Enterococcus faecium* cells, and synthetic liposomes, after enterocin P addition, showed leakage only in wild-type cells. This indicating the possibility of a receptor-type factor present in class IIa bacteriocin activity [35]. Furthermore, the enantiomeric form of leucocin A has been shown to lack bioactivity suggestive of a chiral recognition feature required for activity of class IIa bacteriocins [58]. This stereospecific requirement is complemented by new findings suggesting involvement of a mannose PTS permease as the putative receptor-type molecule in class IIa bacteriocin activity [14, 28]. Moreover, evidence regarding a recognition feature for activity of class IIa bacteriocins, include suggestions of functional binding of class IIa bacteriocins to anionic phospholipid headgroups, which could be mediated by the positively charged and polar residues of the bacteriocin [10, 9, 38, 8]. There has also been speculation that the cell outer surface polymers like teichoic acids and lipo-teichoic acids may also function as receptors [24].

Therefore, it would be interesting to ascertain the exact role of a receptor-type molecule in the molecular mechanism underlying class IIa bacteriocin resistance, in order to understand what role it may play in pore complex formation.

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2.3 Tertiary structure and its impact on bacteriocin activity

Biophysical studies on a few class IIa bacteriocins, using nuclear magnetic resonance and circular dichroism in membrane-mimicking environments, have allowed predictions of basic structure conformations for class IIa bacteriocins [56, 25, 57, 33]. A functional structure of class IIa bacteriocins, is suggested to occur with an N-terminal YGNGV motif forming a β-turn structure as part of the hydrophilic N-terminal antiparallel β-sheet structure, followed by a central more amphiphilic α-helical region and then a hydrophobic α-helical C-terminus (see Fig. 2).

The YGNGV motif forms a β-turn structure [2, 25, 10]. The β-turn folding of the YGNGV consensus sequence is thought to be involved in recognition of a receptor molecule [2, 24]. Mutations of positively charged residues in this N-terminal region beyond the YGNGV motif show a larger binding of pediocin PA-1 to phospholipid vesicles than the YGNGV motif itself [10], indicating a more important role for electrostatic interaction in binding to target membranes [10]. Several other studies of substitution mutations [45, 51] and deletions [51, 24] of specific residues in the N-terminus, show reduced or abolished activity of the bacteriocin, indicating the significance of this sequence for bacteriocin activity.

The N-terminus, including residues 1 to 17 or 18, form an amphiphilic triple-stranded β-sheet containing a positively charged patch at the tip of the β-hairpin loop was suggested to be involved in bacteriocin-membrane electrostatic interactions [10, 25]. A hydrophobic region is also found in the N-terminus and is probably significant for membrane insertion of the peptide [9, 19]. Mutational analysis of various residues in this region conveys the importance of the cationic nature of the N-terminus in influencing the target cell specificity through initial electrostatic binding of the bacteriocin to target cells [39].

Residues 15 or 16 to 27 or 28 are responsible for forming an α-helix, characterized as being oblique oriented and thus efficient at causing membrane destabilization and effective peptide insertion [19, 4]. Fleury et al. [24] showed that in a peptide variant of mesentericin Y105, with a deletion in the central residues 15 to 27, there was a subsequent loss of activity. This loss of activity was speculated to occur due to the

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entire length of the peptide not being intact, but this may also be due to inability of the peptide to cause destabilization of the membrane.

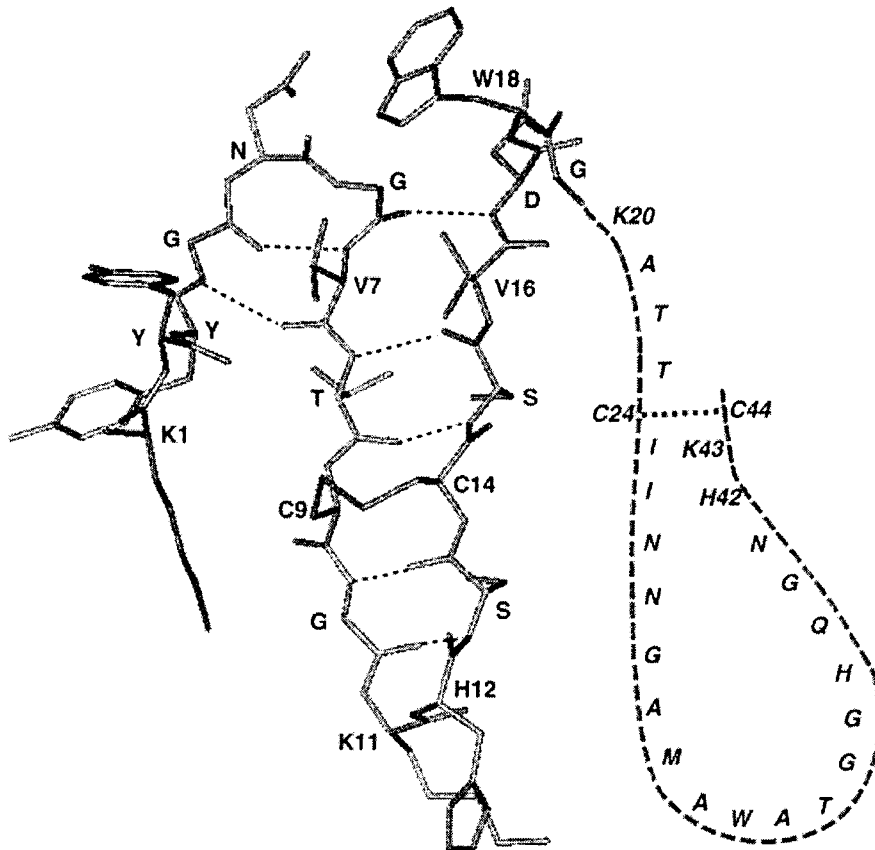

Fig. 2. N-terminal 19 residue predicted tertiary structure of pediocin PA-1. No structure is shown for the C-terminus, except for the C24-C44 disulphide bridge that is required for activity (after Chen et al., 1997 [10]).

Residues spanning the C-terminal end of class IIa bacteriocins forms a hydrophobic α-helical conformation [25, 57, 33] in membrane-mimicking environments, facilitating bacteriocin insertion into the membrane and formation of pores. While there have been indications of the C-terminus being involved in target cell specificity [21] *via* recognition of a key membrane target region [22], other studies suggest a non-critical role for the C-terminus, consisting only of modulation of the activity potency [24, 2]. Miller et al. [45] also suggested, from studies done on pediocin substitution mutants that the C-terminal hydrophobicity is important for activity and speculate that the amphipathicity of the region may also be essential for activity if proper helix folding occurs.

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3. Factors modulating class IIa bacteriocin sensitivity.

It makes sense to consider factors modulating bacteriocin sensitivity and factors associated with resistance to class IIa bacteriocins together since they influence each other. Therefore, one will notice some repetition of perspectives on factors modulating bacteriocin activity in the following section on resistance.

3.1 Possible roles for membrane phospholipids and/or membrane proteins in activity of bacteriocins

The predicted mode of action of class IIa bacteriocins involves electrostatic and hydrophobic interactions, because of the cationic nature of the molecules [19, 48]. As described in the previous section on mode of action of class IIa bacteriocin, it is possible for the anionic phospholipid head groups to behave as a docking or receptor site [10, 38], thus modulating bacteriocin activity. Alternative evidence also points to the requirement of a receptor-type molecule [35, 11] and this is complemented by reports of a mannose phosphotransferase system permease as the putative receptor-type molecule for class IIa bacteriocin activity [14, 28]. Evidence for both these factors provide uncertainty regarding what factors actually influence the activity of bacteriocins. It is likely that all these factors are requirements for efficient bacteriocin activity and should all be taken into consideration in determining the exact mechanism of action of class IIa bacteriocins. The contribution of other factors impacting on bacteriocin binding may also be important here, for example, Fleury et al. [24] suggested a role for teichoic acids as potential binding sites for bacteriocin since they pose as a net negatively charged structure. Our study, outlined in Chapter 5, observing the charge influences afforded by D-alanylation of the teichoic acid, and lysinylation of membrane phospholipid on class IIa bacteriocin activity, serves as an investigation into other potential factors directly modulating the electrostatic interaction of bacteriocin with the target cell membrane.

3.2 Effects of pH on activity of class IIa bacteriocins

Bacteriocin binding to liposomes, derived from target listerial membranes, was increased at lower pHs [38, 9]. Pediocin PA-1 showed increased carboxyfluorescein efflux from lipid vesicles as pH decreased from 7.5 to 5.5 [9] Also, bavaricin MN

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induced carboxyfluorescein efflux from listerial liposomes was found to occur maximally at a pH optimum of 6.0 [38]. Additionally, leucocin A was shown to be unstable at pHs above 5.0 [30]. The pI values for bavaricin MN, pediocin PA-1 and leucocin A are 9.3, 8.8 and 8.6, and the net charge of these bacteriocins is therefore pH dependent. [19]. The ionisation of the positively charged amino acids of class IIa bacteriocins occurs at lower pH values and may improve their electrostatic interaction with anionic phospholipid head groups, in support of an electrostatic-type binding in class IIa bacteriocin activity.

3.3 Effects of lipid composition

Another potential determining factor in modulating class IIa bacteriocin activity is the lipid composition of target organisms. There are only a limited number of studies published that have investigated the effect of the lipid composition on bioactivity of class IIa bacteriocins. Chen et al. [8] reported increased binding of pediocin PA-1 to anionic phospholipid vesicles compared to zwitterionic lipid vesicles. They also suggested a minor effect of saturated or less fluid dimyristoyl-phosphatidylglycerol vesicles showing greater pediocin PA-1 insertion, compared to insertion into the more fluid oleoyl fatty acid chain environment of dioleoyl-phosphatidylglycerol. The first report on alterations in phospholipid composition of class IIa bacteriocin resistant strains were in *Pediococcus* and *Lactobacillus sake* strains [3]. This study indicated that there was increased fluidity due to increased saturated fatty acids in sensitive *Pediococcus* strains and contrasting greater saturated fatty acid content in the insensitive strains, which was the opposite in *L. sake* strains [3]. However, this may be as a result of inherent differences in lipid composition as observed from the higher percentage of short-acyl-chains and saturated fatty acids in *L. sake* compared to the *Pediococcus* strains. Another study on member strains of the *Leuconostoc* and *Weisella* genera, showed significant increases in saturated fatty acids and decreases in unsaturated fatty acid content in response to class IIa bacteriocin mes52A treatment [41]. The *Leuconostoc citreum* strain with the lowest unsaturated and highest saturated fatty acid content, was suggested to be insensitive to mes52A because of its rigid membrane [41]. Previously, a more rigid membrane was suggested to affect easy insertion and/or association of nisin in the membrane [47]. One of the major studies in

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this thesis is on phospholipid compositional changes modulating activity of bacteriocin from the perspective of class IIa bacteriocin resistance.

4. Resistance to class IIa bacteriocins

Resistance development of food-borne pathogens to class IIa bacteriocins provides an impediment to the potential use of these bacteriocins as bio-preservatives. Resistance may develop naturally or through exposure to a bacteriocin [19, 53, 27]. Strains within a particular species may also exhibit differences in their relative susceptibilities to a particular bacteriocin [37], but this may be accounted for by strain specific differences. It does not however, explain the existence of different level sensitivities of members of one particular strain. Therefore, it may be important to compare factors affecting different levels of resistance within a specific strain for possible clues on development of resistance populations. In addition, high frequencies (about 10^{-6}) and stability of class IIa resistance have been reported [27; 53, 17], and this heightens concern about resistance in food-borne pathogens. Furthermore, there is also only limited information available on class IIa bacteriocin resistance.

The class I bacteriocin nisin, currently being used as a bio-preservative worldwide, is however a subject of ongoing research on understanding resistance to this molecule. This has resulted in the generation of massive amounts of information with regards to its bioactivity and mechanism of resistance. With the vast amount of information regarding resistance to nisin, and the almost similar pore formation mode of action described for nisin and class IIa bacteriocins, it would be valuable to consider approaches and findings used in characterisation of resistance to nisin, in studies related to class IIa bacteriocin resistance in *L. monocytogenes.*

4.1 The role of the cell envelope in resistance to nisin

It seems logical to investigate the first barriers of permeability of the cell in search of protective mechanisms against activity of antimicrobial agents. Moreover, with general consensus in pore formation as part of mode of membrane action of nisin and class IIa bacteriocins, it makes sense to investigate changes in the cell envelope. The passage of the bacteriocin through the cell would be to first encounter the cell wall and then the cell membrane.

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Evaluation of the cell wall in nisin resistance, implicated factors such as decreased cell surface hydrophobicity that would affect hydrophobic interaction between the polar residues of nisin and the cell surface [15, 47, 42]. Modifications of components of the cell surface, like teichoic acids, lipo-teichoic acids, membrane proteins and anionic phospholipids that influence cell surface hydrophobicity properties have been shown in nisin-resistant variants [43, 47]. Differing response in wild-type and resistant cells to cell wall acting antibiotics (e.g. D-cycloserine, ampicillin) and lytic enzymes (e.g. lysozyme), were also suggested to be due to changes in cell wall structure and function [13, 41, 15]. It is likely that cell wall structural changes may prevent accessibility of nisin into the target cell. The above findings implicated the cell wall, in acquisition of nisin resistance. This is significant considering the recent observation that the first monomeric cell wall precursor, the lipid II molecule, serves as the docking molecule for nisin activity and pore formation [5, 6]. It is also evident that electrostatic interactions play a central role in lipid II and nisin interaction [36]. Charged molecules on the cell surface like teichoic acids, lipo-teichoic acids and anionic phospholipids may affect these interactions.

Changes in the composition of the cell membrane of nisin resistant *L. monocytogenes* strains, were first observed by Ming and Daeschel [46, 47]. A nisin resistant variant showed a higher phase transition temperature, higher percentage of straight-chain fatty acids, and a lower percentage of branched chain fatty acids [44], than the wild-type. Similar alterations of membrane fatty acid composition indicated by lower C15/C17 fatty acid ratios, increases in straight long-chain and fewer short-chain fatty acids in another nisin resistant strain of *L. monocytogenes* have also been shown [44]. These membrane changes are consistent with decreased membrane fluidity, indicating a rigid membrane affecting insertion of nisin. Other studies have also shown that *L. monocytogenes* nisin resistant strains have significantly less anionic phospholipid [47, 13]. The interactions of nisin with membranes is influenced by anionic phospholipids [16, 26], since a decrease in net negative charge of the lipid bilayer would hinder ability of nisin to bind and insert into the membrane. Additionally, Verheul et al. [55] observed increased diphosphatidylglycerol in sensitive *L. monocytogenes* membranes, which may interact more strongly with cationic nisin than phosphatidylglycerol because of its high charge density and specific charge distribution. It is interesting that changes in the cell wall and phospholipid of resistant strains can be related to

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hindrance of binding and insertion of the nisin molecule. Given this, it seems likely that similar changes may occur in membranes of class IIa bacteriocin resistant *L. monocytogenes* strains, as is described in Chapter 3.

4.2 Resistance phenomena observed in class IIa resistant listerial strains

4.2.1 The mannose-phosphotransferase system enzyme IIAB

The first study, using a genetic approach to gain insight into resistance to class IIa bacteriocins, demonstrated the association of a σ^{54} transcriptional factor to resistance in *L. monocytogenes* [54]. Another report using a proteomic approach showed the absence of a mannose-specific phosphotransferase (PTS) system Enzyme IIAB component ($EIIAB^{Man}$) in a spontaneous leucocin resistant variant of *L. monocytogenes* B73 [52]. Subsequent genetic studies described genes of the mannose-specific PTS to be controlled by the σ^{54} transcriptional factor, and that when insertionally inactivated resulted in resistance to the class IIa bacteriocin mesentericin Y105 in *L. monocytogenes* and *E. faecalis* [14, 31]. The mannose PTS was observed to consist of a tricistronic operon, *mptACD* coding for a complex permease, which showed resistance to mesentericin following inactivation of each gene in the operon. Additionally, deletion of a putative extracellular domain of the MptD membrane component of the permease also resulted in resistance [14]. This permease was speculated to be the docking molecule for class IIa bacteriocin activity [14]. Moreover, shut-down of *mptA* ($EIIAB^{Man}$) was demonstrated to be the one general mechanism associated with high levels of class IIa resistance in *L. monocytogenes* strains isolated form various sources [28]. The most recent findings indicate reversion of an insensitive *Lactococcus lactis* strain to a sensitive phenotype following heterologous expression of the *L. monocytogenes* mannose PTS operon (unpublished results, M. Ramnath). This supports a role for the mannose PTS permease as a docking molecule facilitating activity of class IIa bacteriocins.

4.2.2 Non-receptor type phenomena in class IIa bacteriocin resistance

An analysis of expressed protein of a divercin V41 resistant *L. monocytogenes* mutant, suggested that transcriptional sigma factor regulators controlled the expression of genes in bacteriocin resistance [17]. This supported the findings on the involvement of a σ^{54} transcriptional factor in mesentericin resistance [54]. Increased

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expression of two β-glucoside-specific phosphotransferase enzymes, β-glucoside enzyme II and a phospho-β-glucosidase homologue has been observed in eight different class IIa cross resistant mutants [28, 29]. The up-regulation of these enzymes were suggested to be a regulatory consequence of the absence of the mannose PTS EIIAB component, since inactivation of either enzyme did not result in resistance to pediocin [28, 29].

Besides studies investigating class IIa bacteriocin resistance from the genetic and proteomic perspectives, another study analysed the membrane composition of class IIa resistant strains of the *Leuconostoc* and *Weisella* genera of lactic acid bacteria. Insensitive wild-type and spontaneous resistant strains showed increased saturated and decreased unsaturated fatty acid contents, in response to bacteriocin addition, resulting in a more rigid membrane [41]. The implication of these findings is that the cell wall does not prevent diffusion of the bacteriocin to the membrane, but that the membrane becomes less sensitive to bacteriocin action. It is also clear here that inherent strain differences influenced findings given the atypical decrease in saturated fatty acid content of the resistant *Weisella paramesenteroides* DSM 20288^T strain in the presence of the class IIa bacteriocin mes 52A [41]. It would be more significant to analyse the membrane composition of resistant *L. monocytogenes* strains since they are food-spoilage organisms, compared to the food-enhancing character of lactic acid bacteria. Chapter 3 describes the membrane compositional differences, in wild-type and resistant *L. monocytogenes* strains, and outlines its potential role in modulating activity of class IIa bacteriocins.

5. Objectives of the study on class IIa bacteriocin resistance in *Listeria monocytogenes*

This study was undertaken to gain more insight into the resistant mechanisms and phenomena displayed by the pathogenic food spoilage organism *L. monocytogenes* to class IIa bacteriocins. Studies focussing on changes in the outer permeability barriers that have provided valuable information on nisin resistance in *L. monocytogenes* have been lacking with respect to class IIa bacteriocin resistance. We have therefore sought to determine the role of potential resistance factors such as membrane phospholipid compositional changes and their effect on bacteriocin activity; alterations in cell

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surface charge (teichoic acids, phospholipids); and responses to a cell wall acting antibiotic in class IIa bacteriocin resistant and wild-type cells. A final study looking at the impact of resistance on glucose metabolism brings a novel perspective to understanding class IIa resistance, and provides a 'reality check' to the feasibility of the use of class IIa bacteriocins as food preservatives.

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Chapter 2

Introduction to class IIa bacteriocin resistant Listeria monocytogenes strains and their response to leucocin A

Abstract

Listeria monocytogenes strains were treated with leucocin A to determine inhibitory concentrations (ICs) using a quantitative and reproducible bioassay. Various levels of resistance were detected. The two resistant strains generated in this study, namely, *L. monocytogenes* B73-V1 and B73-V2 were found to be 2 and 4 times more resistant than their parental strain *L. monocytogenes* B73, and were referred to as intermediate resistant strains. In comparison, the *L. monocytogenes* B73-MR1 and *L. monocytogenes* 412P were greater than 500 times more resistant to their corresponding wild-type strains, *L. monocytogenes* B73 and 412 respectively, and were referred to as highly resistant mutants. The evaluation of the growth patterns of all of the above strains, at 37 °C, 30 °C and 10 °C showed some common trends: the highly resistant strain B73-MR1 had a decreased growth rate but increased growth yield at all temperatures compared to the rest of the strains in the B73 family; the 412P strain showed the same growth pattern as B73-MR1 at 37 °C; and the intermediate resistant strains also showed a decreased growth rate and increased biomass at 37 °C and 30 °C, but a similar growth rate to the wild-type B73 and the lowest growth yield at 10 °C. These preliminary results provided evidence for possible effects of membrane adaptation and metabolic changes in the resistant organisms and prompted further investigation.

Introduction

Possibly, the most disconcerting factor regarding class IIa bacteriocin resistance is the frequency at which it occurs and its stability over successive generations [17, 9]. Additionally, it has been shown that resistance to the class IIa bacteriocin, pediocin develops to higher concentrations required for killing, in comparison to nisin resistance [8]. It has also been suggested that the high level resistance may occur by

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an 'on-off' type mechanism [8]. The observation of one prevalent mechanism involving shut-down of the mannose PTS permease in high level resistant *L. monocytogenes* strains, could play a role in this hypothesized 'on-off' mechanism [11]. If the mannose PTS permease is the docking molecule of class IIa bacteriocins [2], this hypothesis would make sense. However, mechanisms of resistance in *L. monocytogenes* strains with lower level susceptibilities to class IIa bacteriocins have not been investigated, although it may be envisaged to provide clues about the development of high levels of resistance.

The advantages of standard and sensitive methods for class IIa bacteriocin IC determinations would be the following: determination of accurate IC values; characterisation of mutants according to these IC values indicating resistance levels; conservation of limited test material (e.g. synthetic bacteriocin); and possible accurate assessment of cross-resistance to other bacteriocins. A standard and sensitive micro-gel diffusion assay enabling precise inhibitory concentration determination of antimicrobial activity and high reproducibility has recently been developed by Du Toit and Rautenbach [3], and may provide adequate assessment of class IIa bacteriocin ICs.

For further characterisation of mutants a simple growth study at various temperatures may provide immediate clues to possible differences existing in mutant strains. Many organisms are known to respond to changes in environmental temperature by changing their membrane phospholipid compositions, and this has been reported as a primary mechanism of the regulation of membrane fluidity [12, 15, 18]. Changes in membrane phospholipid affecting membrane fluidity can occur at lower temperatures and this affects growth patterns [10, 14, 15].

In addition, growth patterns may provide information about specific growth rate as a measure of the relative fitness of the strains [4, 9], and growth yields indicating metabolic changes [19, 6]. This would then enable observation of the physiological changes associated with resistance and possibly its stability and frequency.

The strains used in this study were obtained from various sources. The wild-type *L. monocytogenes* B73 strains and spontaneous mutant B73-MR1 have been described in leucocin A resistance studies previously [4, 16] in our laboratory. The wild-type *L.*

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monocytogenes 412 and the 412P mutant have also been previously studied in pediocin resistance [10]. The B73-MR1 and 412P mutants have shown, high levels of resistance to leucocin A using spot-on-lawn assays, absence of the mannose PTS EIIAB subunit, and increased expression of two enzymes of the β-glucoside PTS [9]. Furthermore, a clinical wild-type isolate *L. monocytogenes* EGDe whose genome has been sequenced [7] was used in this study since it displays sensitivity to mesentericin Y105. A defined mutant of *L. monocytogenes* EGDe, named EGK54 contained an insertional inactivation of the mannose PTS EIIAB component, and showed resistance to mesentericin Y105 [2]. These previously described mutants and the mutants generated in this study were chosen to understand resistance-associated phenomena associated with different class IIa bacteriocins and different levels of resistance.

