Differential protein expression focusing on the mannose phosphotransferase system, in Listeria monocytogenes strains with class IIa bacteriocin resistance.

by

Manilduth Ramnath

M.Sc. (Genetics)

Dissertation approved for the degree

Doctor of Philosophy (Biochemistry)

in the

Faculty of Science

at the

University of Stellenbosch

Supervisor: Prof. J.W. Hastings

Co-supervisor: Dr M. Rautenbach

Department of Biochemistry

University of Stellenbosch

December 2003

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.

Manilduth Ramnath

Summary

Bacteriocins produced by lactic acid bacteria are potentially attractive as food biopreservatives as they target food pathogens, without known toxic and other adverse effects. Some of the class IIa sub-set of bacteriocins are of interest because of their high-level of antimicrobial activity against the potential food pathogen *Listeria monocytogenes*. Development of resistance by *L. monocytogenes* toward class IIa bacteriocins would, however, severely compromises the use of this sub-class of antimicrobial compounds in the food industry. This study was initiated to gain insight into the molecular basis of high-level class IIa resistance of *L. monocytogenes*.

A reproducible protein isolation method and two dimensional (2-D) protocol was optimised. In order to facilitate the analysis of membrane proteins, a protein compartmentalization procedure was assessed, which provided only partial fractionation of membrane and cytosolic proteins. A partially annotated proteome reference map, containing 33 identified spots of *L. monocytogenes* EGDe, was developed. The 2-D profiles from food-isolated strains from serotype 1/2a and 1/2b were compared to that of strain EGDe. The results show that the *L. monocytogenes* EGDe reference map could be a valuable starting point for the analysis of *L. monocytogenes* strains from varying origins.

Numerous studies on the molecular basis of resistance have reported seemingly diverse results. Eight highly resistant *L. monocytogenes* mutants (showing an increase in minimum inhibitory concentration of at least 1000), originating from five wild type strains, isolated after exposure to four different class IIa bacteriocins were screened *via* 2-D and northern blot analysis. One prevalent mechanism of resistance was observed, irrespective of wild-type strain, class IIa bacteriocin, or the environmental conditions under which the resistant strain was isolated. The changes include the shut-down of the mannose phosphotransferase system (PTS), encoding EII_t^{man}, and the up-regulation of the β-glucoside PTS. The inactivation of the β-glucoside PTS in a bacteriocin resistant and sensitive strain did not alter the sensitivity to class IIa bacteriocins, implying that the β-glucoside PTS was not directly associated with resistance acquisition. Other studies using defined genetic mutants of *L. monocytogenes* have also implicated EII_t^{man} in the sensitivity to class IIa bacteriocins. To assess the role that EII_t^{man} plays in sensitivity, it was heterologously expressed in the class IIa bacteriocin insensitive

Lactococcus lactis MG 1363 strain. The resultant conversion of the phenotype, strongly suggests the direct involvement of the EII_t^{man} in class IIa bacteriocin activity. Furthermore, to determine the changes in protein expression induced by exposure to the bacteriocin, a wild type, intermediate and resistant *L. monocytogenes* 412 strains were exposed to a sub-lethal final concentration (9.8 ng/ml) of leucocin A and its D-enantiomer. No changes in protein expression levels were detected in the 2-D gels, after the exposure of any of the strains to either enantiomer. Comparisons of the 2-D protein profiles of the unchallenged wild type and intermediate resistant strains showed that the intensity of the spot corresponding to $EIIAB^{man}$ was enhanced 6-fold in the gel showing the proteins from the resistant strain. This indicated an up regulation of EII_t^{man}.

Opsomming

Bakteriosiene word geproduseer deur melksuurbakterieë en kan dien as potensiële voedsel biopreserveerders, juis omdat dit voedselpatogene teiken sonder om enige toksiese of nadelige effekte tot gevolg te hê. Sommige klas IIa bakteriosiene toon 'n hoë mate van antimikrobiese aktiwiteit teen die potensiële voedselpatogeen, *Listeria monocytogenes*. Die ontwikkeling van weerstandbiedendheid deur *L. monocytogenes* teen klas IIa bakteriosiene kan die gebruik van dié antimikrobiese verbindings in die voedselbedryf erg kompromitteer. Tydens hierdie studie is onderneem om die molekulêre basis van hoë vlak klas IIa weerstandbiedendheid van *L. monocytogenes* te ondersoek en belangrike inligting rondom hierdie aspek in te win.

'n Herhaalbare proteïen isoleringsmetode en 2-dimensionele (2-D) protokol was geoptimiseer. 'n Proteïen kompartementaliseringsproses, wat slegs gedeeltelike fraksionering van membraan en sitosoliese proteïene teweegbring, is verder ondersoek om die analises van membraanproteïene te fasiliteer. 'n Gedeeltelik geannoteerde proteoom verwysingskaart, bevattende 33 identifiseerbare kolle van *L. monocytogenes* EGDe, was ontwikkel op 'n 2-D gel. Die 2-D profiele vanaf voedsel geïsoleerde rasse vanaf sereotipe 1/2a en 1/2b was vergelyk met dié van die EGDe ras. Hierdie resultate toon dat die *L. monocytogenes* EGDe verwysingskaart 'n waardevolle beginpunt vir die analise van *L. monocytogenes* rasse van verskillende oorspronge kan wees.

Verskeie studies op die molekulêre basis van weerstandbiedendheid het klaarblyklik verskillende resultate getoon. Agt hoogs weerstandbiedende *L. monocytogenes* mutante (wat 'n styging in MIC van ten minste 1000 aantoon), het ontstaan vanaf 5 wilde tipe rasse. Hierdie mutante was geïsoleer na blootstelling aan 4 verskillende klas IIa bakteriosiene en was verder ondersoek met behulp van 2-D en Northern klad analise. Een oorheersende meganisme van weerstandbiedendheid is waargeneem, ongeag van die wilde tipe ras, klas IIa bakteriosien, of die omgewingskondisies waaronder die weerstandbiedende ras geïsoleer is. Veranderinge behels die afsluitingsmeganisme van die mannose fosfotransferase sisteem (PTS), koderend vir EII_t^{man}, en die oormatige ekspressie van die β-glukosied PTS. Die inaktivering van die β-glukosied PTS in 'n bakteriosien weerstandbiedende en sensitiewe ras het geensins die sensitiwiteit tov die klas IIa bakteriosiene gewysig nie. Hierdie resultate impliseer dat die β-glukosied PTS nie direk geassosieer is met die weerstandbiedendheidomskakeling nie.

Verdere studies, waar gebruik gemaak is van gedefinieerde genetiese mutante van *L. monocytogenes,* het ook geïmpliseer dat EII_t^{man} 'n rol speel in die sensitiwiteit tov klas IIa bakteriosiene. EII_t^{man} is heteroloog uitgedruk in klas IIa bakteriosien onsensitiewe *Lactococcus lactis* MG 1363 ras om sodoende die sensitiwiteit hiervan te assesseer. Die gevolglike omskakeling van die fenotipe dui op 'n sterk direkte betrokkenheid van EII_t^{man} in klas IIa bakteriosien aktiwiteit. Verder is die veranderinge in proteïen ekspressie bepaal met behulp van induksie deur die blootstelling aan die bakteriosien, 'n wilde tipe, intermediaat en weerstandbiedende *L. monocytogenes* 412 rasse. Dit is bewerkstellig deur blootstelling aan 'n sub-dodelike finale konsentrasie (9,8 ng/ml) van leucocin A en sy D-enantiomeer. Na die blootstelling van enige van dié rasse aan beide enantiomere, was geen veranderinge in die proteïen ekspressie vlakke waargeneem in die 2-D gels nie. Vergelykings van die 2-D proteïen profiele van die ongebruikte wilde tipe en intermediêre weerstandbiedende rasse het getoon dat die intensiteit van die kolle ooreenstemmend tot EII_t^{man} 6-voudig verhoog het in die gel, wat die proteïene vanaf die weerstandbiedende ras aantoon. Hierdie resultate dui die oormatige uitdrukking van EII_t^{man} aan.

With love to Dadhi and Dadha

Acknowledgements

I would like to express my thanks and gratitude to the following persons:

- my supervisor Prof. J. W. Hastings for his guidance, supervision, as well as his ingenious suggestions and encouragement during the trying times;
- Dr. Marina Rautenbach, my co-supervisor, for her insightful discussions and motivation;
- Dr. A. Gravesen, for her excellent guidance, support and for going out of her way to make me feel at home during my time in Denmark;
- Prof. Y. Héchard, for his constant help and supervision during the time that I spent in France and for not giving up on me during the cloning work;
- Dr. K. B. Rechinger, for the demonstration of the two-dimensional electrophoresis procedure and for his constant inspiration;
- Lene Gertman, for her excellent technical assistance for her assistance with the two-dimensional gels;
- the staff and students of the fourth floor in the Department of Dairy and Food Science at The Royal Veterinary and Agricultural University in Denmark, for their friendship and support;
- Prof. J. M. Berjeaud, from the Black Cat office in IBMIG for his constant brainstorming and enthusiasm;
- Safia Arous, for her help with the real-time PCR analysis;
- the staff and students of Labortorie de Microbiologie Fondamentale et Appliquée, IBMIG, for there assistance and friendship;
- the personnel and students of the Biochemistry department at the University of Stellenbosch, for there assistance, friendship and support during the writing up of this thesis;

- Dr. Bhoola (Jnr.) and Parisha, for their buoyant enthusiasm, never fading friendship and for been a source of constant encouragement;

- Kushal and Vishad Deepaul, for keeping me sane during the rough patches and been a source of constant entertainment;

- my colleague Viveka Vadyvaloo, for her constant friendship, brainstorming sessions, encouragement and critical reading of my thesis;

- The National Research Foundation, Danish Rectors' Conference and E.G.I.D.E, for financial support during this degree;

- Petite Fée, for having revolutionised my life and for the never ending encouragement and support;

- my sister and brother for their constant support;

- and last but definitely not least, I would like to thank my parents, for their endless love, absolute belief in my abilities and providing me with opportunities to further myself.

Table of Contents

List of Abbreviations and Acronyms

2D	two-dimensional
BHI	brain heart infusion
CF	carboxy fluorescein
CHAPS	3-[(3-cholamidopropyl)-dimethylammonio] 1-propane sulfonate
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EI	Enzyme I
EII	Enzyme II
E_t^{man}	mannose phosphotransferase system II (composed of EII^{AB}, EII^{C} and EII^{D})
GRAVY	grand average hydropathy
IEF	isoelectric focusing
LAB	lactic acid bacteria
MeOH	methanol
MS/MS	tandem mass spectrometry
OD	optical density
PEP	phosphoenol-pyruvate
PMF	proton motor force
PTS	phosphotransferase system
Q-TOF-MS	quadropole time-of-flight mass spectrometer
SDS	sodium dodecyl sulfate
TE	Tris-EDTA
TFA	trifluoroacetic acid
TMD	transmembrane domains
Tris-HCl	2-amino-2-(hydroxymethyl)-1,3-propandiol-hydrochloride
TSA	tryptone soy agar
Tyr	tyrosine
UV	ultraviolet
ΔpH	pH gradient
$\Delta\psi$	transmembrane potential

Chapter 1

Class IIa bacteriocins and resistance phenomena

1. General introduction to bacteriocins

Ribosomally synthesised antimicrobial peptides are produced by a broad spectrum of living organisms ranging from prokaryotes to higher eukaryotes [74, 93]. Bacterial ribosomally synthesized antimicrobial polypeptides are generally referred to as bacteriocins. They differ from traditional antibiotics in at least two ways in that they have relatively narrow killing spectrum as they are only toxic to bacteria closely related to the producing strain [85] and they are ribosomally synthesised whereas antibiotics are generally secondary metabolites [87]. Although bacteriocins may be found in numerous Gram-positive and Gram-negative bacteria, those produced by lactic acid bacteria (LAB) are of particular interest due to their potential application in the food industry as natural preservatives due to their ability to target food pathogens without toxic and other adverse effects [88].

1.1 Classification of LAB bacteriocins

Bacteriocins from LAB are commonly divided into three or four groups [56, 72]. Class I are lantibiotics and are characterized by their unusual amino acids such as lanthionine, methyl-lanthionine, dehydrobutyrine and dehydroalanine as a result of extensive post-translational modifications. Examples of class I bacteriocins are nisin, lactocin S, carnocin, cinnanycin, daramycin, and mersacidin. Class II bacteriocins consist of small heat-stable, non-modified peptides that can be further subdivided into class IIa, IIb and IIc. Class IIa include *Listeria* active peptides, which contain a conserved N-terminal sequence, YGNGV, and two cysteines forming a disulphide bridge in the N-terminal half of the peptide. Approximately twenty class IIa bacteriocins have been identified, examples of which are pediocin PA-I/AcH, sakacin P, leucocin A, mesentericin Y105, bavaricin MN, enterocin P and curvacin. Class IIb bacteriocins require two peptides for full activity, examples of which are lactacin F, lactococcin M, and lactococcin G. Class IIc bacteriocins are secreted by the general sec-system. However, it has been shown that

class IIa bacteriocins can also be secreted by this system and consequently the sub-class may not be necessary [23]. Large and heat labile bacteriocins make up the Class III bacteriocins, examples of which are helveticin J and enterolysin A. A fourth class has been proposed that consists of bacteriocins that form large complexes with other macromolecules [56] but bacteriocins belonging to this class are still to be purified.

1.2 Use of bacteriocins in the food industry

Food-borne diseases account for 76 million illnesses, 325 000 hospitalisations and 5 000 deaths in the United States of America alone [101]. *Listeria monocytogenes* has been recognized as an important food-borne pathogen since the early 1980's and is the causative agent of listeriosis [4]. Listeriosis has emerged as an atypical food-borne illness of major public health concern because of the severity of the disease, the high case fatality, the long incubation time, and the predilection for individuals who are immuno-compromised [4].

Artificial chemical additives such as sulphur dioxide, benzoic acid, sorbic acid, and nitrate are currently added to food to suppress microorganisms. However, increasing customer awareness of the risks derived not only from food-borne pathogens, but also from artificial chemical preservatives used to control them has resulted in an increased interest in naturally produced antimicrobial agents [88].

Nisin has been approved for use in the food industry as a bio-preservative by "The Joint Food and Agriculture Organization/World Health Organization Committee" in 1969 and is currently in use in at least 48 countries [28]. However, class IIa bacteriocins are more interesting anti-listerial agents than class I bacteriocins such as nisin, because they do not have as broad an inhibitory spectrum as nisin and thus may not kill starter cultures while they are also more effective at killing *Listeria* strains [75]. Furthermore, it has been demonstrated that class IIa bacteriocins are more effective at killing pathogens in meat products, where nisin is less effective [54, 103].

A potential problem associated with using bacteriocins as bio-preservatives is the development of resistant populations of problematic bacteria. Such an occurrence may severely compromise the use of bacteriocins in food preservation. Recently, there have been an increasing number of studies focusing on the resistance of *L. monocytogenes* to

nisin and class IIa bacteriocins. The understanding of the resistance mechanism resulting in the protection of strains against bacteriocins may help in the understanding of how bacteriocins mechanistically kill sensitive bacteria.

The aim of this study is to gain an improved understanding of class IIa bacteriocin resistance. The information should also enhance our understanding of the class IIa bacteriocin mechanism of action. In light of the almost similar pore formation mode of action of the well characterised class I bacteriocin 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*.

2. Mode of action

2.1 Mechanism of action of class I and IIa bacteriocins

The permeablization of the membranes of susceptible microorganisms is the primary mechanism by which LAB bacteriocins affect their lethal activity. Bacteriocins may dissipate either or both transmembrane potential ($\Delta\psi$) and pH gradient (ΔpH) components of the proton motor force (PFM). Nisin completely dissipates both $\Delta\psi$ and ΔpH in sensitive cells [14, 89]. Class IIa bacteriocins on the other hand cause the total dissipation of ΔpH, with a partial dissipation of the $\Delta\psi$ [6, 14, 55, 61]. However, mundticin, a class IIa bacteriocin causes the complete dissipation of $\Delta\psi$ [4].

The interaction of class IIa bacteriocins with the cytoplasmic membrane of sensitive cells is generally different to that of nisin as they interact regardless of the energization state of the membrane [14, 22, 53, 99]. This suggests that the loss of permeability of the cytoplasmic membrane of class IIa sensitive cells occurs in a voltage-independent manner, while nisin acts on the target cell in a membrane-potential-dependant manner [70, 90]

The initial electrostatic interaction between the bacteriocin and the cytoplasmic membrane can be adversely affected by the presence of charged ions or at pH values that change the net charge of bacteriocin molecules, thereby affecting the antimicrobial activity [2, 21, 36, 55].

The resultant pore formation in the cytoplasmic membrane induces the leakage of a number of small intracellular substances from sensitive cells. Nisin, for example causes the efflux of amino acids, potassium, inorganic phosphate and partial efflux of ATP [89] from the target cell. The formation of poration complexes by class IIa bacteriocins, causes an ionic imbalance and leakage of inorganic phosphate and UV-absorbing materials [1, 6, 14, 21, 22, 53, 55, 56, 61,]. Unlike nisin, leakage of ATP does seem to occur when cells are treated with class IIa bacteriocins [4, 36]. The depletion of ATP levels in class IIa treated cells [4, 17] was probably due to the shift in ATP consumption as the cell tries to regenerate the PMF [17]. The active transport of amino acids in class IIa bacteriocin cells is also inhibited [22, 61]. The efflux of pre-accumulated amino acids [6, 22] in class IIa bacteriocin treated cells was proposed to also be due to the reflux via the PMF transport systems in combination with the diffusion through the bacteriocin pores [61, 71].

2.2 Models for bacteriocin-membrane interaction and pore formation

The hypothesis that nisin and class II bacteriocins permeablize target membranes through a multi-step process of binding, insertion and pore formation, has provided the conceptual framework for studies on the molecular mechanism of bacteriocin action [31, 76, 97]. Precisely how pore complexes are formed is a major focus of ongoing research. Models for pore formation are largely based on studies of nisin with membranes. These include synthetic phospholipid vesicles or planer lipid bilayers [10, 31, 44, 45, 46, 98], phospholipid monolayers [29], detergent micelles [97], or lipid vesicles derived from sensitive microorganisms [104].

Two alternative mechanism were proposed to describe the detailed steps involved in membrane permeation for nisin and class IIa bacteriocins, namely, the "barrel-stave" [34, 76] and the "wedge" model [31, 97]. Nisin and class IIa bacteriocins are both, water-soluble and possess membrane-binding ability, which are essential for either model.

The "wedge" model for pore formation has been proposed for nisin [31, 51, 97]. The model takes into account nisin's flexibility in aqueous solutions and the defined structural elements of the thio-ether rings [51]. Due to the amphiphilic nature of nisin, it was found that nisin does not only interact with the phospholipid head groups *via* ionic forces, but also inserts with its hydrophobic side into the outer leaflet of a bilayer [90]. The orientation of the nisin to the head groups does not change. The resulting pore has the

hydrophobic side of nisin and the attached lipid head-groups facing the centre of the water filled pore [51, 97]. Due to this arrangement, both polar sides of the cationic nisin and the anionic lipids face the lumen of the pore, which may explain the non-selective efflux of ions and small solutes [57, 89].

The presence of amphiphilic segments that are putative transmembrane helices indicate that class IIa bacteriocins may form pores via the "barrel-stave" model [36, 91]. Pore formation by class IIa bacteriocins may be initiated by the N-terminal region of class IIa bacteriocins interacting electrostatically with the membrane surface in an non-specific manner [38, 39]. It was hypothesised that a hydrophobic interaction occurs between residues in the c-terminal specificity–determining region of the bacteriocin and a membrane component, thereby resulting in a membrane that is more susceptible to permeabilization [39]. However, it has been shown that there may not be an absolute requirement for membrane a receptor to facilitate pore formation by class IIa bacteriocins [21, 55]. Following the interaction between the N-terminal region and membrane, hydrophobic interactions between the hydrophobic/amphiphilic domain of the C-terminal half of the bacteriocin and the lipid acyl chains, have been demonstrated to be essential for the pore formation process [18, 39, 41, 55]. Furthermore, the C-.terminal domain could be the cell-specificity-determining region for class IIa bacteriocins [38, 39]. The formation of pores would occur by formation of bundles of amphipathic α-helices, such that their hydrophobic surfaces interact with the lipid core of the membrane and their hydrophilic surfaces point inward, producing an aqueous pore. Both, the size and the stability of a water filled pore would depend on the number of peptides involved in pore formation.

2.3 Putative docking molecules

2.3.1 Evidence of specific docking of nisin to a receptor-type molecule

Pore formation by nisin was thought not to require a receptor because it was capable of dissipation of the PMF and caused carboxy fluorescein (CF) efflux from lipid vesicles, which lacked proteins and other cellular components [9, 43, 44, 64, 98, 104]. The level of activity of nisin, observed in the model membrane experiments, did not correspond to its *in vivo* action. *In vitro*, nisin was 1 000-fold less active on membranes solely composed of phospholipids compared with the nanomolar concentrations required for *in vivo* activity

[11]. From these results, it is evident that the interaction of nisin with bacterial cells was more specific and that this specificity could not be explained by a mechanism of action for which the presence of anionic lipids is the only prerequisite.

It was well known that nisin also inhibits peptidoglycan synthesis [60] and that it interacts with cell wall precursors, Lipid I or II [83]. The presence of Lipid II in liposomes substantially increased the susceptibility of the liposomes to nisin [11]. Lipid II not only acts as a receptor for nisin, but is also an intrinsic component of the pore formed by nisin [8]. The role of Lipid II is to switch the orientation of nisin from parallel to perpendicular with respect to the cell membrane surface and this is crucial for pore formation [8]. It has been suggested that the pore was formed by five to eight nisin molecules and an identical number of Lipid II molecules [8]. Mutations in the N-terminal rings of nisin indicated that these rings are involved in the specific binding to Lipid II [11] and corroborated an earlier observation of a peptide comprising the twelve N-terminal amino acids of nisin showing antagonism to nisin activity [16].

Nisin resistance might be altered by the degree of the biosynthesis and/or accessibility of Lipid II molecules. This may explain the observed changes in the bacterial cell wall of nisin resistant strains [24, 62]. The over-expression of a penicillin binding protein may shield Lipid II, resulting in the hindrance of nisin to its receptor [48]. Conversely, the inhibition of the transglycoslyation and the subsequent concomitant accumulation of Lipid II in the membrane *via* pre-treatment with mersacidin, resulted in greater sensitivity of pre-treated cells as additional binding sites for nisin were made available [13]. Furthermore, the low level of Lipid II in yeasts and fungi may explain the low activity of nisin against these microorganisms [102].

2.3.2 Evidence for specific docking of class IIa bacteriocins to a putative receptor-type molecule

Initial work suggested that a protein-based receptor mediates pediocin PA-1 pore formation [22]. However, subsequent studies have demonstrated that a protein receptor was not essential for the activity of pediocin PA-1/AcH [21], as pediocin was able to induce CF efflux from complex lipid vesicles derived from *L. monocytogenes*, as well as from pure phospholipid vesicles [21]. Other class IIa bacteriocins, bavaricin MN and enterocin P, also induced CF leakage from lipid vesicles [52, 55]. These observations confirmed that pore formation could take place in the absence of a protein receptor.

Consequently, anionic lipids were proposed to act as functional binding sites for pediocin [18, 20]. It has also been suggested that cell surface polymers such as lipoteichoic acid and teichoic acid could act as receptors for class IIa bacteriocins [41]. However, a protein receptor was indispensable for membrane permeability of the class IIc bacteriocin, lactococcin A, as observed by the resultant insensitivity observed in protease treated membrane vesicles derived from sensitive cells [96]. The narrow spectrum of activity could also be attributed to the interaction of the bacteriocin with a specific receptor found only in lactococcal strains. The class IIb bacteriocin, lactococcin G, also requires a specific receptor for activity as it was only active against whole cells and inactive against membrane or lipid vesicles [69].

Further evidence for receptor mediation was obtained when a peptide fragment derived from pediocin PA-1 (residues 20 to 34) was found to specifically inhibit the bacterial activity of pediocin PA-1 and to a lesser extent that of enterocin A [39]. This indicated that pediocin interacts specifically with a cell surface entity on the target membrane. As mentioned before, a similar study conducted with nisin was one of the first indications that nisin specifically interacts with a particular component of the cell membrane [16]. Antimicrobial peptides from eukaryotes, which do not have receptors, are equally active in their D and L forms [105]. The all D-enantiomer of leucocin A has been found to be inactive, indicating a stereospecific interaction with a target molecule for the bacteriocin that strongly suggests the presence of a receptor or docking molecule [105].

Inactivation of the *rpoN* gene, encoding the σ^{54} subunit of the RNA polymerase resulted in insensitivity of *L. monocytogenes* [86] and *Enterococcus faecalis* [25] to mesentericin Y105 and other class IIa bacteriocins. Subsequently, the inactivation of the σ^{54} controlled mannose PTS in both organisms also resulted in the insensitivity to mesentericin Y105 [26, 50]. In addition, an increase in the expression of the *mpt* operon (*mptA*, *mptC*, and *mptD*), encoding EII_t^{man}, with increasing concentrations of glucose or mannose resulted in an enhanced susceptibility of the cells suggesting that the expression level of *mpt* influences sensitivity [26, 50]. The deletion of one of the membrane-associated subunits of the permease also resulted in insensitivity of both *L. monocytogenes* and *E. faecalis* cells [26, 50]. Furthermore, deletion of an extra domain from the $EIID^{man}$ in *L. monocytogenes*, which was found almost exclusively on the $EIID^{man}$ of strains that were sensitive to class IIa bacteriocins, also resulted in insensitivity [26]. Finally, it was

hypothesised that $EIID^{man}$ could act as a docking molecule, *via* an interaction between its additional domain and mesentericin Y105 [26].

In all the above-mentioned studies, however, the expression of the downstream genes (*mpt*A and *mpt*C) was not confirmed in an attempt to negate their association in possible class IIa bacteriocin docking. An exclusive interaction of a class IIa bacteriocin and the $EIID^{man}$ can only be established if the remainder of the operon was indeed expressed. Assuming the remainder of the operon was not expressed then the EII_t^{man} as an entire unit may act as a docking complex or it may regulate the expression of the docking molecule. This may be likely since the EIIAB component of the mannose PTS has been known to control the expression of a number of operons in oral streptococci [94].

2.3.3 Docking molecules and model of class IIa bacteriocin pore formation.

Based on recent evidence, a model for the mode of action for class IIa bacteriocins has been proposed [50]. The interaction of the bacteriocin with a docking molecule was essential for the *in vivo* activity against whole bacterial cells.

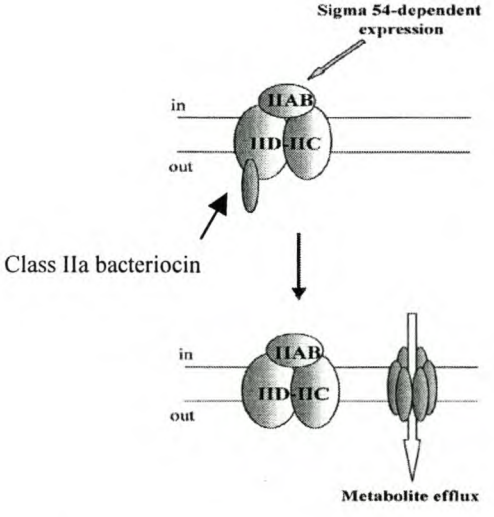


Fig. 1. A model for the mode of action of subclass IIa bacteriocins. IIAB, IIC and IID represent the subunits of the EII_t^{Man} mannose permease (see text) (after Héchard and Sahl, 2002 [51]).

The docking complex has been speculated to be the EII_t^{man} permease. The interaction between the bacteriocin and EII_t^{man} may result in the permease switching to a permanent open state or the bacteriocin may interact with the cytoplasmic membrane leading to pore formation or disruption of the membrane [51] (Fig. 1). Alternatively, the disruption of the

membrane may not require the docking molecule to facilitate pore formation as this may depend on electrostatic and/or hydrophobic interactions with the membrane instead. Both cases it would result in the permeabilization of the bacterial cell.

The lack of expression of the mannose PTS in a class IIa bacteriocin resistant *L. monocytogenes* strain [80], indicates that spontaneous high-level resistance to class IIa bacteriocins may be achieved by the absence of the of EII_t^{man} permease. Furthermore, the mannose PTS acts as a receptor for lambda phage, facilitating the translocation of the phage DNA across the inner membrane of *Eschericha coli* [37]. It remains to be determined whether both phage and class IIa bacteriocins may interact with the mannose PTS using a similar mechanism.

2.3.3.1General overview of mannose phosphotransferase system (PTS)

The PTS uses phosphoenol-pyruvate (PEP) in a group translocation process to phosphorylate incoming sugars *via* a phosphoryl-transfer process involving the general energy coupling, non-sugar-specific proteins, Enzyme I (EI) and HPr, and subsequently a sugar-specific membrane bound Enzyme II (EII) complex that catalyses the transport and the phosphorylation of the specific carbohydrate (Fig. 2) [78, 94]. During the translocation process, HPr is transiently phosphorylated by P~EI (Fig. 2). The phosphate group from HPr is then transferred to the membrane bound EII complex (Fig 2). The EII complex usually consists of three functional domains that can be a single protein or on separate polypeptides: (1) the IIA domain possess the first phosphorylation site; (2) the IIB domain bears the second phosphorylation site; and, (3) the IIC domain which is not phosphorylated, provides the sugar-binding site and forms the translocating transmembrane channel [78, 94]. The mannose PTS differs from most other PTS EIIs in combining the EIIA and EIIB domains in a single hydrophobic protein and having two proteins, EIIC and EIID making up the integral membrane part of the complex [78].

Studies carried out on oral streptococci have provided evidence suggesting that its EIIAB protein from the mannose PTS may be involved in the regulation of gene expression. Some of these genes are known to code for cytoplasmic as well as membrane proteins, including enzymes involved in the metabolism of fructose, lactose, galactose and melibiose [7,12, 58]. This indicates that the EIIAB of the mannose PTS forms part of the regulatory components that allows streptococci to select rapidly metabolizable sugars.