This chapter serves as a preliminary data chapter that focuses on highlighting: 1) generation of class IIa resistant strains; 2) simple characterisation of the strains using growth studies; 3) and dose response determinations of the class IIa bacteriocin, leucocin A (leuA) against these strains. The purity of leuA was also assessed to ensure that extraneous contaminants did not influence the activity of the peptide. Comparisons of solvent conditions that influence bioactivity of leuA was outlined, and reasons for the choice of analytical grade water as the most favourable solvent discussed. Data presented in this chapter was generated to gain initial insight into the growth changes associated with, and response of target cells, to bacteriocin. This insight assisted in determining approaches to the investigation of class IIa bacteriocin resistance. The determination of dose response of the *Listeria monocytogenes* resistant variants helped in characterisation of the level of resistance prior to further investigation of factors influencing resistance. Since this data warranted further investigations into particular phenomena associated to class II resistance, it will be reproduced in other chapters, which it complemented.

MATERIALS AND METHODS

Purity analysis of leuA

LeuA was synthesized by a solid phase Fmoc-based peptide methodology and purified as described by Ramnath et al. [16]. The purified synthetic leuA was donated by K. Tamura and S. Aimoto. The peptide purity was verified by high performance liquid chromatography (HPLC) and electrospray mass spectrometry (ESMS).

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The HPLC analysis was performed on a Waters HPLC system using a Novapak C_{18} HPLC column (3.9x150 mm). The solvent system was 0.1 % trifluoroacetic acid (TFA) in analytical water (solvent A) and 90 % acetonitrile plus 10 % solvent A (solvent B). In order to accomplish chromatography, a linear gradient was developed over 13 minutes from 10 % to 100 % solvent B at a flow rate of 1.0 ml min^{-1}. The elution of the peptide was monitored at 254 nm.

ESMS of the synthetic leuA preparation was performed on a Micromass triple-quadrupole mass spectrometer fitted with an electrospray ionisation source. The preparation was resuspended in 50 % acetonitrile at a concentration of approximately, 1 mg ml^{-1} and 5 µl was injected via a Rheodyne valve per analysis. The carrier solvent was 50 % acetonitrile/0.5 % TFA and was delivered at a flow rate of 20 µl min^{-1} during the analysis. A capillary voltage of 3.5 kV was applied, with the source temperature at 120 ^{o}C and the cone voltage at 50 V. Data acquisition was in the positive mode, scanning the first analyser (MS_1) through m/z = 200-2000 at a scan rate of 100 atomic mass units per minute. Combining the scans across the elution peak and subtracting the background produced representative scans.

Bacterial strains and culture conditions

The strains used in most of the studies are listed in Table 1. *L. monocytogenes* B73 (leuA sensitive) and *L. monocytogenes* B73-MR1 (leuA resistant) have been previously described [16]. *L. monocytogenes* B73-V2 *and L. monocytogenes* B73-V1 are leuA intermediate resistant strains of the parental *L. monocytogenes* B73 strain that have been generated by leuA exposure (see hereafter). The pediocin sensitive, *L. monocytogenes* 412 strain and the pediocin resistant *L. monocytogenes* 412P strain have been previously described by Gravesen et al. [10]. All *L. monocytogenes* strains were grown on BHI (Biolab, Midrand, South Africa) agar or broth and at 37 ^{o}C, unless otherwise stated

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Table 1. *Listeria monocytogenes* strains

Listeria monocytogenes	**Description**	**Reference of strain**
Wild-type isolates		
412	Sensitive; isolated from raw salted pork	Gravesen et al. [10]
B73	Sensitive; isolated from meat	Dykes and Hastings [4]
EGDe	Sensitive; clinical	Glaser et al. [7]
Spontaneous mutants		
412P	Highly resistant mutant of 412 isolated on pediocin PA-1	Gravesen et al. [10]
B73-MR1	Highly resistant mutant of B73 isolated on leucocin A	Ramnath et al. [16]
B73-V1	Intermediate resistant mutant of B73 isolated on leucocin A	
B73-V2	Intermediate resistant mutant of B73 isolated of leucocin A	
Defined mutant		
EGK54 (EGDe-*mptA*)	Highly resistant; insertional inactivation of *mptA* in EGDe	Dalet et al. [2]

Isolation of leucocin resistant strains *L. monocytogenes* B73-V1 and *L. monocytogenes* B73-V2

A 1 % inoculum of *L. monocytogenes* B73 was added to BHI broth containing leuA at a concentration of 100 μg ml^{-1}. After 36 hours, the broth culture was serially diluted and plated on BHI agar plates free of leuA. Following incubation for 24 hours, colonies were selected randomly, and their inhibitory concentrations determined by agar well diffusion assay, to assess resistance development. The stability of the resistant mutant phenotype of the selected mutants *L. monocytogenes* B73-V1 and *L.*

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monocytogenes B73-V2, was monitored over ten successive subcultures in BHI broth, free of leuA.

Growth curves of *L. monocytogenes* strains

A 0.1 % inoculum from an overnight culture was added to fresh BHI broth and the growth was monitored at OD_{600} at 37 °C, 30 °C and 10 °C for all the strains.

Antilisterial activity determinations

The 50 % inhibitory concentration, IC_{50}, was determined using the agar well diffusion assay, as described by Du Toit and Rautenbach [3], with the following modifications: The cells were not washed and resuspended in phosphate-buffered saline; and a 0.7 % BHI agar, was used in the wells. All dose response data from the agar well diffusion assays was analysed using Graphpad Prism version 3.0 for Windows (GraphPad Software, San Diego, California, USA, www.graphpad.com) as described by Du Toit and Rautenbach [3]. A sigmoidal curve with variable slope and constant top of 100 and variable bottom was fitted to each of the data sets using the following equation: $Y = \text{Bottom}+[100-\text{Bottom}]/[1+10^{[\log IC_{50} \times \text{Hill slope}]}]$. For curve fitting only the mean value of each data point, without weighting, was considered. The IC_{50} was calculated from the x-value of the response halfway between top and bottom plateau. The IC_{min} value corresponded to the concentration of leuA at the onset of growth inhibition, while the IC_{max} corresponded to the leuA concentration resulting in 0 % relative growth.

RESULTS AND DISCUSSION

Purity of leuA

HPLC analysis (Fig. 1A) showed that the leuA preparation used in this study contained one major UV absorbing component (>95 %). ESMS analysis of this synthetic preparation confirmed the chemical purity in terms of molecular mass, as the expected molecular ions ($3H^+$, $4H^+$ and $5H^+$), corresponding to Mr of 3930.3 (3930.3 expected) was found (Fig. 1B). From these analyses it could thus be deduced that the purity of the synthetic leuA is very high (>95 %) and that the biological and biophysical effects observed in this and subsequent chapters in this thesis are predominantly due to the presence of leuA and not to unknown contaminants.

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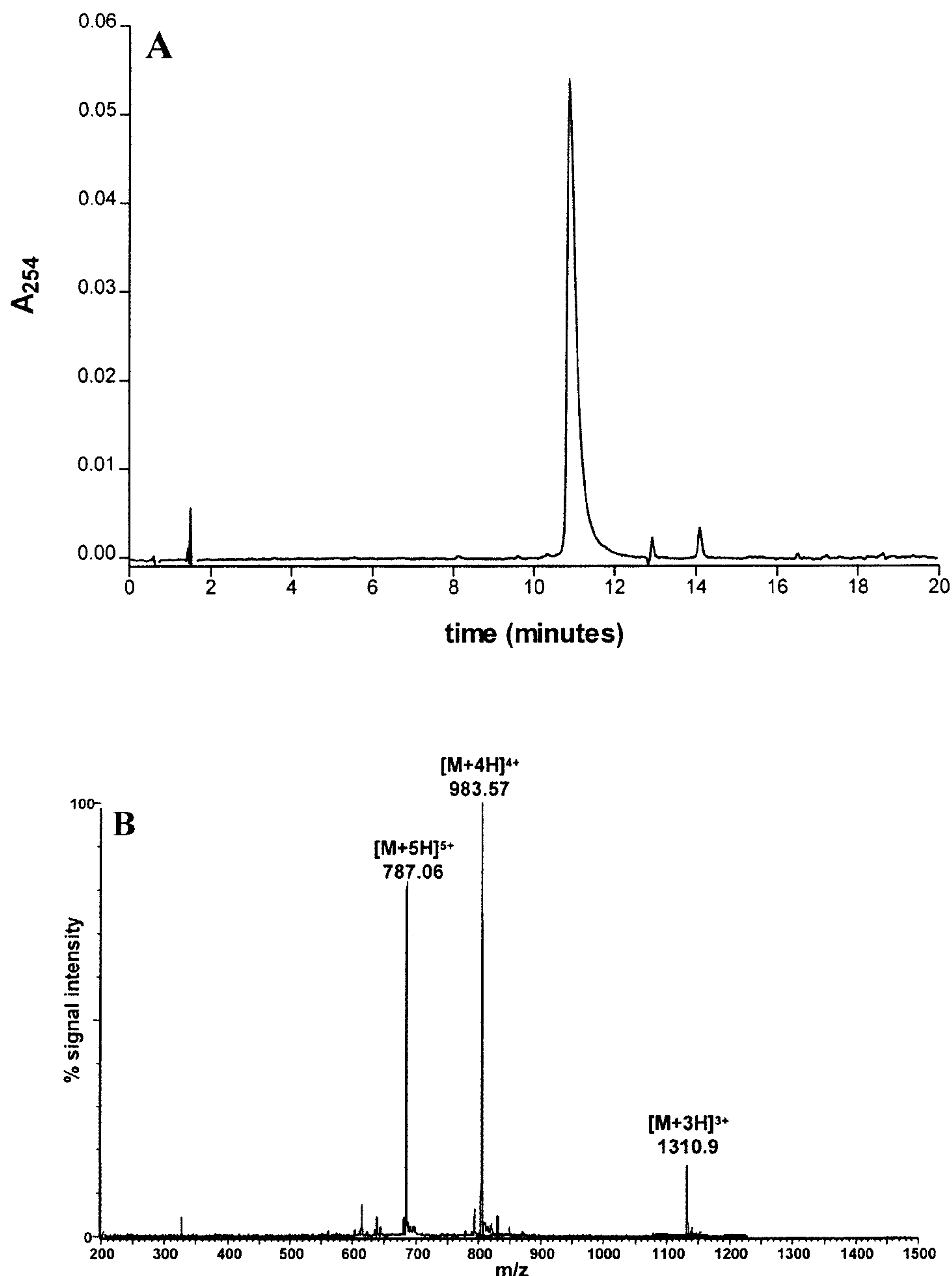

Fig. 1. HPLC (A) and raw electrospray mass ionisation spectrum (B) of leucocin A.

Effect of solvent on the activity of leuA.

Three different solvents were tested to assess the influence of the solvent environment on antilisterial activity of leuA against the wild-type *L. monocytogenes* B73 strain.

If leuA was dissolved beforehand in 50 % acetonitrile, a shift to the left in the dose response curve, if compared to H_2O as solvent, indicated an improved inhibition of 223 % (Fig. 2). The final acetonitrile concentration per cell was >5% and no

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inhibition of growth was observed in the control wells that received the solvent without leuA. Proteins in hydrophobic solvents (e.g. acetonitrile) are thought to retain their native structure due to strong hydrogen bonding between protein atoms and a more rigid structure in the absence of water [13]. The increase in leuA's activity in the presence of the less polar (δ=37.5) acetonitrile may be the consequence of an increase in the availability of monomeric leuA to bind to the target cell. The 50% acetonitrile in the initial mixture would decrease the hydrophobic effect and thus the interaction between leuA's hydrophobic C-terminal, but because acetonitrile is highly diluted in the assay, the very important hydrophobic interactions with the target cell would not be compromised. The importance of this type of interaction may support the findings of Fimland et al. [5], who showed that the C-terminal hydrophobic α-helical region of the class IIa bacteriocin, pediocin PA-1 is important for the recognition of a specific hydrophobic interacting entity of the target membrane.

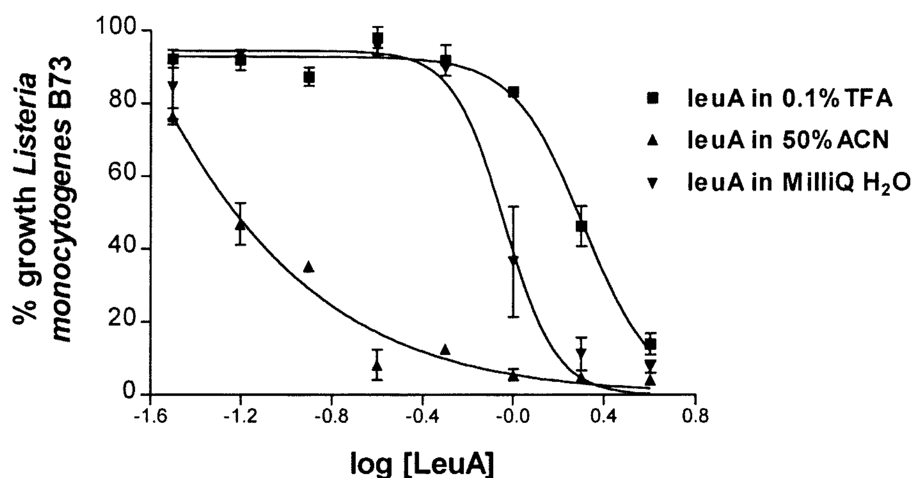

Fig. 2. Dose-response of *L. monocytogenes* B73 to leucocin A resuspended in varying media. Data are a combination of results of duplicate experiments. The error bars represent the standard error of the mean for each concentration value.

When leuA was dissolved in H_20 containing 0.1 % TFA, a shift of the dose-response curve to the right, compared to that of H_2O as solvent, indicated a less effective inhibition of 0.92 % in the wild-type *L. monocytogenes* B73 strain (Fig. 2). However, 0.1 % TFA on its own did not influence the growth of *L. monocytogenes* B73. Although TFA will promote protonation and the cationic nature of the peptide, it will also tend to form ionic interactions with the positive groups of the peptide and thus neutralise the peptide and limit the electrostatic interaction with the target cell. Also,

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it was recently found that TFA tends to promote self-association of amphipathic cationic peptides [Naidoo et al., 2003, unpublished results]. Both these factors may therefore explain the decrease in leuA's activity in the presence of 0.1 % TFA.

These results show that the solvent environment of the peptide seems to have a significant influence on the activity of the peptide even though the solvent components are highly diluted (>10 times) in the assay. Analytical grade water provided the most neutral environment for leuA and target cell interaction by not providing extraneous factors, like charge to influence activity, but rather promoting hydrophobic interaction. We, therefore, used analytical grade water in our standard procedure for determination of critical inhibitory concentrations of leuA against *L. monocytogenes* strains.

Antilisterial activity determinations

The results of this study were discussed in detail in Chapter 3. In brief we found that *L. monocytogenes* B73-MR1 and 412P showed no decrease in growth with increasing concentrations of leuA up to 120 μg ml^{-1} (maximum concentration in our assay), while *L. monocytogenes* B73-V1 and B73-V2 were found to be 2 to 3 times and 3.5 to 4 times more resistant to leuA respectively than *L. monocytogenes* B73 (Fig. 3A and Table 2). In keeping with the nomenclature for vancomycin resistance in *Staphylococcus aureus* [1], we referred to *L. monocytogenes* B73-V1 and *L. monocytogenes* B73-V2 as intermediate resistant strains.

With a limited availability of leuA, we did not determine the complete dose response for the two highly resistant strains. It could, however, be calculated from their insensitivity at 120 μg ml^{-1} leuA that *L. monocytogenes* B73-MR1 is >1000 times more resistant than *L. monocytogenes* B73, while *L. monocytogenes* 412P is >500 times more resistant than *L. monocytogenes* 412P. *L. monocytogenes* B73-MR1 and *L. monocytogenes* 412P have subsequently been referred to as highly leucocin resistant. One noteworthy aspect of the dose-response of *L. monocytogenes* 412P towards increasing concentrations of leuA is an unexpected ±30% increase in growth (Fig. 3B). This improved growth may be an indication of a strain specific resistance response, as this strain was selected under pediocin-PA1 pressure and showed cross-resistance to leuA.

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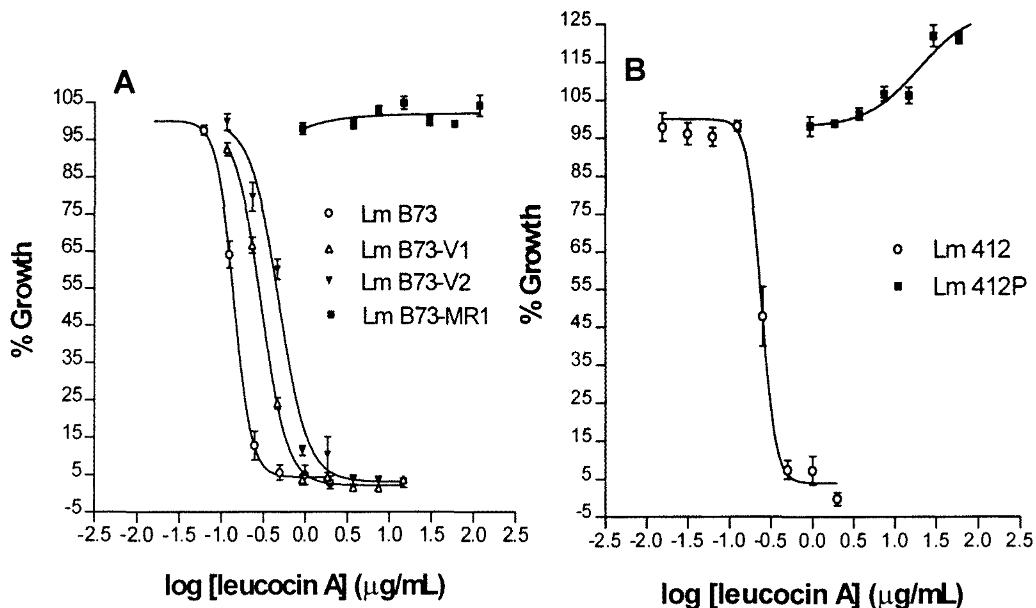

Fig. 3. Dose-response of leucocin A sensitive *L. monocytogenes* B73 [○], intermediate leucocin resistant mutants *L. monocytogenes* B73-V1 [▵] and *L. monocytogenes* B73-V2 [▼], and highly leucocin resistant *L. monocytogenes* B73-MR1 [■]. **B**. Dose-response of pediocin PA-1 and leucocin A sensitive *L. monocytogenes* 412 [○] and the highly pediocin PA-1 and leucocin A resistant *L. monocytogenes* 412P [■]. The 100 % growth was taken, as growth of each strain in the absence of leucocin A. Data on the graphs is a combination of duplicate experiments. The error bars represent the standard error for the mean for each concentration value (eight determinations).

The *L. monocytogenes* EGDe family did not grow well in the microtitre plate assays, and their inhibitory concentrations could therefore not be determined. Spot-on-lawn assays with leuA were used in another study to determine minimal inhibitory concentration of this family [8].

Growth patterns of *L. monocytogenes* strains

In Fig. 4 and Table 3 the growth patterns, growth rates and growth yields of all the wild-type and resistant strains of the *L. monocytogenes* B73 family and *L. monocytogenes* 412 families are shown respectively. It is clear that the growth patterns for the strains differ, with the wild-type showing the fastest growth rate at all temperatures, and the highly resistant strains showing the slowest growth rate. The intermediate resistant strains show a corresponding intermediate growth rate compared to their wild-type and highly resistant corresponding strains at 30 °C and 37 °C. At 10 °C the wild-type and intermediate variants show similar growth rates however the intermediates strains have a much lower biomass at the beginning of log phase. The highly resistant strains and wild-type strains showed the highest biomass

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at all temperatures. At 10 °C, however, the intermediate strains accomplish the lowest growth yield and the highly resistant strain, still accomplishes the largest growth yield.

Table 2. Critical inhibitory concentrations of leucocin A against the six *L. monocytogenes* strains in this study as deduced from the dose response curves.

***L. monocytogenes* strains**	**IC_{50} [µg ml^{-1}]**	**IC_{min} [µg ml^{-1}]**	**IC_{max} [µg ml^{-1}]**
B73	0.14±0.007	0.06 ±0.001	0.28±0.015
B73-V1	0.30±0.023	0.12±0.006	0.78±0.090
B73-V2	0.49±0.066	0.18±0.015	1.10 ±0.115
B73-MR1		>120	
412	0.24±0.020	0.16±0.011	0.48±0.050
412P		>120	

Mazzotta and Montville [14, 15] observed changes in resistant *L. monocytogenes* strain lipids in the presence of nisin, which was consistent with homeoviscous adaptation, which causes decreased growth rates but does not prevent growth at low temperatures. We therefore hypothesized that the growth patterns at different temperatures could result in the membrane phospholipid composition being different in our various resistant strains. It would be interesting therefore to determine whether alteration of the lipid composition has an effect on the resistance to the class IIa bacteriocin. Studies on the changes in phospholipid and their impact on resistance of *L. monocytogenes* strains to class IIa bacteriocins have not previously been characterised. It has however been shown that membrane fluidity and membrane composition may influence activity of nisin, and is implicated in nisin resistance in *L. monocytogenes* [14, 15]. Therefore growth pattern differences exhibited by the various resistant strains, at different temperatures (Fig. 4) warranted further investigation of phospholipid changes in the membrane, and this is discussed in detail in Chapters 3 and 4.

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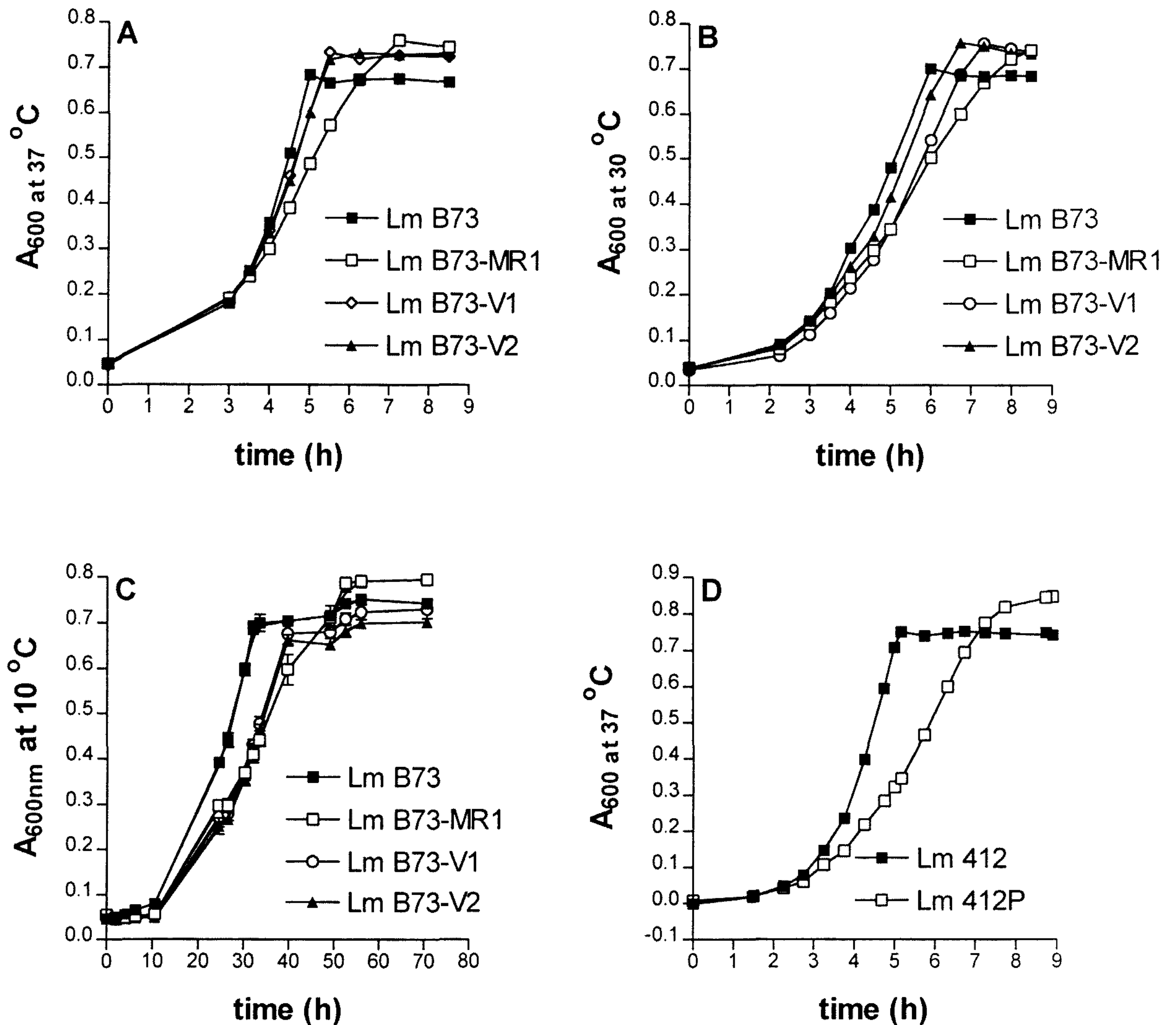

Fig. 3. Growth curves of *Listeria monocytogenes* strains. Curves [**A**], [**B**] and [**C**] represent the growth pattern of the *L. monocytogenes* B73 family of strains at 37 °C, 30 °C and 10 °C, respectively. Curve [**D**] represents the growth pattern of the *L. monocytogenes* 412 family of strains at 37 °C. All curves represent a combination of two separate experiments.

Additionally, previous studies have related a lower growth rate to a decrease in relative fitness in class IIa resistant strains of *L. monocytogenes* B73 [4]. It was also suggested that this could be attributed to the use of energy-expensive metabolic pathways in resistant strains, thus decreasing the likelihood of stable resistant populations [4]. It is apparent, however, that this fitness cost determined by lower growth rate was accompanied by a gain in growth yield in the resistant strains. This contrasting evidence for fitness costs related to class IIa bacteriocin resistance is further investigated by analysis of the glucose metabolism in the highly resistant and

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wild-type strains. This study is outlined in Chapter 6 and attempts to clarify what the potential losses and gains are as a result of resistance in the target organism.

Table 3. Specific growth rates and stationary-phase biomass of *L. monocytogenes* strains at different temperatures.

***Listeria monocytogenes* strains**	**Specific growth rates μ [h^{-1}] and biomass [A_{600}]**		
	37 °C	**30 °C**	**10 °C**
B73	0.294 h^{-1}	0.270 h^{-1}	0.042 h^{-1}
	0.669	0.685	0.743
B73-V1	0.254 h^{-1}	0.260 h^{-1}	0.042 h^{-1}
	0.726	0.740	0.730
B73-V2	0.255 h^{-1}	0.251 h^{-1}	0.042 h^{-1}
	0.733	0.736	0.701
B73-MR1	0.206 h^{-1}	0.228 h^{-1}	0.039 h^{-1}
	0.747	0.743	0.795
412	0.417 h^{-1}	Nd*	Nd*
	0.737		
412P	0.315 h^{-1}	Nd*	Nd*
	0.832		

*Nd, not determined

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Chapter 3

Membranes of class IIa bacteriocin-resistant Listeria monocytogenes cells contain increased levels of desaturated and short-acyl-chain phosphatidylglycerols

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Membranes of Class IIa Bacteriocin-Resistant *Listeria monocytogenes* Cells Contain Increased Levels of Desaturated and Short-Acyl-Chain Phosphatidylglycerols

Viveka Vadyvaloo,[1] John W. Hastings,[1] Marthinus J. van der Merwe,[2] and Marina Rautenbach[1]*

Department of Biochemistry[1] and Electrospray Mass Spectrometry Unit, Central Analytical Facility,[2] University of Stellenbosch, Matieland 7602, Republic of South Africa

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A major concern in the use of class IIa bacteriocins as food preservatives is the well-documented resistance development in target *Listeria* strains. We studied the relationship between leucocin A, a class IIa bacteriocin, and the composition of the major phospholipid, phosphatidylglycerol (PG), in membranes of both sensitive and resistant *L. monocytogenes* strains. Two wild-type strains, *L. monocytogenes* B73 and 412, two spontaneous mutants of *L. monocytogenes* B73 with intermediate resistance to leucocin A (±2.4 and ±4 times the 50% inhibitory concentrations [IC_{50}] for sensitive strains), and two highly resistant mutants of each of the wild-type strains (>500 times the IC_{50} for sensitive strains) were analyzed. Electrospray mass spectrometry analysis showed an increase in the ratios of unsaturated to saturated and short- to long-acyl-chain species of PG in all the resistant *L. monocytogenes* strains in our study, although their sensitivities to leucocin A were significantly different. This alteration in membrane phospholipids toward PGs containing shorter, unsaturated acyl chains suggests that resistant strains have cells with a more fluid membrane. The presence of this phenomenon in a strain (*L. monocytogenes* 412P) which is resistant to both leucocin A and pediocin PA-1 may indicate a link between membrane composition and class IIa bacteriocin resistance in some *L. monocytogenes* strains. Treatment of strains with sterculic acid methyl ester (SME), a desaturase inhibitor, resulted in significant changes in the leucocin A sensitivity of the intermediate-resistance strains but no changes in the sensitivity of highly resistant strains. There was, however, a decrease in the amount of unsaturated and short-acyl-chain PGs after treatment with SME in one of the intermediate and both of the highly resistant strains, but the opposite effect was observed for the sensitive strains. It appears, therefore, that membrane adaptation may be part of a resistance mechanism but that several resistance mechanisms may contribute to a resistance phenotype and that levels of resistance vary according to the type of mechanisms present.

Bacteriocins of lactic acid bacteria are ribosomally synthesized peptides that show antimicrobial activity in their mature form, usually against a narrow spectrum of closely related species. Several classes have been described, including lantibiotics (class I), small heat-stable non-lanthionine peptides (class II), and large heat-labile bacteriocins (class III). The class IIa bacteriocins are known as "pediocin-like" and show strong antilisterial activity (23, 25). Due to the recurrence of serious listeriosis outbreaks caused by the food-borne pathogen *Listeria monocytogenes*, these class IIa bacteriocins have become a major focus in the search for novel naturally occurring biopreservatives. It is estimated that between 1,100 and 2,500 people in the United States develop listeriosis each year and that 20 to 25% of these *Listeria* infections are fatal (http://www.fsis.usda.gov; http://www.cdc.gov).

Reduced sensitivity or resistance to these bacteriocins may compromise the antimicrobial efficiency of these peptides. Resistance has been found to be spontaneous or can be induced by exposure to the bacteriocin. Of concern is the relatively high frequency (10^{-3} to 10^{-4}) at which *L. monocytogenes* develops resistance to class IIa bacteriocins (33). Mechanisms contributing to class IIa bacteriocin resistance have included factors under the influence of the σ^{54} factor (34) and σ^{54}-dependent genes, specifically the mannose phosphotransferase system (PTS) permease for *Enterococcus faecalis* (15, 22) and *L. monocytogenes* (16) and the mannose PTS enzyme IIAB component of *L. monocytogenes* sensitivity to leucocin A (32). The upregulation of a β-glucoside-specific PTS has also been reported in pediocin-resistant *L. monocytogenes* (21).

Class IIa bacteriocins are currently thought to act primarily by permeabilizing the target membrane by the formation of pores. It has been hypothesized that these pores cause leakage of ions and inorganic phosphates and subsequently dissipate the proton motive force (4, 8, 10, 14, 27). The requirement of a receptor-type molecule (5, 37) and a general electrostatic functional binding of these cationic peptides to the anionic head groups of phospholipids in membranes (11, 12, 24, 27) are involved in the mediation of class IIa bacteriocin activity. Further, it has been shown that the lipid composition of the target cell membrane plays an important role in modulating the membrane interaction of the bacteriocin (13, 14). Hydrophobic interactions between the hydrophobic part of the bacteriocin and the lipid fatty acid chains, resulting in insertion, follow the electrostatic binding of the peptide to the membrane (17, 19). This interaction of the bacteriocins with phos-

* Corresponding author. Mailing address: Department of Biochemistry, University of Stellenbosch, Private Bag X1, Matieland 7602, Republic of South Africa. Phone: 27-21-8085872. Fax: 27-21-8085863. E-mail: mra@sun.ac.za.

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pholipids has been reported to largely influence membrane permeability (14).

It is also possible that different levels of resistance are associated with different resistance mechanisms. The aim of this study was therefore to elucidate changes associated with bacteriocin resistance by looking at changes occurring in the phospholipid composition, in particular the phosphatidylglycerol (PG) composition of cells with different levels of resistance and a bacteriocin-sensitive strain of the same listerial species. Electrospray mass spectrometry (ESMS) was used as a tool to study phospholipid composition. Sterculic acid methyl ester (SME), a cyclopropene fatty acid previously reported to be a specific inhibitor of the stearoyl coenzyme A desaturase system (36), was used to determine the effect of inhibiting the monodesaturation of fatty acids on the resistance levels to leucocin A.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *L. monocytogenes* B73 (leucocin A sensitive) and *L. monocytogenes* B73-MR1 (leucocin A resistant) have been described previously (32). *L. monocytogenes* B73-V2 and *L. monocytogenes* B73-V1 are mutants of the parental *L. monocytogenes* B73 strain with intermediate resistance to leucocin A and were generated by leucocin A exposure (see below). The pediocin-sensitive *L. monocytogenes* 412 strain and the pediocin-resistant *L. monocytogenes* 412P strain have been previously described by Gravesen et al. (21). All *L. monocytogenes* strains were grown on brain heart infusion (BHI) agar or broth (Biolab, Midrand, South Africa) at 37°C.

Generation of the leucocin-resistant strains *L. monocytogenes* B73-V1 and B73-V2. Leucocin A was synthesized as described previously (32). A 1% inoculum of *L. monocytogenes* B73 was added to BHI broth containing leucocin A at 100 μg/ml. After 36 h, the broth culture was serially diluted and plated on BHI agar plates free of leucocin. Following incubation for 24 h, colonies were selected randomly, and the 50% inhibitory concentrations (IC_{50}s) of leucocin A for these colonies were determined by agar well diffusion assay, to assess resistance development. The stability of the phenotype of the selected mutants *L. monocytogenes* B73-V1 and B73-V2 was monitored over 10 successive subcultures in BHI broth, free of leucocin.

Antilisterial activity determinations. The IC_{50} was determined using the agar well diffusion assay, as described by Du Toit and Rautenbach (18), with the following modifications. The cells were not washed and resuspended in phosphate-buffered saline, and 0.7% BHI agar was used in the wells. All dose-response data from the agar well diffusion assays was analyzed using Graphpad Prism version 3.0 for Windows (GraphPad Software, San Diego, Calif.) as described by Du Toit and Rautenbach (18). A sigmoidal curve with variable slope and constant top of 100 and variable bottom was fitted to each of the data sets by using the equation $Y = \text{bottom} + (100 - \text{bottom})/[1 + 10^{(\log IC_{50})} \times \text{hill slope}]$. For curve fitting only, the mean value of each data point, without weighting, was considered. The IC_{50} was calculated from the x value of the response halfway between the top and bottom plateau.

Extraction of *L. monocytogenes* phospholipids. *L. monocytogenes* strains were grown in 1-liter broth cultures to early stationary phase (optical density at 600 nm, 0.7 to 0.75) and were then harvested for phospholipid extraction. Phospholipid was extracted using the Bligh and Dyer method as described by Cabrera et al. (9). Cells were lysed in a sonicating water bath for 2 h, instead of by vortexing. The phospholipid standards (Sigma Chemical Co., St. Louis, Mo.) used were dimyristoyl-phosphatidylglycerol (DMPG), dioleoyl-phosphatidylglycerol (DOPG), dipalmitoyl-phosphatidylglycerol (DPPG), and distearoyl-phosphatidylglycerol (DSPG).

Inhibition of PG desaturation by sterculic acid methyl ester. SME, a desaturase inhibitor obtained from Sigma Chemical Co., was prepared and stored in the same way as previously described (36). It was added, to reach a final concentration of 0.025 mM, to a 1% inoculum of the *L. monocytogenes* strains to be tested, in a broth culture. For the agar diffusion assay, a final concentration of 0.05 mM SME was used in the agar.

Electrospray mass spectrometry. Mass spectrometry was performed using a Micromass triple-quadrupole mass spectrometer with an electrospray ionization source. Dried phospholipid was diluted with 100 μl of methanol-chloroform (2:1, vol/vol) and then diluted 1:10 with methanol-chloroform (1:1, vol/vol). A 5-μl volume of the sample solution was introduced into the electrospray ionization mass spectrometer via a Rheodyne injector valve. Methanol-chloroform (1:1, vol/vol) was the carrier solvent, delivered at a flow rate of 20 μl/min during each analysis. A capillary voltage of 3.5 kV was applied, with the source temperature at 120°C and the cone voltage at 100 V. Data acquisition was in the negative mode, scanning the first analyzer (MS_1) through $m/z = 200$ to 2,000 at a scan rate of 100 atomic mass units/s. Combining the scans across the elution peak and subtracting the background produced representative scans. For fragmentation analysis, precursor ions were selected in MS_1 and product ions were detected in MS_2 after decomposition at a collision energy of 40 eV and with the argon pressure in the collision cell at 0.2 Pa.

Statistical evaluation. Tukey's comparative test, using Prism 3.0, was used to statistically evaluate all results and to calculate significant differences in the ratios of unsaturated and short-acyl-chain fatty acid species between the susceptible and resistant strains. P values for the dose-response curves were also generated using this method.

RESULTS

Antilisterial activity determinations. *L. monocytogenes* B73-MR1 showed no decrease in growth with increasing concentrations of leucocin A up to 120 μg/ml (the maximum concentration in our assay) (Fig. 1). However, the other highly leucocin-resistant strain, *L. monocytogenes* 412P, showed an unexpected increased in growth (±30%) with increasing concentrations of leucocin A (Fig. 1). This increase in growth may be an indication of a strain-specific resistance response. This strain was selected under pediocin-PA1 pressure and showed cross-resistance to leucocin A.

The shifting of the dose-response curves (Fig. 1) of the two spontaneous mutant strains, *L. monocytogenes* B73-V1 and B73-V2, to the right indicated their increased resistance to leucocin A. The IC_{50}s (Table 1) of leucocin A for *L. monocytogenes* B73-V1 and B73-V2 indicate that these strains are ±2.4 times and ±4 times more resistant, respectively, to leucocin A than is *L. monocytogenes* B73. According to Tukey's comparison test, the strains with intermediate resistance to leucocin A had a significantly different response from that of *L. monocytogenes* B73 (leucocin susceptible) ($P < 0.001$). The IC_{50}s for *L. monocytogenes* 412 and B73 have also been determined to be significantly different ($P < 0.001$). In keeping with the nomenclature for vancomycin resistance in *Staphylococcus aureus* (6), we have referred to *L. monocytogenes* B73-V1 and B73-V2 as intermediate leucocin resistant strains.

Due to the limited amount of leucocin A available, we did not determine its IC_{50}s for the two highly resistant strains. It has, however, been determined that *L. monocytogenes* B73-MR1 is more than 1,000 times as resistant as *L. monocytogenes* B73, while *L. monocytogenes* 412P is more than 500 times as resistant as *L. monocytogenes* 412P, using the maximum concentration of 120 μg of leucocin A per ml. *L. monocytogenes* B73-MR1 and *L. monocytogenes* 412P have subsequently been referred to as highly leucocin resistant.

ESMS identification and profiles of phospholipids of *L. monocytogenes* strains. Correlating the fragmentation mass spectra of standard PGs with that of the *L. monocytogenes* PG fragmentation patterns allowed the identification of the fatty acyl moieties in the observed PG species (Table 2). Our data correlated with the fragmentation data reported for PGs from *Bacillus* (7). The identification of the fatty acyl moieties of the PG species (Table 2) as mainly C_{14}, C_{15}, C_{16}, and C_{17} is also corroborated by the findings of Mastronicolis et al. (28) in their study of the diversity of the polar lipids of *L. monocytogenes*.

ESMS spectra of the phospholipids of all the *L. monocyto-*

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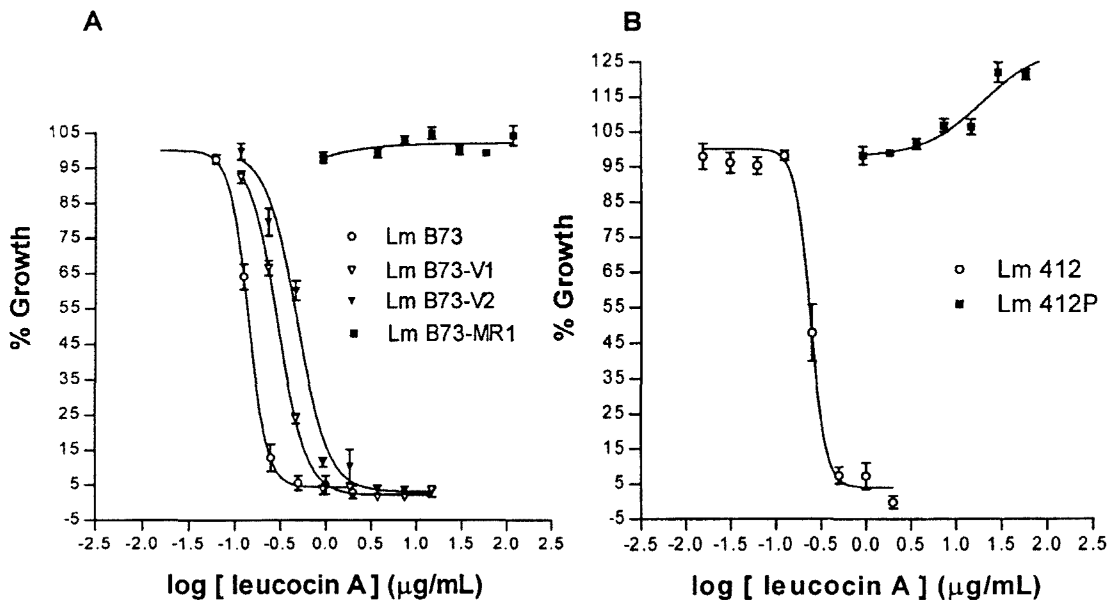

FIG. 1. (A) Dose-response of leucocin A-sensitive *L. monocytogenes* B73, intermediate-resistance mutants *L. monocytogenes* B73-V1 and *L. monocytogenes* B73-V2, and highly leucocin-resistant *L. monocytogenes* B73-MR1. (B) Dose-response of pediocin PA-1- and leucocin A-sensitive *L. monocytogenes* 412 and the highly pediocin PA-1- and leucocin A-resistant *L. monocytogenes* 412P. The 100% growth level was taken as the growth of each strain in the absence of leucocin A. Data are a combination of the results of duplicate experiments. The error bars represent the standard error of the mean for each concentration value (eight determinations).

genes strains (Fig. 2) showed the abundant presence of PG, the major phospholipid of *L. monocytogenes* (20, 26). The ESMS spectrum in Fig. 2A is representative of pediocin-sensitive *L. monocytogenes* strain and the spectrum in Fig. 2B represents the general profile found for the leucocin intermediate resistant and pediocin-resistant strains analyzed in this study. Approximately four major PG molecular species are observed in the ESMS data (Fig. 2), and these are found in both the saturated and unsaturated forms. In the sensitive *L. monocytogenes* B73 strain, the PG species with *m/z* 722, correlated with PG containing C_{15} and C_{17} fatty acid chains, was the most abundant (Fig. 2A). The PG containing C_{15} and $C_{16:1}$ (*m/z* 706) was the most abundant in highly resistant and intermediate resistant strains (Fig. 2B). Similar differences were found between the *L. monocytogenes* 412 and 412P strains. It is apparent that the resistant strains seem to have an observable increase in PGs containing an unsaturated fatty acyl chain.

Saturation differences of PGs. The ratios of the unsaturated to saturated molecular species of PG for all the resistant strains (Fig. 3A) were higher than those in the sensitive strains. Although the resistant strains have an increased level of the unsaturated PG component, there is no clear correlation with the resistance level. For example, the unsaturated/saturated PG ratios were greater for the intermediate-resistance strains (Fig. 3A) than for the highly resistant strains for both [*m/z* 678]/[*m/z* 680] and [*m/z* 706]/[*m/z* 708]. This change, however, correlates with increasing levels of resistance in the intermediate-resistance strain group (Fig. 3A). There was also a significant increase ($P < 0.05$) in the unsaturated PG species (*m/z* 706) in comparison to the major PG species (*m/z* 722), for the intermediate-resistance strains, and this increase is more significant than that for the highly resistant *L. monocytogenes* B73-MR1 strain.

Differences in fatty acyl chain length in PGs. More short-acyl-chain PGs than the PG species with *m/z* 722 were observed for the intermediate-resistance and highly resistant strains (Fig. 3B). *L. monocytogenes* B73-MR1 contained more of all short-acyl-chain PGs, specifically the species with *m/z* 692, than

TABLE 1. IC_{50}s of leucocin A for the *L. monocytogenes* strains tested[a]

L. monocytogenes strain	Leucocin A IC_{50} (μg/ml) (95% confidence range)
B73	0.14 (0.13–0.16)
B73-V1	0.30 (0.28–0.31)
B73-V2	0.49 (0.39–0.60)
B73-MR1	>100
412	0.24 (0.22–0.27)
412P	>100

[a] Two independent experiments were performed, and each concentration was determined in quadruplicate per experiment.

TABLE 2. Molecular species of PG and the corresponding fatty acid species from *L. monocytogenes* strains, identified by ESMS in negative-ion mode

$[M - H]^-$ *m/z*	Calculated atomic mass (Da)	Fatty acyl combinations
678	678.88	$C_{15:1}/C_{14}$
680	680.89	C_{15}/C_{14}
692	692.91	$C_{16:1}/C_{14}$
694	694.92	C_{16}/C_{14}
706	706.93	$C_{15}/C_{16:1}$
708	708.95	C_{15}/C_{16}
722	722.97	C_{15}/C_{17}

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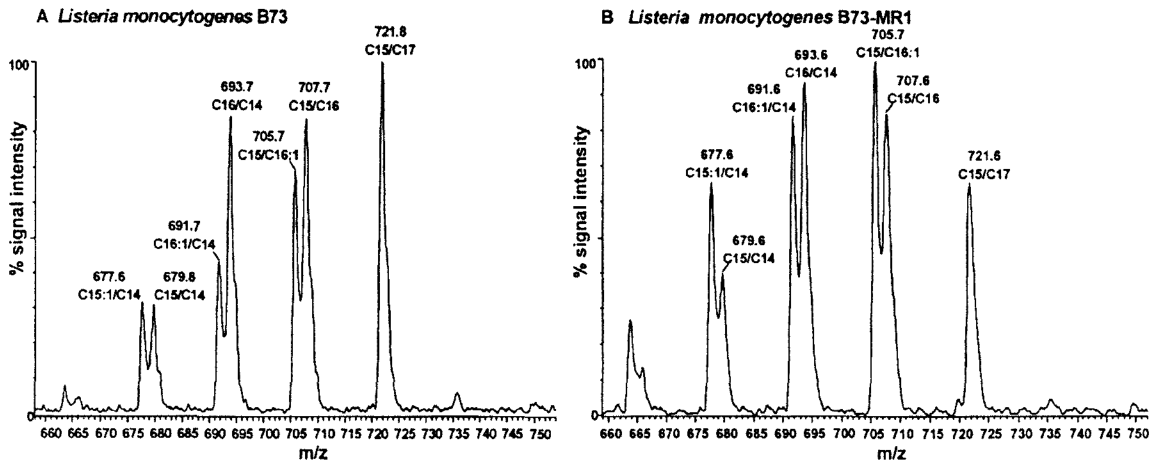

FIG. 2. ESMS spectrum of the PG region of *L. monocytogenes* B73 (leucocin sensitive) (A) and *L. monocytogenes* B73-MR1 (highly resistant) (B). The ESMS data for *L. monocytogenes* B73 are representative of *L. monocytogenes* 412 too, and the ESMS data of *L. monocytogenes* B73-MR1 are representative of the intermediate-resistance strains and *L. monocytogenes* 412P.

did the intermediate-resistance strains (Fig. 3B). It is, however, difficult to detect a clear correlation between the level of resistance and amounts of short-acyl-chain PGs, considering that the highly resistant strains are at least 200 times more resistant than the intermediate-resistance strains.