The role of the mannose PTS in the regulation of gene expression in *Listeria* remains to be determined.

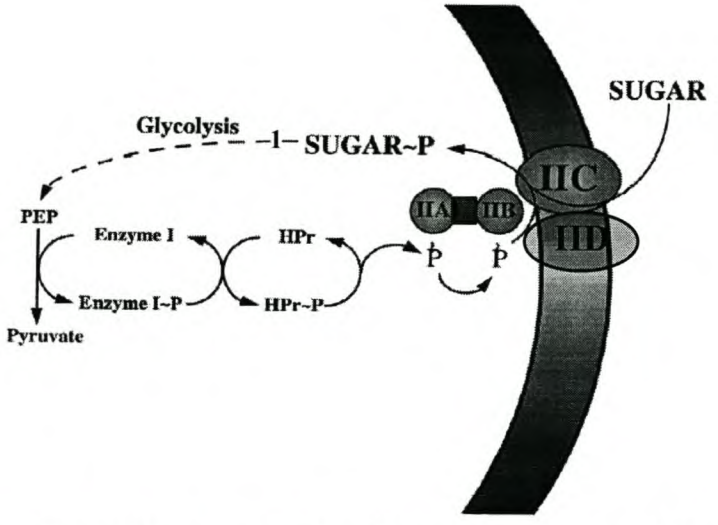


Fig. 2. Organisation of the mannose phosphotransferase system (PTS). Enzyme I and HPr are the general proteins for all PTSs. P~indicates the phoshorylated forms of the various proteins (after Vadeboncoeur and Pelletier, 1997 [94]).

2.4 Structure function relationship of class IIa bacteriocins relating to a receptor/docking molecule.

Certain structural features of class IIa bacteriocins may be necessary for receptor recognition, membrane interaction and insertion. These features include the N-terminal consensus motif which forms a β-turn, a hydrophilic N-terminal portion forming amphiphilic β-sheets, essential amino acids prior to the consensus motif, a central domain forming a hydrophilic/slightly amphiphilic α-helix, the disulphide bridges, the positively charged amino acids and other basic amino acids (Fig. 3)[36, 71, 73].

The specificity of action of class IIa bacteriocins may be attributed in part to their C-terminal region (Fig. 3) [38, 39, 42]. As mentioned earlier, pre-treatment of sensitive cells with a C-terminal fragment of pediocin was capable of inhibiting the activity of the peptide. The C-terminal portion of the bacteriocin was shown to be the main determinant of target cell specificity in studies using hybrid bacteriocins [38].

Fig. 3. Schematic representation of a model class IIa bacteriocin and the predicted structural domains. The hydrophobic face of the peptide is shaded dark and the hydrophilic face is shaded light (after Ennahar et al., 2000 [37]).

The second disulphide bond was shown to increase the potency and spectrum of activity of bacteriocins [35, 53, 71]. The exposure of a second positive patch by the formation of the C-terminal disulphide bond has been proposed to enhance activity of by the formation of a tighter junction between the bacteriocin and the lipid head groups [21]. Alternatively, the second non-conserved C-terminal disulfide bridge may stabilize a structure that may be particularly important for receptor recognition.

The critical role of the N-terminal KYY motif (Fig. 3) was demonstrated for mesentericin Y105 since the removal of only these amino acids from the peptide resulted in a dramatic loss of activity [41]. Numerous analogues of mesentericin Y105 were chemically synthesised and when the C-terminal Trp was absent the antimicrobial activity of the molecule was lost [38, 39, 40]. These results suggest that residues 1-14 form part of a recognition structure for a receptor and that the amphiphilic-helical domain (Fig. 3) would then interact with the lipid bilayer. There is, however, no corroborative data from other bacteriocins to support this hypothesis.

Due to the β-turn structure the consensus motif may be readily exposed (Fig. 3) and recognised by a putative docking molecule [5, 21, 42, 71]. Modifications and deletions of this sequence reduce the activity of the bacteriocin toward *L. monocytogenes* [66, 79]. The YGNGV motif was therefore coined as the *Listeria* active portion of the peptide. The β-turn may be disturbed upon changes to the YGNGV sequence, which may then result in changes to the N-terminal β-sheet conformation. These changes would ultimately affect bacteriocin activity toward all sensitive strains and not only to *Listeria*.

3. Class IIa bacteriocin resistance

Resistance to class IIa bacteriocins has been reported to be a stable phenomenon [47, 80, 84]. However, some strains did show reversion to a sensitive phenotype following growth

in bacteriocin free media [33]. Mutants generated to a single class IIa peptide also exhibit cross-resistance to other bacteriocins belonging to the same subclass [33, 80, 81, 84]. Resistant strains were found to have a lower growth rate (thus relative fitness), and were unable to invade populations of the sensitive strain [33]. In addition, the frequency of pediocin resistance development is not influenced by environmental stresses and the acquisition of the resistance phenotype results in a reduction in the specific growth rate [49]

The investigations of class IIa bacteriocin resistance at the molecular level have yielded unrelated observations. It has been found that a leucocin A resistant mutant of *L. monocytogenes* does not synthesize the EIIAB component of the mannose phosphotransferase system (PTS) [80]. The over-expression of two β-glucoside PTS genes was observed in twelve independent pediocin resistant mutants of *L. monocytogenes* [47]. A mutant with resistance to divercin V41 had several changes in protein synthesis, which was suggested to be due to a mutation in a sigma factor [32]. Changes in membrane phosphoglycerols, indicating increased membrane fluidity for two independently generated resistant strains has been recently reported [95]. The divergence in the molecular basis of resistance could be ascribed to each wild type strain utilising a different mechanism to acquire resistance. Alternatively, the difference in the techniques used to analyse the mutants could be "snap shots" into the same complex resistance mechanism. Molecular work done so far focused only on strains derived from one wild type strain and they were always generated by exposure to a single class IIa bacteriocin.

An increase in cytoplasmic membrane fluidity has also been observed in leucocin A intermediate resistant strains of *L. monocytogenes* [95]. These strains also showed down-regulation of the *mpt* operon (Vadyvaloo et al., submitted) that correlated with the level of resistance. The expression of a limited number of genes associated, possibly with class IIa bacteriocin resistance was shown to be altered in intermediate resistant strains (Vadyvaloo et al., submitted). However, more extensive monitoring of gene expression levels in resistant strains may provide more insight into the global regulatory changes that occur upon intermediate resistance development.

4. Factors influencing bacteriocin activity and resistance

Pore formation by bacteriocins is complicated by a number of *in vivo* effects, which also have to be considered. In this section each *in vivo* factor and its modulation to influence bacteriocin resistance, will be discussed for nisin and class IIa bacteriocins.

4.1 The cell wall

The bacteriocin would have to first traverse the cell wall of a Gram-positive bacterium before it reaches the membrane. The cell wall of Gram-positive bacteria forms a web that is composed of peptidoglycan and teichoic acids, which confers rigidity and shape to the cell. The basic structure consists of repeating units of sugar forming glycan chains [15]. The mechanism(s) of bacteriocin passage through the cell wall has not as yet been studied in detail [53].

Bacteriocins were found to adsorb to the cell surface of Gram-positive bacteria in a pH dependent manner, irrespective of whether strains are bacteriocin producers, non-producers, sensitive or resistant [106]. This supports the idea that initial adsorption occurs through electrostatic attraction between the bacteriocin molecule and the cell surface. It was suggested that the class IIa bacteriocin mesentericin $Y105^{37}$ may bind to the anionic cell surface polymers like teichoic acid that this may be critical for peptide targeting [42].

The tolerance of Gram-negative bacteria and yeast to nisin and pediocin AcH could be due to the relative impermeability of their outer membranes. Both Gram-negative bacteria and resistant Gram-positive bacteria were made sensitive to pediocin AcH and nisin following sub-lethal stress [57, 82, 92]. Intact yeast cells are insensitive to nisin, but removal of the cell wall facilitated access of nisin to the membrane and resulted in the rupturing of the cells [30].

4.1.1 Modulation of the cell wall in nisin resistance

A number of studies have shown that cell wall changes contributed to the acquisition of resistance to nisin. The removal of the cell wall from resistant strains resulted in an identical susceptibility to nisin as a wild-type strain [27]. Altered sensitivity to cell wall hydrolysing enzymes was observed in nisin resistant strains [24, 62]. However, certain resistant strains were equally susceptible to cell wall hydrolysing enzymes as their wild-

type strains [100], indicating that modifications to the cell wall was not the only factor that was responsible for nisin resistance

4.1.2 Modulation of the cell wall in class IIa bacteriocin resistance

Class IIa high-level and intermediate resistant *L. monocytogenes* strains contained an increase in D-alanine content of their teichoic acids (Vadyvaloo et al., submitted). This would result in a decrease in the anionic property of the cell wall and in this way reduce the initial electrostatic interaction of the bacteriocin with the cells. However, the intermediate resistant strains possessed a greater ratio of alanine:phosphorus than the highly resistant strains, which may indicate that alteration of the charge of the cell wall may not account for high-level class IIa bacteriocin resistance. In addition, the net charge of teichoic acids has been shown to play a role in the susceptibility of staphylococci to cationic antimicrobial peptides [77].

Due to the complex structure of Gram-positive bacteria, all the changes that occur upon acquisition of bacteriocin resistance are not yet known. Research into the length of the peptidoglycan chains and the extent and manner of cross-linking of the chains, which may result in greater rigidity of the wall and in turn inhibit the passage of the bacteriocin molecules, would aid in the understanding of the role played by the cell wall in class IIa bacteriocin activity.

4.2 Role of lipid composition

The primary target of bacteriocins appears to be the bacterial cytoplasmic membrane [14, 36, 53, 73]. Cell membranes consist mainly of phospholipids, which have a hydrophobic tail composed of two fatty acid chains and a hydrophilic phosphate group, attached to a small hydrophilic compound such as ethanolamine, choline, glycerol or serine. The major phospholipids in the cell membrane of Gram-positive bacteria are the anionic phospholipids namely cardiolipin and phosphatidylglycerol. Some organisms also contain phosphatidylethanolamine and phosphatidylcholine, which are zwitterionic. The relative amounts in which these phospholipids occur vary from one species to another.

Nisin integrates more tightly with membranes containing the negatively charged phosphatidylglycerol, and has little affinity for zwitterionic lipids predominantly found in Gram-negative bacteria, yeast and human cells [29]. In lipid vesicles with varying ratios

of anionic and zwitterionic phospholipids, nisin induced the highest levels of potassium and carboxyfluorescein (CF) release from vesicles with higher anionic lipid contents [9]. Furthermore, anionic phospholipids, in particular cardiolipin, interact strongly with nisin and encourage nisin insertion [46, 63]. However, the role of the negatively charged phospholipids in nisin interaction with lipid vesicles remains somewhat unclear. This is because nisin was reported to cause the increased efflux of CF from vesicles composed of zwitterionic lipids while anionic lipids were considered to inhibit CF efflux [31, 44, 97].

An increase in the affinity of pediocin PA-1 for lipid vesicles containing higher negatively charged phospholipid content has also been reported. Furthermore, no binding of pediocin PA-1 to zwitterionic lipid vesicles was observed [20]. Such evidence, coupled with the finding that electrostatic interactions, and not the YGNGV consensus motif, govern pediocin binding to the target membrane [18] strongly suggests that class IIa bacteriocins may also bind to membranes in an electrostatic manner, as has been determined for nisin.

The saturation state of the phosphatidylglycerol chains had little effect on the binding affinity of pediocin for the lipid vesicle [20]. However, in the same study fluorescence data indicated that penetration of the bacteriocin was greater for a saturated rather than an unsaturated phosphatidylglycerol. These results may indicate that membrane permeabilization by class IIa bacteriocins is also influenced by membrane fluidity.

4.2.1 Modulation of the cytoplasmic membrane for nisin resistance acquisition

The reductions in phosphatidylglycerol and cardiolipin levels have been reported for *L. monocytogenes* strains with increased resistance to nisin [24, 68]. Phospholipid head groups in nisin resistant variants of *L. monocytogenes* have been altered, *via* a decrease in diphoshatidylglycerol content [100]. Nisin penetrated more deeply into the lipid monolayers of diphosphatidylglycerol compared to monolayers of other lipids. This could be explained by the high charge density and specific charge distribution of diphosphatidylglycerol [100, 29, 46].

Modifications to the composition of the fatty acid chains have been reported for nisin resistant *L. monocytogenes* cells and in all cases there was a decrease in the cell membrane fluidity [24, 65, 67]. A more rigid membrane was suggested to therefore affect nisin activity [65, 68]. Additionally, the decreased fluidity of the cytoplasmic membranes

of resistant *L. monocytogenes* may resist the insertion of nisin and in this way achieve an increase in resistance.

4.2.2 Modulation of the cytoplasmic membrane for acquisition of class IIa bacteriocin resistance

Both high and intermediate resistant strains of *L. monocytogenes* to leucocin A contained an increased amount of phosphatidylglycerol with desaturated fatty acid acyl chains [95]. This could cause an increase in membrane fluidity that would probably result in a reduction in the ability of the peptide to form stable pores. These results are in contrast to that found for nisin resistance, but correlates with previous findings with pediocin insertion in lipid vesicles [20]. Intermediate Mes52A resistant strains of *Leuconostoc* and *Weissella* possessed more rigid cytoplasmic membranes [59]. These conflicting results indicate that different bacterial strains may attain intermediate resistance to class IIa bacteriocins in a strain specific manner.

5. Objectives of study

The aim of this study was to obtain a greater understanding of the molecular basis of high level resistance to class IIa bacteriocins and the involvement of the mannose PTS in this phenomena. The monitoring of gene expression levels within bacteriocin resistant cells would identify genes which are involved in the resistance mechanism. A proteomic approach utilising two-dimensional electrophoresis (2-D) could facilitate the monitoring of the expression level of numerous genes simultaneously *via* protein expression. A protocol that would be reliable and reproducible had to be optimised to ensure that modifications in protein expression were not due to experimental variation, but rather to biological differences. The applicability of proteomic maps, generated for a clinical *L. monocytogenes* strain, had to be assessed in order to gauge its usefulness in comparing it to strains isolated from food. Seemingly unrelated molecular modifications in expression levels of gene products have been reported for high-level resistance to class IIa bacteriocins [32, 47, 80]. Therefore, a need existed to reconcile these differences and to determine if the molecular bases of resistance acquisition was strain specific. The EII_t^{man} has been proposed to be the putative docking molecule for class IIa bacteriocins. However, the possibility that the operon itself regulated the actual docking molecule could not be excluded. Therefore, the role of EII_t^{man} was assessed by heterologous

expression of the *mpt* operon in a bacteriocin insensitive strain. Elucidation of the stress responses induced in cells exposed to class IIa bacteriocins would contribute to the understanding of the mechanistic action of class IIa bacteriocins. Sub-lethal levels of bacteriocin would allow the cells to revive themselves instead of been lethally injured. In order to gain further insight into the molecular bases of intermediate resistance, the proteome of an intermediate strain was compared to a wild type strain.

In summary, this thesis focussed on the mechanism of high-level resistance acquisition by *L. monocytogenes* strains to class IIa bacteriocins to gain a better understanding of the mode of action of class IIa bacteriocins and resistance phenomena.

6. Reference List

1 **Abee, T.** 1995. Pore-forming bacteriocins of gram-positive bacteria and self-protection mechanisms of producer organisms. FEMS Microbiol. Lett. **129**:1-10.

2 **Abee, T., T. R. Klaenhammer, and L. Letellier**. 1994. Kinetic studies of the action of lactacin F, a bacteriocin produced by *Lactobacillus johnsonii* that forms poration complexes in the cytoplasmic membrane. Appl. Environ. Microbiol. **60**:1006-1013.

3 **Allerberger, F.** 2003. *Listeria*: growth, phenotypic differentiation and molecular microbiology. FEMS Immunol. Med. Microbiol. **35**:183-189.

4 **Bennik, M. H., B. Vanloo, R. Brasseur, L. G. Gorris, and E. J. Smid**. 1998. A novel bacteriocin with a YGNGV motif from vegetable-associated *Enterococcus mundtii*: full characterization and interaction with target organisms. Biochim. Biophys. Acta **1373**:47-58.

5 **Bhugaloo-Vial, P., X. Dousset, A. Metivier, O. Sorokine, P. Anglade, P. Boyaval, and D. Marion**. 1996. Purification and amino acid sequences of piscicocins V1a and V1b, two class IIa bacteriocins secreted by *Carnobacterium piscicola* V1 that display significantly different levels of specific inhibitory activity. Appl. Environ. Microbiol. **62**:4410-4416.

6 **Bhunia, A. K., M. C. Johnson, B. Ray, and N. Kalchayanand**. 1991. Mode of action of pediocin AcH from *Pediococcus acidilactici* H on sensitive bacterial strains. J. Appl. Bacteriol. **70**:25-33.

7 **Bourassa, S. and C. Vadeboncoeur**. 1992. Expression of an inducible enzyme II fructose and activation of a cryptic enzyme II glucose in glucose-grown cells of spontaneous mutants of *Streptococcus salivarius* lacking the low-molecular-mass form of III^{man}, a component of the phosphoenolpyruvate:mannose phosphotransferase system. J. Gen. Microbiol. **138**:769-777.

8 **Breukink, E., H. E. Van Heusden, P. J. Vollmerhaus, E. Swiezewska, L. Brunner, S. Walker, A. J. Heck, and B. De Kruijff**. 2003. Lipid II is an intrinsic component of the pore induced by nisin in bacterial membranes. J. Biol. Chem. e-copy, prior to publication

9 **Breukink, E., C. van Kraaij, R. A. Demel, R. J. Siezen, O. P. Kuipers, and B. de Kruijff**. 1997. The C-terminal region of nisin is responsible for the initial interaction of nisin with the target membrane. Biochemistry **36**:6968-6976.

10 **Breukink, E., C. van Kraaij, A. van Dalen, R. A. Demel, R. J. Siezen, B. de Kruijff, and O. P. Kuipers**. 1998. The orientation of nisin in membranes. Biochemistry **37**:8153-8162.

11 **Breukink, E., I. Wiedemann, C. van Kraaij, O. P. Kuipers, H. Sahl, and B. de Kruijff**. 1999. Use of the cell wall precursor lipid II by a pore-forming peptide antibiotic. Science **286**:2361-2364.

12 **Brochu, D., L. Trahan, M. Jacques, M. C. Lavoie, M. Frenette, and C. Vadeboncoeur**. 1993. Alterations in the cellular envelope of spontaneous IIIManL-defective mutants of *Streptococcus salivarius*. J. Gen. Microbiol. **139**:1291-1300.

13 **Brötz, H., G. Bierbaum, K. Leopold, P. E. Reynolds, and H. G. Sahl**. 1998. The lantibiotic mersacidin inhibits peptidoglycan synthesis by targeting lipid II. Antimicrob. Agents Chemother. **42**:154-160.

14 **Bruno, M. E. and T. J. Montville**. 1993. Common mecanistic action of bacteriocins from lactic acid bacteria. Appl. Environ. Microbiol. **59**:3003-3010.

15 **Bugg, T. D. and C. T. Walsh**. 1992. Intracellular steps of bacterial cell wall peptidoglycan biosynthesis: enzymology, antibiotics, and antibiotic resistance. Nat. Prod. Rep. **9**:199-215.

16 **Chan, W. C., M. Leyland, J. Clark, H. M. Dodd, L. Y. Lian, M. J. Gasson, B. W. Bycroft, and G. C. Roberts**. 1996. Structure-activity relationships in the peptide antibiotic nisin: antibacterial activity of fragments of nisin. FEBS Lett. **390**:129-132.

17 **Chen, Y., and T. J. Montville**. 1995. Efflux of ions and ATP depletion induced by pediocin PA-1 are concomitant with cell death in *Listeria monocytogenes* Scott A. J. Appl. Bacteriol. **79**:684-690.

18 **Chen, Y., R. D. Ludescher, and T. J. Montville**. 1997. Electrostatic interactions, but not the YGNGV consensus motif, govern the binding of pediocin PA-1 and its fragments to phospholipid vesicles. Appl. Environ. Microbiol. **63**:4770-4777.

19 **Chen, Y., D. A. Case, J. Reizer, M. H. Saier, Jr., and P. E. Wright**. 1998. High-resolution solution structure of *Bacillus subtilis* IIAglc. Proteins **31**:258-270.

20 **Chen, Y., R. D. Ludescher, and T. J. Montville**. 1998. Influence of lipid composition on pediocin PA-1 binding to phospholipid vesicles. Appl. Environ. Microbiol. **64**:3530-3532.

21 **Chen, Y., R. Shapira, M. Eisenstein, and T. J. Montville**. 1997. Functional characterization of pediocin PA-1 binding to liposomes in the absence of a protein receptor and its relationship to a predicted tertiary structure. Appl. Environ. Microbiol. **63**:524-531.

22 **Chikindas, M. L., M. J. Garcia-Garcera, A. J. Driessen, A. M. Ledeboer, J. Nissen-Meyer, I. F. Nes, T. Abee, W. N. Konings, and G. Venema**. 1993. Pediocin PA-1, a bacteriocin from *Pediococcus acidilactici* PAC1.0, forms hydrophilic pores in the cytoplasmic membrane of target cells. Appl. Environ. Microbiol. **59**:3577-3584.

23 **Cintas, L. M., P. Casaus, L. S. Havarstein, P. E. Hernandez, and I. F. Nes**. 1997. Biochemical and genetic characterization of enterocin P, a novel sec-dependent bacteriocin from *Enterococcus faecium* P13 with a broad antimicrobial spectrum. Appl. Environ. Microbiol. **63**:4321-4330.

24 **Crandall, A. D. and T. J. Montville**. 1998. Nisin resistance in *Listeria monocytogenes* ATCC 700302 is a complex phenotype. Appl. Environ. Microbiol. **64**:231-237.

25 **Dalet, K., C. Briand, Y. Cenatiempo, and Y. Héchard**. 2000. The *rpoN* gene of *Enterococcus faecalis* directs sensitivity to subclass IIa bacteriocins. Curr. Microbiol. **41**:441-443.

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

27 **Davies, E. A., M. B. Falahee, and M. R. Adams**. 1996. Involvement of the cell envelope of *Listeria monocytogenes* in the acquisition of nisin resistance. J. Appl. Bacteriol. **81**:139-146.

28 **Delves-Broughton, J., P. Blackburn, R. J. Evans, and J. Hugenholtz**. 1996. Applications of the bacteriocin, nisin. Antonie Van Leeuwenhoek **69**:193-202.

29 **Demel, R. A., T. Peelen, R. J. Siezen, B. de Kruijff, and O. P. Kuipers**. 1996. Nisin Z, mutant nisin Z and lacticin 481 interactions with anionic lipids correlate with antimicrobial activity. A monolayer study. Eur. J. Biochem. **235**:267-274.

30 **Dielbandhoesing, S. K., H. Zhang, L. H. P. Caro, J. M. van der Vaart, F. M. Klis, C. T. Verrips, and S. Brul**. 1998. Specific cell wall proteins confer resistance to nisin upon yeast cells. Appl. Environ. Microbiol. **64**:4047-4052.

31 **Driessen, A. J., H. W. van den Hooven, W. Kuiper, K. M. van de, H. G. Sahl, R. N. Konings, and W. N. Konings**. 1995. Mechanistic studies of lantibiotic-induced permeabilization of phospholipid vesicles. Biochemistry **34**:1606-1614.

32 **Duffes, F., P. Jenoe, and P. Boyaval**. 2000. Use of two-dimensional electrophoresis to Study differential protein expression in divercin V41-resistant and wild-type Strains of *Listeria monocytogenes*. Appl. Environ. Microbiol. **66**:4318-4324.

33 **Dykes, G. A. and J. W. Hastings**. 1998. Fitness costs associated with class IIa bacteriocin resistance in *Listeria monocytogenes* B73. Lett. Appl. Microbiol. **26**:5-8.

34 **Ehrenstein, G. and H. Lecar**. 1977. Electrically gated ionic channels in lipid bilayers. Q. Rev. Biophys. **10**:1-34.

35 **Eijsink, V. G., M. Skeie, P. H. Middelhoven, M. B. Brurberg, and I. F. Nes**. 1998. Comparative studies of class IIa bacteriocins of lactic acid bacteria. Appl. Environ. Microbiol. **64**:3275-3281.

36 **Ennahar, S., T. Sashihara, K. Sonomoto, and A. Ishizaki**. 2000. Class IIa bacteriocins: biosynthesis, structure and activity. FEMS Microbiol. Rev. **24**:85-106.

37 **Esquinas-Rychen, M. and B. Erni**. 2001. Facilitation of bacteriophage lambda DNA injection by inner membrane proteins of the bacterial phosphoenol-pyruvate: carbohydrate phosphotransferase system (PTS). J. Mol. Microbiol. Biotechnol. **3**:361-370.

38 **Fimland, G., O. R. Blingsmo, K. Sletten, G. Jung, I. F. Nes, and J. Nissen-Meyer**. 1996. New biologically active hybrid bacteriocins constructed by combining regions from various pediocin-like bacteriocins: the C-terminal region is important for determining specificity. Appl. Environ. Microbiol. **62**:3313-3318.

39 **Fimland, G., R. Jack, G. Jung, I. F. Nes, and J. Nissen-Meyer**. 1998. The bactericidal activity of pediocin PA-1 is specifically inhibited by a 15-mer fragment that spans the bacteriocin from the center toward the C terminus. Appl. Environ. Microbiol. **64**:5057-5060.

40 **Fimland, G., L. Johnsen, L. Axelsson, M. B. Brurberg, I. F. Nes, V. G. Eijsink, and J. Nissen-Meyer**. 2000. A C-terminal disulfide bridge in pediocin-like bacteriocins renders bacteriocin activity less temperature dependent and is a major determinant of the antimicrobial spectrum. J. Bacteriol. **182**:2643-2648.

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

42 **Fregeau Gallagher, N. L., M. Sailer, W. P. Niemczura, T. T. Nakashima, M. E. Stiles, and J. C. Vederas**. 1997. Three-dimensional structure of leucocin A in trifluoroethanol and dodecylphosphocholine micelles: spatial location of residues critical for biological activity in type IIa bacteriocins from lactic acid bacteria. Biochemistry **36**:15062-15072.

43 **Gao, F. H., T. Abee, and W. N. Konings**. 1991. Mechanism of action of the peptide antibiotic nisin in liposomes and cytochrome c oxidase-containing proteoliposomes. Appl. Environ. Microbiol. **57**:2164-2170.

44 **Garcera, M. J., M. G. Elferink, A. J. Driessen, and W. N. Konings**. 1993. In vitro pore-forming activity of the lantibiotic nisin. role of protonmotive force and lipid composition. Eur. J. Biochem. **212**:417-422.

45 **Giffard, C. J., H. M. Dodd, N. Horn, S. Ladha, A. R. Mackie, A. Parr, M. J. Gasson, and D. Sanders**. 1997. Structure-function relations of variant and fragment nisins studied with model membrane systems. Biochemistry **36**:3802-3810.

46 **Giffard, C. J., S. Ladha, A. R. Mackie, D. C. Clark, and D. Sanders**. 1996. Interaction of nisin with planar lipid bilayers monitored by fluorescence recovery after photobleaching. J. Membr. Biol. **151**:293-300.

47 **Gravesen, A., P. Warthoe, S. Knøchel, and K. Thirstrup**. 2000. Restriction fragment differential display of pediocin-resistant *Listeria monocytogenes* 412 mutants shows consistent overexpression of a putative beta-glucoside-specific PTS system. Microbiology **146**:1381-1389.

48 **Gravesen, A., K. Sørensen, F. M. Aarestrup, and S. Knøchel**. 2001. Spontaneous nisin-resistant *Listeria monocytogenes* mutants with increased expression of a putative penicillin-binding protein and their sensitivity to various antibiotics. Microb. Drug Resist. **7**:127-135.

49 **Gravesen, A., A. M. Jydegaard Axelsen, D. S. Mendes, T. B. Hansen, and S. Knøchel**. 2002. Frequency of bacteriocin resistance development and associated fitness costs in *Listeria monocytogenes*. Appl. Environ. Microbiol. **68**:756-764.

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

51 **Héchard, Y. and H. G. Sahl**. 2002. Mode of action of modified and unmodified bacteriocins from Gram-positive bacteria. Biochimie **84**:545-557.

52 **Herranz, C., L. M. Cintas, P. E. Hernandez, G. N. Moll, and A. J. Driessen**. 2001. Enterocin P Causes Potassium Ion Efflux from *Enterococcus faecium* T136 Cells. Antimicrob. Agents Chemother. **45**:901-904.

53 **Jack, R. W., J. R. Tagg, and B. Ray**. 1995. Bacteriocins of gram-positive bacteria. Microbiol. Rev. **59**:171-200.

54 **Jack, R. W., J. Wan, J. Gordon, K. Harmark, B. E. Davidson, A. J. Hillier, R. E. Wettenhall, M. W. Hickey, and M. J. Coventry**. 1996. Characterization of the chemical and antimicrobial properties of piscicolin 126, a bacteriocin produced by *Carnobacterium piscicola* JG126. Appl. Environ. Microbiol. **62**:2897-2903.

55 **Kaiser, A. L. and T. J. Montville**. 1996. Purification of the bacteriocin bavaricin MN and characterization of its mode of action against *Listeria monocytogenes* Scott A cells and lipid vesicles. Appl. Environ. Microbiol. **62**:4529-4535.

56 **Klaenhammer, T. R.** 1993. Genetics of bacteriocins produced by lactic acid bacteria. FEMS Microbiol. Rev. **12**:39-85.

57 **Kordel, M. and H. G. Sahl**. 1986. Susceptibility of bacterial, eukaryotic and artificial membranes to the disruptive action of the cationic peptides Pep 5 and nisin. FEMS Microbiol. Lett. **34**:139-144.

58 **Lapointe, R., M. Frenette, and C. Vadeboncoeur**. 1993. Altered expression of several genes in III_L^{Man}-defective mutants of *Streptococcus salivarius* demonstrated by two-dimensional gel electrophoresis of cytoplasmic proteins. Res. Microbiol. **144**:305-316.

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

60 **Linnett, P. E. and J. L. Strominger**. 1973. Additional antibiotic inhibitors of peptidoglycan synthesis. Antimicrob. Agents Chemother. **4**:231-236.

61 **Maftah, A., D. Renault, C. Vignoles, Y. Héchard, P. Bressollier, M. H. Ratinaud, Y. Cenatiempo, and R. Julien**. 1993. Membrane permeabilization of *Listeria monocytogenes* and mitochondria by the bacteriocin mesentericin Y105. J. Bacteriol. **175**:3232-3235.

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

63 **Martin, I., J. M. Ruysschaert, D. Sanders, and C. J. Giffard**. 1996. Interaction of the lantibiotic nisin with membranes revealed by fluorescence quenching of an introduced tryptophan. Eur. J. Biochem. **239**:156-164.

64 **Matsuzaki, K., O. Murase, N. Fujii, and K. Miyajima**. 1995. Translocation of a channel-forming antimicrobial peptide, magainin 2, across lipid bilayers by forming a pore. Biochemistry **34**:6521-6526.

65 **Mazzotta, A. S. and T. J. Montville**. 1997. Nisin induces changes in membrane fatty acid composition of *Listeria monocytogenes* nisin-resistant strains at 10° C and 30° C. J. Appl. Microbiol. **82**:32-38.

66 **Miller, K. W., R. Schamber, O. Osmanagaoglu, and B. Ray**. 1998. Isolation and characterization of pediocin AcH chimeric protein mutants with altered bactericidal activity. Appl. Environ. Microbiol. **64**:1997-2005.

67 **Ming, X. and M. A. Daeschel**. 1993. Nisin resistance of foodborne bacteria and the specific resistance responses of *Listeria monocytogenes* Scott A. Journal of Food Protection **56**:944-948.

68 **Ming, X. and M. A. Daeschel**. 1995. Correlation of cellular phospholipid content with nisin resistance of *Listeria monocytogenes* Scott A. Journal of Food Protection **58**:416-420.

69 **Moll, G., T. Ubbink-Kok, H. Hildeng-Hauge, J. Nissen-Meyer, I. F. Nes, W. N. Konings, and A. J. Driessen**. 1996. Lactococcin G is a potassium ion-conducting, two-component bacteriocin. J. Bacteriol. **178**:600-605.

70 **Moll, G. N., G. C. Roberts, W. N. Konings, and A. J. Driessen**. 1996. Mechanism of lantibiotic-induced pore-formation. Antonie Van Leeuwenhoek **69**:185-191.

71 **Montville, T. J. and Y. Chen**. 1998. Mechanistic action of pediocin and nisin: recent progress and unresolved questions. Appl. Microbiol. Biotechnol. **50**:511-519.

72 **Nes, I. F., D. B. Diep, L. S. Havarstein, M. B. Brurberg, V. Eijsink, and H. Holo**. 1996. Biosynthesis of bacteriocins in lactic acid bacteria. Antonie Van Leeuwenhoek **70**:113-128.

73 **Nes, I. F. and H. Holo**. 2000. Class II antimicrobial peptides from lactic acid bacteria. Biopolymers **55**:50-61.

74 **Nissen-Meyer, J. and I. F. Nes**. 1997. Ribosomally synthesized antimicrobial peptides: their function, structure, biogenesis, and mechanism of action. Arch. Microbiol. **167**:67-77.

75 **O'Sullivan, L., R. P. Ross, and C. Hill**. 2002. Potential of bacteriocin-producing lactic acid bacteria for improvements in food safety and quality. Biochimie **84**:593-604.

76 **Ojcius, D. M. and J. D. Young**. 1991. Cytolytic pore-forming proteins and peptides: is there a common structural motif? Trends Biochem. Sci. **16**:225-229.

77 **Peschel, A.** 2002. How do bacteria resist human antimicrobial peptides? Trends Microbiol. **10**:179-186.

78 **Postma, P. W., J. W. Lengeler, and G. R. Jacobson**. 1993. Phosphoenolpyruvate:carbohydrate phosphotransferase systems of bacteria. Microbiol. Rev. **57**:543-594.

79 **Quadri, L. E., L. Z. Yan, M. E. Stiles, and J. C. Vederas**. 1997. Effect of amino acid substitutions on the activity of carnobacteriocin B2. Overproduction of the antimicrobial peptide, its engineered variants, and its precursor in *Escherichia coli*. J. Biol. Chem. **272**:3384-3388.

80 **Ramnath, M., M. Beukes, K. Tamura, and J. W. Hastings**. 2000. Absence of a putative mannose-specific phosphotransferase system enzyme IIAB component in a leucocin A-resistant strain of *Listeria monocytogenes*, as shown by two-dimensional sodium dodecyl sulfate- polyacrylamide gel electrophoresis. Appl. Environ. Microbiol. **66**:3098-3101.

81 **Rasch, M. and S. Knøchel**. 1998. Variations in tolerance of *Listeria monocytogenes* to nisin, pediocin PA- 1 and bavaricin A. Lett. Appl. Microbiol. **27**:275-278.

82 **Ray, B. and D. G. Hoover**. 1993. Pediocins (mode of action), p. 192-204. *In* D. G. Hoover and L. R. Steenson (eds.), Bacteriocins of Lactic Acid Bacteria. Academic Press inc..

83 **Reisinger, P., H. Seidel, H. Tschesche, and W. P. Hammes**. 1980. The effect of nisin on murein synthesis. Arch. Microbiol. **127**:187-193.

84 **Rekhif, N., A. Atrih, and G. Lefebvre**. 1994. Selection and properties of spontaneous mutants of *Listeria monocytogenes* ATCC 15313 resistant to different bacteriocins produced by lactic acid bacteria strains. Curr. Microbiol. **28**:237-241.

85 **Riley, M. A. and J. E. Wertz**. 2002. Bacteriocin diversity: ecological and evolutionary perspectives. Biochimie **84**:357-364.

86 **Robichon, D., E. Gouin, M. Debarbouille, P. Cossart, Y. Cenatiempo, and Y. Héchard**. 1997. The rpoN (sigma54) gene from *Listeria monocytogenes* is involved in resistance to mesentericin Y105, an antibacterial peptide from Leuconostoc mesenteroides. J. Bacteriol. **179**:7591-7594.

87 **Rodriguez,J.M., Martinez,M.I., and Kok,J.** (2002) Pediocin PA-1, a wide-spectrum bacteriocin from lactic acid bacteria Crit Rev.Food Sci.Nutr. **42**: 91-121.

88 **Rodriguez, J. M., M. I. Martinez, N. Horn, and H. M. Dodd**. 2003. Heterologous production of bacteriocins by lactic acid bacteria. Int. J. Food Microbiol. **80**:101-116.

89 **Ruhr, E. and H. G. Sahl**. 1985. Mode of action of the peptide antibiotic nisin and influence on the membrane potential of whole cells and on cytoplasmic and artificial membrane vesicles. Antimicrob. Agents Chemother. **27**:841-845.

90 **Sahl, H. G. and G. Bierbaum**. 1998. Lantibiotics: biosynthesis and biological activities of uniquely modified peptides from gram-positive bacteria. Annu. Rev. Microbiol. **52**:41-79.

91 **Shai, Y.** 1999. Mechanism of the binding, insertion and destabilization of phospholipid bilayer membranes by alpha-helical antimicrobial and cell non-selective membrane-lytic peptides. Biochim. Biophys. Acta **1462**:55-70.

92 **Stevens, K. A., B. W. Sheldon, N. A. Klapes, and T. R. Klaenhammer**. 1991. Nisin treatment for inactivation of *Salmonella* species and other gram-negative bacteria. Appl. Environ. Microbiol. **57**:3613-3615.

93 **Tossi, A., L. Sandri, and A. Giangaspero**. 2000. Amphipathic, alpha-helical antimicrobial peptides. Biopolymers **55**:4-30.

94 **Vadeboncoeur, C. and M. Pelletier**. 1997. The phosphoenolpyruvate:sugar phosphotransferase system of oral streptococci and its role in the control of sugar metabolism. FEMS Microbiol. Rev. **19**:187-207.

95 **Vadyvaloo, V., J. W. Hastings, M. J. van der Merwe, and M. Rautenbach**. 2002. Membranes of class IIa bacteriocin-resistant *Listeria monocytogenes* cells contain increased levels of desaturated and short-acyl-chain phosphatidylglycerols. Appl. Environ. Microbiol. **68**:5223-5230.

96 **van Belkum, M. J., J. Kok, G. Venema, H. Holo, I. F. Nes, W. N. Konings, and T. Abee**. 1991. The bacteriocin lactococcin A specifically increases permeability of lactococcal cytoplasmic membranes in a voltage-independent, protein- mediated manner. J. Bacteriol. **173**:7934-7941.

97 **van den Hooven, H. W., C. A. Spronk, K. M. van de, R. N. Konings, C. W. Hilbers, and F. J. Van De Van**. 1996. Surface location and orientation of the lantibiotic nisin bound to membrane-mimicking micelles of dodecylphosphocholine and of sodium dodecylsulphate. Eur. J. Biochem. **235**:394-403.

98 **van Kraaij, C., E. Breukink, M. A. Noordermeer, R. A. Demel, R. J. Siezen, O. P. Kuipers, and B. de Kruijff**. 1998. Pore formation by nisin involves translocation of its C-terminal part across the membrane. Biochemistry **37**:16033-16040.

99 **Venema, K., G. Venema, and J. Kok**. 1995. Lactococcins: mode of action, immunity and secretion. Int. Dairy J. **5**:815-832.

100 **Verheul, A., N. J. Russell, H. R. Van'T, F. M. Rombouts, and T. Abee**. 1997. Modifications of membrane phospholipid composition in nisin-resistant *Listeria monocytogenes* Scott A. Appl. Environ. Microbiol. **63**:3451-3457.

101 **White, D. G., S. Zhao, S. Simjee, D. D. Wagner, and P. F. McDermott**. 2002. Antimicrobial resistance of foodborne pathogens. Microbes Infect. **4**:405-412.

102 **Wiedemann, I., E. Breukink, C. van Kraaij, O. P. Kuipers, G. Bierbaum, B. de Kruijff, and H. G. Sahl**. 2001. Specific binding of nisin to the peptidoglycan precursor lipid II combines pore formation and inhibition of cell wall biosynthesis for potent antibiotic activity. J. Biol. Chem. **276**:1772-1779.

103 **Winkowski, K., A. D. Crandall, and T. J. Montville**. 1993. Inhibition of *Listeria monocytogenes* by *Lactobacillus bavaricus MN* in beef systems at refrigeration temperatures. Appl. Environ. Microbiol. **59**:2552-2557.

104 **Winkowski, K., R. D. Ludescher, and T. J. Montville**. 1996. Physiochemical characterization of the nisin-membrane interaction with liposomes derived from Listeria monocytogenes. Appl. Environ. Microbiol. **62**:323-327.

105 **Yan, L. Z., A. C. Gibbs, M. E. Stiles, D. S. Wishart, and J. C. Vederas**. 2000. Analogues of bacteriocins: antimicrobial specificity and interactions of leucocin A with its enantiomer, carnobacteriocin B2, and truncated derivatives. J. Med. Chem. **43**:4579-4581.

106 **Yang, R., M. C. Johnson, and B. Ray**. 1992. Novel method to extract large amounts of bacteriocins from lactic acid bacteria. Appl. Environ. Microbiol. **58**:3355-3359.

Chapter 2

Development of a Listeria monocytogenes EGDe proteome reference map and comparison with protein profiles of food isolates

This chapter has been accepted for publication in Applied and Environmental Microbiology, volume 69, number 6, page 3368-3376 in 2003 with Manilduth Ramnath as first author and K. Björn Rechinger, Lothar Jänsch, John W. Hastings, Susanne Knøchel, and Anne Gravesen as co-authors.

Abstract

A partially annotated proteome reference map of the food pathogen *Listeria monocytogenes* was developed for exponentially growing cells under standardized, optimal conditions using the sequenced strain EGDe (serotype 1/2a) as a model organism. The map was developed by a reproducible total protein extraction and two-dimensional (2-D) polyacrylamide gel electrophoresis analysis procedure, and contained 33 identified proteins representing the four main protein functional classes. In order to facilitate analysis of membrane proteins, a protein compartmentalization procedure was assessed. The method provided a partial fractionation of membrane and cytosolic proteins. The total protein 2-D profiles of three serotype 1/2a strains and one serotype 1/2b strain isolated from food were compared to the *L. monocytogenes* EGDe proteome. An average of 13% of the major protein spots in the food strain proteomes were unmatched in strain EGDe. Variation was greater in the less intense spots with an average 28% beginning unmatched. Two of the proteins identified in *L. monocytogenes* EGDe were missing in one or more of the food isolates. The two proteins were from the main glycolytic pathway and from the metabolism of coenzymes and prosthetic groups. The two corresponding genes were

indicated, by PCR amplification, to be present in the four food isolates. The results show that the *L. monocytogenes* EGDe reference map is a valuable starting point for analyses of strains of varying origin, and could be useful for analyzing the proteomes of different isolates of this pathogen.

Introduction

Listeria monocytogenes is a gram-positive facultative intracellular pathogen mainly associated with infections in certain human risk groups, including pregnant woman, newborns, and immuno-compromised patients [40]. It is wide-spread in nature and may be transferred to humans by contaminated foods [12]. This pathogen is able to survive food-processing technologies such as high concentrations of salt and relatively low pH, as well as being capable of multiplication at refrigeration temperatures [24]. Many of the preservation methods and cleaning compounds in the food industry target the bacterial cell membrane, and furthermore many successful drugs act by modulating the activity of membrane proteins [37]. Methods that especially monitor membrane proteins are therefore of vital interest.

Two-dimensional (2-D) polyacrylamide gel electrophoresis (PAGE) of bacterial proteins was first introduced more than 25 years ago [29]. The principle is based upon the separation of proteins by their isoelectric point (pI) in the first dimension and according to molecular weight (MW) in the second dimension. Over the years, the technique has been improved, and presently has the potential to resolve thousands of proteins from a complex sample [15].

Previous 2-D analysis on *L. monocytogenes* proteins has focused on responses to stress, including resistance to antimicrobial compounds [9, 17, 34], pH stress [7, 28, 32, 35], high salinity [11], or cold shock [2, 19, 42]. We know of no studies using 2-D analysis that focused on membrane proteins in *L. monocytogenes*. Two-dimensional electrophoretic analysis has also been used for the identification and classification of *Listeria* [16]; the authors observed that proteome similarity was highest for strains of the same serovar, with larger variation occurring between serovars.

The general knowledge about the molecular constituents of *L. monocytogenes* has been greatly enhanced by the recent release of the genomic sequence of *L. monocytogenes* EGDe [14]. The

release of this sequence provides a resource for the comparison of genomes and proteomes of strains of *L. monocytogenes* from various sources. Strain EGDe is an animal isolate of serotype 1/2a, which is the only serotype that is prevalent in illness as well as in foods and food processing facilities [see 22, for a recent review]. The strain may therefore be expected to be a good reference organism for clinical as well as food isolates. There is, however, a relatively high genetic diversity within serotype 1/2a, and a pronounced diversity in food strains in general [22].

In this study, we present a total protein 2-D reference map of abundant proteins in exponentially dividing cells under standardized, optimal growth conditions using *L. monocytogenes* EGDe as model organism. Furthermore, we evaluate a membrane protein extraction procedure for *L. monocytogenes* based on the method developed for *Escherichia coli* by Ames and Nikaido [1]. In order to assess how well the reference map represents strains originating from food with focus on serotype 1/2a, we compared the *L. monocytogenes* EGDe proteome reference map with the 2-D profiles of a serotype 1/2b and several serotype 1/2a food isolates.

Materials and methods

Bacterial strains and growth conditions

L. monocytogenes EGDe (animal isolate; serotype 1/2a) [14], B73 (meat isolate; serotype 1/2a) [10], 412 (isolated from raw salted pork; serotype 1/2a) [18], 386 (isolated from heat-treated pork; Danish Meat Research Institute, Roskilde, Denmark; serotype 1/2b), and O57 (isolated from lightly pickled salmon; serotype 1/2a)[3] were maintained on Brain Heart Infusion (BHI) broth or agar at 37°C.

Preparation of cells prior to protein isolation

L. monocytogenes strains were grown without shaking until mid-exponential phase (optical density at 600 nm, 0.45-0.5). Chloramphenicol (Sigma, St. Louis, MO) was added to a final concentration of 20 µg/ml to halt protein synthesis. Bacterial cells were harvested by centrifugation (8,000 x *g*, 15 min, 4°C). The cell pellet was washed once using 10 mM phosphate buffered saline (pH 7.0) and twice in 32 mM Trizma® Pre-Set Crystals (pH 7.5) (Sigma). Wash buffers were also supplemented with chloramphenicol to the same final concentration as above. The washed cell pellet was resuspended in TE (10 mM Tris, 1 mM EDTA, pH 7.5) containing a

Complete™ Mini tablet (protease inhibitors; one cocktail mini tablet per five ml TE; Roche, Mannheim, Germany) and stored at –80°C. Cell suspensions were thawed on ice and transferred to FastProtein™ Blue tubes (Bio 101, Carlsbud, CA). The cells were disrupted, using the FastPrep® Instrument FP 120 (Bio 101) at a maximum tube velocity of 6.5 m/s for 45 seconds, and subsequently chilled on ice. This cycle was repeated five times. All chemicals and materials were obtained from Amersham Biosciences (Buckinghamshire, UK), unless otherwise indicated.

Fractionation of cellular proteins

Fractionation was based on the method of Ames and Nikaido [1], modified as follows: unbroken cells and cellular debris were sedimented by centrifugation (16,000 x *g*, 4°C, 25 min); the supernatant was treated with DNase I at 85 µg/ml and RNase I at 4.2 µg/ml (Boehringer Mannheim, Mannheim, Germany) and incubated at 37°C for 30 min. In order to separate the membrane from the cytosolic fraction, the homogenate was centrifuged (100,000 x *g*, 4°C, 80 min). The clarified supernatant, which was considered as the cytosolic fraction, was removed and stored at –20°C. The yellow pellet was resuspended in 200 µl 1% (w/v) sodium dodecyl sulfate (SDS) solution containing 100 mM dithiothreitol (DTT) (Sigma) and boiled for five min. To the boiled sample, 9.5 M urea, 100 mM (DTT), 8% (w/v) 3-[(3-cholamidopropyl)-dimethylammonio] 1-propane sulfonate (CHAPS), and 4% (v/v) Pharmalyte™ (pH 3-10) were added to the final concentrations indicated. The mixture was incubated at 30°C for two hours. Insoluble material was removed by centrifugation (18,000 x *g*, 45 min, 28°C), and the resulting supernatant was considered as the membrane fraction. The membrane fraction was stored at –80°C or used immediately for isoelectric focusing (IEF).

Total protein isolation

Cell lysates were treated with nucleases as described above. To the homogenate, 9.5 M urea, 100 mM DTT, 4% (w/v) CHAPS, and 2% (v/v) Pharmalyte™ (pH 3-10) were added to the final concentrations indicated. The mixture was incubated at 30°C for two hours. Insoluble material was removed by centrifugation (18,000 x *g*, 85 min, 28°C). The clarified supernatant was carefully removed and stored at –80°C or used immediately for IEF.

First-dimension electrophoresis

IEF was carried out using 11 cm precast Immobline DryStrips with a linear 4 to 7 pH gradient on a Multiphore II apparatus according to the manufacturers instructions. For total protein gels, approximately 75 µg or 7.5 µg of protein were cup-loaded at the anodic end for Coomassie or silver-stained gels, respectively. Prior to loading of the cytosolic fraction, 15 µl of this protein preparation was added to 85 µl of solubilization solution [9.5 M Urea, 100 mM DTT, 4% (w/v) CHAPS, 2% (v/v) Pharmalyte™ (pH 3-10) and incubated at 30°C for one hour. When a similar protein load was used for the membrane fraction gels as described for the total protein samples, a low numbers of spots was visualized. In order to increase the number of visualized spots, approximately double the protein load was utilized for compartmentalized gels, i.e. approximately 150 µg or 15 µg of protein for Coomassie or silver-stained gels, respectively. Protein concentrations were determined by the PlusOne™ 2-D Quant Kit (Amersham Biosciences). The following voltage gradient was applied: from 0 to 300 V in 0.01 h; 300 V for 6.5 h; from 300 V to 3500 V for 5 h; and 3500 V for 8 h.

Second-dimension electrophoresis

Electrophoresis in the second dimension was done on precast ExcelGel XL SDS 12-14 gels on a Multiphore II apparatus as described in the manufacturers instructions. IEF strips were equilibrated in SDS equilibration buffer as recommended with the following modifications: (i) the concentration of SDS and iodoacetamide (Sigma) was increased from 2 to 4% (w/v); (ii) each of the equilibration steps were carried out for 30 min instead of 15 min. MultiMark™ Multi-Colored Standards (Novex, San Diego, CA) were run in the second dimension to determine the relative molecular mass of proteins. Gels were stained either with Coomassie R250 or silver, the latter with an automated silver stainer. Gels that were prepared for mass spectrometry were stained with Coomassie colloidial blue G250 according to a previously described procedure [26].

Image Analysis

Coomassie-stained gels were scanned at a resolution of 200 dots per inch, and analyzed using the Z3 2-D gel image analysis system version 2.00 (Compugen Ltd., Jamesburg, NJ). Total spot numbers were determined by automated spot detection followed by manual editing. Major spots for analysis were defined as all spots with a minimum spot area of 50 pixels and a minimum spot

contrast of 25. For the comparison of total protein gels, a cut-off value of 200 predominant spots was used in order to standardize the number of spots being compared. Spots not fitting the criteria for major spots were considered as minor spots. A minimum of three Coomassie-stained gels was run of each sample, and a typical gel was used for computer-aided analysis. Absence of spots was visually verified on all Coomassie-stained gels and in addition on silver-stained gels for each sample.

Protein identification.

For N-terminal sequencing, the electroblotting, staining, and storage of the blot were done as described previously [36]. N-terminal sequencing was performed on a 491 Procise automated sequencer (Perkin-Elmer, Wellesley, MA).

Sequence data of internal peptides were acquired with a quadropole time-of-flight mass spectrometer (Q-TOF-MS) after electrospray ionisation performing tandem mass spectrometry (MS/MS). The membrane or cytosolic fraction gels of *L. monocytogenes* EGDe were destained and cut away from the backing, and selected spots were excised and dried at room temperature under reduced pressure. In-gel digestions were done on the dried gel pieces by treatment with 30-60 µl trypsin (2 µg/ml) in 50 mM NH_4HCO_3 (sequencing grade; Promega, Madison, WI) overnight at 37°C. The supernatant was removed and stored, and the gel pieces were incubated again with 40 µl to 80 µl 5% formic acid for 30 min. The same volume acetonitrile was added and the incubation was continued for 15 min. The supernatant was removed and pooled with the first supernatant, and the volume was reduced in a Speed Vac to approximately 15 µl. The generated peptides were desalted using C18-ZipTip™ (Millipore, Bedford, MA) as recommended by the manufacturer. The elution of the purified peptides was carried out with 5 µl of 65% MeOH, 0.5% formic acid. For electrospray analysis and subsequent peptide sequencing, 3 µl of ZipTip™ purified sample was filled into Au/Pd-coated nanospray glass capillaries (Protana, Odense, Denmark). The tip of the capillary was placed orthogonally in front of the entrance hole of a Quadrupole Time-of-Flight mass spectrometry instrument (Q-TOF II ™, Micromass, Manchester, UK) equipped with a nanospray ion source. A capillary voltage between 750 V and 1000 V and a cone voltage of 35 V were applied. Doubly and triply charged peptides were chosen for collision-induced MS/MS fragmentation experiments and the

corresponding parent ions were selectively transmitted from the quadrupole mass analyzer into the collision cell. Argon was used as collision gas and the kinetic energy was set between 20 eV and 35 eV. The resulting daughter ions were separated with an orthogonal time-of-flight mass analyzer. Peptide micro-sequencing and protein identification was carried out with the program "Peptide-Sequencing" within the BioLynx software (version 3.4, Micromass) and with the program "Sonar" (Proteometrics, New York, NY), respectively. The obtained trypsin fragment sequences were compared to the proteins predicted from the *L. monocytogenes* EGDe genome sequence (http://genolist.pasteur.fr/ListiList/).

Bioinformatics

The theoretical MW and pI of identified proteins were calculated from the predicted amino acid sequence using the ProtParam tool at the Expasy site (http://www.expasy.ch/tools/protparam.html). The grand average hydropathy (GRAVY) values were calculated according to Kyte and Doolittle [23], also using ProtParam. Transmembrane domains (TMD) were predicted using TMpred with default settings at http://www.ch.embnet.org/software/TMPRED_form.html. Only TMD values above 500 were considered to be significant.

The *L. monocytogenes* EGDe total protein reference map is available at http://www.mli.kvl.dk/foodmicro/special/index.htm.

PCR of the glyceraldehyde 3-phosphate dehydrogenase and phosphomethylpyrimidine kinase genes

The presence of the two genes was investigated by colony PCR using primers designed from the *L. monocytogenes* EGDe genome sequence (*lmo2459* and *lmo0662*, respectively) to cover a substantial part of the respective reading frames. For *lmo2459*, primers gapF1, 5'-GTCTAGCATTCCGTCGTATTC-3' and gapR1, 5'-AGCTCATTTCGTTATCGTACC-3', were used, giving 915 bp of the 1011 bp coding region (nucleotide 44-958). For *lmo0662*, primers thiDF1, 5'-CAATGGACCCAGACAACAAC-3' and thiDR1, 5'-TGCGACAGCTTCTTCAAC-3', were used, giving 584 bp of the 816 bp coding region (nucleotide 128-711). The PCR was run for 30 cycles with annealing at 52°C for 1 min.

Results

Total protein profile

A total of 261 spots were detected in the *L. monocytogenes* EGDe total protein Coomassie-stained gel (Fig. 1a). The reproducibility of the total protein profiles was evaluated from 2D gels of proteins extracted from two independent cultures of *L. monocytogenes* EGDe. The logarithms of the spot intensities from the two gels are plotted in Fig. 2. Most of the spots (97%) had a less than two and a half-fold difference in intensity (within the area delineated by the first set of dashed lines in Fig. 2). Moreover, no outliers were observed. The results indicate that, when using this procedure for proteome comparison, differences in spot intensity in excess of five fold (delineated by the second set of dashed lines in Fig. 2) would be due to biological rather than experimental variation.

Compartmentalization of cellular proteins

Visual comparisons of the 2-D profiles of the membrane and cytosolic fractions of *L. monocytogenes* EGDe suggested a distinctive overall protein pattern for each of the fractions (Fig. 3). Image analysis of the Coomassie-stained membrane fraction gel detected a total of 208 spots, of which 106 were considered to be major (see section on image analysis for definition of major spots). Similar analysis of the cytosolic fraction gel showed 420 spots of which 236 were major. A higher protein load was added to gels of the compartmentalized fractions than of the total protein samples. Computer-aided comparison of the membrane and cytosolic fraction gels showed that only 27 of the major spots in the membrane fraction were unique, i.e. were not discernible in the cytosolic fraction, whereas 161 of the major spots were unique in the cytosolic fraction. However, many of the common spots were enriched, having a substantially higher intensity in one of the two fractions.

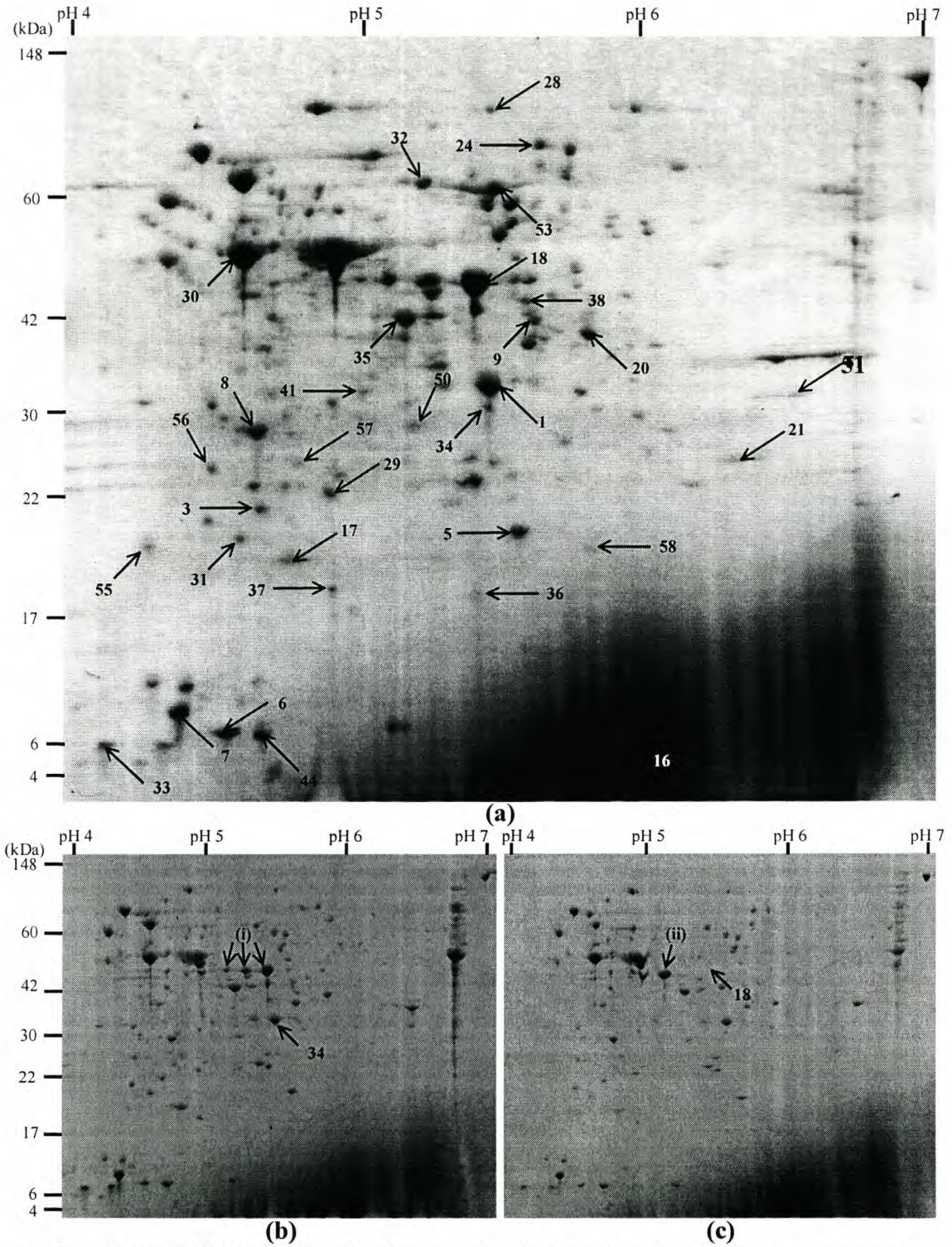


Fig. 1. Coomassie R250 stained 2-D gels of total cellular proteins from *Listeria monocytogenes* EGDe (a), B73 (b) and 412 (c). The numbered spots represent identified proteins in the *L. monocytogenes* EGDe total protein reference map. The identified proteins are described in Table 1. Strain B73 and 412 lacked spots 34 and 18, respectively. Arrows (i) show spots in strain B73 corresponding to glyceraldehyde 3-phosphate dehydrogenase identified by Michel Hebraud (personal communication), the leftmost of which is shown by (ii) in strain 412 (see text for details).