IC_{50} determinations for SME-treated cells. SME has been used previously as a desaturase inhibitor in prokaryotes (36). A final concentration of 0.025 mM in broth was determined to be most favorable since it caused no significant growth inhibition (data not shown). No significant growth inhibition was found with 0.05 mM SME in agar, while inhibition was observed with 0.05 mM SME in broth. The higher tolerance to the inhibitor could probably be attributed to decreased diffusion in a solid (agar) environment. The sensitivity of *L. monocytogenes* B73 to leucocin A was not affected by the presence of SME (data not shown). The inhibitor, however, affected the response of the two intermediate-resistance strains to leucocin A (compare Table 1 and Fig. 3). The 2-fold-resistant *L. monocytogenes* B73-V1 displayed an unexpected 2.5-fold increase in resistance at 0.33 μg of leucocin A per ml (compare Table 1 and Fig. 4). The fourfold-resistant *L. monocytogenes* B73-V2 displayed a decrease of ±50% in resistance (compare Table 1 and Fig. 4)

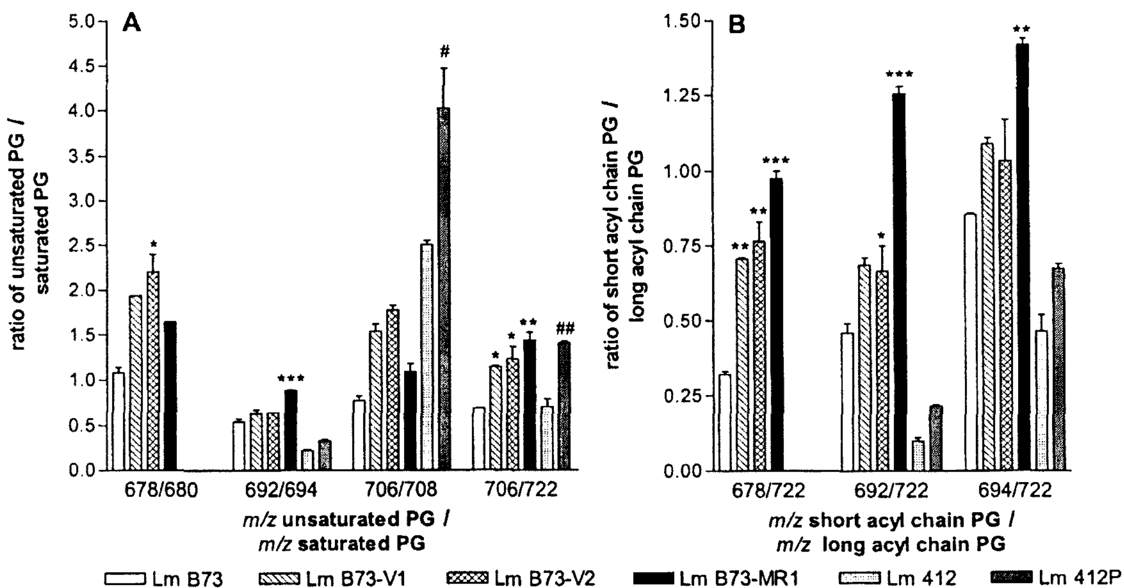

FIG. 3. PG composition of *L. monocytogenes* sensitive, (strains B73 and 412), intermediate-resistance (strains B73-V1 and B73-V2), and highly resistant (strains B73-MR1 and Lm 412P) strains depicted as ratios of unsaturated PGs to saturated PGs (A) and short-acyl-chain PGs to the long-acyl-chain PG (*m/z* = 722), (B). Statistical comparison between *L. monocytogenes* B73 and its resistant strains: *, $P < 0.05$, **, $P < 0.01$; ***, $P < 0.001$; comparison between *L. monocytogenes* 412 and 412P: #, $P < 0.05$; ##, $P < 0.01$. Note that only one value for 678/680 was determined for *L. monocytogenes* B73-V1 and so it could not be statistically compared.

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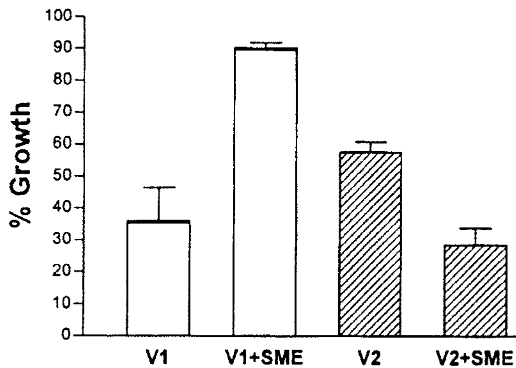

FIG. 4. Influence of SME on the leucocin A resistance of intermediate-resistance *L. monocytogenes* strains. *L. monocytogenes* B73-V1 and B73-V2 were incubated for 17 h with 0.33 and 0.65 μg of leucocin A per ml, respectively, without (control) or with 0.05 mM SME added to the growth media. The 100% growth level was taken as growth in the absence of leucocin A, without (control) or with 0.05 mM SME added to the growth media. Data are a combination of results of duplicate experiments. The error bars represent the standard error of the mean for eight determinations. Means are significantly different ($P < 0.0005$) between the SME-treated and nontreated cultures.

to leucocin A at 0.65 μg/ml. No decrease in resistance was observed for the highly resistant *L. monocytogenes* B73-MR1 at the maximum of 2.6 μg/ml leucocin A concentration. We did not determine IC_{50}s for the highly resistant strains in the presence of SME due to the limited availability of leucocin A.

Changes in PG composition of cells treated with SME. The desaturase inhibitor affected the sensitive and the intermediate-resistance and highly resistant strains in different ways. The PG profiles in Fig. 5A and B are representative of the pediocin-sensitive *L. monocytogenes* 412 and the resistant *L. monocytogenes* 412P strains, respectively, after addition of desaturase inhibitor. The major difference exhibited in phospholipid profiles of SME-treated highly resistant strains was the replacement of the unsaturated PG (*m/z* 706) by a saturated PG (*m/z* 722) as the major PG (Fig. 5B). This result indicated that the precursor PG species might have been channeled into an alternative reaction in which an extra methyl group was added, because the desaturation reaction was inhibited.

ESMS profiles (Fig. 5C and D) and percent change (Fig. 6A) of the intermediate-resistance strains show the significant de-

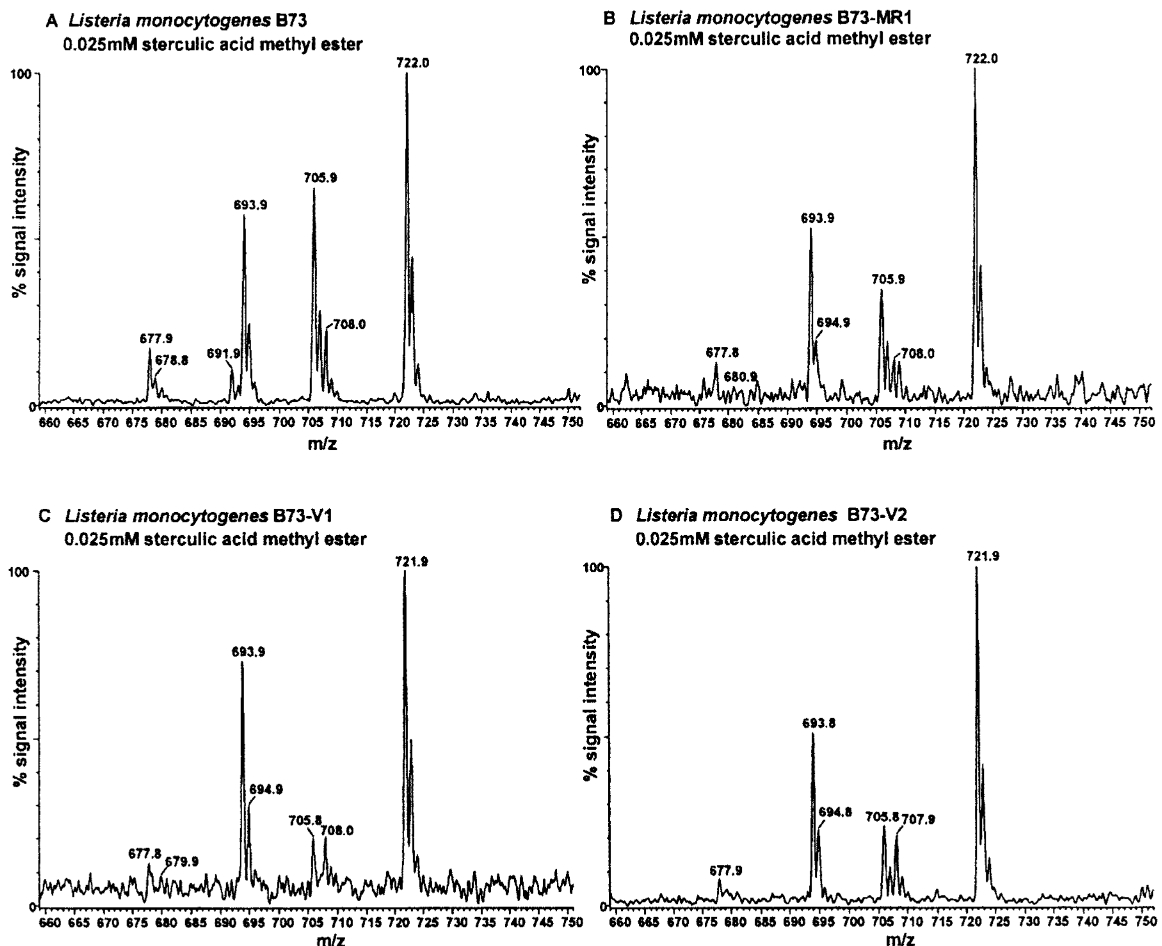

FIG. 5. ESMS spectrum of the PG region of *L. monocytogenes* B73, B73-V1, B73-V2, and B73-MR1 after treatment with SME. The spectral data for *L. monocytogenes* B73 are similar to those for *L. monocytogenes* 412, and the data for *L. monocytogenes* B73-MR1 are similar to those for *L. monocytogenes* 412P.

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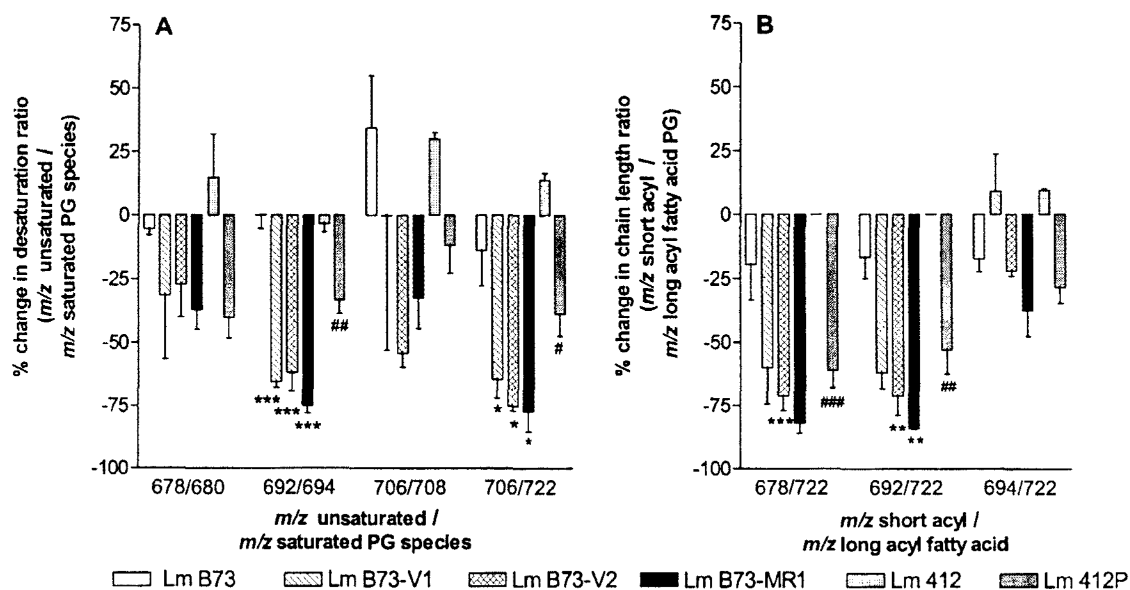

FIG. 6. Percent change in the ratio of unsaturated to saturated PGs (A) and in the ratio of short-acyl-chain to long-acyl-chain PGs (B) after treatment with 0.025 mM SME in *L. monocytogenes* strains. The means and standard deviations from for independent experiments are given. Comparison between *L. monocytogenes* B73 and its resistant strains: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Comparison between *L. monocytogenes* 412 and the resistant *L. monocytogenes* 412P: #, $P < 0.05$; ##, $P < 0.01$.

creases in the levels of two unsaturated PGs species, namely, the *m/z* 706 ($P < 0.05$) and *m/z* 692 ($P < 0.001$) species.

The percent decrease for all the *L. monocytogenes* B73 resistant strains was greater than 50% for the unsaturated PG species with *m/z* 692 and *m/z* 706. We found no significant difference between the intermediate-resistance and highly resistant strains in the percent change of unsaturated/saturated PG ratios. *L. monocytogenes* B73 showed the opposite effect, namely, an increase in the unsaturated PG/saturated PG ([*m/z* 706]/[*m/z* 708]) ratio and very small decreases in the rest of the unsaturated/saturated PG ratios analyzed (Fig. 6A). The sensitive *L. monocytogenes* 412 strain also showed the opposite effect to that observed for *L. monocytogenes* 412P, namely, significant ($P < 0.05$) increases in several unsaturated PG/saturated PG ratios ([*m/z* 706]/[*m/z* 708], [*m/z* 678]/[*m/z* 680], and [*m/z* 706]/[*m/z* 722]). After multiple assessments, inconclusive data for the PG ratio [*m/z* 706]/[*m/z* 708] in *L. monocytogenes* B73-V1 were found (error bar in Fig. 6A).

SME also had an influence on the length of the esterified fatty acids in the PG population. The intermediate-resistance strain, *L. monocytogenes* B73-V1, showed a possible increase in the short-acyl/long-acyl fatty acid PG ratio ([*m/z* 694]/[*m/z* 722]) after treatment with SME (Fig. 6B). Again, the highly resistant *L. monocytogenes* B73-MR1 displayed the largest decreases in the short-acyl/long-acyl chain PG ratios (Fig. 6B). *L. monocytogenes* B73 showed less than a 25% decrease for all the short-acyl/long-acyl chain PG ratios determined. The resistant strains showed a greater than 50% decrease in the ratios of [*m/z* 678]/[*m/z* 722] PG and [*m/z* 692]/[*m/z* 722] PG (Fig. 6B). The sensitive *L. monocytogenes* 412 strain showed no change in the [*m/z* 678]/[*m/z* 722] and [*m/z* 692]/[*m/z* 722] ratios and an increase in the [*m/z* 694]/[*m/z* 722] ratio, with *L. monocytogenes* 412P showing decreases in the same ratios.

DISCUSSION

This study showed that there are significant differences in the PG content of class IIa bacteriocin-resistant *L. monocytogenes* strains compared to the wild type. Differences in the PG composition of the resistant *L. monocytogenes* cell membrane include an increase in the concentration of unsaturated fatty acyl chains of PG, as well as an increase in the concentration of short acyl chains of PG, for all the leucocin-resistant strains. Both of these phospholipid adaptations should result in an increase in membrane fluidity. In contrast to our findings that resistant strains have larger amounts of desaturated and shorter PGs in their membranes (indicating more fluid membranes), it has been shown that nisin-resistant *L. monocytogenes* has a more rigid membrane (29, 30, 31). Verheul et al. (35), however, observed no differences in fatty acid content between nisin-resistant and -sensitive *Listeria* strains. Chen et al. (13) also reported that the saturation state of the PG acyl chains in vesicles had little effect on the binding affinity of pediocin PA-1 for the vesicle. However, their fluorescence results indicated that the penetration of the bacteriocin into a bilayer of the saturated dimyristoyl-phosphatidylglycerol was deeper than into a bilayer of unsaturated DOPG (13). The DOPG is therefore thought to be less favorable for efficient membrane permeabilization due to its higher fluidity. A weaker insertion ability of class IIa bacteriocins into unsaturated PG could point to the role of increased amounts of unsaturated PGs in the membranes of resistant strains. In contrast, the larger amounts of longer and saturated PGs in the sensitive *L. monocytogenes* B73 and 412 strains may enhance membrane insertion by bacteriocin and thus increase sensitivity. A less fluid membrane has also been shown to be a factor influencing the resistance of *Staphylococcus aureus* to the cat-

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ionic antimicrobial peptide thrombin-induced platelet microbicidal protein (3). Similar to our findings, it has been observed that *Kluyveromyces lactis* mutant cells with reduced amphotericin B sensitivity have a higher unsaturated fatty acid/saturated fatty acid ratio than do wild-type *K. lactis* cells (38). It is also known that the increased levels of monounsaturated fatty acids in the membrane phospholipid influence the overall decrease in the membrane molecular order (38). These findings indicate the significant roles played by unsaturated phospholipids and membrane fluidity in antibiotic or antimicrobial peptide association with membranes and consequently in resistance mechanisms. Membrane fluidity could be an important contributing factor to class IIa bacteriocin resistance by affecting the insertion of these bacteriocins into the membrane and consequently the formation and stability of pores. Any increase in membrane fluidity could decrease class IIa bacteriocin insertion into the phospholipid membrane and pore or permeability complex stability and could therefore contribute to resistance.

By treating strains with SME, a putative inhibitor of *Listeria* desaturase, we were able to determine a possible correlation between unsaturated PGs and resistance of *L. monocytogenes* to class IIa bacteriocins. Desaturase enzymes are responsible for the production of unsaturated acyl chains in phospholipids. SME has reportedly been used as a desaturase inhibitor in prokaryotes (36). In our study we saw a marked influence of the inhibitor on the PG phospholipid composition of *L. monocytogenes*. The levels of the major unsaturated PG molecular species, *m/z* 692 and *m/z* 706, were decreased in the resistant strains. The percent decrease of unsaturated/saturated PG ratios in the highly resistant strains was greater than in the intermediate-resistance strains and significantly different from that in the sensitive strains. Sensitive strains showed small decreases and even increases in unsaturated/saturated PG ratios after addition of the desaturase inhibitor.

Our results indicated that the resistant *L. monocytogenes* probably contains an SME-sensitive desaturase while the sensitive strains probably contain a less responsive desaturase. The only bacterial desaturase (except for those in cyanobacteria) described to date has been the *Bacillus subtilis* $\Delta 5$ desaturase (1). No significant homologues to the *Bacillus* desaturase were found after scanning the *L. monocytogenes* genome (http://genolist.pasteur.fr/Listilist/index.html.). A two-component signal transduction system for this single desaturase in *B. subtilis* was also described recently (2). The environment finely controls this two-component signal transduction system. Unsaturated fatty acids reportedly act as negative signaling molecules of *des* (desaturase gene) transcription (2). A similar system could exist for the control of the desaturase activity in *L. monocytogenes*.

One of the strains, *L. monocytogenes* B73-VI, displaying intermediate resistance, showed an anomalous response to SME. Rather than the expected increase in leucocin A sensitivity, it became 2.5 times more resistant after treatment. The PG profile of this strain also showed some anomalies after treatment; for example, we found both increases and decreases in the levels of the major unsaturated PG (*m/z* 706) and a possible increase in the level of the major short-acyl-chain PG (*m/z* 694) in different culture batches of this strain. This could mean that the mechanism involved in membrane adaptation of this strain is highly sensitive to extremely small changes in its lipid metabolism and/or culture conditions. It is also possible that some undetected membrane adaptation, occurring after SME treatment, may be important in the increased resistance of *L. monocytogenes* B73-V1. *L. monocytogenes* B73-V2, however, showed the expected increase ($\pm 50\%$) in leucocin A sensitivity with SME treatment, which coincided with decreases in the levels of unsaturated and short-acyl-chain PGs. This may indicate that both the levels of unsaturated PGs and short-acyl-chain PGs and therefore the activity of a desaturase and fatty acid elongation and branching may influence the resistance of this particular strain.

We did not observe a decrease in resistance after addition of the desaturase inhibitor to the highly resistant *L. monocytogenes* B73-MR1 strain. However, analysis of the PG profiles of *L. monocytogenes* B73-MR1 showed no significant differences from those of the intermediate-resistance *L. monocytogenes* B73-V2 strain after inhibitor addition. It is apparent from this result that an additional factor(s), besides increases in the levels of short-acyl-chain and unsaturated PGs, also contributes to resistance in the highly resistant cells.

In summary, our findings indicate that there is an association between increased amounts of unsaturated and short-acyl-chain PGs in cell membranes and resistance to class IIa bacteriocins. Moreover, the PG composition may be regulated differently, as seen for the differing effects of the desaturase inhibitor on the sensitive and resistant strains. The resistance of the intermediate-resistance strains could be modulated by changing the PG composition of their membranes by treatment with SME. However, we observed no changes in the resistance of highly resistant strains after the same treatment.

Membrane adaptation is probably only one of several mechanisms involved in resistance, and our present results show clearly that other mechanisms are necessary for the development of complete resistance. For example, the absence of a putative mannose-specific PTS enzyme IIAB component under σ^{54} control has been noted in *L. monocytogenes* B73-MR1 (32) and *L. monocytogenes* 412P (M. Ramnath and A. Gravesen, personal communication). The absence of this membrane-bound enzyme may be associated with resistance, but it may also influence membrane fluidity and lipid ordering. Further consideration should also be given to the role of desaturase(s) and the influence of membrane composition on bacteriocin resistance.

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Chapter 4

The interaction of leucocin A with phospholipid liposomes from wild-type and class IIa bacteriocin resistant Listeria monocytogenes strains

Abstract

We have used Fourier transform infrared (FTIR) spectroscopy and circular dichroism (CD) spectroscopy to characterise the functional structure of the class IIa bacteriocin, leucocin A and to determine the role of lipid composition on leucocin A bioactivity. Phospholipid was extracted from both *Listeria monocytogenes* wild-type and class IIa bacteriocin resistant strains. FTIR of phospholipid extracts indicated a more disordered hydrocarbon acyl chain conformation, and thus greater fluidity for all the resistant strain phospholipids, as observed by the 1 cm^{-1} increase in the phase transitional sensitive antisymmetric CH_2 stretching vibrational frequency of class IIa bacteriocin resistant lipids. A shift from 2921 cm^{-1} to 2922 cm^{-1} of the antisymmetric CH_2 stretching vibrational frequency, was also observed in wild-type lipids only, on addition of leucocin A, indicating insertion of leucocin A into the phospholipid. With CD it was observed that leucocin A has an unordered structure in aqueous buffer and 13 % α-helix, and 31 % β-sheet in 50 % trifluoroethanol. A more defined structure of leucocin A, indicated by 21 % α-helix and approximately 27 % β-sheet conformation, was induced in liposomes from membrane lipids of sensitive *L. monocytogenes* B73 and liposomes from synthetic dioleoyl-phosphatidylglycerol. The leucocin A in liposomes from membrane lipids of resistant *L. monocytogenes* B73-MR1 showed a lower 18 % α-helical and greater random coil content. From these results it is clear that the increased α-helical content and more defined structure of leucocin A and the higher hydrocarbon acyl chain ordering may be contributing factors in the greater activity of leucocin A in sensitive cell membranes.

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Introduction

The 37 residue peptide, leucocin A (leuA), isolated from the lactic acid bacterium, *Leuconostoc gelidum*, has been characterised as a class IIa bacteriocin, on the basis of its N-terminal YGNGV motif and antilisterial bioactivity [10, 17].

The proposed active structure of leuA in membrane mimicking environments (Refer to Fig. 1 for predicted tertiary structure) suggests that the class IIa bacteriocins have a three-stranded antiparallel β-sheet at the N-terminus, which is supported by a disulphide bridge (residues 9 and 14), followed by a central amphipathic α-helix (starting at residue 17 or 18 to 31), and an unordered C-terminal end [4, 14, 15]. CD analysis of the structure of closely related class IIa bacteriocins, mesentericin Y105 and pediocin AcH, in lipophilic environments indicated 30 to 40 %, and 32 % α-helicity, respectively [14, 29]. Features of bacteriocins include: presence of amphiphilic segments, which may form putative transmembrane helices; and water solubility plus membrane-binding ability suggestive of a potential to form pore complexes [10].

The specific mode of action of class IIa bacteriocins has not been elucidated. However, there are general features of the basic mechanism that have been described. Research showing the need for a chiral receptor-type molecule [6, 30] and elucidation of a possible receptor-type molecule [18, 8, 16, 25] in class IIa bacteriocin mechanism of action is juxtaposed by evidence showing pore formation in liposomal environments without a receptor requirement [4, 20]. The two cysteines in the N-terminus are thought to stabilise the β-sheets in a β-hairpin conformation at the YGNGV motif [4, 10]. This YGNGV sequence was thought to be involved in initial electrostatic interaction with the anionic phospholipid head groups of the cell membrane [4, 5, 10] or interaction with a receptor [14]. Following binding, the hydrophobic C-terminal segment, which is thought to be the cell-specificity determining region, interacts hydrophobically with the lipid acyl chains of the membrane [12, 11]. Pore formation for class IIa bacteriocins has been suggested to follow the 'barrel-stave' model [10, 22, 7] for action of cationic antimicrobial peptides. This model describes production of an aqueous pore through the formation of transmembrane channels from bundles of amphipathic α-helices, such that their

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hydrophobic regions interact with the lipid core of the membrane and their hydrophilic surfaces are directed inwards [22, 7].

In light of our recent findings regarding differences in the phospholipid composition of wild-type and class IIa resistant variant listerial cells ([28], refer to Chapter 3) and its impact on alteration of sensitivity of class IIa resistant strains, we compared the interaction of wild-type and class IIa resistant *L. monocytogenes* cell membranes with leuA using CD and FTIR. This study was done in order to complement our study on the membrane changes previously observed in class IIa bacteriocin resistant cells ([28], Chapter 3).

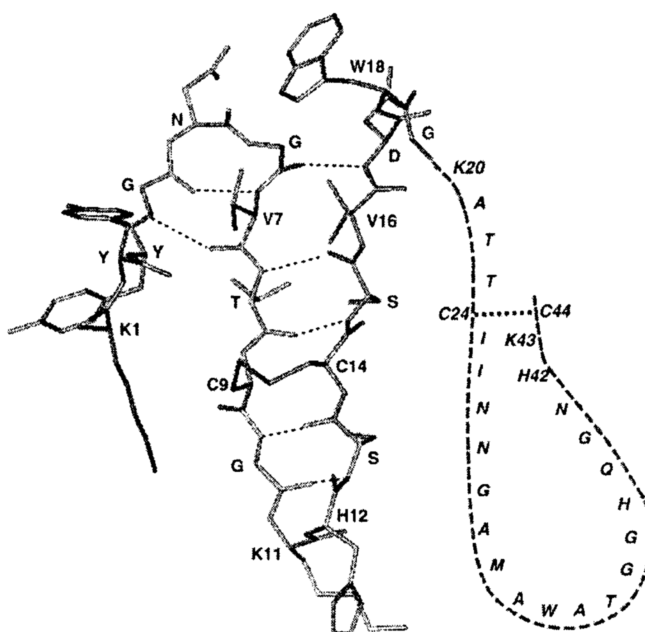

Fig. 1. N-terminal 19 residue predicted tertiary structure of pediocin PA-1. No structure is shown for the C-terminus, except for the C24-C44 disulphide bridge that is required for activity (after Chen et al. [4]).

MATERIALS AND METHODS

Strains and culture conditions

A list of strains used in this study appears in Table 1 of Chapter 2. All wild-type and spontaneous mutant *L. monocytogenes* strains were grown in brain heart infusion (BHI) broth (Biolab) at 37 °C. The *mptA* insertional mutant of *L. monocytogenes*

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EGDe, called *L. monocytogenes* EGK54, was supplemented with erythromycin (Sigma Chemical Co.; South Africa) at 5 μg ml^{-1}.

Phospholipid isolation and quantification of organic phosphorus

Phospholipid was isolated according to the method described by Bligh and Dyer [3] as previously modified by Vadyvaloo et al. [28]. The chloroform fraction containing the phospholipid was dried using vacuum rotary evaporation. Organic phosphate from the lipids was quantified using the method described by Ames [1]. Phosphorus contained in the phospholipid, forms a phosphomolybdate complex with ammonium phosphomolybdate in 1N H_2SO_4, after ashing under a direct flame. The phosphomolybdate complex is reduced by ascorbic acid to yield a blue colour complex with absorbance maximum at 820 nm.

Fourier Transform Infrared (FTIR) Spectroscopy

For the lipid isolations for each *L. monocytogenes* strain (B73, 412, and EGDe families, see Table 1 in Chapter 2) 5 mg lipid was resuspended in approximately 120 μl deuterium oxide (D_2O) (Aldrich Chemical Co., Milwaukee, Wi, USA), to give an approximate final concentration of 5 mg ml^{-1} lipid/D_2O. This mixture was vortexed for 1 minute and divided equally with leuA being added to a final concentration of 0.1 mg ml^{-1} into one half of the preparation. Both samples were then incubated at 37 °C for 1 hour. A volume of 40 μl sample was placed on CaF_2 windows separated by a 25 μm teflon spacer, placed in the chamber and equilibrated for 2 minutes and then subjected to 264 infrared (IR) scans. FTIR spectra were obtained on a Shimadzu FTIR 8900 spectroscope (Shimadzu Laboratory Instruments, Japan). The FTIR spectroscope Hyper-IR software (Shimadzu Laboratory Instruments, Japan) for IR data analyses was used to calculate difference spectra and identify peaks.

Preparation of crude phospholipid liposomes

Liposomes were prepared by dissolving approximately 5 mg of phospholipid (dioleoyl-phosphatidylglycerol (DOPG), *L. monocytogenes* B73 or *L. monocytogenes* B73-MR1 lipid extracts) in 2 ml chloroform in a detergent free glass test tube. The chloroform was evaporated using a rotary evaporator to leave a thin film of lipid on the glass. The lipid was resuspended in 2-5 ml 5 mM Na-MES buffer, pH 6.5, at room temperature (25 °C), and sonicated in a sonic water bath until a clear solution was obtained. The lipid was quantified as described above and used for CD analysis.

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Circular Dichroism (CD) Spectroscopy

Synthetic leuA was added to a final concentration of 24 μM to 50 % trifluoroethanol (TFE), 5 mM Na-MES pH 6.5, and 240 μM of DOPG, *L. monocytogenes* B73 or *L. monocytogenes* B73-MR1 liposomes. CD spectra were recorded between 180 nm to 260 nm for leuA in TFE, 5 mM Na-MES and DOPG liposomes; and from 200 nm to 260 nm for the listerial liposomes, in keeping with the requirement for a scanning voltage of 600 V or below. This allowed more accurate peptide conformation assessment. Analysis was done at room temperature (25 ^{o}C) in a quartz cell (path length of 1 mm), on a J-810 spectropolarimeter (Jasco, Tokyo, Japan). Data was digitally collected every 0.2 nm at a scanning speed of 100 nm min^{-1}. All spectra presented here are the average of 10 scans. Results are expressed in molar ellipticity (θ) and the Na-MES buffer background was subtracted from the samples. Secondary structure predictions of leuA were carried out using the web-based program (K2D) for analysis of CD data (http://www.embl-heidelberg.de/~andrade/k2d/).

RESULTS AND DISCUSSION

FTIR of listerial lipids in the presence and absence of leuA

We observed in all the lipid extracts the major bands associated with lipids, namely the antisymmetric CH_2 and symmetric CH_2 stretching vibrations located at frequencies of approximately 2921 cm^{-1} and 2851 cm^{-1}, respectively (Fig. 2). The C-CH_3 antisymmetric stretching vibration at 2962 cm^{-1} was also observed for all the listerial lipids. These vibrations are used to detect lipid hydrocarbon chain-melting phase transitions, which are determined by increases in absorption maxima of the vibrational mode and reflects increases in hydrocarbon chain conformational disorder and mobility [26, 23].

The lipids from wild-type strains of all three families had a lower antisymmetric CH_2 stretching vibration frequency, indicating a more ordered conformation of the hydrocarbon acyl chains, and a higher phase transition temperature. An increase in wave number of 1 cm^{-1} for the antisymmetric CH_2 stretching vibration from 2921 cm^{-1} to 2922 cm^{-1} was, however, consistently observed in the class IIa resistant listerial phospholipids (Fig. 2). This is in contrast to the wild-type strains and indicates more disordered hydrocarbon acyl chains for the class IIa resistant *L. monocytogenes* lipids, and lower phase transition temperature. A more ordered conformation of the lipid

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hydrocarbon acyl chains is suggestive of a more rigid or less fluid membrane, and the disordering of the lipid hydrocarbon acyl chains suggests increased fluidity and mobility of the phospholipid membrane at the same temperature. Double bonds in the acyl chains would result in lower melting temperatures, and higher fluidity than in saturated analogues and thus more disorder. These results correlated well with our previous findings that showed increases in unsaturated and short-acyl-chain lipids suggesting greater fluidity, in resistant *L. monocytogenes* phospholipid membranes, in a comparative study on wild-type and class IIa resistant *L. monocytogenes* phospholipid membranes ([28], see Chapter 3).

A shift in the antisymmetric CH_2 stretching vibration frequency from 2921 cm^{-1} to 2922 cm^{-1}, upon addition of leuA, indicated insertion of the bacteriocin into the phospholipid core of wild-type *L. monocytogenes* lipids (Fig. 2). This suggested disruption of the hydrocarbon acyl chain order by leuA, as a consequence of the interaction of leuA with deeper layers of the phospholipid membrane. Similarly, a 0.5 cm^{-1} vibrational frequency change of the antisymmetric acyl chain CH_2 stretching was observed in 1, 2 dipalmitoyl-phosphatidylserine monolayers upon interaction with the cationic antimicrobial peptide, mellitin [13]. Resistant strains, however, did not show any change of the antisymmetric CH_2 stretching vibration after leuA addition. It is not clear whether insertion of leuA occurred in the resistant cell phospholipids since they already contained more disordered acyl chains.

Acyl chain order in membranes with decreased fluidity may be more sensitive to insertion of leuA (as found with the liposomes of the wild-type strains) and the formation of possible pores and consequent permeabilisation of the lipid membrane. A fluorescence spectroscopy study investigating the binding of pediocin PA-1, another, class IIa bacteriocin, to DOPG and dimyristoly-phosphatidylglycerol (DMPG) liposomes, showed a larger blue-shift thus greater insertion of the peptide, as measured by tryptophan fluorescence of the peptide, into less fluid DMPG vesicles. A larger blue-shift of the tryptophan fluorescence in the membrane would indicate a deeper translocation to a more hydrophobic, non-polar environment [5].

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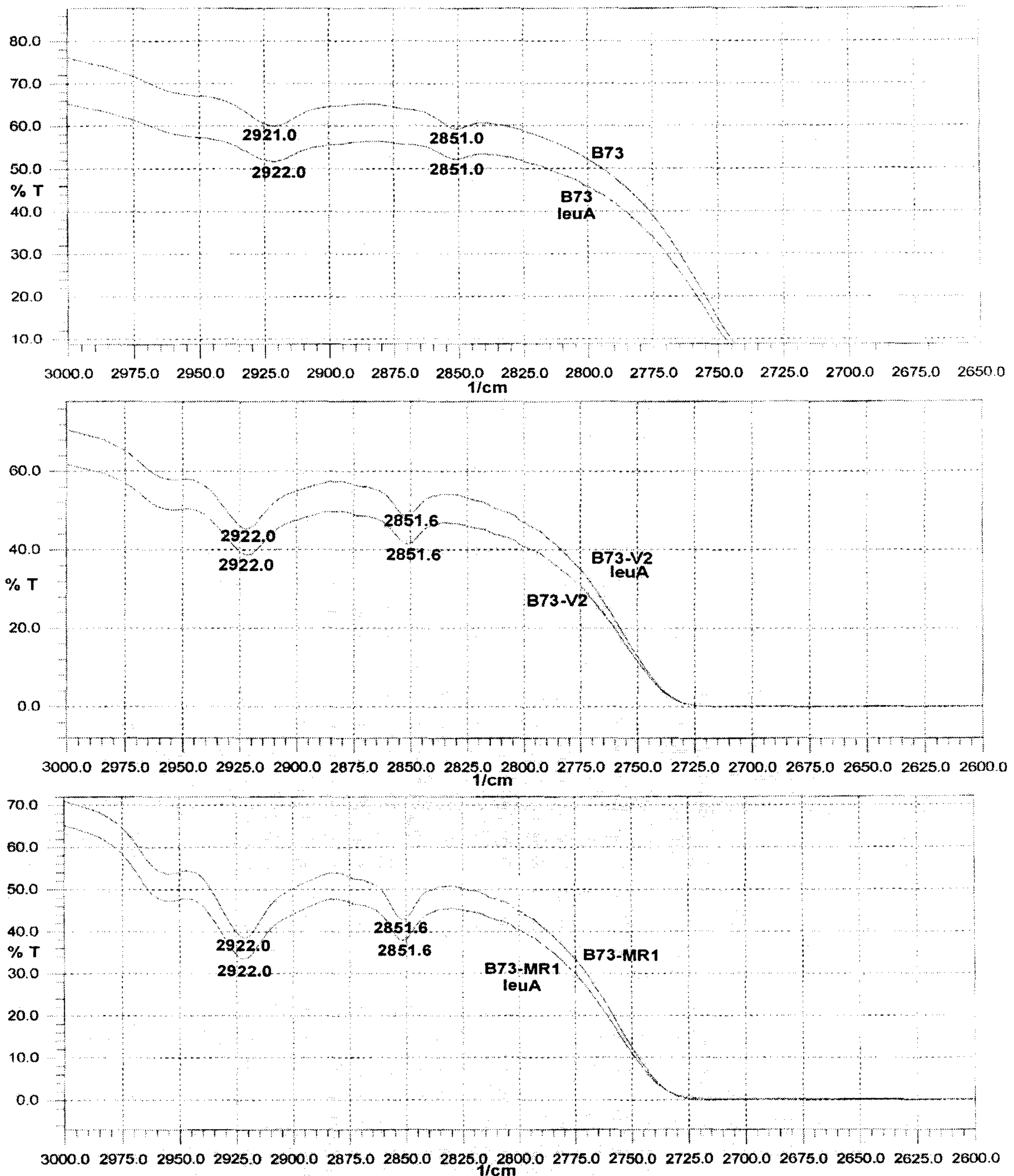

Fig. 2. FTIR transmittance spectra showing the lipid stretching vibration region of *L. monocytogenes* phospholipids. The spectrum of wild-type *L. monocytogenes* B73 is representative of the spectra for wild-type EGDe and 412 strains; while the spectrum of highly-resistant *L. monocytogenes* B73-MR1 represents 412P and EGK54; and the spectrum of intermediate-resistant *L. monocytogenes* B73-V2 is representative of B73-V1.

This therefore indicated a greater hydrophobic environment of the DMPG for pediocin PA-1 insertion although both lipid environments had comparable affinities

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for the bacteriocin [5]. Similarly, the less fluid wild-type membranes could be a more suitable environment for interaction of leuA.

CD of leuA in liposomes from wild-type *L. monocytogenes* B73 and resistant *L. monocytogenes* B73-MR1

CD was performed to obtain conformational data on leuA in the presence of the secondary structure promoting solvent TFE, aqueous buffer, and phospholipid liposomes derived from both *L. monocytogenes* sensitive (B73) and resistant (B73-MR1) strains, and synthetic DOPG lipid.

A strong negative Cotton effect at 200 nm characterised the structure of leuA in Na-MES aqueous buffer, and indicated a significant, unordered structure of the peptide (Table 1; Fig. 3). However, a high percentage (48%) of the peptide was in β-sheet conformation characterised by 214 nm, and had a 203 nm minimum, which could also indicate β-turn, while there was almost no α-helical conformation. In the hydrophobic environments, such as the TFE and liposomes, there was an expected transition to an α-helix conformation, probably from random structures, and the β-sheet conformation was mostly retained (Table 1). The observation that leuA displayed mostly β-sheet conformation regardless of the environment (Table 1) correlated well with previous observations [15, 24], on the predicted tertiary structure estimations of leuA, using CD and NMR. This is an indication of β-sheet structure being the most stable conformation of leuA in any medium. Our results also confirm a previous observation that a combined α-helix/β-sheet conformation is a characteristic of class IIa bacteriocins in a membrane-mimicking environment [29].

The antiparallel β-sheet minimum at 214 nm [27] and broader minimum of an unknown structure between 217-218 nm is also a common signature displayed by leuA in all environments (Table 1). The most significant difference between the CD signature of leuA in B73 and B73-MR1 liposomes is the absence of the minimum for an α-helix at 222 nm [19] for B73-MR1, which may be obscured by the higher β-sheet and random coil content in the resistant liposomes. This is corroborated by the K2D programs prediction of a 21 % α-helix and 25 % β-sheet in the structure of leuA in wild-type lipids as compared to 18 % α-helix and 27 % β-sheet in the resistant lipids (Table 1).

It is also clear from the large minima at 203.2 nm, which may also be indicative of β-turn [27], and 213.4 nm minima, indicative of an antiparallel β-sheet [27], that these type of conformations are also present in the leuA structure induced by resistant B73-MR1 phospholipids. There is also slightly greater β-sheet conformation retention from aqueous structure in resistant lipids as compared to sensitive lipids (Table 1). In general the induced structures of leuA in TFE and B73-MR1 liposomes, showed great similarity, and this was observed by a common minimum at around 206 nm, lower α-helix, greater β-sheet, and greater random coil structures predicted for them (Table 1).

Table 1 Percentage estimations and minima of the secondary structure of leucocin A in 50 % TFE, 5 mM Na-Mes aqueous buffer, and 5 mM Na-Mes containing 240 μM liposomes, as determined by CD.

STRUCTURES	SAMPLES				
	Na-Mes buffer	**TFE**	**DOPG**	**Lm B73**	**Lm B73-MR1**
% α helix	**0.8**	**13**	**21**	**21**	**18**
nm min (θ)	207 (0.26)	205.8 (-2.98)	222 (-4.22); 220.2 (-4.30) 207.6 (-5.84)	221.8 (-5.83) 207.4 (-8.87)	208.4 (-9.31) 206 (-9.96)
% β-sheet	**48**	**31**	**28**	**25**	**27**
nm min (θ)	204 (-0.75) 216.4 (0.22)	214.4 (-1.17)	214 (-4.50)	214.4 (-7.86)	213.4 (-8.04)
% Random coil	**44**	**56**	**52**	**53**	**55**
nm min (θ)	200.6 (-0.63)	199.6 (-5.18) 201.8 (-4.5)*	ND	203.6 (-5.49)*	203.2 (-8.66)*
unknown structures nm min (θ)	212.4 (0.28)	218.8 (-0.61) 217 (-0.68)	218 (-4.67)	217.8 (-6.26)	218 (-7.11)

N.B. The average of duplicate values for the determinations of secondary structure for the listerial liposomes is shown.
The K2D tertiary structure prediction program only estimated percentages of α helix, β-sheet and random coil.
* could also indicate β-turn structure (Urry, 1985 [27])
ND, not determined

Alternately, DOPG and B73 liposomes induced a similar type of signature and percentage defined structure of leuA.

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CONCLUSIONS

The CD results may indicate that the greater α-helical conformation of leuA in lipids of sensitive strains allows better insertion of leuA, which correlates to the FTIR

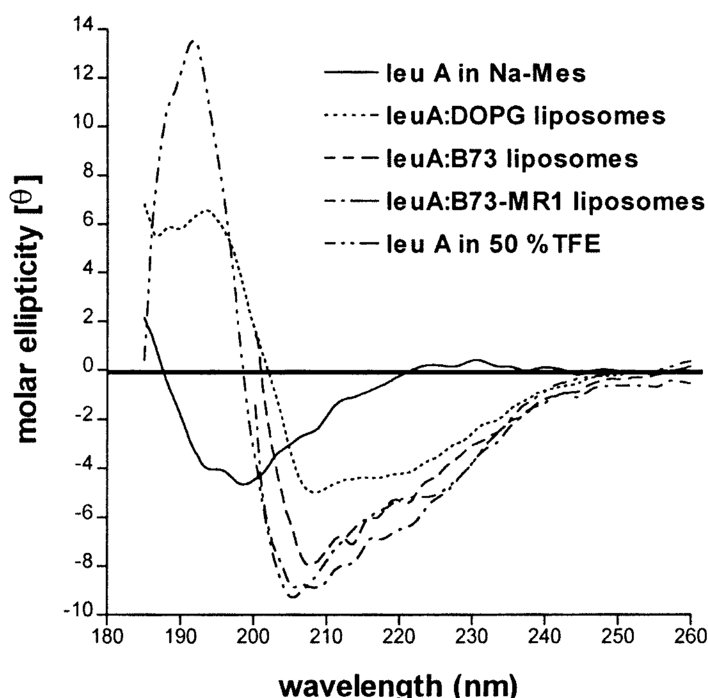

Fig. 3. CD spectra of leucocin A in 50 % TFE, 5 mM Na-Mes aqueous buffer, and 5 mM Na-Mes containing 240 μM liposomes. The experiments have been performed in duplicate for the CD of leucocin A in the presence of *L. monocytogenes* lipid.

results showing disordering of the hydrocarbon acyl chains upon leuA addition. It has been suggested that the central helix of piscicocins V1a and V1b anchors the class IIa bacteriocin to the membrane surface [2], and several reports have indicated accountability of the helix in the mode of action by formation of pores for class IIa bacteriocins [4, 15, 12]. The hydrophobic and amphipathic properties and correct spatial arrangements of key amino acid side chains of the α-helix would be determining factors in cell specificity and potency of the bacteriocin molecule [10]. It is possible that the electrostatic interaction between leuA and the phospholipid membrane is also affected considering the increased lysine positive charge content observed for B73-MR1 lipids (refer to Chapter 5). This would weaken association of leuA and the resistant membrane and affect proper α-helix induction. The net negative

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charge of the phospholipid bilayer was suggested to be important for the induction of bioactive structure in class IIa bacteriocins [21].