Table 1. Proteins identified from 2-D gels of *Listeria monocytogenes* EGDe.

Spot number	Protein identification	mol wt (kDa)		pI		Functional class[1]	TMD prediction[2]	GRAVY value
		Observed	Predicted	Observed	Predicted			
Spots excised from membrane fraction gels								
1	FbaA - *lmo2556* Similar to fructose-1,6-bisphosphate aldolase	23	30.0	5.47	5.20	2.1.1	1/0	-0.034
3	*lmo2829* Similar to yeast protein Frm2p in fatty acid signaling	20	22.2	4.77	4.70	2.4	1/1	-0.174
5	RplJ - *lmo0250* Ribosomal protein L10	16	17.7	5.60	5.36	3.7.1	1/0	-0.080
7	RplL - *lmo0251* Ribosomal protein L12	10	12.5	4.54	4.54	3.7.1	1/1	0.143
16	*lmo2149* Similar to proteins with no known function	9.25	11.9	6.00	5.53	5.2	0/0	-0.488
29	ClpP - *lmo2468* ATP-dependent Clp protease proteolytic subunit	18.5	21.6	5.06	4.94	4.1	3/2	-0.072
36	TufA – *lmo2653* Highly similar to translation elongation factor EF-Tu	13.5	43.34	5.49	4.81	3.7.4	1/0	-0.272
37	*lmo1580* Similar to proteins with no known function	14	16.9	5.05	4.98	5.2	1/0	-0.192

38	MreB – *lmo1548* Similar to cell-shape determining protein	35	35.5	5.66	5.16	1.1	2/1	0.061
41	*lmo1011* Similar to tetrahydrodipicolinate succinylase	24	24.8	5.14	4.95	2.2	6/2	0.193
55	LmaA - *lmo0118* Antigen A	16.1	18.1	5.40	4.47	4.5	1/0	-0.093
58	*lmo0273* Similar to proteins with no known function	16	18.8	5.80	5.45	5.2	0/0	-0.387
Spots excised from cytosolic fraction gels								
1	FbaA - *lmo2556* Similar to fructose-1,6-bisphosphate aldolase	23	30.2	5.5	5.20	2.1.1	1/0	-0.034
17	Fri - *lmo0943* Non-heme iron-binding ferritin	14.7	18.1	4.83	4.86	4.1	0/0	-0.371
18	GAPDH - *lmo2459* Similar to glyceraldehyde 3-phosphate dehydrogenase	38	36.3	5.3	5.20	2.1.2	1/0	-0.115
20[3]	Pfk - *lmo1571* Similar to 6-phosphofructokinase	30	34.4	5.79	5.46	2.1.1	4/1	-0.104
21	Upp - *lmo2538* Similar to uracil phosphoribosyltransferase	30	22.9	5.77	5.70	2.3	1/1	-0.035
24	PykA - *lmo1570* Similar to pyruvate kinases	64	62.6	5.64	5.39	2.1.1	3/1	-0.034

28	PnpA - *lmo1331* Polynucleotide phosphorylase (PNPase)	85	79.6	5.49	5.23	2.3	2/1	-0.308
29	ClpP - *lmo2468* ATP-dependent Clp protease proteolytic subunit	18.5	21.6	4.96	4.94	4.1	3/0	-0.072
30	Eno - *lmo2455* Similar to enolase	43	46.5	4.71	4.70	2.1.2	1/0	-0.244
31	*lmo0796* Similar to proteins with no known function	16	19.5	4.71	4.69	5.2	0/0	-0.460
32	Pgm – *lmo2456* Similar to phosphoglycerate mutase	53	56.1	5.29	5.10	2.1.2	0/0	-0.342
33	CspB –*lmo2016* Similar to major cold-shock protein	8.5	7.29	4.24	4.44	4.1	0/0	-0.398
34	ThiD – *lmo0662* Similar to phosphomethylpyrimidine kinase ThiD	22	28.8	5.41	5.25	2.5	2/1	-0.025
35	Tsf – *lmo1657* Translation elongation factor	32.5	32.6	5.20	5.11	3.5.3	0/0	-0.430
44	PtsH - *lmo1002* PTS phosphocarrier protein (Hpr)	9	9.4	4.73	4.81	1.2	1/0	-0.067
50	Adk - *lmo2611* Similar to adenylate kinases	22	24.2	5.23	5.08	2.3	0/0	-0.586
51	SerS - *lmo2747* Seryl-tRNA synthetase	22	49.2	6.26	5.26	3.7.2	1/0	-0.553

53	*lmo0355* Similar to flavocytochrome C fumarate reductase chain A	49	54.6	5.54	5.71	1.4	2/2	-0.489
56	*lmo2376* Similar to peptidyl-prolyl cis-trans isomerase	19.5	21.5	4.57	4.59	3.9	0/0	-0.327
57	DeoD - *lmo1856* Purine nucleoside phosphorylase	20	25.5	4.84	4.87	2.3	2/1	0.073
Spots excised from total protein gels								
6	*GroES* – lmo2069 Class I heat shock protein	8.50	10.05	4.72	4.59	3.9	0/0	-0.104
7	*RplL* – lmo0251 Ribosomal protein L12	10.85	12.44	4.57	4.54	3.7.1	1/1	0.143
8	*Tpi* – lmo2457 Triosephosphate isomerase	27.42	26.86	4.77	4.78	2.1.2	2/1	0.096
9[3]	MptA - *lmo0096* Similar to mannose-specific PTS enzyme IIAB	35	34.99	5.35	5.32	1.2	1/1	-0.123
9[3]	Pfk - *lmo1571* Similar to 6-phosphofructokinase	35	34.42	5.35	5.46	2.1.1	1/1	-0.104

Spots 6, 7, and 8 were identified by N-terminal sequencing, and all others by Time-of-Flight mass spectrometry.

[1] Functional classification codes according to genolist (http://genolist.pasteur.fr/ListiList/help/function-codes.html)

1. Cell envelope and cellular processes
1.1 Cell wall
1.2 Transport/binding proteins and lipoproteins
1.3 Membrane bioenergetics
2. Intermediary metabolism
2.1 Metabolism of carbohydrates and related molecules
2.1.1 Specific pathways
2.1.2 Main Glycolytic pathways
2.2 Metabolism of amino acids and related molecules
2.3 Metabolism of nucleotides and nucleic acids
2.4 Metabolism of lipids
2.5 Metabolism of coenzymes and prosthetic groups
3. Information pathways
3.5 RNA synthesis
3.5.3 RNA synthesis – Elongation
3.7 Protein synthesis
3.7.1 Protein synthesis - Ribosomal proteins
3.7.2 Protein synthesis - Aminoacyl-tRNA sysnthetases
3.7.3 Protein synthesis - Protein synthesis Initation
3.9 Protein folding
4. Other functions
4.1 Adaptation to atypical conditions
4.5 Miscellaneous
5. Similar to unknown proteins
5.2 From other organisms

[2]The detected / significant TMDs as calculated at http://www.ch.embnet.org/software/TMPRED_form.html
[3]Identified previously [17]

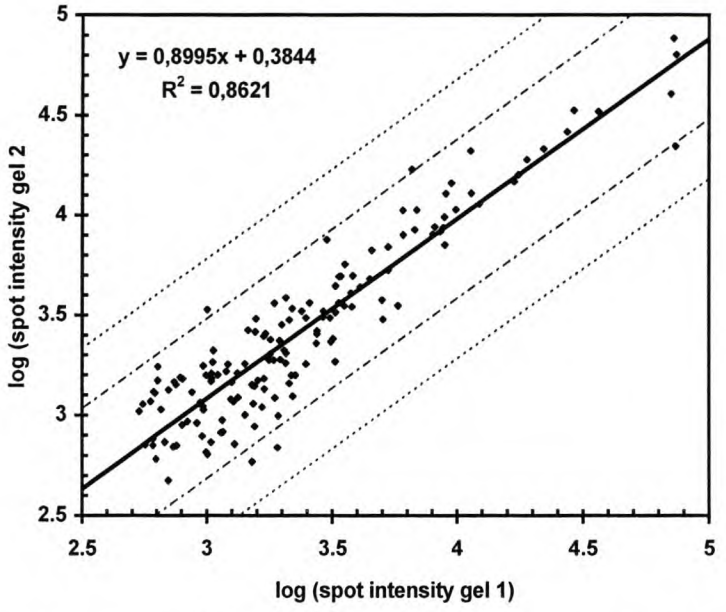


Fig. 2. Double logarithmic plot showing reproducibility of two independent protein extractions and 2-D analyses of *Listeria monocytogenes* EGDe total protein isolations. The logarithms of the spot intensities are plotted, and the regression line and R^2 value are presented. The two sets of dashed lines represent 2.5 and five-fold differences in intensities.

Identification of proteins

A total of 12 unique and enriched spots from the membrane and 20 from the cytosolic fraction (Fig. 3) were excised and identified. Additionally, four spots from a total protein gel were analyzed. Identified proteins and their characteristics are presented in Table 1. All sequenced trypsin fragments from each of the analyzed spots had 100% identity with the corresponding region in the predicated amino acid sequence of the protein. The theoretical pI and MW values were generally in good agreement with the experimentally observed values. Clear deviations in theoretical and experimentally observed pI could be due to post-translational modifications.

From the 36 spots that were analyzed, 33 different proteins were identified, as three proteins were found in more than one fraction. Spots 1 (fructose-1,6-bisphosphate aldolase) and 29 (ClpP) were excised from both the membrane and the cytosolic fraction gels, and spot 7 (ribosomal protein L12) was isolated from the membrane fraction and total protein gels. The identified proteins represented each of the four major functional classes. Four were cell envelope and cellular process category proteins (class 1), 14 were intermediary metabolism class proteins (class 2), seven were information pathways class proteins (class 3), three were considered to be

involved in adaptation to atypical conditions (subclass 4.1), and one was antigen A (miscellaneous functions subclass, 4.2). Four proteins had no known function in *Listeria* (Table 1).

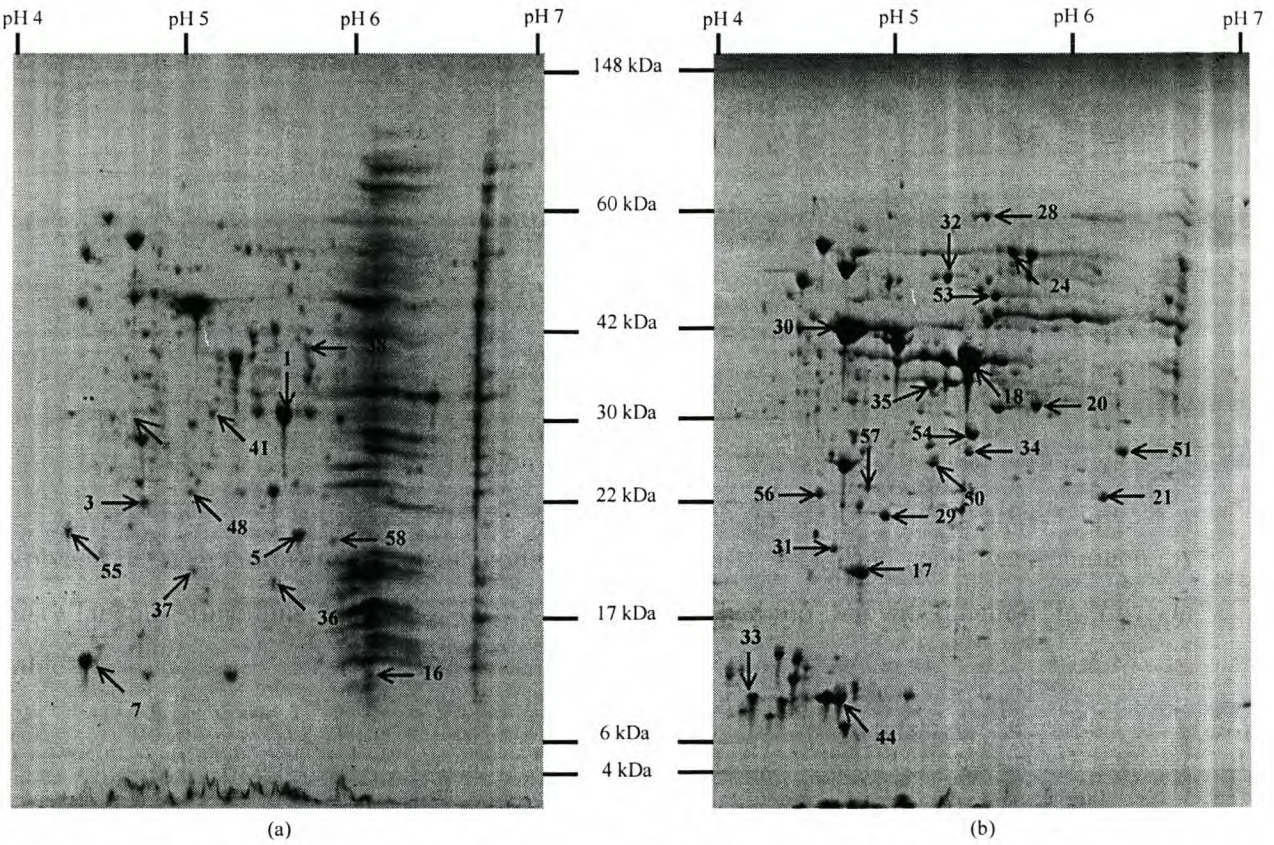


Fig. 3. Coomassie R250 stained 2-D gels of the membrane fraction (a) and cytosolic fraction (b) protein extracts of *Listeria monocytogenes* EGDe. Spots from the two fractions that were excised and identified are numbered. Identified proteins are described in Table 1.

Membrane proteins generally contain hydrophobic domains. A measure of the average protein hydrophobicity is the GRAVY value with hydrophobic and hydrophilic proteins possessing positive and negative GRAVY values, respectively. A total of five proteins had a positive GRAVY value. These were the ribosomal protein L12, the cell shape determining protein MreB, and tetrahydrodipicolinate succinylase (spots 7, 38 and 41, respectively) from the membrane fraction and purine nucleotide phosphorylase, DeoD (spot 57) from the cytosolic fraction. One protein from the total protein gels, spot 8 corresponding to triosephosphate isomerase, Tpi, also had a positive GRAVY value.

The TMpred program predicts the likelihood that a protein traverses a membrane, as well as the most likely orientation of the protein in the membrane. TMDs were detected in 21 of the 33 identified proteins. Of the detected TMDs, the only significant ones were for four proteins excised from the membrane fraction (spots 3, 7, 38, and 41), eight from the cytosolic fraction (spots 20, 21, 24, 28, 29, 34, 53, and 57), and two from the total protein gel (spots 8 and 9). Spot 53 (a homologue of the flavocytochrome C fumarate reductase chain A) from the cytosolic fraction had the highest predicted TMD score of 2305. All proteins with a positive GRAVY value also had at least one significant predicted TMD.

Five of the sequenced spots (17, 18, 24, 34, and 50) were unique to the cytosolic fraction; two of these (24 and 34) contained proteins with significant predicted TMDs. In the membrane fraction, spots 16, 36, 37, and 38 were unique, and MreB (spot 38) had a significant TMD and a positive GRAVY value. One of the five proteins with positive GRAVY values, DeoD (spot 57) was enriched in the cytosolic fraction. There was therefore no distinct correlation between GRAVY value or predicted TMD and observed fraction.

Proteome reference map of *L. monocytogenes* EGDe

Comparison of the membrane and cytosolic fraction gels with total protein gels revealed that all spots found in each of the respective fractions were present in total protein gels stained either with Coomassie or silver. Subsequently, a total protein 2-D reference map of *L. monocytogenes* EGDe was constructed containing the 33 identified proteins (Fig. 1).

Comparison of food isolates with *L. monocytogenes* EGDe

The reference map was used to assess the similarity between *L. monocytogenes* serotype 1/2a and 1/2b strains isolated from food and the serotype 1/2a animal strain EGDe. Visual comparison indicated that the total protein profiles of the food-isolated strains were highly similar to strain EGDe (Fig. 1). The number of spots that were not matched in *L. monocytogenes* EGDe, after computer aided analysis of gels with total protein samples, are presented in Table 2. From four to eight of the major spots in the food strains were unmatched in *L. monocytogenes* EGDe. *L. monocytogenes* 412 (serotype 1/2a) had the highest level of unmatched major proteins, and also lacked one of the identified proteins in *L. monocytogenes* EGDe (see below). Comparison of minor spots resulted in an up to ten-fold increase in the number of unmatched spots in *L.*

monocytogenes EGDe. Strain O57 (serotype 1/2a) had the highest number of unmatched minor spots. The proteome of strain 386 (serotype 1/2b) did not differ more from the reference strain EGDe than the serotype 1/2a food isolates.

Table 2. Comparison of 2-D total protein profiles of *Listeria monocytogenes* food isolates with *L. monocytogenes* EGDe. The numbers of major and minor spots in the food isolates that were unmatched in strain EGDe (serotype 1/2a) are shown.

L. monocytogenes Strain and serotype	Unmatched major spots[1]	Unmatched minor spots[2]
B73 (1/2a)	4 out of 57 (7%)	43 out of 143 (30%)
412 (1/2a)	8 out of 42 (19%)	40 out of 158 (25%)
O57 (1/2a)	7 out of 46 (15%)	52 out of 154 (34%)
386 (1/2b)	5 out of 54 (9%)	32 out of 146 (22%)

[1] Only spots with a minimum spot area of 50 pixels and a minimum spot contrast of 25 were considered.

[2] Minor spots were considered as those of the 200 most significant spots that did not fit the criteria for major spots.

Of the 33 spots identified in *L. monocytogenes* EGDe, only two were not detected in the total protein profiles of all the food isolates analyzed. Spot 18 (glyceraldehyde 3-phosphate dehydrogenase, GAPDH) was absent from *L. monocytogenes* 412, and spot 34 (phosphomethylpyrimidine kinase) was missing from strains B73, O57, and 386. The presence of the two corresponding genes was tested by colony PCR using internal primers covering a substantial part of the reading frame. For each gene, strains B73, 412, O57, and 386 gave PCR products of the same size as strain EGDe, showing that all four food strains contained the corresponding chromosomal regions without discernible insertions or deletions.

Discussion

Proteome reference map of *L. monocytogenes* EGDe

A partially annotated total protein 2-D map of *L. monocytogenes* EGDe was constructed from the identified proteins (Fig. 1a and Table 1). The map represents proteins from each of the four major functional classes defined for *L. monocytogenes* EGDe [14]. Only six of the 33 identified proteins, Fri [9, 17, 19, 31, 32], GAPDH [8], Pfk [17, 39], Pgm [8], TufA [8], and the mannose-specific PTS enzyme IIAB [8, 17, 34] have been identified before in *L. monocytogenes* by 2-D analysis.

Previously generated total protein 2-D maps of other microorganisms contained very few if any proteins with an overall hydrophobic amino acid composition [25]. In the 2-D reference map presented in this study, five of the identified proteins had a positive GRAVY value and 42% had significant predicted TMDs.

Evaluation of procedures

Our data analysis showed that the procedure, i.e. total protein isolation, IEF, second dimensional electrophoresis, and spot quantification, had a good reproducibility, and that we with confidence can consider above five-fold differences in spot intensities as meaningful variations in protein expression.

The *L. monocytogenes* genome contains 2853 annotated open reading frames [14]. It has been estimated that 30% of the open reading frames from previously sequenced organisms encode transmembrane proteins [30, 41], which would correspond to approximately 850 transmembrane proteins in the *L. monocytogenes* EGDe proteome. Under the experimental window of molecular mass 4 to 148 kDa and pI range 4-7, we detected 261 spots in Coomassie stained gels of total protein samples and 208 spots in gels of membrane fraction extractions from *L. monocytogenes* EGDe. The difference in coding capacity and visualized proteins could possibly be explained by some of the following: (i) some proteins are not in the experimental window; (ii) insolubility of certain proteins in the IEF sample buffer [37]; (iii) less sensitive detection limit of Coomassie staining; (iv) some proteins were not expressed under the growth conditions employed or at the

growth phase at the time of cell harvesting; and, (v) membrane proteins are generally low copy number proteins so they are not very abundant.

This is, to our knowledge, the first report on the use of 2-D gel electrophoresis to specifically assess membrane protein profiles of *L. monocytogenes*. A modification of one of the first protocols used to visualize membrane proteins [1] was employed, in that we ran the first dimensional electrophoresis on immobilized pH gradients which yield highly reproducible protein profiles between laboratories [5] and increased protein loading capacity [33]. The compartmentalization procedure was assessed from the 33 identified proteins (Fig. 3 and Table 1). We compared the GRAVY values and predicted TMDs with the spot intensity in the two fractions, however, no direct correlation was observed. For example, only one (spot 7, ribosomal protein L12) of the five proteins with positive GRAVY values was substantially enriched in the membrane fraction, and ClpP (spot 29) having two predicted TMDs was distinctly more intense in the cytosolic than in the membrane fraction (excised and identified from both). However, three of the five proteins with positive GRAVY values were from the membrane fraction. Although the methods only give an indication of cellular location, since proteins with negative GRAVY values may contain hydrophobic domains and the TMD prediction algorithm is not ideal for prokaryotes [27], the observations suggest that the procedure provides a partial but incomplete fractionation and does not clearly reflect the cellular location of the proteins.

Comparison of food isolates with strain EGDe

L. monocytogenes EGDe has in several occasions been noticed to be less robust than a number of other strains when subjected to stresses including acid [6] or carbon dioxide (Jydegaard-Axelsen and Knøchel, unpublished data). Nonetheless, our results indicate that *L. monocytogenes* EGDe would be a useful reference organism for the study of the proteomes of strains isolated from food. Our results do not, however, permit speculation on the cause of the different phenotypes or on the adaptation to different environments.

We compared both major and minor proteins of three serotype 1/2a *L. monocytogenes* strains and one serotype 1/2b strain originating from food with strain EGDe (Table 2). An average of 13% of the major proteins of the food strains were not detected in strain EGDe. When the intensity of

the spots being compared was decreased, the average percentage of unmatched spots in strain EGDe increased to 28%.

Two of the 33 spots identified from strain EGDe were missing in one or more of the food isolates. The two proteins were glyceraldehyde 3-phosphate dehydrogenase (GAPDH) from the main glycolytic pathway and phosphomethylpyrimidine kinase from the metabolism of coenzymes and prosthetic groups. PCR analyses indicated that the two corresponding genes were present in all five strains. The absence of the spots could thus be due to either an extremely low expression level (below the detection limit of silver-stained gels), or a change in pI or MW giving rise to a different location on the gel. GAPDH is an essential enzyme in the glycolytic pathway and expected to be highly expressed in all organisms, even though a reduced expression may in some cases be sufficient to sustain normal growth [38]. The *L. monocytogenes* EGDe genome sequence does not contain a reading frame corresponding to an auxiliary GAPDH protein, as seen in some Gram-positive bacteria [13, 43]. The GAPDH protein has been reported to exist in several forms with conserved molecular weight but differing pI [4, 42]. Similarly, three forms of GAPDH have been identified from 2D gel analysis of *L. monocytogenes* (Michel Hebraud, personal communication). In Fig. 1b, arrows (i) indicate the corresponding locations of the three forms of GAPDH in strain B73, the rightmost being spot 18. Comparison of Fig. 1b and 1c shows that strain 412 has an increased intensity of the leftmost of the putative GAPDH forms (Fig. 1c, arrow (ii)), corresponding to the missing spot 18. This indicates that 412 possess only one form of GAPDH as opposed to the other tested strains, which all had three possible forms of the protein. A functional role of the different forms of the GAPDH protein has as yet not been proved [43].

In our study, we compared strains of serotype 1/2a and 1/2b. Variations between serotypes were shown in a previous study [16] to be larger than those within serotypes. Only 46.7% of the spots were common amongst *L. monocytogenes* strains across serotypes, and serotypes 1/2a and 1/2b were in two different major clusters [16]. In our hands, the difference between serotype 1/2b and 1/2a strains was not greater than the variation within serotype 1/2a. Genomic comparisons by subtractive hybridization showed that 5% of the genome of a serotype 4b strain did not hybridize to the genome of *L. monocytogenes* EGD, a variant of strain EGDe and also serotype 1/2a [20]. The larger variability observed at the proteomic level can be attributed to the fact that while a

single amino acid change in a protein can result in a shift in pI giving a detectable modification in the 2-D pattern [21], the corresponding DNA change would not be registered by hybridization.

We have by construction of the *L. monocytogenes* EGDe total protein reference map, based on a reproducible protein extraction and 2-D analysis procedure, established a platform for further work on protein expression in this pathogen. The observed variations indicate that most of the predominant proteins in food isolates could be identified with a degree of confidence from the EGDe map, while the identification of less intense spots would require greater caution.

ACKNOWLEDGEMENTS

We wish to thank Carmen Buchrieser and Philippe Glaser for the serotyping of *L. monocytogenes* B73, 412, 386, and O57, Henrik Siegumfeldt for construction of the reference map website, and Lene Gertman for expert technical assistance. This work was supported by the Food Biotechnology Program of the Danish Ministry for Food, Agriculture, and Fisheries (BIOT99-8), and by a grant from The Danish Rectors' Conference and the National Research Foundation (South Africa) to MR and JWH.

Reference List

1 **Ames, G. F. and K. Nikaido**. 1976. Two-dimensional gel electrophoresis of membrane proteins. Biochemistry **15**:616-623.

2 **Bayles, D. O., B. A. Annous, and B. J. Wilkinson**. 1996. Cold stress proteins induced in *Listeria monocytogenes* in response to temperature downshock and growth at low temperatures. Appl. Environ. Microbiol. **62**:1116-1119.

3 **Ben Embarek, P. K. and H. H. Huss**. 1993. Heat resistance of *Listeria monocytogenes* in vacuum packaged pasteurized fish fillets. Int. J. Food Microbiol. **20**:85-95.

4 **Cash, P., E. Argo, L. Ford, L. Lawrie, and H. McKenzie**. 1999. A proteomic analysis of erythromycin resistance in *Streptococcus pneumoniae*. Electrophoresis **20**:2259-2268.

5 **Corbett, J. M., M. J. Dunn, A. Posch, and A. Görg**. 1994. Positional reproducibility of protein spots in two-dimensional polyacrylamide gel electrophoresis using immobilised

pH gradient isoelectric focusing in the first dimension: an interlaboratory comparison. Electrophoresis **15**:1205-1211.

6 **Cotter, P. D., C. G. Gahan, and C. Hill**. 2001. A glutamate decarboxylase system protects *Listeria monocytogenes* in gastric fluid. Mol. Microbiol. **40**:465-475.

7 **Davis, M. J., P. J. Coote, and C. P. O'Byrne**. 1996. Acid tolerance in *Listeria monocytogenes*: the adaptive acid tolerance response (ATR) and growth-phase-dependent acid resistance. Microbiology **142**:2975-2982.

8 **Duché, O., F. Tremoulet, P. Glaser, and J. Labadie**. 2002. Salt stress proteins induced in *Listeria monocytogenes*. Appl. Environ. Microbiol. **68**:1491-1498.

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

10 **Dykes, G. A. and J. W. Hastings**. 1998. Fitness costs associated with class IIa bacteriocin resistance in *Listeria monocytogenes* B73. Lett. Appl. Microbiol. **26**:5-8.

11 **Esvan, H., J. Minet, C. Laclie, and M. Cormier**. 2000. Protein variations in *Listeria monocytogenes* exposed to high salinities. Int. J. Food Microbiol. **55**:151-155.

12 **Farber, J. M. and P. I. Peterkin**. 1991. *Listeria monocytogenes*, a food-borne pathogen. Microbiol. Rev. **55**:476-511.

13 **Fillinger, S., S. Boschi-Muller, S. Azza, E. Dervyn, G. Branlant, and S. Aymerich**. 2000. Two glyceraldehyde-3-phosphate dehydrogenases with opposite physiological roles in a nonphotosynthetic bacterium. J. Biol. Chem. **275**:14031-14037.

14 **Glaser, P., L. Frangeul, C. Buchrieser, C. Rusniok, A. Amend, F. Baquero, P. Berche, H. Bloecker, P. Brandt, T. Chakraborty, A. Charbit, F. Chetouani, E. Couve, A. de Daruvar, P. Dehoux, E. Domann, G. Dominguez-Bernal, E. Duchaud, L. Durant, O. Dussurget, K. D. Entian, H. Fsihi, F. G. Portillo, P. Garrido, L. Gautier, W. Goebel, N. Gomez-Lopez, T. Hain, J. Hauf, D. Jackson, L. M. Jones, U. Kaerst, J. Kreft, M. Kuhn, F. Kunst, G. Kurapkat, E. Madueno, A. Maitournam, J. M. Vicente, E. Ng, H. Nedjari, G. Nordsiek, S. Novella, B. de Pablos, J. C. Perez-Diaz, R. Purcell, B. Remmel, M. Rose, T. Schlueter, N. Simoes, A. Tierrez, J. A. Vazquez-Boland, H. Voss, J. Wehland, and P. Cossart**. 2001. Comparative genomics of *Listeria* species. Science **294**:849-852.