It should also be made clear that the point of the K2D program analysis in this work is not to obtain accurate values for secondary structure predications, but rather to generate information on trends in the secondary structure in different environments. It is also difficult to get accurate data especially since the secondary structure predictions programs use model proteins (not peptides) to determine secondary structure. The inherent problems in these types of analyses include blocking of the solvent by bulky side chains, thus preventing interaction with the polypeptide backbone responsible for absorption in the far-UV. In summary, our results indicate that defined secondary structure of leuA is best induced in the sensitive target cell phospholipid environment, and that α-helical conformation is an important tertiary structure for activity in sensitive membranes. Factors like membrane fluidity and phospholipid composition are important for induction of bioactive structure and for mediation of bacteriocin insertion and pore formation. Our results suggest that the sensitive cell membranes contain lipids that are more likely to enhance bacteriocin bioactive structure and insertion into the membrane. Recently a permease molecule, MptD that resides in the phospholipid membrane of *L. monocytogenes* was speculated to be the target, docking molecule for class IIa bacteriocins [8, 16, 18]. How the membrane phospholipid structure and lipid acyl chain ordering, which would affect internal lateral packing forces and thus functionality of a protein receptor [9], in wild-type and resistant *L. monocytogenes* strains would therefore also be significant in affecting interaction of the bacteriocin with the receptor.

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Chapter 5

Cell surface changes and mannose PTS EIIAB are associated with intermediate class IIa bacteriocin resistance

A modified version of this chapter has been submitted to the journal, Applied and Environmental Microbiology.

Abstract

Strains of *Listeria monocytogenes* showing either intermediate or high level resistance to class IIa bacteriocins were investigated to determine characteristics that correlated with their sensitivity levels. Characterising intermediate resistance was a primary focus particularly to determine whether the 'one general mechanism' associated with the mannose PTS *mptA* ($EIIAB^{Man}$ component) gene (Gravesen et al. [15]) was implicated in all levels of resistance and/or whether there were other contributing factors, including changes in cell surface properties. This complemented a previous study where cell membrane changes were investigated (Vadyvaloo et al. [30]). Two intermediate and one highly resistant spontaneous mutant of *L. monocytogenes* B73, a highly resistant mutant of *L. monocytogenes* 412, and a highly resistant, defined (*mptA*) mutant of *L. monocytogenes* EGDe, were compared with their respective wild-type strains. The alanine:phosphorus ratios of teichoic acid showed significant increases ($P<0.05$) in alanine in all resistant strains, especially for the intermediate resistant strains. There was a tendency towards slightly increased lysinylation of membrane phospholipid in highly resistant strains only. D-cycloserine inhibition effects were varied in the resistant strains with the most rapid inhibition seen in the *mptA* mutant. Real time PCR of the *dltA* (D-alanine esterification), *lmo1695* (putative membrane phospholipid lysinylation), and *dal* and *ddlA* genes (peptidoglycan biosynthesis) showed no change in transcriptional levels. Decreases in *mptA* transcriptional levels correlated well with the levels of resistance, and provided evidence for MptA down-regulation in intermediate class IIa resistance. The contribution of several mechanisms to intermediate resistance is discussed.

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Introduction

Class IIa bacteriocins are a homologous subgroup of bacterial antimicrobial peptides that display strong antilisterial activity [10, 18]. Most, class IIa bacteriocins known are produced by food-associated lactic acid bacteria [10], and the compounds are seen as potentially useful for food preservation because of their antilisterial activity.

Reports of resistance development by listerial strains against class IIa bacteriocins in laboratory systems has implied that increased resistance may compromise the potential role of these antimicrobial compounds in biopreservation. This has in turn spurred interest into understanding resistance phenomenon displayed by the pathogen. There is cross-resistance between different class IIa bacteriocins, indicating common or similar resistance mechanisms in *L. monocytogenes* [15, 30]. A prevalent mechanism involving the loss of the enzyme IIAB subunit of a mannose-specific phosphotransferase system ($EIIAB^{Man}$ PTS, encoded by *mptA*) was recently described [27, 8, 15]. An up-regulation of two β-glucoside specific PTS genes, possibly associated with the absent $EIIAB^{Man}$, was also demonstrated [16, 15]

Studies on structure and activity of class IIa bacteriocins suggest that the cationic nature of the peptides enables interactions with negatively charged cell surfaces, while the hydrophobic region of the peptide induces membrane permeabilisation [6, 10]. Modification of the bacterial surface charge could, therefore, be expected to affect the initial electrostatic interaction between peptide and the membrane that is required for pore formation, with or without mediation by a membrane-bound receptor-type molecule [10, 8]. Two important options for modulating the charge of the cell envelope are D-alanylation of teichoic acid (TA) and lipo-teichoic acid (LTA) in the cell wall and lysinylation of the cell membrane phospholipids. Previous reports have indicated a role of D-alanine esterified TA and LTA in conferring sensitivity to cationic peptides and other cationic antimicrobial compounds in various Gram-positive pathogenic bacteria, including induced nisin sensitivity in D-alanine deficient LTA of *L. monocytogenes* [23, 13, 21, 28, 1]. The description of lysine-deficient phospholipid due to insertional inactivation of the *mprF* (multiple peptide resistance factor) gene in *S. aureus,* was the first study indicating an influential role of charge modification of membrane phospholipids in resistance to antimicrobial peptides [24]. The lysinylated phospholipids, L-lysyl-phosphatidylglycerol and L-lysyl-cardiolipin

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are among the four major phospholipids of the *Listeria* spp. [12], and it is conceivable that these phospholipids could display differences in lysinylation in the different strains. Changes of the cell surface charge by D-alanyl esterification of TA and lysinylation of membrane phospholipid were determined.

The effect of the cell wall acting antibiotic D-cycloserine (DCS) was also analysed, since the cell wall of nisin-resistant *L. innocua* has been shown to be thickened and more resistant to cell wall acting antibiotics, like DCS [20]. In addition, alanine that is incorporated in TA is also the substrate for DCS enzyme targets [31], *dal* [28] and *ddlA* [26]. The *dal* gene product plays a role in converting L-alanine to D-alanine, which is channelled into TA and cell wall biosynthesis [29]. The *ddlA* gene product catalyses amide-bond formation between two D-alanines, for incorporation into peptidoglycan [26]. Furthermore, the expression of genes that potentially influence cell surface charge (*dltA, lmo1695*) and DCS inhibitory activity (*dal, ddlA*) were also analysed by real-time PCR.

In a previous study, we investigated the compositional changes in the cell membrane associated with intermediate resistant and highly resistant strains. We observed an overall increase in short-acyl and unsaturated phosphatidylglycerol (PG) species for all resistant strains. However, upon treatment with a desaturase inhibitor the intermediate resistant strains responded differently from the highly resistant and wild-type strains by showing changes in their resistance levels [30].

In this study we report the results of a parallel study, which focussed on the intermediate resistant strains and investigating the general alteration in the cell surface charge, gene expression changes associated with this alteration and whether or not the 'one general mechanism' associated with the *mptA* gene was also a critical factor influencing intermediate resistance.

MATERIALS AND METHODS

Bacterial strains and growth conditions

A list of the strains used in this study appears in Table 1. All wild-type and spontaneous mutant *L. monocytogenes* strains were grown in brain heart infusion (BHI) broth (Biolab) at 37 °C. The insertional mutant of *L. monocytogenes* EGDe was supplemented with erythromycin (Sigma Chemical Co.) at 5 μg ml^{-1}.

Table 1. *Listeria monocytogenes* strains

Listeria monocytogenes	**Description**	**IC_{50}[a] (µg ml^{-1})**	**Reference of strain and IC[a] value**
Wild type isolates			
412	Sensitive; isolated from raw salted pork	0.24	Gravesen et al. [16] Vadyvaloo et al. [30]
B73	Sensitive; isolated from meat	0.14	Dykes and Hastings [9] Vadyvaloo et al. [30]
EGDe	Sensitive; clinical	1.95 [b]	Glaser et al. [14] Gravesen et al. [15]
Intermediate spontaneous mutants			
B73-V1	Intermediate resistant mutant† of B73 isolated on leucocin A	0.30	Vadyvaloo et al. [30]
B73-V2	Intermediate resistant mutant† of B73 isolated of leucocin A	0.49	Vadyvaloo et al. [30]
Highly resistant spontaneous mutants			
B73-MR1	Highly resistant* mutant of B73 isolated on leucocin A	> 100	Ramnath et al. [27] Vadyvaloo et al. [30]
412P	Highly resistant* mutant of 412 isolated on pediocin PA-1	>100	Gravesen et al. [16] Vadyvaloo et al.[30]
Defined mutant			
EGK54	Highly resistant; insertional inactivation of *mptA* in EGDe	> 100 [b]	Dalet et al. [8] Gravesen et al. [15]

†, 2-4 fold increase in 50 % inhibitory concentration of class IIa bacteriocin
*, >1000 fold increase in 50 % inhibitory concentration of class IIa bacteriocin
[a], IC_{50} stands for 50 % inhibitory concentration.
[b] The MIC for EGDe and EGK54 are shown.

Isolation of cell wall teichoic acid

The TA was isolated according to the procedure of Webster et al. [32] with modification, as follows: cells in the mid-exponential phase of growth (OD_{600} approx. 0.5) were harvested at 7500 x *g*; the bacterial pellets were washed twice in 0.1 M Na-acetate, pH 5, and resuspended in the same buffer; DNase and RNase were added to the bacterial cell suspension, which was then sonicated in a sonic water bath, cooled with ice; a Gram stain was carried out to verify that the cells had been broken; the

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disrupted cell suspension was centrifuged at 1500 x *g* for 5 min to remove unbroken cells and debris; the supernatant was next added drop-wise into a boiling 8 % SDS solution of the same volume and left to simmer for a further 30 min; once the solution had cooled to room temperature, it was centrifuged at 20 000 x *g* for 15 min at 15 °C; the pellet was washed five times in analytical grade water until SDS was removed, and lyophilised.

Separation of TA from peptidoglycan cell wall was done according to the method described by Kaya et al. [19]. The lyophilised material (0.2 ml mg^{-1} in 25 mM glycine-HCl, pH 2.5) was heated to 100 °C for 10 min. The suspension was cooled to room temperature and centrifuged at 20 000 x *g* at 4 °C for 45 min. The TA extraction from the cell wall was repeated once. The supernatant was ultrafiltrated using a polyethersulfone membrane (Millipore Corporation) with a 10 000 MW limit. The ultrafiltrate or TA suspension was lyophilised and resuspended in analytical grade water.

Phospholipid isolation

Phospholipid was isolated according to the method described by Bligh and Dyer [5] as previously modified by Vadyvaloo et al. [30]. The chloroform fraction containing the phospholipid was dried using vacuum rotary evaporation.

Quantitation of phosphorus

Organic phosphate was quantified using the method described by Ames [2]. Phosphorus present in the ribitol-phosphate backbone of TA, and in the phospholipid, forms a phosphomolybdate complex with ammonium phosphomolybdate in 1N H_2SO_4, after ashing under a direct flame. The phosphomolybdate complex is reduced by ascorbic acid to yield a blue colour complex with absorbance maximum at A_{820}.

Quantitation of alanine and lysine

Alanine and lysine were quantified on a Waters® Breeze high performance liquid chromatograph using the PicoTag® method [3]. Alanine was liberated from TA by base hydrolysis in 4 N NaOH at 112 °C for 24 hours. Lysine was liberated from phospholipid by acid hydrolysis in 6 N HCl containing 0.5 % phenol at 112 °C for 18 hours.

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Cationic cytochrome C binding to whole cells

This method was carried out as described by Peschel et al. [25] with some modifications. Cells were harvested at mid exponential phase (OD_{600} approx. 0.5) and washed twice by centrifugation in 20 mM MOPS buffer, pH 7. The cells were resuspended in the neutral pH MOPS buffer to a final OD_{600} of 0.150 before interaction with the positively charged cytochrome c, to counteract pH influence on charge. Cationic cytochrome c was added to the cells at a concentration of 0.5 mg ml^{-1} and the mixture was incubated at room temperature for 10 min. The solution was centrifuged at 8000 r.p.m. in an Eppendorf benchtop centrifuge for 5 min and unbound cationic cytochrome c in the supernatant was quantified photometrically at A_{530}.

D-cycloserine inhibition assay

A 1 % inoculum of the bacterial culture was added to a tube containing 10 ml of media and growth at OD_{600} was monitored. At mid-exponential phase (OD_{600} approx. 0.5), DCS was added to the culture at approximately 100 µg ml^{-1}. After addition of DCS the OD_{600} was monitored over 10 h for the *L. monocytogenes* B73 and 412 families and 6 h for the *L. monocytogenes* EGDe family. Control cultures were made without antibiotic addition. All data was analysed on Graphpad Prism version 3.0 for Windows (Graphpad software, San Diego, CA). A sigmoidal plot (% relative survival *vs* log time) was constructed to calculate the 50 % survival/inhibition time. The sigmoidal curve of variable slope, and constant top of 100, and bottom of 0 was fitted to each of the data sets. For curve fitting only, the mean value of each data point, without weighting was considered. The time taken to achieve 50 % survival was calculated from the 50 % inhibition value halfway between top and bottom. The intersection between the tangent across the top of each curve and the tangent of the plateau, of a log-log plot, was used to calculate the onset time of DCS inhibition. Curve fitting was carried out as described above, with no top and bottom constants.

cDNA synthesis

Total RNA was isolated from a mid-exponential phase culture (10 ml) using the RNAwiz kit (Ambion), according to the manufacturer's instructions. The extracted RNA was treated with DNase-RNase free (Invitrogen) and its quality was assessed, by running samples on a 1 % formaldehyde agarose gel. RNA was quantified spectrophotometrically. cDNA was synthesised from 2 µg total RNA using random

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hexamers, and the Superscript II Kit (Gibco), according to the manufacturers instructions. A reaction containing all the components but omitting reverse transcriptase was included in order to assess DNA contamination.

Real-time PCR

The primers used for the real-time PCR are listed in Table 2. They were designed using Primer Express software version 1.0 (Applied Biosystems), from the gene sequences of the *L. monocytogenes* EGDe genome (http//genolist.pasteur.fr/Listilist/). The real-time PCR was carried out using the SYBR Green PCR Core Reagent kit (Applied Biosystems), as recommended. PCR reactions were run on the ABI Prism 7700 sequence detector (Applied Biosystems) under the following conditions: 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles at 95 °C for 10s and 60 °C for 1 min. Each assay included, in triplicate, either a serial two-fold dilution of *L. monocytogenes* genomic DNA, a control without template, or the cDNA from the same sample.

Table 2. List of genes and primers used in the real-time quantitative PCR experiments

Gene	Gene product	Primer name	Primer sequence (5'-3')	Product size (bp)
dltA	D-alanine-D-alanyl carrier protein ligase	DLTAF1	CACAAGATCAGCTAATGGACGC	51
		DLTAR1	CTGGAACTTCTCCGAAATGTTTT	
dal	D-alanine racemase	DALF1	GCCAGCACTTGCGCTCTATAC	51
		DALR1	GGTGCGAGTTCTTTCACATGA	
ddlA	D-alanine-D-alanine ligase	DDLAF1	CCCACTTTTACATGGTCCAAAC	51
		DDLAR1	AACAATCCTTGAACAGTGCCATC	
lmo1695	putative phospholipid lysinylation	1695F1	GGGATTGACTATCCGTCGCTA	51
		1695R1	TTCCGGGCTTTGAGAAGTTAA	
mptA	EIIAB^{Man} of mannose-specific PTS	MPTAF1	CAGGACTTAATTTGCCAATGTTG	110
		MPTAR1	CGCGAACACCTTCTTGGAGCT	
rpoD	sigma 70 subunit of RNA polymerase	RPOD1	ACTGAAAAAGTTCGGGAAATCCT	92
		RPOD2	TCGCCTAGATGTGAATCGTCTTC	

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Data analysis of real-time PCR

Data acquisition and subsequent data analyses were done using the ABI Prism 7700 sequence detector. The analysis gave a threshold cycle (C_T) value for each sample, which is defined as the cycle at which a significant increase in amplification product occurs, for each sample. The C_T values are inversely related to the target cDNA copy number. The mean C_T value was calculated for each triplicate. A ΔC_T value was then calculated, for each sample by subtracting the mean C_T value of the sample in the wild-type strain from the mean C_T value in the mutant strain. Each ΔC_T value was then normalised by subtracting the ΔC_T value of a constitutively expressed reference gene, (*rpo*D gene encoding the sigma 70 subunit of the RNA polymerase) to give the $\Delta\Delta C_T$ value. The $\Delta\Delta C_T$ value represents, for a given gene, the difference of its expression in a mutant strain, compared to the wild-type. The difference could be quantitatively expressed as $2^{\Delta\Delta C_T}$. The expression of each gene was monitored in two independent experiments.

RESULTS

Alanine content of teichoic acid

Incorporation of D-alanine esters into the ribitol-phosphate backbone of TA decreases the anionic nature of TA. The D-alanine content was quantified as the alanine:phosphorus ratio (Fig. 1). All spontaneous resistant mutants had a significantly ($P<0.05$) higher alanine:phosphorous ratio than the corresponding wild-type strain. However, the intermediate resistant mutants showed a slightly greater alanine:phosphorus ratio than the highly resistant mutant, in comparison to the corresponding wild-type B73 strain.

Lysine content of phospholipid membranes

Lysine esters in PG species cause neutralisation of the negative charge of these phospholipids. The results of the phospholipid lysine:phosphorus ratios are presented in Fig. 2. The highly resistant strains had increased ratios of lysine:phosphorus compared to the corresponding wild-type strain, while little or no change was observed for the intermediate strains.

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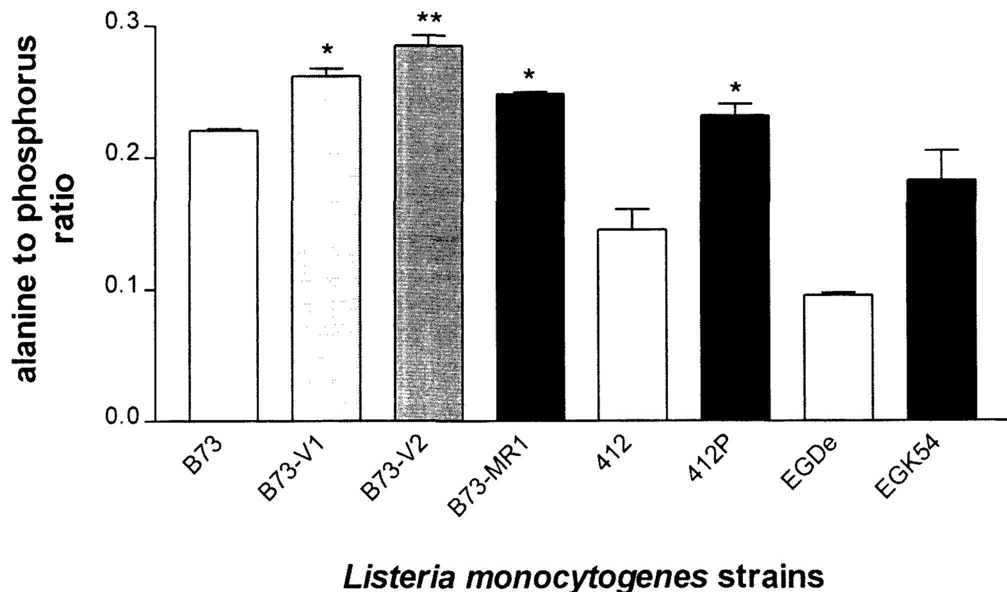

Fig. 1. Alanine to phosphorus ratio in teichoic acid isolated from *Listeria monocytogenes* strains. The data are the averages of duplicate experiments, with error bars representing the standard error for each mean value. Comparison between *L. monocytogenes* wild-type and its corresponding resistant strains: * P<0.05; ** P<0.01.

Cationic cytochrome c binding to whole cells

To determine whether an alteration in the electrostatic nature of the cell surface affects binding of a cationic compound, cationic cytochrome c was added to whole cells. Because cytochrome c has a very characteristic absorption spectrum, the determination of unbound cytochrome c can be done with relative ease and accuracy. The data showing residual cytochrome c in the supernatant of the cells is presented in Fig. 3. The spontaneous highly resistant mutants 412P and B73-MR1, had a significantly greater (P<0.05) than 10 % quantity of unbound cytochrome c in their supernatants, than their corresponding wild-type strains. The EGK54 defined mutant had a significantly (P<0.05) greater than 5 % unbound cytochrome c than its corresponding wild-type. The two intermediate mutants of strain B73 showed a slight increase in unbound cytochrome c, but the differences were, however, not statistically significant.

D-cycloserine inhibition assay

Table 3 and Fig. 4 show that the wild-type strains have different responses to DCS activity. The longest lag time to initiate DCS inhibition was found with all the strains in the B73 family, but followed with a rapid inhibition to 50 % relative survival. The

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highly resistant B73-MR1 showed the greatest lag in DCS inhibition onset and continued to take the longest to be reduced to 50 % relative survival. The two intermediate resistant strains had the shortest DCS inhibition onset, but differing response times to reach 50 % relative inhibition. Overall, the resistant strains of the B73 family, took almost two times longer from inhibition onset to 50 % relative inhibition. Although the 412 family showed a rapid inhibition onset, they displayed the longest lag time to reach 50 % relative inhibition. Here the highly resistant 412P, showed quickest onset of inhibition, and inhibition occurred faster than the wild-type, even when 50 % relative inhibition was reached. The EGK54 mutant was inhibited to 50 % relative survival in a third of the time of the wild-type EGDe, and more rapidly than all the other strains, and was thus the most sensitive to DSC of all the strains tested.

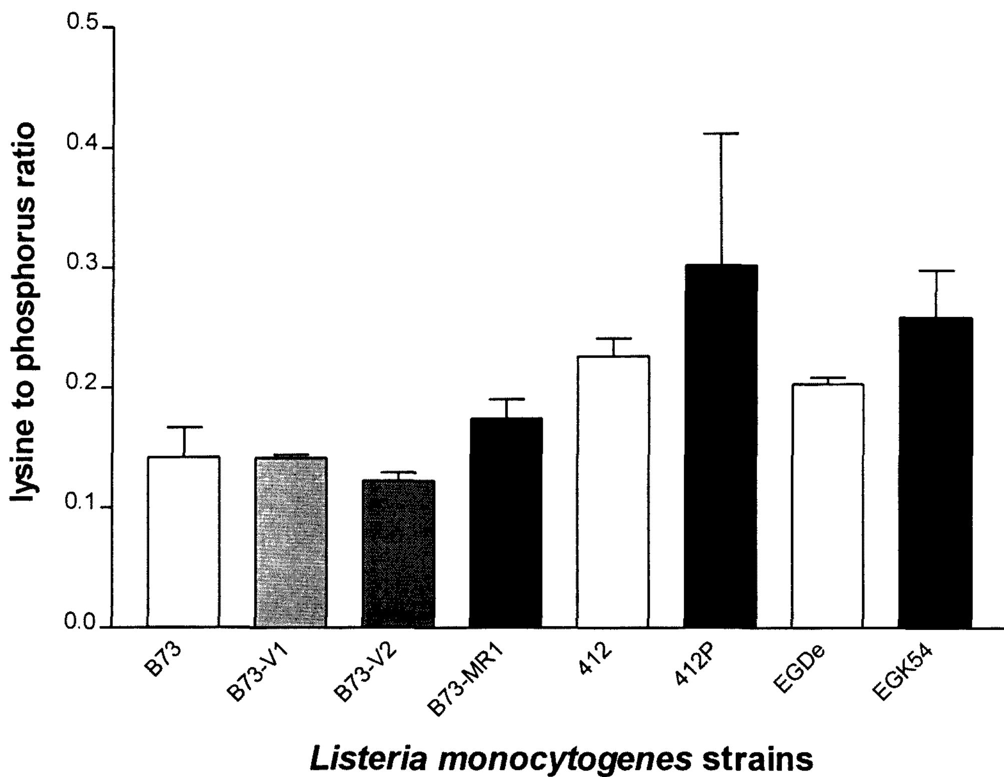

Fig. 2. Lysine to phosphorus ratio in membrane phospholipid isolated from *Listeria monocytogenes* strains. The data are averages of duplicate experiments with error bars representing the standard error for each mean value. Differences between *L. monocytogenes* wild-type and its corresponding resistant strains were not statistically significant (P>0.05).

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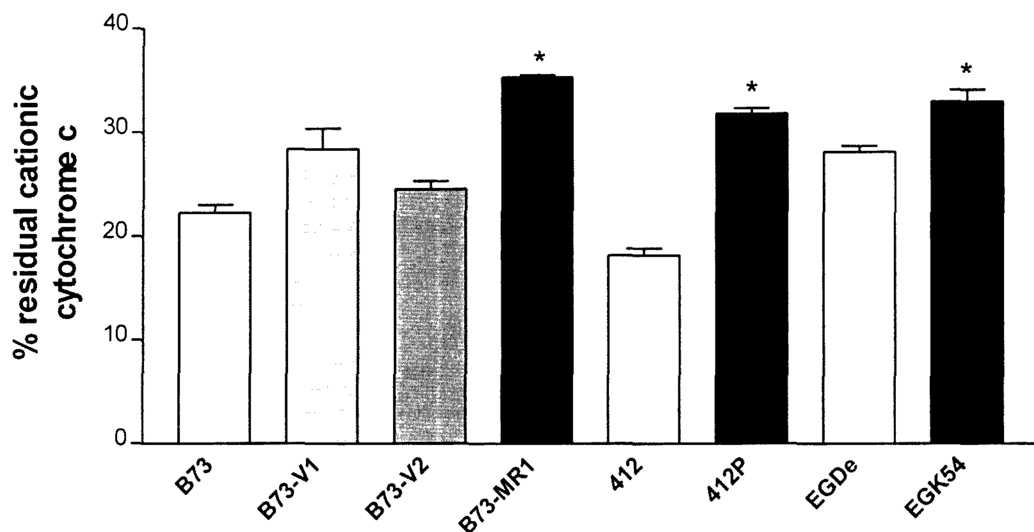

Fig. 3. Residual amount of cationic cytochrome c in the supernatant after binding to whole *Listeria* cells. The data are the averages of duplicate experiments with error bars representing the standard error for the mean for each ratio value. Comparison between *L. monocytogenes* wild-type and its corresponding resistant strains: * P<0.05.

Table 3. Inhibition responses to 100 μg ml^{-1} D-cycloserine

Listeria monocytogenes strains	Response time after D-cycloserine addition (h)	Time from onset to 50 % relative inhibition (h)
B73	4.4	4.7
B73-V1	4.3	5.1
B73-V2	4.1	4.7
B73-MR1	5.4	6.1
412	0.9	16.8
412P	0.5	11.5
EGDe	0.5	7.0
EGK54	1.3	3.1

The values represent an average of duplicate experiments.

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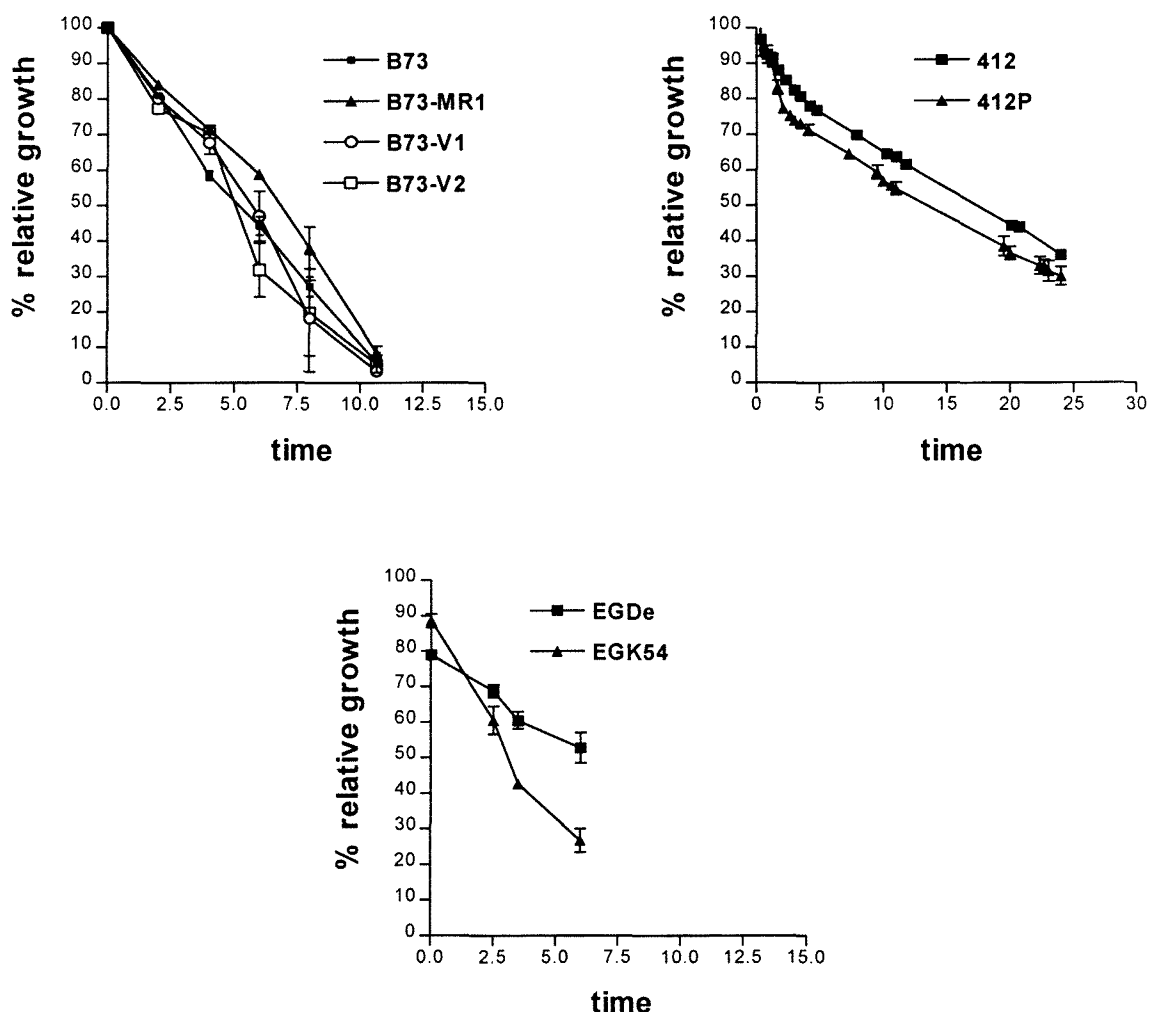

Fig. 4. Inhibitory effects on wild-type and class IIa bacteriocin resistant *Listeria monocytogenes* strain growth after addition of 100 μg ml^{-1} D-cycloserine at mid-log phase. The data are averages of duplicate experiments with error bars representing the standard error for each mean value.

Real-time PCR analysis

The expression of four genes that putatively influence cell surface charge (*dltA*, *lmo1695*) and are targets for DCS (*dal, ddlA*) was analysed by real-time PCR. This was done to determine whether cell surface modifications that were observed could be related to the transcriptional level of these specific genes.

We considered a three-fold difference in expression ($2^{\Delta\Delta C_T}$) as a significant change (i.e. $\Delta\Delta C_T$ >1.6 or <-1.6) in expression. There was thus no change in expression of *dltA*, *dal, ddlA* and *lmo1695* in any of the resistant mutants (see fold expression change results in Table 4). Results for the expression analysis of the *mptA* gene appear in Fig. 5 for all the spontaneous resistant strains.

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Table 4. Calculated fold expression change of genes (relative to the wild-type) analysed by real-time quantitative PCR

***L. monocytogenes* strains**	***genes***				
	mptA	***dal***	***ddlA***	***dltA***	***lmo1695***
B73-V1	**-3.96**	1.37	-1.03	-1.00	-1.08
B73-V2	**-3.72**	2.03	1.57	1.15	1.28
B73-MR1	**-3601084**	1.03	-1.37	1.35	-1.28
412P	**-1070**	-1.54	-1.22	-1.05	-1.30
EGK54	-	-1.29	-1.75	1.64	1.26

Values represent the average of duplicate experiments.
Negative values indicate a decrease in expression and positive values indicate increases in expression.
Expression was considered significant if expression values were $-3\leq$ or ≥ 3 (see values in bold font).

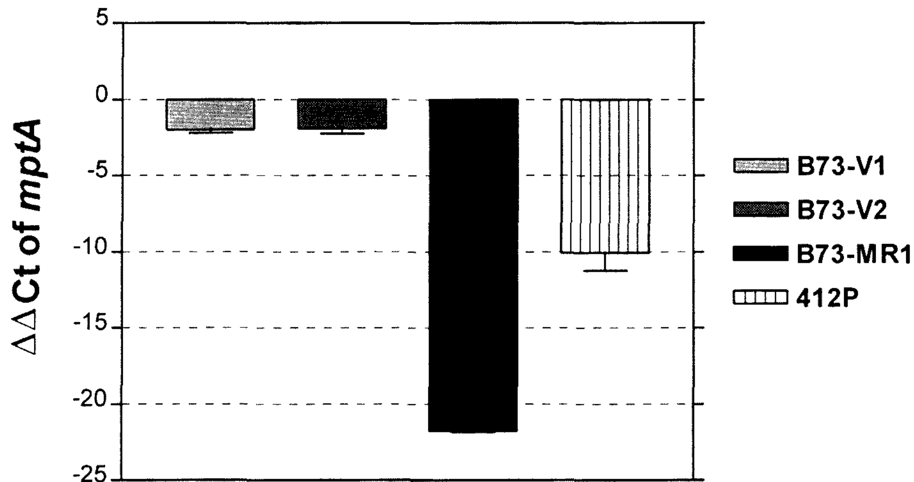

Fig. 5. Quantitative real-time PCR, of the *mptA* gene, representing the enzyme IIAB component of the mannose PTS. The results are a duplicate of two experiments, with each individual experiment consisting of two pooled RNA extractions and triplicate quantitative PCR reactions. Error values are represented by the standard deviation between two experiments. The corresponding wild-type strains have not been included since the resistant strains $\Delta\Delta C_T$ value is calculated relative to the wild-type (see text for details).

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There was a large decrease in expression of *mptA* in the highly resistant strains. There was also a decrease in expression of *mptA* in the intermediate resistant strains, B73-V1 and B73-V2. The average change in expression is represented as the calculated fold change in expression in Table 4, and this shows an approximately four-fold decrease in expression for the *mptA* gene in B73-V1 and B73-V2, and a >1000-fold and >3.6 million-fold decrease in expression of *mptA* in 412P and MR1, respectively.

DISCUSSION

Cell surface changes in Class IIa bacteriocin resistant bacteria

The ribitol-phosphate backbone of the cell wall polymer, TA, can be substituted with D-alanine forming a D-alanyl ester bond [11]. In this form the D-alanine presents a positive charge resulting in neutralisation of the anionic polymer. The cationic lantibiotics, Pep5 and nisin, have been shown to adsorb to the bacterial surface in the presence of the TA, and this is influenced by D-alanine incorporation [4]. Unlike previous reports in which TA is deficient in D-alanine, because of insertional inactivation [1] or deletion mutation [25] of *dltA*, this study shows small increases in D-alanylation of the TA. These small increases are similar to increases seen in wild-type and *dltA* insertional mutant *S. aureus* cells, which were complemented with a plasmid encoding the *dlt* operon [25, 22], and that displayed resistance to cationic peptides.

The possibility that the outer cell surface charge could be influenced by L-lysine in the phospholipid membrane, also suggests possible involvement in susceptibility to cationic antimicrobial compounds [13, 24], and has not been explored in *L. monocytogenes*. Cardiolipin and PG in *L. monocytogenes* are normally negatively charged, whereas the lysinylated forms of these two phospholipids bears a net positive charge [25]. Our results indicate that in addition to the increase in D-alanine in TA, there may also be a tendency for an increase in the lysine in the phospholipid, in highly resistant strains. The increase in positive charge due to alanine and possibly also lysine incorporation would decrease the anionic property of the cell permeability barriers, which would interfere with the initial and subsequent electrostatic interaction of the cationic bacteriocin with the cells. The combined increased charge from both alanine and lysine (from cell wall and membrane), could explain why there was least binding of the cytochrome c to the highly resistant cells. Additionally, other factors

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that could influence net surface charge, like the zwitterionic phosphatidylethanolamine content of *L. monocytogenes* phospholipid [7] have not been investigated. The genes involved in D-alanylation and lysinylation (*dltA* and *lmo1695*), did not display increased expression in any of the resistant mutant strains studied. This may suggest that the, regulation of the activity of these enzymes is not at the transcriptional level.

DCS effects on class IIa bacteriocin resistant *L. monocytogenes* strains

DCS affected the strains differently, but we were unable to show any clear relationships between DCS sensitivity and resistance phenomena that we studied. The onset of DCS inhibition was different for the intermediate resistant strains of the B73 family, compared to the highly resistant and wild-type strains. There could be a relationship between MptA absence and DCS activity since the *mptA* mutant shows the most dramatic inhibition by DCS. The reason for this relationship is, however, not clear.

There were no alterations in the levels of transcription of the *dal* and *ddlA* genes between the resistant and sensitive strains indicated that the DCS targets were not implicated in the variances in DCS sensitivity unless there is interference with regulation at post transcriptional level. DCS sensitivity could be affected by differences in abilities to transport DCS into the cells. DCS, is transported into the cell by the same transporter as alanine [31].

Different mechanisms may contribute to intermediate class IIa bacteriocin resistance

Present evidence suggests that prevention of the *mptACD* expression conferring resistance in *L. monocytogenes* strains, is due to a potential interaction or docking of the class IIa bacteriocin on the MptC-MptD membrane complex of the mannose PTS [8, 15]. Additionally, the requirement of stereospecificity for leucocin A activity supports the presence of a docking molecule [33]. We found a clear correlation between levels of transcription of the *mptA* gene and level of resistance to class IIa bacteriocins in strains B73-V1 and B73-V2, which were observed to be 2 and 4 times more resistant than the wild-type respectively [30], and showed a four-fold decrease in transcription level of this gene. Furthermore, the highly resistant spontaneous mutants, B73-MR1 and 412P showed a decrease of greater than a thousand-fold in

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transcription levels of the *mptA* gene. Our study shows that MptA is also associated to intermediate class IIa bacteriocin resistance.

In our previous study on the PG composition of the same *L. monocytogenes* strains described in this study [30], we observed an overall increase in unsaturated and short-acyl-chain PG species in all class IIa resistant strains of *L. monocytogenes*. The increases of unsaturated acyl chain was however significantly greater in the intermediate resistant strains, B73-V1 and B73-V2, while the highly resistant showed significantly greater increases in short-acyl-chain PG species [30]. In addition, the decrease in desaturated PG after treatment with a desaturase inhibitor, resulted in a 2.5-fold increase in resistance of B73-V1, a 50 % decrease in resistance of B73-V2, and no changes in sensitivity of the wild-type and highly resistant strains, to leucocin A [30]. Additionally, a nisin resistant mutant, 412N [16], also had a two-fold increase in resistance to pediocin [A. Gravesen, unpublished results], and may therefore be regarded as an intermediate class IIa resistant strain. *L. monocytogenes* 412N became as sensitive to pediocin as the wild-type, 412, upon inactivation of the penicillin-binding protein associated with nisin resistance. These findings suggest various mechanisms present in intermediate class IIa bacteriocin resistance.

In summary, our results show a clear correlation between MptA down-regulation and resistance levels in the intermediate resistant strains we studied. Moreover these intermediate resistant strains had larger increases in D-alanine esterification of TA than the highly resistant strains. The fact that the D-alanine content was increased in all the studied class IIa mutants compared to the wild-type strains indicates that cell surface charge may play a complementary role in modulating class IIa resistance in general. It is important to consider at this point, that electrostatic interactions have been identified to play a central role in the interaction of nisin with its docking molecule, lipid II [17]. Our results therefore indicate that several factors may contribute to intermediate class IIa bacteriocin resistance, and that in at least some of these mutants, an increased D-alanine content may be a relatively important factor.

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Chapter 6

Metabolic changes associated with class IIa bacteriocin resistance in Listeria monocytogenes strains

Abstract

High-level resistance to class IIa bacteriocin has been directly associated with the absent $EIIAB^{Man}$ (MptA) subunit of the mannose-specific PTS in *Listeria monocytogenes* strains (Gravesen et al. [12]). Class IIa bacteriocin resistant strains used in this study were a spontaneous resistant, *L. monocytogenes* B73-MR1, and a defined mutant, *L. monocytogenes* EGDe-*mptA*. Both strains were previously reported to have $EIIAB^{Man}$ PTS components missing (Gravesen et al. [12]). In this study we show that these class IIa bacteriocin resistant strains have a significantly ($P<0.05$) decreased growth and glucose consumption rates, but they also have a significantly ($P<0.05$) higher growth yield than their corresponding wild-type strains, *L. monocytogenes* B73 and *L. monocytogenes* EGDe, respectively. In the presence of glucose, the strains showed a shift from a predominantly lactic acid to a mixed acid fermentation. This metabolic shift could be correlated to reduced glucose consumption and growth rates as a consequence of the missing $EIIAB^{Man}$ PTS. This is in agreement with the observed growth yield increase observed in class IIa bacteriocin resistant strains, with respect to their ATP yields. This study suggests that class IIa resistance development in *L. monocytogenes* does not necessarily bring a cost implication to the cell.

Introduction

Food-associated strains of lactic acid bacteria frequently produce antimicrobial compounds referred to as class IIa bacteriocins [8]. Class IIa bacteriocins have been grouped based on their high homology, conserved N-terminal YGNGV motif and effective antilisterial activity [15, 7, 13]. The potential application of class IIa bacteriocins as food preservatives has been extensively studied in the search for safe, non-toxic antimicrobial food additives. However, the frequent occurrence of

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resistance has become an increasingly important concern, since it reduces the value of adding class IIa bacteriocins to foods [11, 7].

Research carried out on understanding the mechanism of class IIa bacteriocin resistance shows strong evidence for one prevalent mechanism among various listerial strains and *Enterococcus faecalis* [12, 14]. This mechanism involves the absence of the EIIAB subunit of a mannose-specific PTS (known as $EIIAB^{Man}$ or *mptA*) [12]. Phosphoenolpyruvate (PEP)-dependent PTSs are sugar transport systems, which simultaneously phosphorylate the sugar with its translocation across the cell membrane [16, 19]. The phosphorylated sugar derivative serves as the first metabolic intermediate, thus coupling sugar uptake and catabolism.

Carbohydrates are required by *L. monocytogenes* as the primary energy source for growth, with glucose being the preferred carbon source [18, 20]. There are evidence for the presence of two glucose transport systems in *L. monocytogenes*, a high affinity PTS and a low affinity proton-motive force (PMF) driven system [17]. Glucose transport inhibition of the high affinity system by competing sugars, mannose and 2-deoxyglucose was also observed [17]. Only two mannose-specific PTSs (PTS^{Man}), coded for by the *mpo* and *mpt* operons, have been described in detail for *L. monocytogenes* [5]. However, three complete mannose-specific PTSs and an incomplete glucose-specific PTS gene sequence have been identified in the *L. monocytogenes* genome [10]. Glucose uptake in *L. monocytogenes* can be attributed to the PTS^{Man} [5], which is also known to transport mannose and 2-deoxyglucose [2, 23]. In many lactic acid bacteria and streptococci, transport and phosphorylation of glucose occurs mainly via a PTS^{Man} [2], which maybe be similar for *L. monocytogenes*.

Studies related to class IIa bacteriocin resistance have involved the insertional inactivation of the *mptA* gene that resides on the *mpt* operon coding for the $EIIAB^{Man}$ subunit of the PTS^{Man} in *L. monocytogenes* [5]. This insertional inactivation has resulted in a high level of resistance to class IIa bacteriocins [12], and it has been suggested that the EII_t^{Man} PTS membrane component or permease could play a role as a possible target for class IIa bacteriocins [5, 13, 11].

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The aim of this study was to investigate the effect of the missing MptA subunit of the PTS^{Man} on glucose metabolism in class IIa bacteriocin resistant *L. monocytogenes* strains. We focussed on glucose uptake and analysis of the end products of glucose metabolism. The growth patterns of *L. monocytogenes* were also analysed in this study in brain heart infusion (BHI) culture medium with or without added glucose as a carbon source. All studies were done on two wild-type, sensitive *L. monocytogenes* strains, and their corresponding class IIa resistant variant, of which one was a spontaneous mutant and the other a genetically defined mutant.

MATERIALS AND METHODS

Bacterial strains and growth conditions

All strains were grown in BHI broth (Difco), supplemented, or not supplemented with 10 mM glucose (Associated Chemical Enterprises, Glenvista, South Africa), according to the requirements of the study. The strains were cultivated at 37 ^{o}C without shaking, in tightly capped Spectronic® tubes or in Schott® bottles. The *L. monocytogenes* strains used were the following: wild-type food isolate *L. monocytogenes* B73, and corresponding class IIa bacteriocin spontaneous mutant, *L. monocytogenes* B73-MR1; wild-type clinical isolate *L. monocytogenes* EGDe, and corresponding insertionally inactivated *mptA* mutant, *L. monocytogenes* EGK54 [14, 12], referred to as *L. monocytogenes* EGDe-*mptA*, which displays resistance to class IIa bacteriocins. Media used to grow *L. monocytogenes* EGDe-*mptA* was supplemented with 5 μg ml^{-1}erythromycin.

Growth analysis

Bacterial growth was monitored using optical density (OD) at 600 nm. Dry weight measurements were calibrated against OD_{600} measurements. An OD_{600} of 1 corresponds to 0.64 g [dry weight]. L^{-1}. Specific growth rates were calculated from the growth absorbance data collected from Spectronic® tube cultures, and the same cultures were sampled for analysis of end-products of fermentation. In a separate experiment, samples were taken from Schott® bottle cultures, at regular intervals from early exponential phase through to stationary phase for monitoring of glucose uptake.

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Quantification of glucose and fermentation end products by HPLC

Samples collected for analysis were prepared as described in Ward et al. [26]. Samples were analysed for lactate, pyruvate, acetate, formate, and ethanol. Product analysis allowed the calculation of carbon recovery, glucose yields, ATP yields and glucose uptake rates.

Calculations and statistical analysis

Calculations of specific growth rates and Student t-tests were done using Graphpad Prism 3.0 (Graphpad software, San Diego, CA, USA).

RESULTS

Growth of strains in BHI containing or lacking glucose

The growth curves (Fig. 1a) indicate a more rapid growth for the wild-type strains in the presence of 10 mM glucose. This is corroborated by the specific growth rate (Table 1), which is significantly ($P<0.001$) higher, for B73, than for B73-MR1. Similarly, the EGDe strain shows a significantly greater ($P<0.05$) specific growth rate in comparison to that of the corresponding mutant strain, EGDe-*mptA*. Growth rates correlated well with rates reported previously for *L. monocytogenes* strains in BHI medium [24, 12]. Specific growth rates (Table 1, Fig. 1b) for the resistant strains, however, were significantly ($P<0.05$) greater than that of their corresponding wild-type strains when grown in BHI lacking glucose as a carbon source.