15 **Görg, A., C. Obermaier, G. Boguth, A. Harder, B. Scheibe, R. Wildgruber, and W. Weiss**. 2000. The current state of two-dimensional electrophoresis with immobilized pH gradients. Electrophoresis **21**:1037-1053.

16 **Gormon, T. and L. Phan-Thanh**. 1995. Identification and classification of *Listeria* by two-dimensional protein mapping. Res. Microbiol. **146**:143-154.

17 **Gravesen, A., M. Ramnath, K. B. Rechinger, N. Andersen, L. Jansch, Y. Hechard, J. W. Hastings, and S. Knøchel**. 2002. High-level resistance to class IIa bacteriocins is associated with one general mechanism in *Listeria monocytogenes*. Microbiology **148**:2361-2369.

18 **Gravesen, A., P. Warthoe, S. Knøchel, and K. Thirstrup**. 2000. Restriction fragment differential display of pediocin-resistant *Listeria monocytogenes* 412 mutants shows consistent overexpression of a putative beta-glucoside-specific PTS system. Microbiology **146**:1381-1389.

19 **Hébraud, M. and J. Guzzo**. 2000. The main cold shock protein of *Listeria monocytogenes* belongs to the family of ferritin-like proteins. FEMS Microbiol. Lett. **190**:29-34.

20 **Herd, M. and C. Kocks**. 2001. Gene fragments distinguishing an epidemic-associated strain from a virulent prototype strain of *Listeria monocytogenes* belong to a distinct functional subset of genes and partially cross-hybridize with other *Listeria* species. Infect. Immun. **69**:3972-3979.

21 **Jungblut, P. R.** 2001. Proteome analysis of bacterial pathogens. Microbes. Infect. **3**:831-840.

22 **Kathariou, S.** 2002. *Listeria monocytogenes* virulence and pathogenicity, a food safety perspective. J. Food Prot. **65**:1811-1829.

23 **Kyte, J. and R. F. Doolittle**. 1982. A simple method for displaying the hydropathic character of a protein. J. Mol. Biol. **157**:105-132.

24 **Lou, Y. and A. E. Yousef**. 1997. Adaptation to sublethal environmental stresses protects *Listeria monocytogenes* against lethal preservation factors. Appl. Environ. Microbiol. **63**:1252-1255.

25 **Molloy, M. P.** 2000. Two-dimensional electrophoresis of membrane proteins using immobilized pH gradients. Anal. Biochem. **280**:1-10.

26 **Neuhoff, V., N. Arold, D. Taube, and W. Ehrhardt**. 1988. Improved staining of proteins in polyacrylamide gels including isoelectric focusing gels with clear background at nanogram sensitivity using Coomassie Brilliant Blue G-250 and R-250. Electrophoresis **9**:255-262.

27 **Nouwens, A. S., S. J. Cordwell, M. R. Larsen, M. P. Molloy, M. Gillings, M. D. Willcox, and B. J. Walsh**. 2000. Complementing genomics with proteomics: the membrane subproteome of *Pseudomonas aeruginosa* PAO1. Electrophoresis **21**:3797-3809.

28 **O'Driscoll, B., C. G. Gahan, and C. Hill**. 1997. Two-dimensional polyacrylamide gel electrophoresis analysis of the acid tolerance response in *Listeria monocytogenes* LO28. Appl. Environ. Microbiol. **63**:2679-2685.

29 **O'Farrell, P. H.** 1975. High resolution two-dimensional electrophoresis of proteins. J. Biol. Chem. **250**:4007-4021.

30 **Paulsen, I. T., M. K. Sliwinski, and M. H. Saier, Jr.** 1998. Microbial genome analyses: global comparisons of transport capabilities based on phylogenies, bioenergetics and substrate specificities. J. Mol. Biol. **277**:573-592.

31 **Phan-Thanh, L. and F. Mahouin**. 1999. A proteomic approach to study the acid response in *Listeria monocytogenes*. Electrophoresis **20**:2214-2224.

32 **Phan-Thanh, L., F. Mahouin, and S. Alige**. 2000. Acid responses of *Listeria monocytogenes*. Int. J. Food Microbiol. **55**:121-126.

33 **Rabilloud, T., C. Valette, and J. J. Lawrence**. 1994. Sample application by in-gel rehydration improves the resolution of two-dimensional electrophoresis with immobilized pH gradients in the first dimension. Electrophoresis **15**:1552-1558.

34 **Ramnath, M., M. Beukes, K. Tamura, and J. W. Hastings**. 2000. Absence of a putative mannose-specific phosphotransferase system enzyme IIAB component in a leucocin A-resistant strain of *Listeria monocytogenes*, as shown by two-dimensional sodium dodecyl sulfate- polyacrylamide gel electrophoresis. Appl. Environ. Microbiol. **66**:3098-3101.

35 **Ravishankar, S., M. A. Harrison, and L. Wicker**. 2000. Protein profile changes in acid adapted *Listeria monocytogenes* exhibiting cross-protection against an activated lactoperoxidase system in tryptic soy broth. Journal of Food Safety **20**:27-42.

36 **Rechinger, K. B., H. Siegumfeldt, I. Svendsen, and M. Jakobsen**. 2000. "Early" protein synthesis of *Lactobacillus delbrueckii* ssp. *bulgaricus* in milk revealed by [35S] methionine labeling and two-dimensional gel electrophoresis. Electrophoresis **21**:2660-2669.

37 **Santoni, V., M. Molloy, and T. Rabilloud**. 2000. Membrane proteins and proteomics: an amour impossible? Electrophoresis **21**:1054-1070.

38 **Solem, C., B. J. Koebmann, and P. R. Jensen**. 2003. Glyceraldehyde-3-phosphate dehydrogenase has no control over glycolytic flux in *Lactococcus lactis* MG1363. J. Bacteriol. **185**:1564-1571.

39 **Trémoulet, F., O. Duché, A. Namane, B. Martinie, The European Listeria Genome Consortium, and J. C. Labadie**. 2002. Comparison of protein patterns of *Listeria monocytogenes* grown in biofilm or in planktonic mode by proteomic analysis. FEMS Microbiol. Lett. **210**:25-31.

40 **Vazquez-Boland, J. A., M. Kuhn, P. Berche, T. Chakraborty, G. Dominguez-Bernal, W. Goebel, B. Gonzalez-Zorn, J. Wehland, and J. Kreft**. 2001. *Listeria* pathogenesis and molecular virulence determinants. Clin. Microbiol. Rev. **14**:584-640.

41 **Wallin, E. and G. von Heijne**. 1998. Genome-wide analysis of integral membrane proteins from eubacterial, archaean, and eukaryotic organisms. Protein Sci. **7**:1029-1038.

42 **Wemekamp-Kamphuis, H. H., A. K. Karatzas, J. A. Wouters, and T. Abee**. 2002. Enhanced levels of cold shock proteins in *Listeria monocytogenes* LO28 upon exposure to low temperature and high hydrostatic pressure. Appl. Environ. Microbiol. **68**:456-463.

43 **Willemoes, M., M. Kilstrup, P. Roepstorff, and K. Hammer**. 2002. Proteome analysis of a *Lactococcus lactis* strain overexpressing *gapA* suggests that the gene product is an auxiliary glyceraldehyde 3-phosphate dehydrogenase. Proteomics. **2**:1041-1046.

Chapter 3

High-level resistance to class IIa bacteriocins is associated with one general mechanism in Listeria monocytogenes

This chapter has been published in, *Microbiology* in 2002, Volume 148, pages 5223-5230. The article as published is enclosed as Chapter 3 of this thesis.

All proteomic investigations and minimum inhibitory concentration determinations was contributed my M. Ramnath

Microbiology (2002), **148**, 2361–2369 Printed in Great Britain

High-level resistance to class IIa bacteriocins is associated with one general mechanism in *Listeria monocytogenes*

Anne Gravesen,[1]† Manilduth Ramnath,[1,2]† K. Björn Rechinger,[1]‡ Natalie Andersen,[1] Lothar Jänsch,[3] Yann Héchard,[4] John W. Hastings[2] and Susanne Knøchel[1]

Author for correspondence: Anne Gravesen. Tel: +45 3528 3272. Fax: +45 3528 3231. e-mail: alg@kvl.dk

[1] Department of Dairy and Food Science, Centre for Advanced Food Studies, LMC, The Royal Veterinary and Agricultural University, Rolighedsvej 30, DK-1958 Frederiksberg C, Denmark

[2] Department of Biochemistry, University of Stellenbosch, Private Bag X1, 7602 Matieland, South Africa

[3] German Research Centre for Biotechnology, Department of Cell Biology, Mascheroder Weg 1, Braunschweig D-38124, Germany

[4] Laboratoire de Microbiologie Fondamentale et Appliquée, CNRS FRE 2224, IBMIG, UFR Sciences, 40 Avenue du Recteur Pineau, 86022 Poitiers Cedex, France

Class IIa bacteriocins may be used as natural food preservatives, yet resistance development in the target organisms is still poorly understood. In this study, the understanding of class IIa resistance development in *Listeria monocytogenes* is extended, linking the seemingly diverging results previously reported. Eight resistant mutants having a high resistance level (at least a 10^3-fold increase in MIC), originating from five wild-type listerial strains, were independently isolated following exposure to four different class IIa bacteriocin-producing lactic acid bacteria (including pediocin PA-1 and leucocin A producers). Two of the mutants were isolated from food model systems (a saveloy-type sausage at 10 °C, and salmon juice at 5 °C). Northern blot analysis showed that the eight mutants all had increased expression of EII^{Bgl} and a phospho-β-glucosidase homologue, both originating from putative β-glucoside-specific phosphoenolpyruvate-dependent phosphotransferase systems (PTSs). However, disruption of these genes in a resistant mutant did not confer pediocin sensitivity. Comparative two-dimensional gel analysis of proteins isolated from mutant and wild-type strains showed that one spot was consistently missing in the gels from mutant strains. This spot corresponded to the MptA subunit of the mannose-specific PTS, EII_t^{Man}, found only in the gels of wild-type strains. The *mptACD* operon was recently shown to be regulated by the σ^{54} transcription factor in conjunction with the activator ManR. Class IIa bacteriocin-resistant mutants having defined mutations in *mpt* or *manR* also exhibited the two diverging PTS expression changes. It is suggested here that high-level class IIa resistance in *L. monocytogenes* and at least some other Gram-positive bacteria is developed by one prevalent mechanism, irrespective of wild-type strain, class IIa bacteriocin, or the tested environmental conditions. The changes in expression of the β-glucoside-specific and the mannose-specific PTS are both influenced by this mechanism. The current understanding of the actual cause of class IIa resistance is discussed.

Keywords: pediocin, PTS, mannose, β-glucoside, sigma-54

INTRODUCTION

Class IIa bacteriocins (also called pediocin-like bacteriocins) constitute an abundant, highly homologous family of antimicrobial peptides that are active against the foodborne pathogen *Listeria monocytogenes* (Cleveland *et al.*, 2001; Ennahar *et al.*, 2000). These compounds are frequently produced by lactic acid bacteria in different types of food. The producer strains or bacteriocins have therefore been suggested as suitable biopreservatives, and some such starter cultures as well as fermentates thereof are now commercially available. However,

† The first two authors contributed equally to this work.

‡ **Present address:** Foss Electric A/S, Slangerupgade 69, DK-3400 Hillerød, Denmark.

Abbreviations: 2D, two-dimensional; PFK, 6-phosphofructokinase; PTS, phosphoenolpyruvate-dependent phosphotransferase system.

0002-5524 © 2002 SGM

subsequent resistance development in the target organisms is poorly characterized, and remains a major concern.

Cross-resistance between different class IIa bacteriocins has frequently been reported (Dykes & Hastings, 1998; Ramnath *et al.*, 2000; Rasch & Knøchel, 1998), indicating an identical or similar resistance mechanism. Previous studies aimed at characterizing mechanisms of class IIa resistance in *L. monocytogenes* have, however, reported seemingly varying results. Spontaneous resistance development resulted in an increase and a decrease of two different phosphoenolpyruvate-dependent phosphotransferase systems (PTSs), which are responsible for the uptake and concomitant phosphorylation of a number of sugars in both Gram-negative and Gram-positive bacteria (for a review, see Postma *et al.*, 1993). A leucocin A-resistant mutant of *L. monocytogenes* B73 no longer synthesized the IIAB subunit of a mannose-specific PTS (Ramnath *et al.*, 2000), and 12 independent mutants of *L. monocytogenes* 412 overexpressed two β-glucoside-specific PTS genes (Gravesen *et al.*, 2000). A mutant with resistance to divercin V41 had several changes in protein synthesis, which was suggested to be due to a mutation in a sigma transcription factor (Duffes *et al.*, 2000). This suggestion was partly based on the fact that transposon mutagenesis of *rpoN*, encoding the σ^{54} transcription factor, conferred resistance to the IIa bacteriocin mesentericin Y105 (Robichon *et al.*, 1997). Recently, the mannose-specific PTS, EII_t^{Man}, was shown by the construction of defined mutants to be directly involved in sensitivity to mesentericin Y105, and a specific domain of the MptD subunit was suggested to be involved in target recognition by the bacteriocin (Dalet *et al.*, 2001).

One explanation for the apparently diverging results may be that the specific strain in each study developed resistance by different mechanisms. Alternatively, the diverse observations could be 'windows' to the same, complex mechanism, revealed by the different experimental approaches. If different mechanisms do exist, it is conceivable that resistance developed in foods would differ from that developed under standard laboratory conditions.

The aim of the present study is to resolve the differences observed in previous work regarding class IIa resistance in *L. monocytogenes*, and to further the understanding of the underlying mechanisms, focusing on food relevance. We have compared IIa resistance developed in a panel of listerial wild-type strains following exposure to different IIa bacteriocins in a range of systems, including food. Our results indicate that one general mechanism is responsible for spontaneous class IIa resistance development in *L. monocytogenes*, and possible models incorporating the different observations are discussed.

METHODS

Bacterial strains and growth conditions. The *L. monocytogenes* wild-type strains and mutants are described in Table 1. Wild-type strains and spontaneous mutants were cultured in brain-heart infusion broth (BHI; Difco) at 30 or 37 °C without agitation, and the bacteriocin resistance or sensitivity phenotypes of harvested cultures were verified by plating on tryptone soya agar (TSA; Oxoid) plates supplemented with 30% pediocin PA-1 fermentate (see below). The deletion and insertional mutants were cultured in BHI at 37 °C, and chromosomal integration in harvested cultures was verified by parallel enumeration at 37 and 42 °C on TSA containing 5 μg erythromycin ml^{-1} or by PCR using primers complementary to the vector and to chromosomal DNA adjacent to the insert.

Class IIa bacteriocins. The class IIa bacteriocins used in this study were all prepared as fermentates of the producer organisms: *Pediococcus acidilactici* PA-2 producing pediocin PA-1 (Chr. Hansen A/S, Hørsholm, Denmark), *Leuconostoc gelidum* UAL 187-22 producing leucocin A (Papathanasopoulos *et al.*, 1997), *Leuconostoc carnosum* 4010 (Danish Meat Research Institute, Roskilde, Denmark) and *Carnobacterium piscicola* A9b (Nilsson *et al.*, 1999). Ammonium sulphate-precipitated fermentate of *C. piscicola* A9b was kindly supplied by Lilian Nilsson (Danish Institute for Fisheries Research, Lyngby, Denmark) and was added at 1% to TSA agar supplemented with 0·1% Tween 80. For the three other producer strains, stationary-phase cultures in de Man, Rogosa, Sharp broth (Oxoid) were catalase-treated and the pH adjusted to 6·5 with 5 M NaOH. The fermentate was subsequently harvested and sterile-filtered, and stored at −80 °C until use. Synthetic leucocin A (Ramnath *et al.*, 2000) was kindly supplied by S. Aimoto and K. Tamura (Osaka, Japan).

MIC of leucocin A. The MICs were determined by a spot-on-lawn assay, essentially as described previously (Ramnath *et al.*, 2000). Five-microlitre spots of a twofold serial dilution of 4 mg synthetic leucocin A ml^{-1} in 0·1% trifluoroacetic acid was spotted onto BHI agar lawns (0·7% agar, 0·1% Tween 80) containing approx. 10^7 c.f.u. listerial cells ml^{-1}. The MIC was determined as the minimal concentration giving a visible zone of inhibition after 20 h at 37 °C, as the median of three to four independent experiments.

Insertional inactivation of the putative β-glucoside-specific PTS genes. The putative β-glucoside-specific PTS enzyme II (EII^{Bgl}, annotated as *lmo00027*) and the phospho-β-glucosidase (*lmo00319*), which were overexpressed following pediocin resistance development in *L. monocytogenes* 412 (Gravesen *et al.*, 2000), were inactivated in *L. monocytogenes* 412 and 412P using the 9 kb temperature-sensitive integration vector pAUL-A (Chakraborty *et al.*, 1992). For EII^{Bgl}, a 1306 bp PCR fragment was made from chromosomal DNA of *L. monocytogenes* 412P using primers P1 (5′-CATCTGCTAAAGTTACG-ATTTCGCC-3′) and X2 (5′-AAYCAYGTNCCNGAYGT-3′, where the mixed bases N and Y correspond to ACGT and CT, respectively). P1 was designed from the previously found C-terminal gene fragment (Gravesen *et al.*, 2000), and the degenerate primer X2 was designed from an N-terminal conserved region in β-glucoside-specific EII permeases. An internal 674 bp *Eco*RI restriction fragment of the PCR product was cloned in pAUL-A, resulting in pAG540. This plasmid was transformed to *L. monocytogenes* 412 and 412P by electroporation (Park & Stewart, 1990) and integrated into the chromosome by propagation at 42 °C in the presence of 5 μg erythromycin ml^{-1} (Chakraborty *et al.*, 1992), resulting in strains *L. monocytogenes* AG122 and AG119, respectively. For inactivation of the phospho-β-glucosidase, a 991 bp PCR product made with primers P3 (5′-GGACTTTCCGTTCAA-GATG-3′) and P4 (5′-GTGGTTTTTGGTATCTATCC-3′) was cloned in pAUL-A, giving plasmid pAG538. The two primers were designed from the two previously found frag-

Table 1. *Listeria monocytogenes* strains

Spontaneous mutants isolated in this study were selected on tryptone soya agar (pH 6·5, 30 °C) containing fermentates from *P. acidilactici* PA-2 (ped PA-1), *Leuconostoc gelidum* UAL 187-22 (leu A), *Leuconostoc carnosum* 4010 (leu 4010) and *C. piscicola* A9b (carn A9b). Mutants in food systems were isolated as survivors following challenge with *Leuconostoc carnosum* 4010 in a saveloy-type meat model at 10 °C (leu 4010), or with *C. piscicola* A9b in salmon juice at 5 °C (carn A9b). *lmo00027* encodes a putative β-glucoside-specific PTS enzyme II, EII^{Bgl}; *lmo00319* encodes a putative phospho-β-glucosidase.

L. monocytogenes	Leucocin A MIC (μg ml^{-1})	Description	Reference or source*
Wild-type isolates			
412	0·98	Wild-type from raw, salted pork	Gravesen *et al.* (2000)
B73	0·98	Wild-type from meat	Dykes & Hastings (1998)
EGDe	1·95	Clinical	Glaser *et al.* (2001)
386	0·98	Wild-type from heat-treated pork	Anette Granly Larsen, DMRI
O57	1·95	Wild-type from lightly pickled salmon	Ben Embarek & Huss (1993)
Spontaneous mutants			
412P	$>4\times10^3$	Mutant of 412 isolated on pediocin PA-1	Gravesen *et al.* (2000)
412L-A1	$>4\times10^3$	Mutant of 412 isolated on leu A	This work
412L2	$>4\times10^3$	Mutant of 412 isolated on leu 4010	This work
412C2	$>4\times10^3$	Mutant of 412 isolated on carn A9b	This work
B73-MR1	$>4\times10^3$	Mutant of B73 resistant to leucocin A	Ramnath *et al.* (2000)
EGDeP4	$>4\times10^3$	Mutant of EGDe isolated on ped PA-1	This work
DMRICC 4053	$>4\times10^3$	Mutant of 386 isolated from a meat model containing leu 4010	Anette Granly Koch, DMRI
3.33A	$>4\times10^3$	Mutant of O57 isolated from salmon juice containing carn A9b	Lilian Nilsson, DIFRES
Defined mutants			
AG115	0·98	Insertional inactivation of *lmo00319* in strain 412	This work
AG117	$>4\times10^3$	Insertional inactivation of *lmo00319* in strain 412P	This work
AG119	$>4\times10^3$	Insertional inactivation of *lmo00027* in strain 412P	This work
AG122	0·98	Insertional inactivation of *lmo00027* in strain 412	This work
EGK51	$>4\times10^3$	Insertional inactivation of *manR* in strain EGDe	Dalet *et al.* (2001)
EGK54	$>4\times10^3$	Insertional inactivation of *mptA* in strain EGDe	Dalet *et al.* (2001)
EGY2	$>4\times10^3$	84 bp in-frame deletion in *mptD* in strain EGDe	Dalet *et al.* (2001)

* DMRI, Danish Meat Research Institute, Roskilde, Denmark; DIFRES, Danish Institute for Fisheries Research, Lyngby, Denmark.

ments of the gene (Gravesen *et al.*, 2000). Chromosomal integration of pAG538 in *L. monocytogenes* 412 and 412P resulted in strains *L. monocytogenes* AG115 and AG117, respectively. Correct integration was verified by PCR using standard primers complementary to the vector sequence combined with a primer recognizing a chromosomal region adjacent to the segment employed for insertion. For the EII gene, primer P1 was used. For the phospho-β-glucosidase, primer P4X (5′-ATAGCGCCAAGTCCGTTCTC-3′), situated 20 bp downstream of P4, was used. The class IIa bacteriocin sensitivity of the insertional mutants was tested by plating on TSA supplemented with 30% pediocin PA-1 fermentate, and by determining the MIC for leucocin A.

Northern hybridization. Expression analysis of the putative β-glucoside-specific PTS genes was modified after Gravesen *et al.* (2000). Cells were harvested from late-exponential cultures (OD_{600} approx. 0·4–0·6, depending on the strain) grown in BHI, pH 7·2, at 30 °C without selection, and lysed with a FastPrep FP 120 instrument (Bio 101) at a power setting of 6·0 m s^{-1} for 45 s. For analysis of insertional and deletion mutants, these strains and the corresponding wild-types were cultured at 37 °C. RNA was purified with the RNeasy Mini kit (Qiagen). The probe for EII^{Bgl} was the fragment A RFDD (restriction fragment differential display) PCR product (Gravesen *et al.*, 2000) encompassing 292 bp of the permease. For the putative phospho-β-glucosidase, the 991 bp P3–P4 PCR product was used. The probes were labelled with [α-^{33}P]dATP, and hybridization was visualized with a STORM 840 PhosphorImager (Molecular Dynamics).

Protein extraction for two-dimensional (2D) gel electrophoresis. *L. monocytogenes* strains were grown until late-exponential phase (OD_{600} 0·45–0·5) in BHI, pH 7·2, 37 °C. Chloramphenicol (Sigma) was added at a final concentration of 20 μg ml^{-1} to halt protein synthesis. Bacterial cells were harvested, washed, resuspended in buffer containing Complete Mini tablets (protease inhibitors; Roche), and stored at −80 °C. Once thawed, cell suspensions were disrupted using the FastPrep FP 120 instrument at a power setting of 6·5 for 45 s. This cycle was repeated five times, with chilling of the tubes on ice between cycles. The cell lysate was treated with DNase I and RNase I, and, subsequently, 9·5 M urea, 100 mM DTT (Sigma), 4% (w/v) CHAPS and 2% (v/v) Pharmalyte (pH 3–10) were added. The supernatant was clarified by centrifugation and stored at −80 °C. All reagents for the

protein isolation and 2D gel electrophoresis were from Amersham Pharmacia Biotech, unless stated otherwise.

First-dimension electrophoresis. Isoelectric focusing was carried out on immobilized pH gradients, pH 4–7 (11 cm Immobiline Drystrips), on a Multiphor II apparatus according to the manufacturers' instructions. The following voltage gradient was applied: from 0 to 300 V in 0·01 h; 300 V for 6·5 h; from 300 to 3500 V in 5 h; and 3500 V for 8 h. Protein samples were cup-loaded at the anodic end. For zoom gels, 18 cm Immobiline Drystrips, pH 5–6, were used, protein samples were loaded via rehydration, and isoelectric focusing was run according to the manufacturer's instructions.

Second-dimension electrophoresis. Prior to the SDS-PAGE run, isoelectric focusing strips were equilibrated in an SDS equilibration buffer as recommended by the manufacturer, with the following modifications: (1) the concentration of SDS in the SDS equilibration buffer was increased to 4% (w/v); and (2) the concentration of iodoacetamide (Sigma) for the second equilibration step was increased to 4% (w/v). Each equilibration step was carried out for 30 min. The second-dimension run was carried out on pre-cast ExcelGel XL SDS 12–14 gels according to the manufacturer's instructions. Gels were stained with either Coomassie brilliant blue or silver, and dried as described previously (Rechinger *et al.*, 2000). For the extended run of the zoom gel, electrophoresis was stopped after the normal run, the buffer strips were replaced, and the run was resumed for another 2 h.

2D gel analysis. For each protein sample, at least one Coomassie-stained and one silver-stained gel were examined. Images of Coomassie-stained gels were scanned at a resolution of 200 d.p.i. Quantification and spot matching between gels were done using Z3 Desk Top version 2.0 (Compugen). Differences of at least fourfold up-regulation or 0·3-fold down-regulation were noted by comparison of the 200 most intense spots on the gels of resistant mutants when compared with the corresponding wild-type strain gels. Differences that were found by Z3 analyses were verified by visual examination.

Identification of protein samples by MS. Protein samples from *L. monocytogenes* EGDe were excised from Coomassie-stained gels, and in-gel tryptic digestions were carried out overnight. The peptides generated were subjected to electrospray analysis and subsequent peptide sequencing using a quadrupole time-of-flight MS instrument (Q-TOF II; Micromass) equipped with a nanospray ion source. Doubly and triply charged peptides were chosen for collision-induced MS/MS fragmentation experiments, and the corresponding parent ions were selectively transmitted from the quadrupole mass analyser into the collision cell. The resulting daughter ions were separated by an orthogonal time-of-flight mass analyser. Peptide micro-sequencing and protein identification was carried out with the Peptide-Sequencing program within the Biolynx software (version 3.4; Micromass) and with the Sonar program (Proteometrics). The trypsin fragment sequences obtained were compared with the proteins predicted from the *L. monocytogenes* EGDe genome sequence (http://genolist.pasteur.fr/ListiList/). Predicted molecular mass and pI values were calculated at the Expasy site (http://www.expasy.ch/tools).

RESULTS

Resistance levels of the isolated mutants

The resistance levels of the spontaneous IIa mutants were assessed by comparing the mutant and wild-type MICs for leucocin A (Table 1). All eight mutants were unaffected by the highest concentration (4 mg synthetic leucocin A ml^{-1}) used. This corresponds to an at least 2×10^3-fold increase in resistance compared to the respective wild-type strain. *L. monocytogenes* EGK51, EGK54 and EGY2, which are defined mutants of strain EGDe, also had a MIC of over 4 mg leucocin A ml^{-1}, in concordance with the previous observation of a high level of resistance to mesentericin Y105 (Dalet *et al.*, 2001). All mutants were also resistant to the fermentates of the four producer strains, showing cross-resistance between the bacteriocins used.

Analysis of expression of the putative β-glucoside-specific PTS genes

The result of the Northern analysis of the spontaneous mutants employing the EII^{Bgl} probe is shown in Fig. 1(a). All eight mutants had a constitutive increase in expression compared to the wild-type strains, which did not have detectable transcription of this gene. The level of the increase varied for the different mutants. There was, however, no correlation between level of increase in expression and level of resistance: the two mutants with low expression, EGDeP4 and DMRICC 4053, had as high resistance to leucocin A as the other mutants. Northern analysis of further 29 high-level-resistant spontaneous mutants of 11 other wild-type strains and of the defined mutants *L. monocytogenes* EGK51, EGK54 and EGY2 showed that these all had increased expression of the EII^{Bgl} (results not shown).

The analysis with the probe for the putative phospho-β-glucosidase showed that this gene also had increased expression in the eight spontaneous mutants (Fig. 1b). Again, varying levels of increase were observed, but there was no correlation to level of resistance or to level of increase of EII^{Bgl} expression. Further studies of another 21 spontaneous mutants showed that 20 of these had increased expression of the phospho-β-glucosidase (results not shown).

Effect of inactivation of the putative β-glucoside-specific PTS genes on IIa sensitivity

EII^{Bgl} and the putative phospho-β-glucosidase were inactivated in *L. monocytogenes* 412 and 412P by plasmid integration using a 674 bp and a 991 bp internal gene fragment, respectively, for homologous recombination. The integration in EII^{Bgl} (giving strains AG122 and AG119, respectively) deleted the C-terminal 194 of the predicted 635 amino acids, including the entire catalytic domain IIA and part of the membrane-spanning domain IIC. In the phospho-β-glucosidase knockouts (strains AG115 and AG117, respectively), the insertion truncated the last 110 of the 474 predicted amino acids, removing the C-terminal glycosylhydrolase motif.

The pediocin sensitivity of the four insertional mutants was determined. All strains had the same phenotype as the respective original strain: *L. monocytogenes* AG122

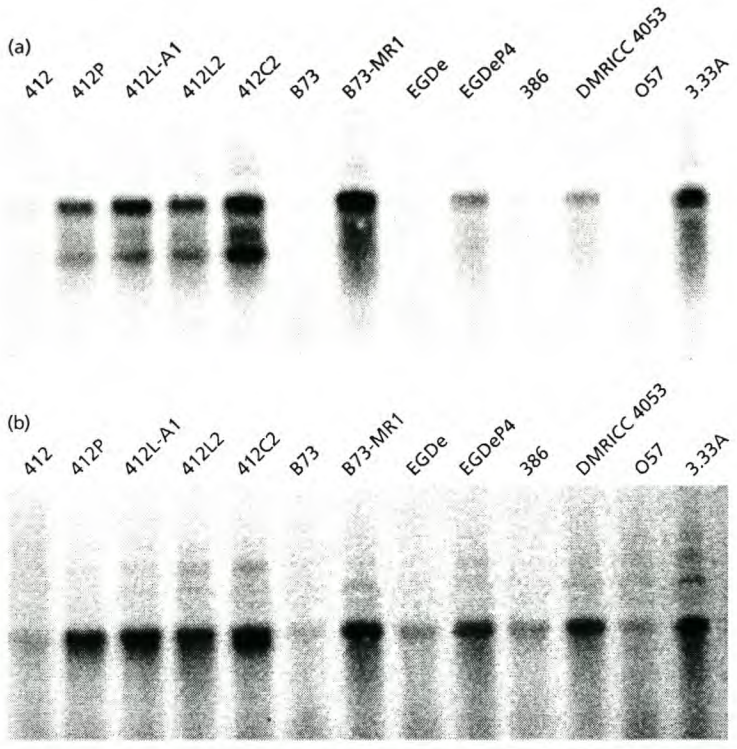


Fig. 1. Northern blot analysis of wild-type *L. monocytogenes* strains and derived spontaneous class IIa bacteriocin-resistant mutants, using internal gene fragments of the putative β-glucoside-specific PTS enzyme II, EII^{Bgl} (a), and the phospho-β-glucosidase homologue (b) as probes.

and AG115 were pediocin-sensitive, like *L. monocytogenes* 412, and *L. monocytogenes* AG117 and AG119 were pediocin-resistant, like *L. monocytogenes* 412P. In concordance, the MIC for leucocin A was unaffected by the insertion (Table 1).

Analysis of protein expression by 2D gel electrophoresis

Comparison of the protein profiles of the wild-type and spontaneous resistant mutants showed that one protein spot consistently disappeared following class IIa resistance development (Fig. 2). This change was observed for all five wild-type strains employed, and following exposure to each of the four tested class IIa bacteriocins. The protein spot also disappeared in resistant strains that had developed in a food system, either a saveloy-type sausage or salmon juice. Additionally, this spot was not detected in the gels of the deletion and insertional mutants *L. monocytogenes* EGK51, EGK54 and EGY2 (Fig. 2).

Initial attempts at identifying the consistently missing spot by N-terminal sequencing were inconclusive. To overcome this problem, zoom gels were made to improve the resolution of the region containing the spot. The results indicated that what appeared to be a single spot on normal gels appeared to consist of several spots on zoom gels. This cluster of spots was consistently present in *L. monocytogenes* EGDe, B73 and 412, and missing in *L. monocytogenes* EGY2, B73-MR1 and 412P, as shown for *L. monocytogenes* EGDe and EGY2 for example (Fig. 3). To further improve the resolution, the second-dimension running time was extended for the *L. monocytogenes* EGDe sample, and this clearly resolved the protein into a cluster of several spots (Fig. 3).

After tryptic in-gel digestion of the whole cluster from a zoom gel of *L. monocytogenes* EGDe and subsequent electrospray ionization, 19 putative peptide ions could be detected. Six ions were selected randomly for Q-TOF-based peptide micro-sequencing, which revealed the presence of two different proteins. One was the MptA subunit of a mannose-specific PTS enzyme II operon, *mptACD* (GenBank accession number AF397145, annotated as *lmo0096*; Dalet *et al.*, 2001), which is identical to 17 of the 20 N-terminal amino acids sequenced from a putative mannose-specific EII in *L. monocytogenes* B73 (Ramnath *et al.*, 2000). The other was the 6-phospho-fructo-kinase (PFK; *lmo1571*). The relative amounts of MptA and PFK in the cluster were estimated to be 8:1. These two proteins have predicted molecular masses of 34·99 and 34·42 kDa, and pI values of 5·32 and 5·46, respectively; this compares with the observed molecular mass of 35 kDa and the pI of 5·35 of the cluster. However, TOF-MS analysis of an adjacent

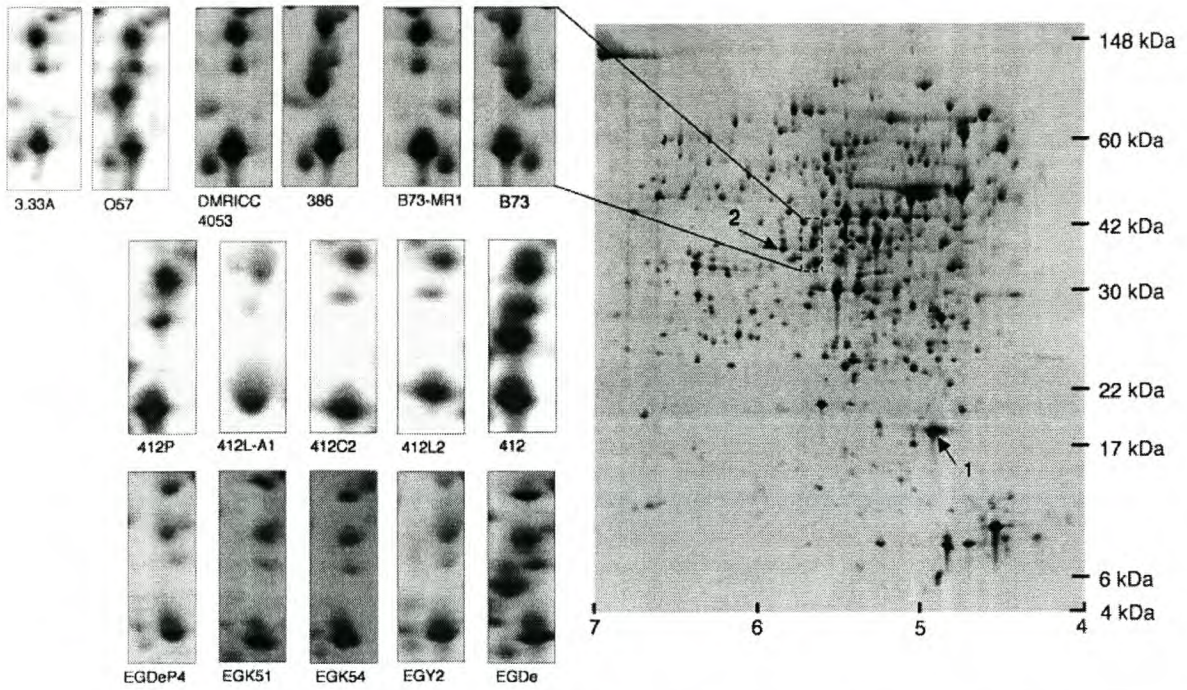


Fig. 2. 2D gel electrophoresis of wild-type *L. monocytogenes* strains and derived class IIa bacteriocin-resistant mutants. The entire gel (pH range 4–7) of *L. monocytogenes* B73 is shown; enlargements of the region encompassing the spot consistently missing following class IIa bacteriocin resistance development are shown for the other strains. Arrows indicate the non-haem-iron-binding ferritin (1) and the 6-phosphofructokinase (2).

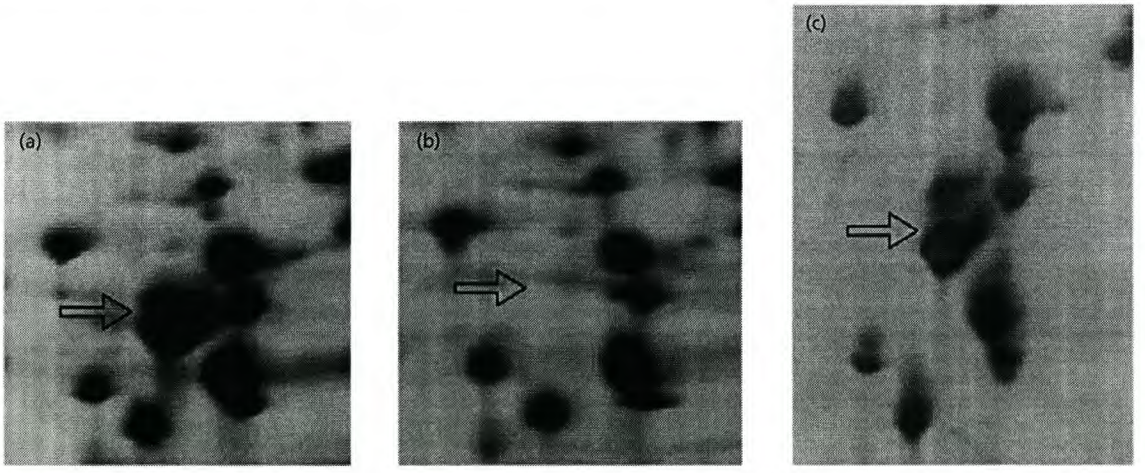


Fig. 3. Enlargements from 2D electrophoresis zoom gels (pH range 5–6) of the region encompassing the spot (arrowed) consistently missing following class IIa bacteriocin resistance development. Proteins were isolated from *L. monocytogenes* EGDe (a) and EGY2 (b). An extended-run zoom gel of *L. monocytogenes* EGDe (c) further resolved the spot into a cluster of several closely situated protein spots.

spot located to the lower left of the cluster (observed molecular mass 30 kDa, pI 5·79; Fig. 2), proved this also to contain PFK.

No other consistent changes were observed in the protein profiles of all the mutants tested. However, all four mutants of *L. monocytogenes* EGDe had an

increased content of three high-molecular-mass protein spots (molecular mass range 78·5–84·8 kDa, pI range 4·53–4·66), which presumably represents a strain-specific effect. In the other strains, no consistent strain-specific changes were found.

A non-haem-iron-binding ferritin was previously observed to be missing in an *L. monocytogenes* mutant with resistance to divercin V41 (Duffes *et al.*, 2000). A protein with similar molecular mass and pI (Fig. 2) was found in all wild-type and mutant strains studied, showing no apparent change in expression. TOF-MS analysis of the corresponding spot from *L. monocytogenes* EGDe confirmed its identity as the non-haem-iron-binding ferritin.

DISCUSSION

The expression changes of different PTS systems are part of the same general resistance mechanism

In previous work, two different changes in PTS expression were correlated to spontaneous development of resistance to class IIa bacteriocins in *L. monocytogenes* (Gravesen *et al.*, 2000; Ramnath *et al.*, 2000). The results presented in this paper show that both changes were evident in all eight independently isolated spontaneous *L. monocytogenes* mutants with high-level resistance to class IIa bacteriocins. All strains showed an increased expression of two putative β-glucoside-specific PTS genes, EII^{Bgl} and a phospho-β-glucosidase homologue, and all strains no longer synthesized the MptA subunit from a mannose-specific PTS, EII^{Man}_{L}. Additionally, the increased β-glucoside-specific PTS expression was seen in numerous other spontaneous mutants (this work and Gravesen *et al.*, 2000). These results strongly indicate that spontaneous class IIa resistance in *L. monocytogenes* is developed by one general mechanism that confers the two diverging PTS expression changes, and that this mechanism would also be expected to prevail in strains from food products.

Expression of *mptACD* is controlled by the σ^{54} transcription factor and the activator ManR (Dalet *et al.*, 2001). Knockout mutants of ManR or MptA, which were resistant to mesentericin Y105 (Dalet *et al.*, 2001) and to all class IIa bacteriocins used in this study, also had increased expression of EII^{Bgl}. This observation corroborates the hypotheses that the mannose and β-glucoside-specific PTS expression changes are part of the same resistance mechanism, and also indicates that the class IIa resistance mechanism conferring spontaneous, high-level resistance in *L. monocytogenes* is related to the mannose PTS-mediated effects described by defined mutations (Dalet *et al.*, 2001).

A divericin 41-resistant mutant of *L. monocytogenes* P lacked at least nine protein spots (Duffes *et al.*, 2000), two of which had a molecular mass and pI very similar to the MptA cluster. It is therefore possible that this mutant also acquired resistance by the same general mechanism; however, verification of this hypothesis would require identification of the proteins in these two spots.

An important aspect of resistance is whether the same mechanism is acquired in different species or genera. *Listeria innocua* is a non-pathogenic species with high genomic similarity to *L. monocytogenes* (Glaser *et al.*, 2001). Northern analysis with *L. monocytogenes* probes showed that a class IIa bacteriocin-resistant mutant of each of five *L. innocua* wild-type strains similarly had increased expression of EII^{Bgl} and the phospho-β-glucosidase (results not shown). In *Enterococcus faecalis*, expression of a mannose PTS enzyme II is also involved in class IIa bacteriocin sensitivity (Héchard *et al.*, 2001). Altogether, the results strongly suggest that class IIa bacteriocin resistance is conferred by the same general mechanism in *L. monocytogenes*, *L. innocua* and *E. faecalis*, and conceivably also in some other Gram-positive organisms.

Direct and indirect effects – how is class IIa bacteriocin resistance acquired?

It was recently reported that the enantiomer of leucocin A was not biologically active (Yan *et al.*, 2000), which strongly indicates that activity of class IIa bacteriocins requires chiral interaction with a docking molecule. It is tempting to speculate that the general resistance mechanism involves elimination of this docking molecule.

EII^{Bgl} and the putative phospho-β-glucosidase were suggested to be encoded in the same operon, based on homology (Gravesen *et al.*, 2000). However, according to the recently released *L. monocytogenes* EGDe genome sequence (Glaser *et al.*, 2001), they are located at separate positions on the chromosome. Interruption of EII^{Bgl} or the phospho-β-glucosidase in the resistant mutant *L. monocytogenes* 412P did not affect IIa resistance. Therefore, the increased expression of either of these genes does not *per se* cause class IIa bacteriocin resistance, but is presumably a natural regulatory consequence of acquired resistance. Prevention of *mptACD* expression directly conferred resistance (Dalet *et al.*, 2001), suggesting that the membrane component, the MptC–MptD complex, could function as target for class IIa bacteriocins. Expression of *mpt* could be prevented through mutation in *rpoN*, *manR* or *mpt*. The multiple possibilities could explain the observation of relatively high IIa resistance frequencies of approximately 10^{-6} (Gravesen *et al.*, 2002). The specific location of the mutation in a resistant strain will determine the extent of the changes in the strain, i.e. a mutation in σ^{54} would have more extensive consequences than a mutation in *mpt*. Abolished *mptACD* expression could cause up-regulation of EII^{Bgl} and the phospho-β-glucosidase expression; this is similar to the observation that a mannose PTS regulates expression of other PTS, including β-glucoside-specific enzymes, as part of the carbon catabolite repression in *Streptococcus salivarius* and *Lactobacillus pentosus* (Bourassa & Vadeboncoeur, 1992; Chaillou *et al.*, 2001; Gauthier *et al.*, 1990).

Supporting this hypothesis, a sequence with only two mismatches to the *cre* (catabolite-responsive element; Stülke & Hillen, 1999) consensus overlaps a putative $-35/-10$ promoter upstream of the EII^{Bgl} reading frame.

An additional 28 aa domain present in the MptD subunit of EII_t^{Man} compared to other mannose PTS EII sequences was indicated to possibly be involved in IIa sensitivity, since *L. monocytogenes* EGY2, which has an in-frame deletion of the additional domain, was resistant to mesentericin Y105 (Dalet *et al.*, 2001). However, the 2D gel analysis showed that *L. monocytogenes* EGY2 did not produce detectable amounts of MptA, indicating that the *mptACD* operon is repressed. The repression could be due to the deletion rendering the permease functionally inactive, which in turn could modify the expression through a regulatory cascade.

The two other candidate proteins potentially involved in class IIa bacteriocin resistance, PFK and the non-haem iron-binding ferritin, are apparently not the causative factor. The presence of PFK in two different protein spots could be due to post-translational modifications of the protein, where phosphorylation and/or truncation could result in the lower molecular mass and higher pI observed in the spot adjacent to the consistently disappearing cluster. However, the adjacent PFK spot had similar intensity in wild-type strains and their derived mutants, indicating that there is no overall change in PFK expression related to class IIa bacteriocin resistance. The non-haem iron-binding ferritin, which was missing in one mutant (Duffes *et al.*, 2000), did not have any difference in expression in the eight mutants in this study. This observation and the strain-specific changes seen in the *L. monocytogenes* EGDe mutants underline the importance of analysing several wild-type and mutant strains.

All in all, we suggest that the presently available knowledge allows the following conclusions. Resistance to IIa bacteriocins is acquired through one general mechanism in *L. monocytogenes* and at least some other Gram-positive organisms. This mechanism is characterized by prevention of EII_t^{Man} synthesis and up-regulation of EII^{Bgl} and the phospho-β-glucosidase. Up-regulated EII^{Bgl} and phospho-β-glucosidase expression is not a direct cause of resistance, but is presumably a regulatory consequence of abolished *mptACD* expression. Prevention of *mpt* expression directly confers resistance. Although present evidence suggests that the MptC–MptD complex interacts as target with class IIa bacteriocins, more work is required to elucidate how shutdown of *mpt* expression actually causes resistance.

ACKNOWLEDGEMENTS

We thank S. Aimoto and K. Tamura for the generous gift of synthetic leucocin A. We also thank Anette Granly Koch for supplying listerial strains, and Lilian Nilsson for strains and bacteriocin fermentate. The expert technical assistance of Hanne Mordhorst and Lene Gertman is greatly appreciated. This work was supported by the Food Biotechnology Programme of the Danish Ministry for Food, Agriculture, and Fisheries (BIOT99-8), and by a grant from The Danish Rectors' Conference and the National Research Foundation (South Africa) to M.R. and J.W.H.

REFERENCES

Ben Ambarek, P. K. & Huss, H. H. (1993). Heat resistance of *Listeria monocytogenes* in vacuum packaged pasteurized fish fillets. *Int J Food Microbiol* **20**, 85–95.

Bourassa, S. & Vadeboncoeur, C. (1992). Expression of an inducible enzyme II fructose and activation of a cryptic enzyme II glucose in glucose-grown cells of spontaneous mutants of *Streptococcus salivarius* lacking the low-molecular-mass form of III^{man}, a component of the phosphoenolpyruvate:mannose phosphotransferase system. *J Gen Microbiol* **138**, 769–777.

Chaillou, S., Postma, P. W. & Pouwels, P. H. (2001). Contribution of the phosphoenolpyruvate:mannose phosphotransferase system to carbon catabolite repression in *Lactobacillus pentosus*. *Microbiology* **147**, 671–679.

Chakraborty, T., Leimeister-Wachter, M., Domann, E., Hartl, M., Goebel, W., Nichterlein, T. & Notermans, S. (1992). Coordinate regulation of virulence genes in *Listeria monocytogenes* requires the product of the *prfA* gene. *J Bacteriol* **174**, 568–574.

Cleveland, J., Montville, T. J., Nes, I. F. & Chikindas, M. L. (2001). Bacteriocins: safe, natural antimicrobials for food preservation. *Int J Food Microbiol* **71**, 1–20.

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

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

Dykes, G. A. & Hastings, J. W. (1998). Fitness costs associated with class IIa bacteriocin resistance in *Listeria monocytogenes* B73. *Lett Appl Microbiol* **26**, 5–8.

Ennahar, S., Sashihara, T., Sonomoto, K. & Ishizaki, A. (2000). Class IIa bacteriocins: biosynthesis, structure and activity. *FEMS Microbiol Rev* **24**, 85–106.

Gauthier, L., Bourassa, S., Brochu, D. & Vadeboncoeur, C. (1990). Control of sugar utilization in oral streptococci. Properties of phenotypically distinct 2-deoxyglucose-resistant mutants of *Streptococcus salivarius*. *Oral Microbiol Immunol* **5**, 352–359.

Glaser, P., Frangeul, L., Buchrieser, C. & 52 other authors (2001). Comparative genomics of *Listeria* species. *Science* **294**, 849–852.

Gravesen, A., Warthoe, P., Knøchel, S. & Thirstrup, K. (2000). Restriction fragment differential display of pediocin-resistant *Listeria monocytogenes* 412 mutants shows consistent overexpression of a putative β-glucoside-specific PTS system. *Microbiology* **146**, 1381–1389.

Gravesen, A., Jydegaard Axelsen, A.-M., Mendes da Silva, J., Hansen, T. B. & Knøchel, S. (2002). Frequency of bacteriocin resistance development and associated fitness costs in *Listeria monocytogenes*. *Appl Environ Microbiol* **68**, 756–764.

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

Nilsson, L., Gram, L. & Huss, H. H. (1999). Growth control of *Listeria monocytogenes* on cold-smoked salmon using a competitive lactic acid bacteria flora. *J Food Protection* **62**, 336–342.

Papathanasopoulos, M. A., Krier, F., Revol-Junelles, A. M., Lefebvre, G., Le Caer, J. P., von Holy, A. & Hastings, J. W. (1997). Multiple bacteriocin production by *Leuconostoc mesenteroides* TA33a and other *Leuconostoc/Weissella* strains. *Curr Microbiol* **35**, 331–335.

Park, S. F. & Stewart, G. S. (1990). High-efficiency transformation of *Listeria monocytogenes* by electroporation of penicillin-treated cells. *Gene* **94**, 129–132.

Postma, P. W., Lengeler, J. W. & Jacobson, G. R. (1993). Phosphoenolpyruvate:carbohydrate phosphotransferase systems of bacteria. *Microbiol Rev* **57**, 543–594.

Ramnath, M., Beukes, M., Tamura, K. & Hastings, J. W. (2000). Absence of a putative mannose-specific phosphotransferase system enzyme IIAB component in a leucocin A-resistant strain of *Listeria monocytogenes*, as shown by two-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis. *Appl Environ Microbiol* **66**, 3098–3101.

Rasch, M. & Knøchel, S. (1998). Variations in tolerance of *Listeria monocytogenes* to nisin, pediocin PA-1 and bavaricin A. *Lett Appl Microbiol* **27**, 275–278.

Rechinger, K. B., Siegumfeldt, H., Svendsen, I. & Jakobsen, M. (2000). 'Early' protein synthesis of *Lactobacillus delbrueckii* ssp. *bulgaricus* in milk revealed by [^{35}S]methionine labeling and two-dimensional gel electrophoresis. *Electrophoresis* **21**, 2660–2669.

Robichon, D., Gouin, E., Debarbouille, M., Cossart, P., Cenatiempo, Y. & Héchard, Y. (1997). The *rpoN* (σ^{54}) gene from *Listeria monocytogenes* is involved in resistance to mesentericin Y105, an antibacterial peptide from *Leuconostoc mesenteroides*. *J Bacteriol* **179**, 7591–7594.

Stülke, J. & Hillen, W. (1999). Carbon catabolite repression in bacteria. *Curr Opin Microbiol* **2**, 195–201.

Yan, L. Z., Gibbs, A. C., Stiles, M. E., Wishart, D. S. & Vederas, J. C. (2000). Analogues of bacteriocins: antimicrobial specificity and interactions of leucocin A with its enantiomer, carnobacteriocin B2, and truncated derivatives. *J Med Chem* **43**, 4579–4581.

Received 13 February 2002; revised 10 April 2002; accepted 17 April 2002.

Chapter 4

Proteomic analysis of class IIa bacteriocin sensitive and resistant strains of Listeria monocytogenes 412 upon sub-lethal exposure to leucocin A and its enantiomer

Abstract

Listeria monocytogenes wild-type, intermediate and totally resistant strains were exposed to a sub-lethal final concentration (9.8 ng/ml) of L-leucocin A and its D-enantiomer. Two-dimensional electrophoresis showed no proteomic changes for any of the tested strains after sub-lethal exposure to either enantiomer. Comparisons of the proteomes of unchallenged wild-type and intermediate resistant strains showed that the intensity of the spot corresponding to the EIIAB component of the mannose phosphotransferase system (PTS) was enhanced 6-fold indicating an up-regulation of this gene. The absence of proteomic changes upon bacteriocin challenge and the significance of the unexpected up-regulation of the mannose PTS in the intermediate resistant strain *L. monocytogenes* 412C, are discussed.

Introduction

Listeria monocytogenes, a Gram-positive bacterium, has frequently been implicated in food-borne disease outbreaks [3]. Bacteriocins produced by lactic acid bacteria (LAB) which have antimicrobial activity, are currently being considered for use as potential biopreservatives [20]. Bacteriocins of LAB can be divided into several classes and these have been reviewed extensively [15, 16, 18]. The class IIa subclass consists of small heat-stable, non-lanthionine peptides with strong anti-listerial activity. Several of the peptides belonging to this subclass are potentially useful as food biopreservatives [7].

A major concern regarding the use of any antimicrobial compound for biopreservation is the development of resistance. Both high and intermediate levels of resistance to class IIa bacteriocins in *L. monocytogenes* have been reported [5, 6, 9, 12, 21, 25]. The shut-down of the mannose phosphotransferase system (PTS) encoded for by the *mpt* operon has been described as the prevalent mechanism by which high-level resistance to class IIa bacteriocins is acquired [12]. Furthermore, the permease of the mannose PTS has been proposed to act as a docking complex for class IIa bacteriocins [4, 13, 14]. On the other hand, intermediate resistance to class IIa bacteriocins has thus far been associated with modifications to the cytoplasmic membrane [17, 25]. Thus far no common mechanism for intermediate resistance acquisition by listerial strains has been determined. Furthermore, little is known about the molecular basis of resistance to class IIa bacteriocins in intermediate resistant strains.

Previous studies have demonstrated that pediocin JD and leuconocin S inhibited phosphoenol-pyruvate (PEP)-mediated glucose uptake *via* a PTS system, independently of PEP efflux [2, 26]. The *mpt* operon was induced by glucose in *L. monocytogenes* EGDe indicating that glucose was actively transported by the mannose PTS [4]. The genome sequence of *L. monocytogenes* EGDe displays homology to a single EIIA component for a dedicated glucose PTS [8]. Other subunits required for a fully functional glucose PTS are not encoded on the genome sequence. This could indicate that the mannose PTS is perhaps one of the main active transporters of glucose. Therefore, monitoring of the protein expression level of the mannose PTS after sub-lethal exposure to class IIa bacteriocin could indicate if this PTS is inhibited at the protein expression level.

In a food system, the target organism may be exposed to a bacteriocin concentration gradient resulting in sub-lethal exposure to a bacteriocin [24]. To our knowledge there has been no reports of studies that have specifically monitored alterations in the proteins expressed in *L. monocytogenes* upon sub-lethal exposure to class IIa bacteriocins. Elucidation of the stress responses induced in cells exposed to class IIa bacteriocins would contribute to the understanding of the mechanistic action of class IIa bacteriocins.

L-leucocin A has strong antimicrobial activity against listerial strains, however the synthesised D-enantiomer of leucocin A has no inhibitory effect at minimum

inhibitory concentration (MIC) levels [27]. However, the exposure to high levels of D-leucocin A (10^5 times the MIC of L-leucocin A) results in a weak inhibitory effect on some strains, indicating non-specific membrane disruption [27]. Cellular responses induced upon exposure to the inactive enantiomer would consequently identify non-specific molecular effects of a cationic peptide on a cell.

To investigate the impact of sub-lethal exposure to bacteriocins and the molecular basis of intermediate resistance, protein expression profiles of strains were assessed using two-dimensional electrophoresis (2-D).

Materials and Methods

Bacterial strains and growth conditions

The wild-type *L. monocytogenes* 412 [9], class IIa bacteriocin resistant, 412P [9] and intermediately resistant 412C (a kind gift from A. Gravesen) strains were all maintained on Brain Heart Infusion (BHI) (Difco) broth or agar at 30 °C without aeration. Strain 412C was isolated on Tryptone Soy Agar (TSA) (Difco) plates with 100 AU/ml carnobacteriocin B2 and 0.1 % Tween 80, incubated at 5 °C for 24 h and hereafter at 30 °C.

Bacteriocin preparation

L-leucocin A was synthesized using Fmoc-peptide chemistry as described previously [21]. The enantomier of L-leucocin A was prepared by coupling D-amino acids also utilising Fmoc chemistry, described by Yan and co-workers [27]. Both forms of leucocin A were reconstituted in 0.1 % trifluoroacetic acid (TFA) and the bacteriocin stocks were stored at –20 °C until used.

MIC determination

The MIC of L-leucocin A was determined by the spot-on-lawn assay as described previously [21]. Five µl of a two-fold serial dilution of the bacteriocins in 0.1 % TFA were transferred onto the surface of BHI agar lawns (0.7 % agar, 0.1 % Tween 80) previously inoculated with *L. monocytogenes* 412, 412C, or 412P to yield a lawn of cells. The MIC was determined as the lowest concentration producing a visible zone after 20 h incubation at 30 °C. The MIC value represents the average of three to four independent experiments.

Determination of the sub-lethal concentration of Leucocin A

A 10 % inoculum of an overnight culture of 412 was transferred to fresh BHI broth and the growth was monitored until an OD_{600} of 0.25 was attained. D or L-leucocin A was added to final concentrations ranging between 980 ng/ml to 9.8 ng/ml into the exponentially dividing culture. The inhibitory effect of the bacteriocins on the growth of the culture was assessed, both spectrophotometrically and by colony forming unit (CFU/ml) counts. The number of injured cells was determined as the number of CFU/ml able to grow on a 6% NaCl supplemented agar plate containing a duplicate aliquot of challenged cells. The osmotic stress imparted by the NaCl may not allow for the survival of bacteriocin-injured cells. This procedure was also used to determine the effect of L-leucocin A to strains 412C and 412P. Exposure to D or L-leucocin A was carried out in duplicate.