A significant ($P<0.05$) difference in growth yield between the wild-type and resistant strains can also be noted in the growth curves. The absolute biomass values shown in Table 1 indicate a higher growth yield for both the spontaneous, and the genetically defined mutant, in comparison to their corresponding wild-type strains, in media supplemented with glucose. In contrast, insignificant changes in the growth yields between the wild-type and resistant variants strains were observed in BHI lacking glucose (Fig 1b, Table 1). The final biomass achieved by all the strains was however lower when glucose was not present.

In keeping with the higher biomass values for the resistant strains, a greater yield on glucose ($Y_{glucose}$) was also observed for these strains in the BHI medium supplemented with glucose (Table 1).

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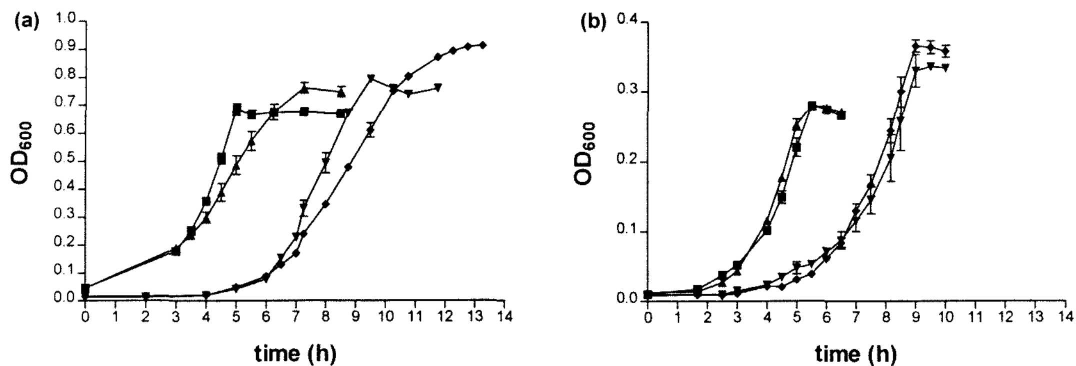

Fig. 1. Growth of *Listeria monocytogenes* strains in BHI supplemented with 10mM glucose **(a)**, and BHI without glucose, **(b)**. Growth studies were carried out at least in duplicate for all the strains. Symbols: ■, wild-type B73; ▲, class IIa resistant mutant B73-MR1; ▼, wild-type EGDe; ♦, class IIa resistant, insertionally inactivated *mptA* mutant, EGDe-*mptA*.

Metabolic end products produced in BHI with 10 mM glucose

Fermentation end-products were analysed to assist in understanding whether the increase in biomass observed for the class IIa resistant *L. monoctyogenes* strains, was due to an increase in ATP production. The wild-type strains, B73 and EGDe, produced lactate as their major product and minor quantities of formate, acetate and ethanol, thus displaying a more homolactic fermentation pattern. In contrast, the class IIa-resistant strains, B73-MR1 and EGDe-*mptA*, were observed (Table 2) to produce larger concentrations of formate, acetate and ethanol than their corresponding wild-type strain, thus displaying a more mixed acid fermentation pattern. In addition, a lower lactate production was observed in the class IIa resistant strains, B73-MR1 (43%) and EGDe-*mptA* (40%), while the calculated lactate production of the wild-type strains was 80 % and 85 % for B73, and EGDe, respectively. It was also interesting to note that EGDe-*mptA* only metabolised approximately half the glucose in the medium. The carbon recovery (Table 2), however, was not in keeping with expected values for glucose metabolism, in all the strains. The calculated average growth yield per mole ATP (Y_{ATP}) for the four strains were as follows: B73, 20.3 g [dry weight]. mol^{-1} ATP; B73-MR1, 16.5 g [dry weight]. mol^{-1} ATP; EGDe, 28.9 g [dry weight]. mol^{-1} ATP; EGDe-*mptA*, 31.3 g [dry weight]. mol^{-1} ATP.

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Table 1. Maximum specific growth rate, biomass and yield on glucose of wild-type and class IIa bacteriocin-resistant *Listeria monocytogenes* strains in BHI broth supplemented, or not supplemented, with glucose.

	Specific growth rate [h^{-1}]		**Biomass [OD_{600}]**		
Listeria monocytogenes	**BHI 10mM**	**BHI no**	**BHI 10mM**	**BHI no**	**$Y_{glucose}$ (g [dw]. mol^{-1} glc‡)**
B73	0.68 [0.018]	0.70 [0.014]	0.68 [0.003]	0.28 [0.006]	25.7
B73-MR1*	0.48 [0.023]	0.90 [0.017]†	0.76 [0.019]	0.28 [0.003]	30.7
EGDe	0.79 [0.029]	0.49 [0.018]	0.79 [0.004]	0.33 [0.023]	29.4
EGDe-*mptA**	0.67 [0.017]	0.67 [0.012]†	0.91 [0.001]†	0.36 [0.009]	35.2

Experimental values represent an average of at least two independent measurements, and standard deviations are shown in parentheses
.* indicates the class IIa-resistant *L. monocytogenes* strains.
† represents a significantly different ($P<0.05$) growth rate or growth yield (biomass) of the resistant strain compared to the corresponding wild-type strain.
‡ represents the yield on glucose value given in the units gram dry weight [dw] per mol glucose (glc).

Metabolic end products produced in BHI lacking glucose

To determine whether a similar growth pattern and metabolism existed for the wild-type and class IIa resistant strains in the absence of glucose, their metabolic end-products were also analysed after growth in BHI lacking glucose. The end-products in BHI without glucose were formate, ethanol and acetate and these were produced in small quantities compared to BHI supplemented with glucose (Table 3). A very small amount of lactate was formed in the B73, B73-MR1 and EGDe strains. A peak co-eluting with glucose, and also present in the BHI lacking glucose control, was detected in all stationary phase samples, whether glucose was supplemented or not. It is assumed that this peak is not glucose, and it was thus subtracted in the analysis.

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Table 2. Product formation and carbon recovery of *Listeria monocytogenes* strains grown in BHI supplemented with 10 mM glucose. The concentration values represent an average of at least three independent measurements and standard deviations are shown in parentheses.

Listeria monocytogenes **strains**	**Glucose concentration [mM]**	**Concentration of products [mM]**				**% carbon recovery**
		lactate	**formate**	**acetate**	**ethanol**	
10mM glucose BHI control *	10.6	5.0			9.3†	
B73	1.8 [0.07]	20.9 [0.13]	6.4 [0.57]	2.9 [0.34]		113.4
B73-MR1	1.8 [0.09]	13.6 [0.59]	11.5 [1.72]	6.6 [0.96]	7.9 [0.77]	126.0
EGDe	1.6 [0.16]	22.0 [1.30]	3.4 [1.97]	0.3 [0.48]		104.2
EGDe-*mptA*	5.4 [0.45]	10.6 [0.38]	9.3 [1.46]	4.2 [0.36]	14.0 [2.90]	140.7

* represents the media without bacterial inoculum.
† indicates ethanol from the erythromycin stock used to supplement the growth of *L. monocytogenes* EGDE-*mptA*.
N.B Media concentrations of lactate, glucose and ethanol (only for EGDe-*mptA*) have not been subtracted from product concentrations values shown here.

Glucose consumption rates

Analysis of the glucose uptake rate was performed to determine the effect of the missing MptA subunit on glucose transport. The following were the calculated glucose uptake rates as mmol glucose g [dry weight]$^{-1}$.h^{-1} for the various *L. monocytogenes* strains: B73, -15.51; B73-MR1, -6.7; EGDe, -10.73; EGDe-*mptA*, -3.3. A significant reduction in the glucose uptake rate was observed for the class IIa resistant strains in comparison to their corresponding wild-type strains.

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Table 3. Product formation of *Listeria monocytogenes* strains grown in BHI not supplemented with glucose. The concentration values represent an average of at least three independent measurements and standard deviations are shown in parentheses.

Listeria monocytogenes strains	Glucose concentration [mM]	Concentration of products [mM]			
		lactate	formate	acetate	ethanol
no glucose BHI control*	1.5‡	4.8			11.0†
B73	1.6 [0.09]	5.7 [0.69]	3.3 [0.32]	1.7 [0.85]	
B73-MR1	1.7 [0.13]	6.0 [0.28]	3.5 [0.86]	2.3 [0.49]	
EGDe	1.8 [0.15]	6.0 [0.18]	2.6 [0.97]	1.5 [0.65]	3.3 [0.41]
EGDe-*mptA*	1.7 [0.88]	4.9 [0.48]	3.7 [0.60]	1.6 [0.15]	10.3 [0.93]

* represents the media without bacterial inoculum
† indicates ethanol from the erythromycin stock used to supplement the growth of *L. monocytogenes* EGDE-*mptA*.
‡ the value for glucose has been subtracted for calculation of carbon recoveries.
Note: Media concentrations of lactate, glucose and, ethanol (only for EGDe-*mptA*) have not been subtracted from product concentrations shown in the table for the different strains.

DISCUSSION

The decreased growth rates shown by class IIa bacteriocin-resistant strains, B73-MR1 and EGDe-*mptA*, in glucose, is similar to growth rate decreases described for another class IIa resistant *L. monocytogenes* strain, 412P, also showing loss of MptA expression [11, 12]. The decreased growth rate in 412P, and in other class IIa bacteriocin resistant B73 strains [6] has been described as a fitness cost associated with class IIa bacteriocin resistance, and this seems to be the same for the B73-MR1 and EGDe-*mptA* strains. This fitness cost was thought to be due to energy-expensive metabolic pathways in resistant strains [6]. Considering the fitness cost implication and the shut-down of the glucose transporter, it is surprising that the higher final biomass for the class IIa resistant strains used for this study, display a more efficient metabolism, in terms of ATP production, and glucose utilisation. This is particularly clear in the spontaneous resistant mutant, B73-MR1, which showed a lower yield on ATP [Y_{ATP}] than the wild-type B73 strain, but >10 % higher biomass. It is not clear,

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however, why both the clinical isolate, EGDe and its defined mutant EGDe-*mptA*, seemed to show a larger Y_{ATP} than the food isolate, B73 and its spontaneous resistant variant. The large yields on ATP could also be explained by the production of ATP from other substrates in the medium besides glucose, as observed by growth in BHI medium without supplemented glucose. Reduced glucose consumption rates for the class IIa resistant strains could be attributed to the loss of the glucose transporter, MptA. Lower growth rates, glucose consumption rates, and glucose limitation are directly implicated in the shift from homolactic behaviour to mixed acid fermentation [4, 9, 1] in *Lactococcus lactis*, which could explain the metabolic shift observed in the class IIa resistant *L. monocytogenes* strains.

Growth observed for all the strains in BHI lacking glucose indicated that these strains are also able to utilise other components of this rich medium for growth, in the absence of glucose as a major carbon source. This could account for the increased carbon recovery observed for all the strains in glucose supplemented BHI medium. The higher growth rates observed for the resistant strains in the absence of glucose supplementation, and the higher Y_{ATP} may also suggest the ability of the resistant strains to more readily utilise other nutrients present in the rich BHI, than the wild-type strains, which may be a regulatory consequence of the missing glucose transporter. Up-regulation of two β-glucoside-specific PTS system enzymes have been suggested to be a possible regulatory consequence of MptA absence in class IIa resistant *L. monocytogenes* strains [12], similar to regulation of other PTSs by the mannose PTS due to carbon catabolite repression in *Lactobacillus pentosus* and *Streptococcus salivarius* [2, 23]

Up-regulation of the *mpt* operon in EGDe in the presence of increasing glucose concentrations, and an increased inhibition by the class IIa bacteriocin mesentericin Y105 [5], has been shown. If the missing MptD, is the docking molecule for class IIa bacteriocins [5, 12], it would implicate a possible competition between glucose and bacteriocin for uptake by the permease, which could provide a glucose limiting environment that may consequently down-regulate *mpt* genes, and produce the shift in fermentation we observed. This may result in the development of spontaneous resistant populations. Inhibition of glucose uptake by the bacteriocin pediocin JD was also suggested to be to the consequence of bacteriocin inhibition of a PTS component

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and may provide further evidence for bacteriocin down-regulation of the PTS [3, 25, 24].

Furthermore, MptA may represent a high-affinity PTS transporter for glucose as indicated by the higher glucose uptake rate that was present for wild-type strains expressing MptA. This supports previous studies showing a PTS as a high affinity glucose transporter in *L. monocytogenes* Scott A [17]. PTS^{Man} could also be the major transporter of glucose into the cell, considering the existence of only the glucose-specific enzyme IIA component and no other functional components of the glucose-specific PTS in *L. monocytogenes* [10], and the greater than 50 % decrease in glucose uptake rate observed for the resistant strains lacking MptA.

The switch to mixed acid fermentation pattern usually occurs when homolactic fermentation is inadequate to yield the necessary ATP for biosynthetic processes and growth [4]. Our study suggests that the shift to mixed acid metabolism in class IIa resistant *L. monocytogenes* strains occurs as a result of reduced glucose uptake as a consequence of the loss of MptA, involved in glucose transport. This results in reduced growth rates but higher biomass of class IIa bacteriocin resistant strains.

L. monocytogenes has been shown to spontaneously develop resistance to class IIa bacteriocins at high frequencies from 10^{-6} to 10^{-8} in food and laboratory media [21, 7]. Additionally, absence of MptA in class IIa bacteriocin resistant strains could further compromise the potential use of class IIa bacteriocins as bio-preservatives, because it is likely to result in more efficient strains with respect to glucose and other carbon source utilisation and biomass yield. Therefore, the use of class IIa bacteriocins as food-preservatives may have severe consequences. Moreover, in natural fermentation conditions are set such that the desirable microorganisms grow preferentially and produce metabolic by-products, which give the unique characteristics of the product. Therefore, the possibility of lactic acid bacterial starter cultures developing this mechanism of resistance in the presence of a class IIa bacteriocin additive could comprise their ability to produce strictly lactic acid fermentation end-products, which are useful in food preservation [http://dna2z.com/project/lacid.htm] and in production of unique food textures and flavours.

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Chapter 7

General discussion

The motivating factor influencing studies on class IIa bacteriocins, and resistance to them by food-borne pathogens, is the possible use of these peptides as food bio-preservatives [7, 12]. With increasing resistance frequency [34, 16] and levels of resistance to traditional antibiotics, there is a need to tap into other sources of potential antimicrobial compounds. Class IIa bacteriocins display many favourable characteristics such as, being produced by 'food grade' bacteria and being non-toxic, as well as, displaying high potency, with low concentrations exacting complete inhibition of pathogens [12, 26, 27]. Unlike, the traditional antibiotics, e.g. penicillin and vancomycin, class IIa bacteriocins are relatively new antimicrobial compounds, having been discovered just over a decade ago [12]. This is also the reason for the limited availability of information regarding their structure-function properties, mode of action and resistance mechanisms.

In an attempt to answer some questions regarding class IIa bacteriocin resistance, this study focussed on factors influencing bacteriocin resistance. The bacteriocin would have to traverse and interact with the cell envelope (cell wall and membrane) permeability barriers to reach its target site. It would make sense that permeability and physicochemical properties would play significant roles in class IIa bacteriocin sensitivity. Accordingly, we studied the phosphatidylglycerol composition of the cell membrane, some factors influencing charge of the cell surface (i.e. D-alanine content of teichoic acids and lysine content of phospholipids) and the effect of a cell wall antibiotic, D-cycloserine in resistant mutants (Chapters 3 and 5). Alteration in cell surface properties could affect the bioactive structure induction of the class IIa bacteriocins, considering that these peptides adopt an active structure only after interaction with membranes or membrane-like environments [19]. FTIR and CD spectroscopy allowed us to assess physical changes in the phospholipids and the peptide structures themselves related to cell alterations in resistance (Chapter 4). Finally, it is expected that class IIa bacteriocin resistance may have some metabolic consequence on the *L. monocytogenes* cell, which may impact on the ecological

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environment of these food-borne pathogens [11]. Here a novel approach of analysing the glucose metabolism of resistant strains to give us clues about their physiology, which may influence their role in food preservation, was used (Chapter 6).

Bacteriocins form part of an even larger family of cationic antimicrobial peptides (CAMPs). Most eukaryotic CAMPs form part of the innate human defence system, protecting the skin and endothelial cell linings and aiding phagocytotic defence cells against pathogenic invasion of the human body [22, 20]. CAMPs are determined to exhibit electrostatic and hydrophobic interaction with the target cell permeability barrier to exert their pore-forming activity [20]. The likelihood of target cells modifying themselves in a similar way to defend action from these peptides is very probable. Our findings of increased D-alanylation of teichoic acid (TA) and other evidence of increased lysinylation of phospholipids could be important. Various reports implicate modification of the cell surface anionic constituents by D-alanylation of TA [30, 29, 32], lysinylation of phospholipid [31], increase in zwitterionic lipid content [8, 36] and aminoarabinose substitution of lipid A of the cell wall [13, 17] in CAMP resistance to pathogenic bacteria like, *Staphylococcus aureus* and *Salmonella enterica* [28]. These changes will result in altered charge properties of the cell surface and therefore influence the initial electrostatic interaction of CAMPs leading to the most basic form of resistance.

The role of the receptor-mediation in activity of CAMPs, has only been suggested for bacteriocins so far [6, 10, 3, 4, 16, 33]. Therefore, although similar mechanisms may exist in resistance for bacteriocins and other CAMPs, negative charge reduction may be a contributing factor for bacteriocin resistance, albeit a minor one. In addition, this type of electrostatic interference may be a more important factor contributing to lower levels of resistance similar to the levels shown by the *L. monocytogenes* B73-V1 and B73-V2 strains of our study, which showed slightly greater D-alanylation of teichoic acid than the highly resistant variant, *L. monocytogenes* B73-MR1. Moreover, most reports on the roles of teichoic acid D-alanylation [29, 32, 1] and phospholipid lysinylation [31] in other CAMPs involved genetic inactivation of genes responsible for these effects, which subsequently brought large changes in sensitivity of these target organisms to the CAMP. Determination of factors influencing permeability barrier charge in the different *L. monocytogenes* spontaneous mutants with various

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levels of class IIa bacteriocin resistance pointed to charge modulation of the cell wall without a detectable genetic regulation.

Besides the effects of charge modifications on the electrostatic and hydrophobic interaction of CAMPs, changes in lipid composition can also affect permeability of the cells [28]. One of the CAMP resistance mechanisms against the Gram negative organism *S. enterica*, involves decreased outer membrane permeability and possible increased stability of the membrane structure by inclusion of an additional fatty acid into lipid A in response to CAMP addition [18, 17, 13]. Changes in membrane fluidity of *L. monocytogenes* cells in nisin resistance [25, 24, 8], and *S. aureus* cells in resistant to thrombin-induced microbicidal protein [2] has also been a factor influencing CAMP activity. Our study confirmed the occurrence of variations in phospholipid content of resistant cell membranes (Chapter 3), and a possible increase in membrane fluidity (Chapter 4). These membrane changes may possibly influence the activity of class IIa bacteriocins. These altered membrane properties were further observed to affect the proper folding of leucocin A, as indicated by the decreased α-helical structure induced in the presence of the *L. monocytogenes* resistant cell-derived liposomes. α-Helicity is necessary for optimal insertion of the α-helical peptide into the target cell membrane [35, 12]. Alterations of the membrane composition could also impact on variations in the hydrophobic [5] and electrostatic nature of the membrane [28, 31]. The tendency of increased lysinylation of highly resistant *L. monocytogenes* membranes (Chapter 5) is an indication of this. The quantification of all the phospholipids in the resistant *L. monocytogenes* membranes including the zwitterionic phospholipids like phosphatidylethanolamine which are known to also be present [8] would shed more light on the charge effect of the membrane.

Various levels of resistance were assessed to determine whether the identified resistance-associated phenomena are particular to a specific resistance level. In general it seems that several phenomena did exist at different levels of resistance, e.g. increased short-acyl-chain and unsaturated fatty acids of phosphatidylglycerol, possible increased membrane fluidity, increased D-alanylation of TA, and decreased mannose PTS enzyme IIAB expression, were shown in all class IIa resistant strains. It is, however, clear that influence of these resistance-associated phenomena varies in the different level resistant strains, e.g.: (1) addition of a desaturase inhibitor

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decreased unsaturated phosphatidylglycerol acyl chain content of all resistant strains, but this caused alteration of the sensitivity of the intermediate resistant mutants only; (2) D-cycloserine caused early onset inhibition in intermediate resistant strains; (3) and slightly increased levels of D-alanine was present in TA of intermediate resistant strains compared to the highly resistant cells of the *L. monocytogenes* B73 family of strains. The various resistance phenomena discussed here indicate that more than one alteration contributes to intermediate resistance.

One of the most important factors to consider here though is the role of the mannose PTS permease as a possible receptor-type molecule mediating class IIa bacteriocin activity [9] Our mutants confirm presence of a mannose PTS (*mptACD*) phenomenon by down-regulation of MptA observed in all the resistant mutants. Additionally, the induction of sensitivity in an insensitive *Lactococcus lactis* strain due to heterologous expression of the *L. monocytogenes* mannose PTS [personal communication, M. Ramnath], suggests a direct interaction between the mannose PTS permease and bacteriocin. Studies involving physical interaction between the permease and bacteriocin may however help in showing an absolute interaction of a receptor-type molecule and class IIa bacteriocin. It is interesting to note here that the lipid II docking molecule's role in nisin sensitivity [3, 4], gives the impression that a receptor requirement can be of great significance to the activity of class IIa bacteriocins and the alternate phenomena exhibited by intermediate resistance strains should not detract from this. It is also interesting that in the interaction of the lantibiotic mersacidin and lipid II, electrostatics play a central role [21], and this may suggest a similar mechanism for the mannose PTS permease and class IIa bacteriocin, thus explaining the charge modulation we observed in our resistant mutants.

Alternatively, microarray gene expression studies showing up-regulation of the *mptA* gene in an intermediate pediocin resistant *L. monocytogenes* (412C) strain and another pediocin intermediate resistant *L. monocytogenes* (412N) strain [15] showing reversion to a sensitive phenotype after inactivation of the penicillin-binding protein [personal communication, A. Gravesen], may support other mechanisms associated with different levels of resistance.

Finally, assessment of the metabolic impact of resistance and its other downstream effects on normal cellular phenomena would probably serve as an informative method to determine the commercial application of class IIa bacteriocins as bio-preservatives.

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Few studies have researched fitness costs associated with class IIa bacteriocin resistance [10, 11] as an indication of stability of resistance and its implication on spreading of resistant populations of the *L. monocytogenes* pathogen. Previous reports suggested energy expensive metabolic processes as an explanation for reduced growth rates of resistant *L. monocytogenes* [11]. The stability of the resistance phenotype and the efficiency of glucose metabolism we have shown (Chapter 6), provides disconcerting evidence regarding capability of resistance in *L. monocytogenes*. These factors may result in unfavourable perceptions on the use of class IIa bacteriocins as food preservatives. Elucidation of factors influencing resistance could provide new ideas on how these peptides can be manipulated for effective use. Perhaps, it would be best to use these bacteriocins in combination with various antimicrobial agents showing different modes of action. This may then decrease the likelihood of a target organism developing defences against a multitude of target sites to prevent antimicrobial action.

In conclusion, this study indicates that class IIa bacteriocin resistance is a multi-faceted phenotype, and gives us the impression that the use of class IIa bacteriocins in food preservation should be viewed with some caution.

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