Exposure of *L. monocytogenes* 412, 412C and 412P to D and L-leucocin A

All three strains were maintained in a continuous exponential state with a maximum OD_{600} of 0.2. A 10% inoculum of the continuous culture was transferred to fresh BHI broth and the growth of the culture was monitored until it attained an OD_{600} of 0.2. The culture was then split into four portions and supplemented to as follows: (i) chloramphenicol (Sigma) at a final concentration of 100 µg/ml to halt protein synthesis (this was referred to as the start protein extract); (ii) L-leucocin A at a final concentration of 9.8 ng/ml; (iii) D-leucocin A at a final concentration of 9.8 ng/ml; and (iv) an equivalent volume of 0.1 % TFA as was present when D and L-leucocin A were supplemented, was added to the fourth portion. The fourth portion was to act as a control to monitor TFA effects on protein expression. Analysis the protein profile of the start gel would allow for the determination of the protein profiles of strains prior to the sub-lethal exposure to the bacteriocin. The remaining portions were incubated for 1 h before protein synthesis of the strains was stopped. Prior to chloramphenicol addition aliquots were removed to determine CFU/ml counts to access the number of survivors. The exposure of the strains to the bacteriocins was carried in duplicate independent experiments for 2-D analysis.

Total protein isolation

Total protein isolation was carried out as described previously by Ramnath and co-workers [22]. Once listerial cells were challenged with the appropriate test compound and protein synthesis stopped, cells were harvested by centrifugation. The pellet was

washed once in 10 mM phosphate buffered saline (pH 7.5) and twice in 32 mM Trizma® Pre-set Crystals (pH 7.5) (Sigma, St.Louis, MO). Wash buffers were also supplemented with chloramphenicol to a final concentration of 100 µg/ml. The washed cell pellet was resuspended in TE (10 mM Tris, 1 mM EDTA, pH 7.5) containing Complete Mini tablets (cocktail of protease inhibitors, Roche, Mannheim, Germany) and stored at –80°C. Thawed cell suspensions were disrupted using the FastPrep FP 120 instrument (Bio 101, Carlsbud, CA). The cell lysate was initially treated with DNase I and RNase I (Boehringer Mannheim, Mannheim, Germany), followed by the addition of 9.5 M urea, 100 mM dithiothreitol (DTT) (Sigma), 4 % (w/v) 3-[(3-cholamidopropyl)-dimethylammonio] 1-propane sulfonate (CHAPS) and 2 % (v/v) Pharmalyte (pH 3-10). The supernatant was clarified by centrifugation and stored at –80 °C. The Plusone™ Quant kit was used to determine protein concentrations of the solublised proteins. All reagents for the protein isolation and 2-D gel electrophoresis were purchased from Amersham Biosciences (Buckinghamshire, UK), unless otherwise stated.

First dimension electrophoresis

Isoelectric focusing (IEF) was carried out on immobilized pH gradients, pH 4-7 (18 cm Immobiline Drystrips), on a Mulitphor II apparatus according to the manufacturer's instructions. The following voltage gradient was applied: 0 to 300 V in 0.01 h; 300 V for 6.5 h; 300 V to 3500 V in 5 h; and 3500 V for 8 h. Protein samples were loaded via rehydration with 150 µg of sample per gel, and the IEF gels were run according to the manufacturer's instructions.

Second dimension electrophoresis

Prior to the SDS PAGE run, IEF strips were equilibrated in a SDS equilibration buffer as recommended by the manufacturer with the following modifications: (i) the concentration of SDS in the SDS equilibration buffer was increased from 2 % to 4 % (w/v); and, (ii) the concentration of iodoacetamide (Sigma) for the second equilibration step was doubled to 4 % (w/v). Each equilibration step of the IEF strip with the SDS buffer was carried out for 30 min, rather than the recommended 15 min. The second dimension run was carried out on pre-cast ExcelGel XL SDS 12-14 gels according to the manufactures instructions. Gels were stained with Coomassie colloidial blue G250 according to Neuhoff and co-workers [19].

2-D gel analysis

Images of Coomassie-stained gels were scanned at a resolution of 200 dots per inch. Quantification and spot matching between gels was done using the Z3 Desk Top Version 2.01 (Compugen Ltd., Jamesburg, NJ). Protein patterns from both D or L-leucocin A were initially compared to the respective TFA controls, this allowed for the identification of spots that were specifically expressed or repressed due to exposure to the different chiral forms of the bacteriocin. The general non-specific reactions of the bacterium to a cationic peptide were identified by the comparison of L and D-leucocin A gels. All supposed spot changes in D and L-leucocin A gels were checked against the respective gels of the start protein extract so that the expression level of the spots could be determined prior to bacteriocin stress. The TFA control gels of strain 412C were compared to that of strain 412.

Changes in the spot intensities below 2.5-fold were considered to be due to experimental variations rather than biological changes, using our 2-D methodology [22]. Therefore, only differences in expression of spots with the minimum of a 5-fold up-regulation or 0.2-fold down-regulation were considered to be genuine regulatory changes. Putative differences detected by Z3 analyses were verified by visual examination. For each of the protein samples, at least two Coomassie-stained gels were examined.

Results and Discussion

MIC of strains

The MIC of *L. monocytogenes* 412 and 412C to L-leucocin A was 480 and 1920 ng/ml respectively, indicating that strain 412C was four times more resistant. No zones of inhibition were observed for strain 412P at the maximum tested concentration of 4×10^6 ng/ml. Similar MIC levels were previously reported for both the 412 and 412P strains [12].

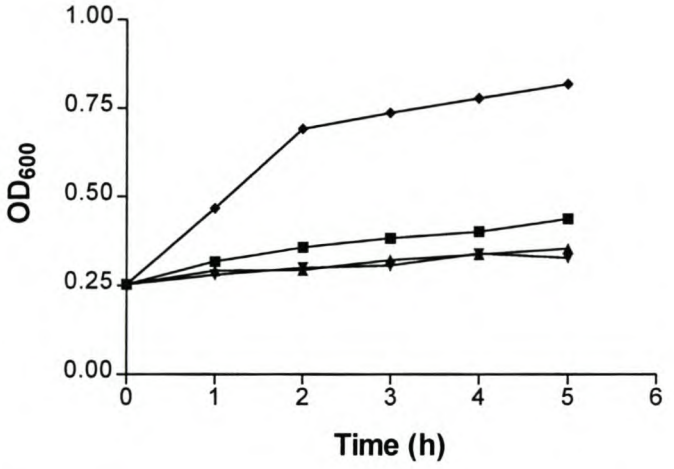


Fig. 1. Growth of *Listeria monocytogenes* 412 in BHI broth at 30°C supplemented with either D or L-leucocin A. The culture was grown until an OD_{600} of 0.25 after which the cultures were supplemented with bacteriocin to following final concentrations: 9.8 ng/ml L-leucocin A (■), 25 ng/ml L-leucocin A (▲), 50 ng/ml L-leucocin A (▼) and 50 ng/ml D-leucocin A (♦). Growth of cells was monitored for five hours after treatment with bacteriocin. Each point represents the mean of a duplicate experiment.

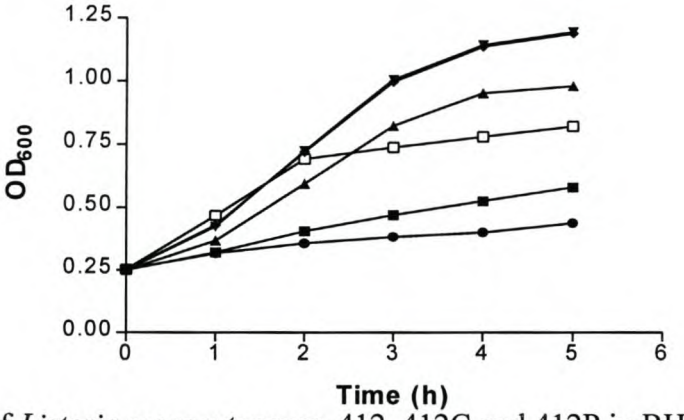


Fig. 2. Growth of *Listeria monocytogenes*, 412, 412C and 412P in BHI broth at 30 °C until an OD_{600} of 0.25. Bacteriocins were then added to obtain the following final concentrations at time 0 h: strain 412, 9.8 ng/ml L-leucocin A (●); strain 412, 9.8 ng/ml D-leucocin A (□); strain 412C, 9.8 ng/ml L-leucocin A (■); strain 412C, 9.8 ng/ml D-leucocin A (▲); strain 412P, 9.8 ng/ml D-leucocin A (▼); and strain 412P, 9.8 ng/ml L-leucocin A (♦). Lines representing 412P D- and L-leucocin A treatments (▼,♦), overlap. Growth of cultures was monitored for five hours after treatment. Each point represents the mean of a duplicate experiment.

Injury levels after sub-lethal exposure to bacteriocins

Partial growth was observed at 9.8 ng/ml for strain 412, but no growth was observed for all concentrations at or above 25 ng/ml of L-leucocin A for 5 h after challenge (Fig. 1). No inhibitory effect was observed for 412P when exposed to either D or L-

leucocin A. Strain 412C was less inhibited by 9.8 ng/ml L-leucocin A than the 412 strain (Fig. 2).

There was no increase in CFU/ml after exposure of *L. monocytogenes* 412 to 9.8 ng/ml L-leucocin A. However, approximately 95 % of the viable population was injured after treatment as determined by the comparison of CFU/ml on agar in the presence or absence of NaCl. For the intermediate resistant 412C strain there was a 1.8 fold increase in CFU/ml after treatment with 9.8 ng/ml L-leucocin A, with 84% of the culture been injured.

No reduction in the CFU/ml for strain 412P was observed after sub-lethal bacteriocin challenge in comparison to the TFA control. As was found previously [27] D-leucocin A had no inhibitory effect on any of the strains in the concentration range tested (results not shown).

Proteome changes upon sub-lethal exposure to bacteriocins

No reproducible changes in the proteome were identified after the exposure of any of the strains to either D or L-leucocin A (Figs. 3 to 5). Possible explanations for the lack of detectable spot changes could be attributed to some of the inherent problems associated with the 2-D procedure, which are as follows: (i) changes in protein expression may not be in the experimental window utilized; (ii) membrane proteins were not monitored due to the insolubility of these proteins in the IEF sample buffer [22, 23]; and finally (iii) the staining procedure does not monitor protein turn over rates.

We were confident that the cells had significant exposure to L-leucocin A because of the high-levels of lethal injury observed for strains 412 and 412C.

Expression levels of MptA

The location of the spot corresponding to MptA was determined from the relative position of the spot in the *L. monocytogenes* EGDe 2-D reference map [22]. MptA encoded by *mptA* is one of three genes, which make up the tricistronic *mpt* operon [4]. Therefore, monitoring the intensity of the spot corresponding to MptA would be indicative of the expression of level the entire *mpt* operon.

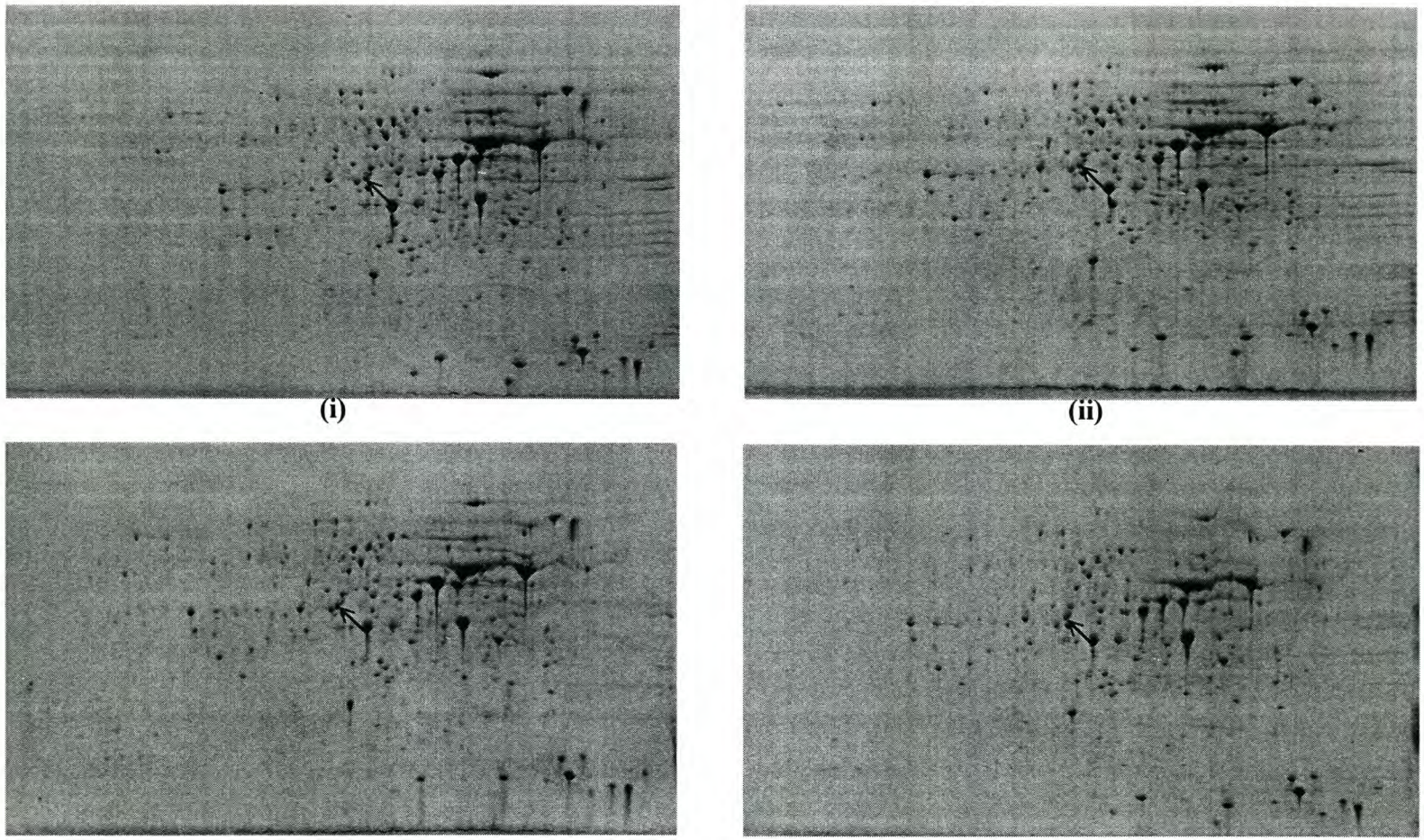


Fig. 3. Coomassie G-250 stained 2-D gels of proteins extracted from wild-type *Listeria monocytogenes* 412 grown in BHI broth at 30°C. The following samples were used for protein extraction: (i), cells prior to exposure to bacteriocins at an OD_{600} of 0.2, (ii) cells after one hour exposure to 9.8 ng/ml L-leucocin A, (iii) cells after one hour exposure to 9.8 ng/ml D-leucocin A, and (iv), cells after one hour exposure to an equal volume of 0.1% TFA used for supplementation of D and L-leucocin A. Arrow indicates the positions of MptA.

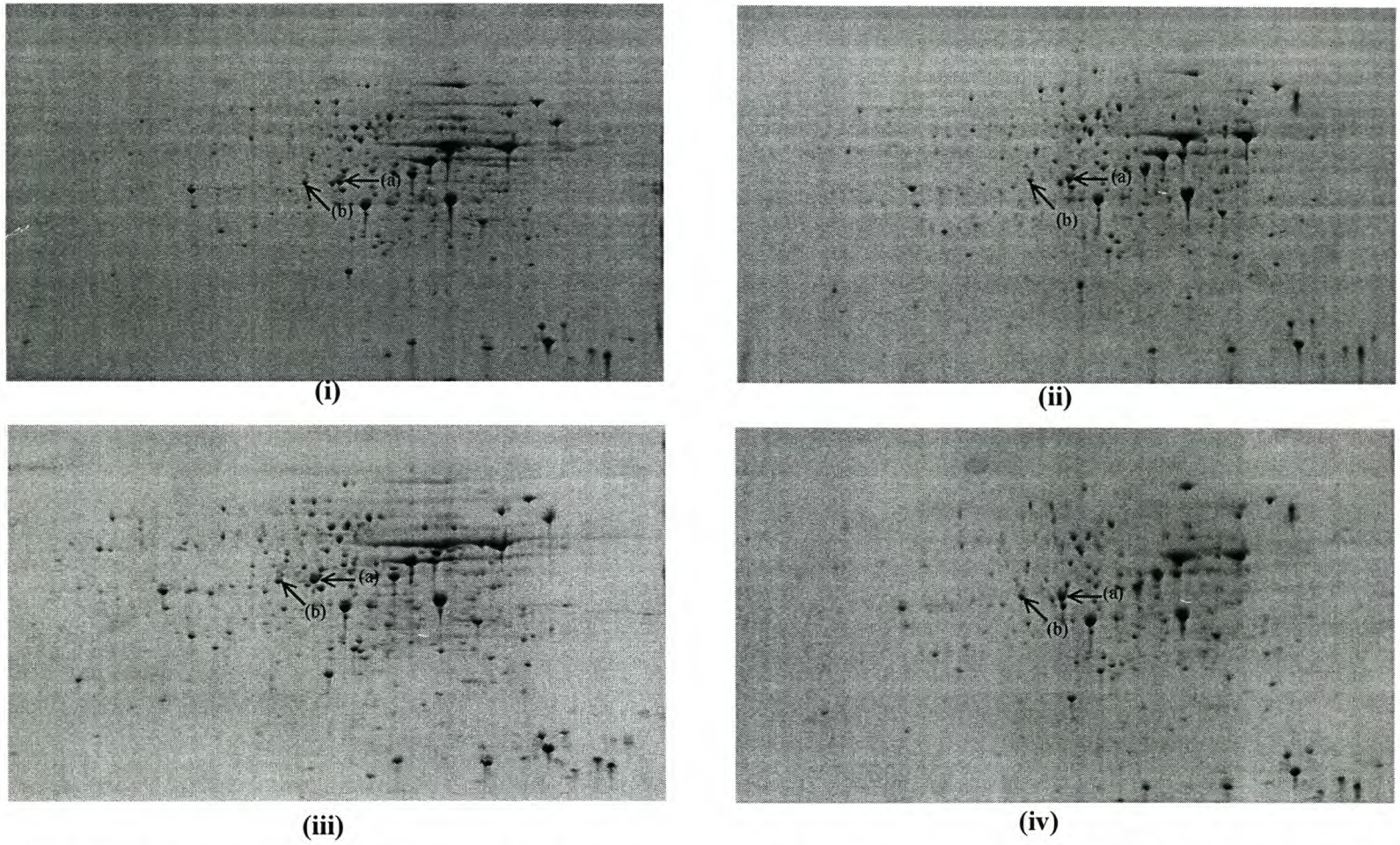


Fig. 4. Coomassie G-250 stained 2-D gels of proteins extracted from the intermediate resistant *Listeria monocytogenes* 412C grown in BHI broth at 30°C. The following samples were used for protein extraction: (i), cells prior to exposure to bacteriocins at an OD_{600} of 0.2, (ii) cells after one hour exposure to 9.8 ng/ml L-leucocin A, (iii) cells after one hour exposure to 9.8 ng/ml D-leucocin A, and (iv), cells after one hour exposure to an equal volume of 0.1% TFA used for supplementation of D and L-leucocin A. Arrows (a) and (b) indicate the positions of MptA and 6-phospho-fructo-kinase respectively.

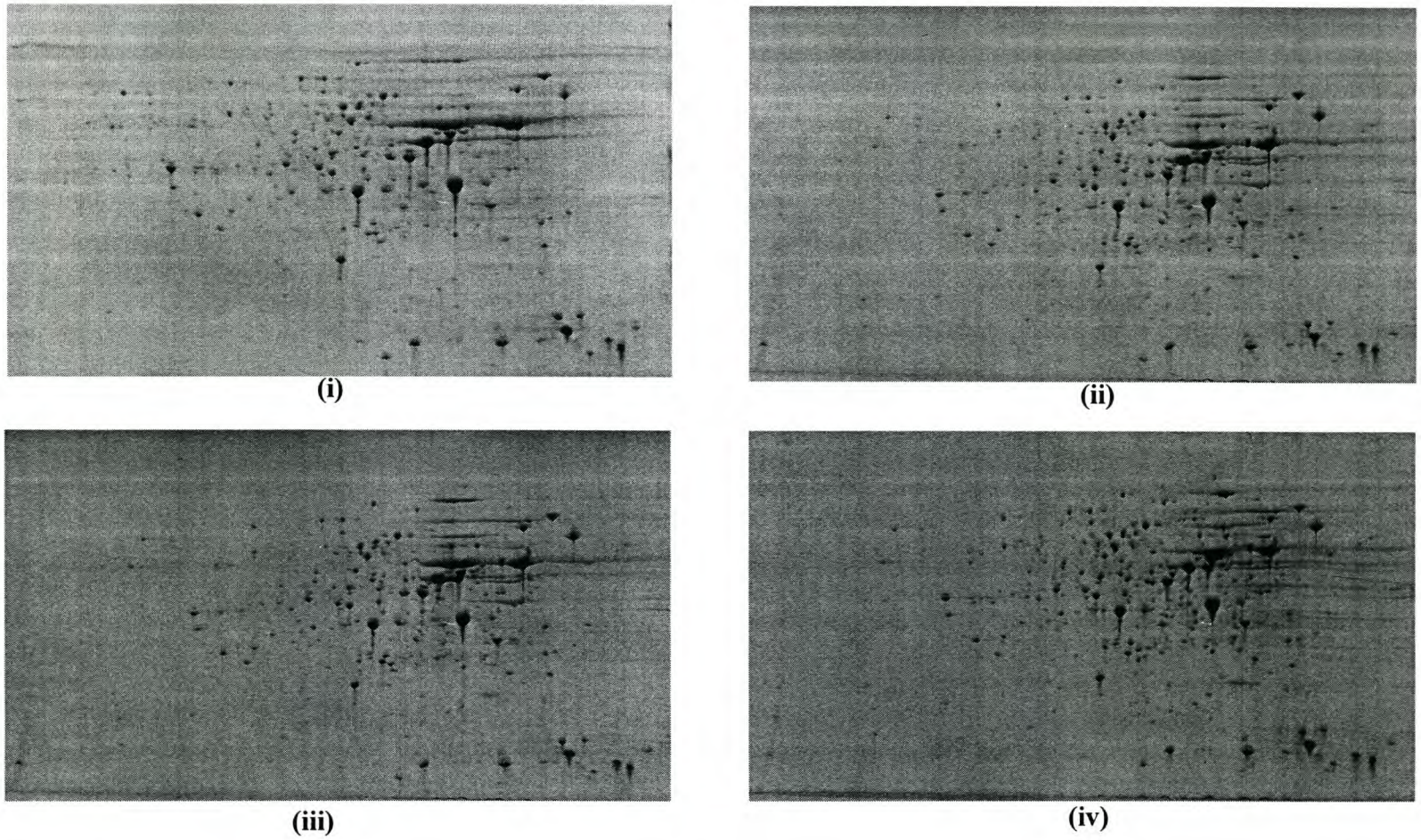


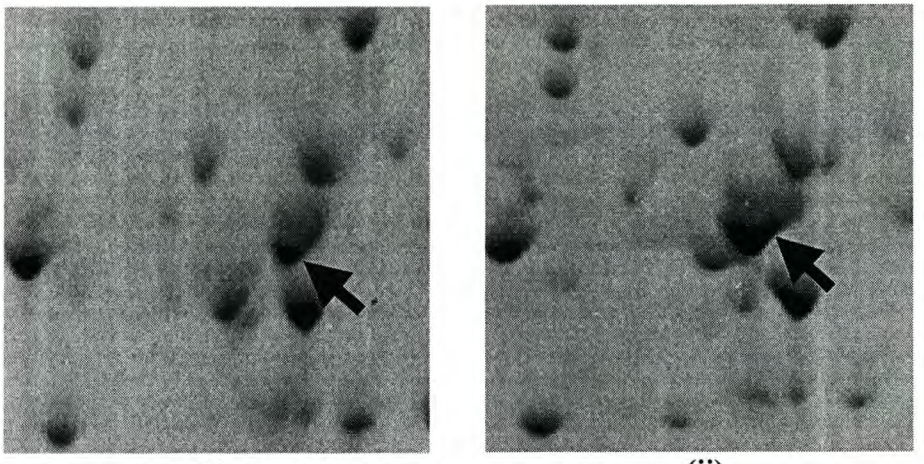
Fig. 5. Coomassie G-250 stained 2-D gels of proteins extracted from wild-type *Listeria monocytogenes* 412P grown in BHI broth at 30°C. The following samples were used for protein extraction: (i), cells prior to exposure to bacteriocins at an OD_{600} of 0.2, (ii) cells after one hour exposure to 9.8 ng/ml L-leucocin A, (iii) cells after one hour exposure to 9.8 ng/ml D-leucocin A, and (iv), cells after one hour exposure to an equal volume of 0.1% TFA used for supplementation of D and L-leucocin A.

No expression level changes were observed for the spot corresponding to MptA following exposure of strains 412 and 412C to L-Leucocin A. As previously shown strain 412P lacks a spot corresponding to MptA [12]. This indicates that leucocin A does not directly modulate the expression of the mannose PTS upon challenge with sub-lethal levels of class IIa bacteriocins. L-leucocin A had no observable impact on the regulation of this glucose transporter. This may indicate that inhibition of this glucose PTS may occur through direct interaction of bacteriocin and a PTS component.

Comparison of the wild-type and intermediate resistant strain

To our knowledge this is the first time that a proteomic comparison between a wild-type and intermediately resistant strain has been made. Comparison of the 2-D gels of the TFA controls of *L. monocytogenes* 412 and 412C strains revealed that no novel spots were synthesized or shut-down in strain 412C. However, a single reproducible up-regulation of a protein spot was detected in *L. monocytogenes* 412C (Fig. 6). There was an average of a 6-fold increase in the spot intensity indicating an increase in expression of this protein in strain 412C when compared to the wild-type strain. The spot was identified as MptA after comparisons of relative spot position and spot pattern to the *L. monocytogenes* EGDe proteome map [12, 22]. The spot corresponding to MptA has previously been shown to consist of two proteins, namely, the MptA and 6-phospho-fructo-kinase (PFK) [12]. In addition a second spot corresponding to PFK was also identified previously, and no change in its expression was associated with the acquisition of high levels of bacteriocin resistance [12]. Similarly, the second spot corresponding to the position of PFK was also monitored in the *L. monocytogenes* 412C gels (Fig. 5, arrow b) and no significant change in its intensity was observed. This indicates that PFK plays no role in intermediate resistance either. A transcriptomic comparison of unchallenged 412 and 412C strains showed an approximate 3.3 fold increase in expression of *mptA* and the downstream *mptD* (Anne Gravesen, personal communication). The increased level of transcription of the *mpt* operon may indicate that the observed increase in intensity of the spot corresponding to MptA and PFK is due to an increase in transcription and thus translation of the *mptA* only.

The up-regulation of the *mpt* operon, encoding EII_t^{man}, has previously resulted in an increased susceptibility to mesentercin Y105 [4, 13]. Furthermore, an increased expression of the *mpt* operon in *Lactoccous lactis* also resulted in an increase in sensitivity to leucocin A (see Chapter 5). This evidence strongly suggests that the level of expression of EII_t^{man} (MptA, MptC and MptD) modulates the sensitivity to class IIa bacteriocins. Our discovery that the MptA was up-regulated in strain 412C was, therefore unexpected, as the down regulation of this protein has been associated with intermediate resistance development.



(i) (ii)

Fig. 6. Magnification of the region of 2-D gels encompassing the up-regulated spot in *Listeria monocytogenes* 412 (i) and 412C (ii). The arrow points out the relative position of the spot with the increased expression.

The resistance phenomena of strain 412C may be due to changes in the cell envelope and/or membrane. Alternatively, the EII_t^{man} may be shielded from the bacteriocin by another membrane protein. *Eschericha coli* also possesses a mannose permease that has high sequence homology to the permease in *L. monocytogenes* [13], yet *E. coli* is insensitive to class IIa bacteriocins [1]. This illustrates the important role that the cell envelope plays in the protection of a bacterium from class IIa bacteriocins. The shielding of the docking molecule form nisin has been proposed to be the mechanism by which some intermediate strains gain resistance to this bacteriocin [10]. A similar mechanism of resistance based on the restriction of accessibility to the docking molecule may also be utilized by strain 412C. Additional analysis of the 412C strain is necessary, as the mechanism of resistance employed by the strain may be more complex than the availability of a docking molecule.

In summary, sub-lethal exposure of sensitive, intermediate and totally resistant cells to D and L-leucocin A did not result in the detectable modulation of any protein expression levels. No changes in the MptA expression were observed when the wild type and intermediate resistant strain were challenged with sub-lethal levels of L-leucocin A. The up-regulation of the *mpt* operon by strain 412C indicates that factors other than the *mpt* operon may also be involved in the acquisition of intermediate resistance.

Reference List

1 **Abee, T., L. Krockel, and C. Hill**. 1995. Bacteriocins: modes of action and potentials in food preservation and control of food poisoning. Int. J. Food Microbiol. **28**:169-185.

2 **Christensen, D. P. and R. W. Hutkins**. 1994. Glucose uptake by *Listeria monocytogenes* Scott A and inhibition by pediocin JD. Appl. Environ. Microbiol. **60**:3870-3873.

3 **Cleveland, J., T. J. Montville, I. F. Nes, and M. L. Chikindas**. 2001. Bacteriocins: safe, natural antimicrobials for food preservation. Int. J. Food Microbiol. **71**:1-20.

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

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

6 **Dykes, G. A. and J. W. Hastings**. 1998. Fitness costs associated with class IIa bacteriocin resistance in *Listeria monocytogenes* B73. Lett. Appl. Microbiol. **26**:5-8.

7 **Ennahar, S., T. Sashihara, K. Sonomoto, and A. Ishizaki**. 2000. Class IIa bacteriocins: biosynthesis, structure and activity. FEMS Microbiol. Rev. **24**:85-106.

8 **Glaser, P., L. Frangeul, C. Buchrieser, C. Rusniok, A. Amend, F. Baquero, P. Berche, H. Bloecker, P. Brandt, T. Chakraborty, A. Charbit,**

F. Chetouani, E. Couve, A. de Daruvar, P. Dehoux, E. Domann, G. Dominguez-Bernal, E. Duchaud, L. Durant, O. Dussurget, K. D. Entian, H. Fsihi, F. G. Portillo, P. Garrido, L. Gautier, W. Goebel, N. Gomez-Lopez, T. Hain, J. Hauf, D. Jackson, L. M. Jones, U. Kaerst, J. Kreft, M. Kuhn, F. Kunst, G. Kurapkat, E. Madueno, A. Maitournam, J. M. Vicente, E. Ng, H. Nedjari, G. Nordsiek, S. Novella, B. de Pablos, J. C. Perez-Diaz, R. Purcell, B. Remmel, M. Rose, T. Schlueter, N. Simoes, A. Tierrez, J. A. Vazquez-Boland, H. Voss, J. Wehland, and P. Cossart. 2001. Comparative genomics of *Listeria* species. Science **294**:849-852.

9 **Gravesen, A., P. Warthoe, S. Knøchel, and K. Thirstrup**. 2000. Restriction fragment differential display of pediocin-resistant *Listeria monocytogenes* 412 mutants shows consistent overexpression of a putative beta-glucoside-specific PTS system. Microbiology **146**:1381-1389.

10 **Gravesen, A., K. Sørensen, F. M. Aarestrup, and S. Knøchel**. 2001. Spontaneous nisin-resistant *Listeria monocytogenes* mutants with increased expression of a putative penicillin-binding protein and their sensitivity to various antibiotics. Microb. Drug Resist. **7**:127-135.

11 **Gravesen, A., A. M. Jydegaard Axelsen, D. S. Mendes, T. B. Hansen, and S. Knøchel**. 2002. Frequency of bacteriocin resistance development and associated fitness costs in *Listeria monocytogenes*. Appl. Environ. Microbiol. **68**:756-764.

12 **Gravesen, A., M. Ramnath, K. B. Rechinger, N. Andersen, L. Jänsch, Y. Hechard, J. W. Hastings, and S. Knøchel**. 2002. High-level resistance to class IIa bacteriocins is associated with one general mechanism in *Listeria monocytogenes*. Microbiology **148**:2361-2369.

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

14 **Héchard, Y. and H.-G. Sahl**. 2002. Mode of action of modified and unmodified bacteriocins from Gram-positive bacteria. Biochimie **84**:545-557.

15 **Jack, R. W., J. R. Tagg, and B. Ray**. 1995. Bacteriocins of gram-positive bacteria. Microbiol. Rev. **59**:171-200.

16 **Klaenhammer, T. R.** 1993. Genetics of bacteriocins produced by lactic acid bacteria. FEMS Microbiol. Rev. **12**:39-85.

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

18 **Nes, I. F. and H. Holo**. 2000. Class II antimicrobial peptides from lactic acid bacteria. Biopolymers **55**:50-61.

19 **Neuhoff, V., N. Arold, D. Taube, and W. Ehrhardt**. 1988. Improved staining of proteins in polyacrylamide gels including isoelectric focusing gels with clear background at nanogram sensitivity using Coomassie Brilliant Blue G-250 and R-250. Electrophoresis **9**:255-262.

20 **O'Sullivan, L., R. P. Ross, and C. Hill**. 2002. Potential of bacteriocin-producing lactic acid bacteria for improvements in food safety and quality. Biochimie **84**:593-604.

21 **Ramnath, M., M. Beukes, K. Tamura, and J. W. Hastings**. 2000. Absence of a putative mannose-specific phosphotransferase system enzyme IIAB component in a leucocin A-resistant strain of *Listeria monocytogenes*, as shown by two-dimensional sodium dodecyl sulfate- polyacrylamide gel electrophoresis. Appl. Environ. Microbiol. **66**:3098-3101.

22 **Ramnath, M, K . B Rechinger, L. Jänsch, S. Knøchel, J. W. Hastings, and A. Gravesen**. 2003. Development of *Listeria monocytogenes* EGDe partial proteome reference map and comparision with the protein profiles of food isolates. Appl.Environ.Microbiol. **69**:3368-3376

23 **Santoni, V., M. Molloy, and T. Rabilloud**. 2000. Membrane proteins and proteomics: an amour impossible? Electrophoresis **21**:1054-1070.

24 **Song, H. J. and J. Richard**. 1997. Antilisterial activity of three bacteriocins used at sub minimal inhibitory concentrations and cross-resistance of the survivors. Int. J. Food Microbiol. **36**:155-161.

25 **Vadyvaloo, V., J. W. Hastings, M. J. van der Merwe, and M. Rautenbach**. 2002. Membranes of class IIa bacteriocin-resistant *Listeria monocytogenes* cells contain increased levels of desaturated and short-acyl-chain phosphatidylglycerols. Appl. Environ. Microbiol. **68**:5223-5230.

26 **Waite, B. L. and R. W. Hutkins**. 1998. Bacteriocins inhibit glucose PEP:PTS activity in *Listeria monocytogenes* by induced efflux of intracellular metabolites. J. Appl. Microbiol. **85**:287-292.

27 **Yan, L. Z., A. C. Gibbs, M. E. Stiles, D. S. Wishart, and J. C. Vederas**. 2000. Analogues of bacteriocins: antimicrobial specificity and interactions of leucocin A with its enantiomer, carnobacteriocin B2, and truncated derivatives. J. Med. Chem. **43**:4579-4581.

Chapter 5

Heterologous expression of a listerial mannose phosphotransferase system II facilitates sensitivity to class IIa bacteriocins in Lactococcus lactis

Abstract

The mannose phosphotransferase system II (EII_t^{man}) was previously implicated in the sensitivity of *Listeria monocytogenes* towards class IIa bacteriocins [Dalet et al Microbiology, 2001 **147**:1575-1580]. To test the hypothesis that EII_t^{man} could act as a docking molecule, the EII_t^{man} was heterologously expressed, using the nisin-controlled expression (NICE) system, in the *Lactoccus lactis* MG 1363 strain. This strain is normally insensitive to the class IIa bacteriocins, but became sensitive upon induction of the NICE system containing the *mpt* operon. Real-time PCR analysis of expression of the *mpt* operon, in the recombinant *L. lactis* strain, showed a 12.5-fold and a 372-fold transcriptional up-regulation upon induction with 0.25 ng/ml and 2.5 ng/ml nisin, respectively. Additionally, the increase in the induction concentration by a factor of 10 resulted in an inhibition of growth by approximately 10 %. The resultant conversion in phenotype towards class IIa bacteriocin sensitivity strongly suggests the direct involvement of the EII_t^{man} in class IIa bacteriocin activity.

Introduction

Class IIa bacteriocins are a subclass of antimicrobial proteinaceous compounds produced by lactic acid bacteria, that inhibits the potential food pathogen *Listeria monocytogenes* [24, 14, 22]. These peptides have been shown to exert their activity by the dissipation of the proton motor force of susceptible cells, possibly *via* membrane pore formation [14, 20, 28]. The initial interaction with the membrane surface is generally suspected to be an electrostatic binding of the bacteriocin to a putative membrane associated component [1, 7, 20]. Protease treatment of membrane vesicles, derived from sensitive cells, resulted in an increased resistance to pediocin. This was

the first indication of a protein docking membrane component [7]. The report of a specific interaction between leucocin A and a chiral molecule at the cell surface as a requirement for antimicrobial activity, further demonstrated the necessity of a receptor type-molecule [33]. However, other studies have indicated that a protein receptor may not be an absolute requirement for pore formation by class IIa bacteriocins [4, 6, 23]. Additionally, it was proposed that anionic lipids in the cytoplasmic membrane were the functional binding sites for class IIa bacteriocins [5, 6]

The mannose phosphotransferase system (PTS), EII_t^{man} (made up of IIAB, IIC, and IID components) was initially shown to be shutdown in a spontaneous mutant of *L. monocytogenes* showing resistance to the class IIa bacteriocin, leucocin A [31]. The same observation was also made for a number of high-level class IIa bacteriocin spontaneous mutants of *L. monocytogenes* [16]. Furthermore, defined genetic inactivation of the EII_t^{man} in *L. monocytogenes* and *Enterococcus faecalis* resulted in resistance to mesentericin Y105 [9, 19]. The EII_t^{man} has thus been proposed to either act as a docking molecule for class IIa bacteriocins or control the expression of a docking molecule [20, 10, 19, 31].

In order to assess the role of EII_t^{man} in bacterial sensitivity, we heterologously expressed the entire *mpt* operon in the class IIa insensitive *Lactococcus lactis* MG 1363 strain, using the nisin-controlled expression (NICE) system. The NICE system consists of two compatible replicons, a plasmid carrying the *nisRK* regulatory genes (regulatory plasmid) and an expression vector carrying the gene of interest under the control of the *nisA* promoter [12, 25, 29]. The expression of the gene of interest from the *nisA* promoter can be varied using different concentrations of the inducer nisin [11, 12]. This expression system was used to express transmembrane proteins previously (for review see, 26), and this ability was essential since the IID component of the permease (IIC-IID) proposed to be the docking molecule [10, 19, 20] is part of a membrane spanning protein complex [30]. Furthermore, the NICE system has been successfully used for the assessment of the role of a gene product implicated in nisin resistance [8].

The aim of this study was to determine if the presence of the EII_t^{man} could induce sensitivity to class IIa bacteriocins in an otherwise bacteriocin insensitive strain, in this case, *L. lactis* MG 1363.

Materials and Methods

Bacterial strains and plasmids

The bacterial strains and plasmids used in this study are listed in Table 1.

Bacterial growth conditions

L. lactis MG 1363 and derivatives were maintained at 30 °C in M17 (Difco) broth or agar, supplemented with 0.5 % glucose (w/v) without agitation. *Escherichia coli* XL-1 Blue, that was used for molecular cloning was grown at 37 °C in Luria-Bertani broth with agitation. *L. monocytogenes* strains were grown at 37 °C in Brain Heart Infusion broth (Difco) without agitation. When appropriate, the media was supplemented with the required antibiotics to the following final concentrations; chloramphenicol (10 μg/ml), erythromycin (5 μg/ml) or ampicillin (100 μg/ml). All antibiotics were purchased from Sigma Chemical Company (St. Louis, Mo., USA).

DNA manipulations

L. monocyogenes EGDe chromosomal DNA was isolated as previously described [27]. The *mpt* operon (*lmo*0096, *lmo*0097, and *lmo*0098) [15] encoding EII_t^{man}, was amplified by PCR using the proof reading polymerase, Pfx (Promega) and the following specific primers: forward primer (AM) 5'-TATATTAGGAGGGAAAAAGATGGTAGG-3' and reverse primer (MptDV) 5'-ATTATACCGTATTCGTTTATCTGTGTC-3'. The annealing and extension temperatures were 50 °C and 68 °C, respectively.

An A-tailing procedure for the addition of A overhangs onto the blunt-ended PCR product for sub-cloning into the pGEM®Easy-T vector (Promega), was carried out as recommended by the manufacturer. The purified blunt-ended PCR product was incubated with 5U Taq polymerase (Promega) in the presence of 0.2 mM dATP at 70 °C for 30 minutes. The A-tailed PCR product was then used as an insert for ligation with pGEM®Easy-T, which was set up as suggested by the manufacturer (Promega).

Table 1. Bacterial strains and plasmids

Strain/plasmid	Features	Selection	Reference or source
E. coli XL-1	Plasmid free cloning host		Stratagene
Lactococcus lactis			
MG 1363	Plasmid free cloning host		NIZO*
MG-Mpt	*L. lactis* MG 1363 containing pNZ-Mpt and pNZ 9530	Cm^r + Amp^r	This study
MG-Con	*L. lactis* MG 1363 containing pNZ 8020 and pNZ 9530	Cm^r + Amp^r	This study
Listeria monocytogenes			
EGDe	Serotype 1/2b, clinical isolate		Dalet et al., 2001[10]
B73-MR1	Spontaneously leucocin A resistant *L. monocytogenes* B73		Ramnath et al., 2000[31]
MR-MG-Mpt	*L. monocytogenes* B73-MR1 containing pNZ-Mpt and pNZ 9530	Cm^r + Amp^r	This study
MR-MG-Con	*L. monocytogenes* B73-MR1 containing pNZ 8020 and pNZ 9530	Cm^r + Amp^r	This study
Plasmids			
p-GEM®-T Easy	*E. coli* cloning vector with 3'-T overhangs	Amp^r	Promega
pMRI	*mpt* operon cloned into the multiple cloning site of p-GEM®-T Easy	Amp^r	This Study
pNZ 8020	*nisA* promoter tanscriptionally fused to a multiple cloning site	Cm^r	NIZO*
pNZ 9530	*nisR* and *nisK* (both expressed from the *rep* promoter)	Ery^r	NIZO*
pNZ-Mpt	*mpt* operon transcriptionally fused to the *nisA* promoter	Cm^r	This study

* Ede, The Netherlands

The ligation mix was electro-transformed into *E. coli* XL-1 using a Gene Pulser apparatus (Bio-Rad, Hercules, CA) with the following settings: 25 μF, 250 Ω, and 2.5 kV. White colonies were selected for further study. Plasmid DNA was extracted using the alkaline lysis method. The pGEM®Easy-T vector containing the *mpt* operon in the correct orientation was designated the name pMRI (Table 1).

The *mpt* operon was excised from pMRI using *Sph*I and *Sac*I and ligated to pNZ 8020 digested at the corresponding sites. Electro-competent *L. lactis* MG 1363 cells were prepared as described previously [18]. Cells were grown until a maximum OD_{600} of 0.5 and placed on ice for 15 minutes; following which they were harvested and resuspended in electroporation buffer (5M sucrose; 2.5 mM magnesium chloride) and incubated on ice for a further 15 minutes. The final step of harvesting and resuspension of cells with electroporation buffer was repeated thrice.

The ligation mix was electro-transformed into electro-competent *L. lactis* MG 1363 cells, utilising the same settings as those used for the electro-transformation of *E. coli*. Plasmids from *L. lactis* transformants were extracted as described previously [2] with the following modifications: incubation with lysozyme (10 mg/ml) was performed at 37°C for 1h; protoplasts were harvested and resuspended in lysis buffer (50 mM Tris-HCl; 1 mM EDTA; and 3 % (w/v) sodium dodecyl sulfate); a high-salt solution (3 M potassium acetate and 1.8 M formic acid) was added prior to phenol extraction. The pNZ 8020 plasmid containing the *mpt* operon in the correct orientation was designated the name pNZ-Mpt (Table 1). The strain harbouring pNZ-Mpt was also electro-transformed with pNZ 9530 yielding the recombinant strain *L. lactis* MG-Mpt (Table 1). Sequential electro-transformations of *L. lactis* MG 1363 with pNZ 8020 and pNZ 9530 yielded the recombinant strain *L. lactis* MG-Con (Table 1), which served as the control strain not containing the *mpt* operon.

Bacteriocin preparation

Nisin, a class I bacteriocin was purchased as a 2.5 % (w/w) powder containing sodium chloride and denatured milk solids (Sigma), which was reconstituted in analytical grade water. Leucocin A was synthesized as described previously [31] and resuspended in 50 % acetonitrile. Pediocin PA-1 [21] and enterocin A [3] were produced by *Pediococcus acidilactici* NRRL B5627 and *Enterococcus faecium* 336,

respectively. These bacteriocins were purified as described previously [17]. All bacteriocin stocks were stored at –20°C until used.

Minimum inhibitory concentration (MIC) of nisin to *L. lactis* MG 1363 and its recombinant derivatives

L. lactis is sensitive to nisin [24], therefore a sub-lethal concentration of nisin had to be determined for the induction of *mpt* expression. The MIC to nisin was determined in microtitre plates by transferring a 1% inoculum of an overnight culture of either *L. lactis* MG 1363, MG-Mpt or MG-Con into fresh media containing the appropriate antibiotics (Table 1). These cultures were then monitored until an OD_{630} of 0.05 was reached. Each well was then supplemented with nisin that had been serially diluted in water. The MIC was considered to be the lowest concentration of the bacteriocin that inhibited growth completely. All MIC determinations are represented by an average of four independent experiments.

Expression of the *mpt* operon

For the expression of the *mpt* operon, microtitre plates were set up as described in the above section using either *L. lactis* MG-Mpt or the control strain (MG-Con) as the inoculum. Once the desired OD_{630} was achieved cultures were induced by addition of stock solutions of nisin at 0.25, 1.25, or 2.5 ng/ml per well. Growth was allowed to continue for a further 1.5 h. After this induction period, each well was supplemented with 20 µg/ml leucocin A and its growth was monitored. To assess the effect of leucocin A alone on growth, controls lacking nisin were monitored simultaneously. All experiments were carried out in triplicate on two independent occasions. The *L. lactis* MG-con served as a negative control for *mpt* expression.

MIC of leucocin A to *L. lactis* MG-Mpt

Microtitre plates were set up as described for the expression of the *mpt* operon, with the exception that *L. lactis* MG-Mpt was induced with 2.5 ng/ml of nisin only. Wells were then supplemented with serially diluted leucocin A that corresponded to a concentration range from 20 to 0.15625 µg/ml. The susceptibility of *L. lactis* MG-Con to leucocin A was determined in microtitre plates as described for strain MG-Mpt, except that 400 µg/ml leucocin A was also added.

Effect of other class IIa bacteriocins on induced *L. lactis* MG-Mpt

The effect of purified pediocin PA-1 and enterocin A on *L. lactis* MG-Mpt induced by nisin was also tested to determine if *L. lactis* MG-Mpt became sensitive to other class IIa bacteriocins. The activity units of the purified bacteriocins and synthetic leucocin A were determined using the spot-on-lawn assay [31]. Five µl of a two-fold serial dilution of the bacteriocins in 50 % acetonitrile were transferred onto the surface of BHI agar lawns (0.7 % agar, 0.1 % Tween 80) previously inoculated with *L. monocytogenes* B73 to yield a lawn of cells. The synthetic leucocin A stock was diluted to the same number of activity units as those of the purified bacteriocins. Microtitre plates were set up as described for the induction of the *mpt* operon. The *L. lactis* MG-Mpt strain was induced with 2.5 ng/ml of nisin followed by the addition of approximately 2 000 AU of leucocin A, pediocin PA-1 or enterocin.

cDNA synthesis

An overnight culture of *L. lactis* MG-Mpt was diluted to the ratio 1:100 in fresh media and its growth was monitored until an OD_{600} of 0.2 was attained. Nisin was then supplemented to the final concentrations of 0.25 and 2.5 ng/ml, respectively. A control not induced with nisin was also prepared. Total RNA was isolated from cells (10 ml) 2 h after the addition of nisin, 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 hexamers, and the Superscript II Kit (Gibco), according to the manufacturer's instructions. A reaction containing all the components, omitting reverse transcriptase, was included in order to assess DNA contamination.

Real-time PCR

The primers to monitor the expression of *mptA* in the real-time PCR were MPTF3, 5'-CAGGACTTAATTTGCCAATGTTG-3' and MPTR3, 5'-CGCGAACACCTTCTTGAGCT-3'. 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 TaqMan Univeral PCR Master 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 10 s 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.

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 uninduced strain from the mean C_T value in the induced strain.

Statistical evaluation

Tukey's comparative test using Prism 3.0 was used to statistically evaluate the change in the level of sensitivity of *L. lactis* MG-Mpt to leucocin A after induction with varying concentrations of nisin.

Results and Discussion

Determination of sub-lethal nisin induction levels for *mpt* expression in *L .lactis*

L. lactis MG1363, MG-Con and MG-Mpt all had MICs of 125 ng/ml for nisin (Fig. 1). Levels of nisin up to 62.5 ng/ml did not significantly affect the growth of MG 1363 and MG-Con (Fig. 1a and b). Nisin concentrations between 0.25 and 2.5 ng/ml were therefore selected for induction of *mpt* expression, since they caused the least effect on *L. lactis* MG-Mpt growth (Fig. 1c). The nisin concentrations chosen for induction ranged between 500 and 50 times below the MIC of the *L. lactis* strains and were similar to levels used previously for *L. lactis* [13, 25, 12]. However, a dose dependent effect on MG-Mpt can be observed in Fig.1c.

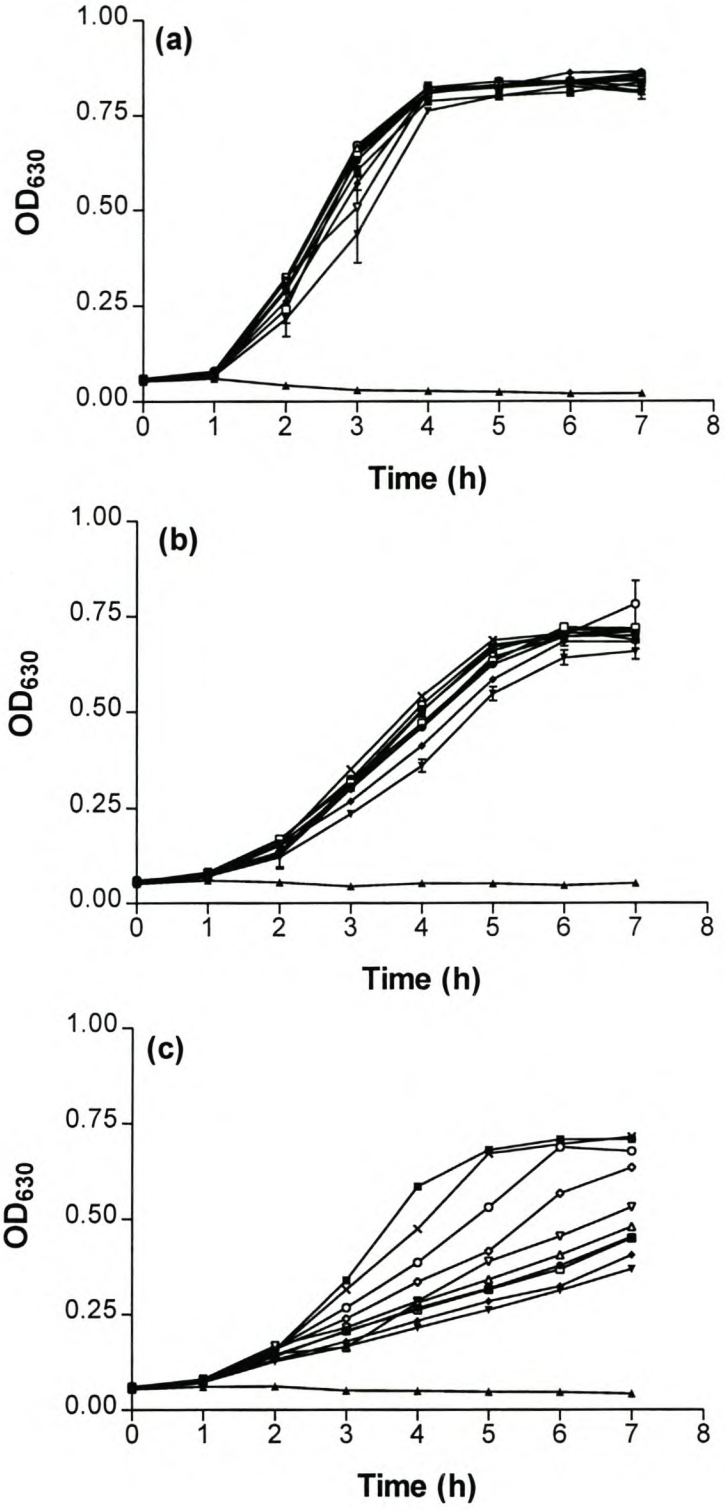


Fig. 1. Growth of *Lactococcus lactis*: MG 1363 (a); MG-Con (no *mpt* operon) (b); and MG-Mpt (containing the *mpt* operon) (c) in BHI broth at 37°C after exposure to doubling dilutions of nisin. Strains were grown up to an OD_{630} of approximately 0.05 before the addition of nisin to the following final concentrations; 0 ng/ml (■), 125 ng/ml (▲), 62.5 ng/ml (▼), 31.25 ng/ml (♦), 15.63 ng/ml (•), 7.81 ng/ml (□), 3.91 ng/ml (Δ), 1.95 ng/ml (∇), 0.98 ng/ml (◊), 0.49 ng/ml (○) and .24 ng/ml (**x**). Time zero represents the point of nisin addition. Error bars represent standard deviations of the mean of quadruplicate experiments.

A similar inhibition of MG-Con by nisin was not observed (Fig. 1b), indicating that components of the NICE expression system were not responsible for the observed increase in sensitivity of the MG-Mpt strain to nisin.

Real-time PCR results indicated that there was a 12.5-fold and a 372-fold up-regulation in expression of the *mpt* operon, upon induction with 0.25 ng/ml and 2.5 ng/ml nisin, respectively, relative to the non-induced control. The observed dose dependent effect by nisin on MG-Mpt growth (Fig. 1c) may be attributed to the energy-consuming steps involved in the transcription and translation of the *mpt* operon.

Induction of the *mpt* operon

Specific growth rate of *L. lactis* MG-Mpt was inhibited approximately >3-fold by leucocin A at all the nisin induction concentrations (Table 2). No inhibition indicated by the decreased growth rate in the presence of leucocin A was observed for *L. lactis* MG-Con (Table 2), indicating that the inhibitory effect observed for *L. lactis* MG-Mpt, was not due to synergistic effect with nisin, nor was it due to components of the NICE expression system. It appears that sensitivity to leucocin A requires the presence of the EII_t^{man}. Independent repetitions showed the same result.

The dose dependent manner in which the sensitivity to a class IIa bacteriocin was enhanced with an increase in expression of the EII_t^{man}, using either mannose or glucose, has been previously reported [19, 10]. However, these studies could not exclude the possibility that the sugars may induce the expression of gene products other than EII_t^{man} that could also contribute to bacteriocin sensitivity. In this study the Tukey's comparison test showed the change in sensitivity between the induction concentration of 0.25 and 2.5 ng/ml nisin to be statistically significant ($P < 0.001$). The increase in the induction concentration by a factor of 10 resulted in a 10 % reduction in growth as indicated by a lower optical density (OD) (Fig. 2). The increase in induction concentration corresponds to a 30-fold increase in the transcriptional levels of the *mpt* operon as determined by real-time PCR. This suggested that the expression level of EII_t^{man} is associated with level of sensitivity displayed by *L. lactis* MG-Mpt to synthetic leucocin A as indicated by the reduction in growth.

Table 2. Specific growth rates (μ) of *Lactococcus lactis* MG-Mpt (*mpt* operon in NICE expression system) and MG.Con (no *mpt* operon in NICE system) after the induction of expression of the NICE system with nisin and exposure to leucocin A. Strains were grown up to an OD_{630} of 0.05 prior to induction of the *nisA* promoter with nisin for 90 minutes. After induction, leucocin A was supplemented into the culture.

Treatment[1]	(μ)MG-Mpt[2] (h^{-1})	(μ)MG-Con[3] (h^{-1})	Fold decrease in growth rate
Control	0.52	0.44	N/A
Acetonitrile	0.52	0.47	N/A
20 μg/ml Leucocin A	0.53	0.47	N/A
2.5 ng/ml nisin	0.37	0.46	1.2
1.25 ng/ml nisin	0.38	0.46	1.2
0.25 ng/ml nisin	0.45	0.44	1.0
2.5 ng/ml nisin + 20 μg/ml leucocin A	0.09	0.46	5.1
1.25 ng/ml nisin + 20 μg/ml leucocin A	0.08	0.47	5.9
0.25 ng/ml nisin + 20 μg/ml leucocin A	0.12	0.51	3.9

[1]final concentrations are indicated

[2](pNZ-Mpt + pNZ 9530)

[3](pNZ 8020 + pNZ 9530)

N/A – not applicable

The observed increase in sensitivity may be explained by the increase in number of potential docking complexs namely, EII_t^{man}, which would probably lead to the increased permeabilization of the membrane.

The two-plasmid NICE system containing the *mpt* operon was transformed into the class IIa bacteriocin resistant *L. monocytogenes* B73-MR1 strain (Table 1). A clear synergistic inhibitory effect between nisin and leucocin A lead to ambiguous results and definite conclusions could not be drawn regarding the B73-MR1 strain's resistance to leucocin A. In future studies, it may be necessary to consider alternative

expression systems in attempting to clarify the role of the *mpt* operon in *L. monocytogenes* resistance.

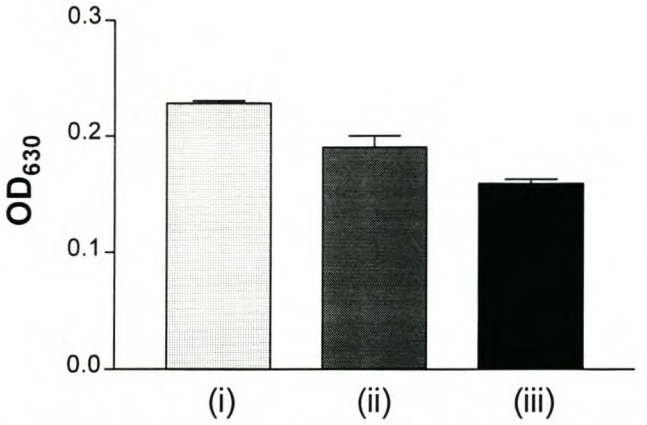


Fig. 2. Bar graph representation of the growth at OD_{630} of *Lactoccus lactis* MG-Mpt (*mpt* operon cloned into NICE system) after 3.5 h of exposure to 20 μg/ml of leucocin A, following induction with 0.25 ng/ml nisin (i), 1.25 ng/ml nisin (ii) and 2.5 ng/ml nisin (iii). Error bars represent standard deviations of the mean of triplicate experiments.

Susceptibility of *L. lactis* MG-Mpt to leucocin A, pediocin and enterocin

The induced *L. lactis* MG-Mpt strain was sensitive to levels of leucocin A below 156.3 ng/ml, (Fig. 3). In contrast, the control strain MG-Con was unaffected by 400 μg/ml leucocin A. The nisin induced *L. lactis* MG-Mpt, therefore, had increased sensitivity in excess of 2500-fold towards leucocin A. Induction of *L. lactis* MG-Mpt with 2.5 ng/ml nisin resulted in a similar level of sensitivity as that of other normally sensitive strains of *L. monocytogenes* towards synthetic leucocin A [16, 32]. *L. lactis* MG-Mpt was also equally sensitive to both pediocin and enterocin (Fig. 4).

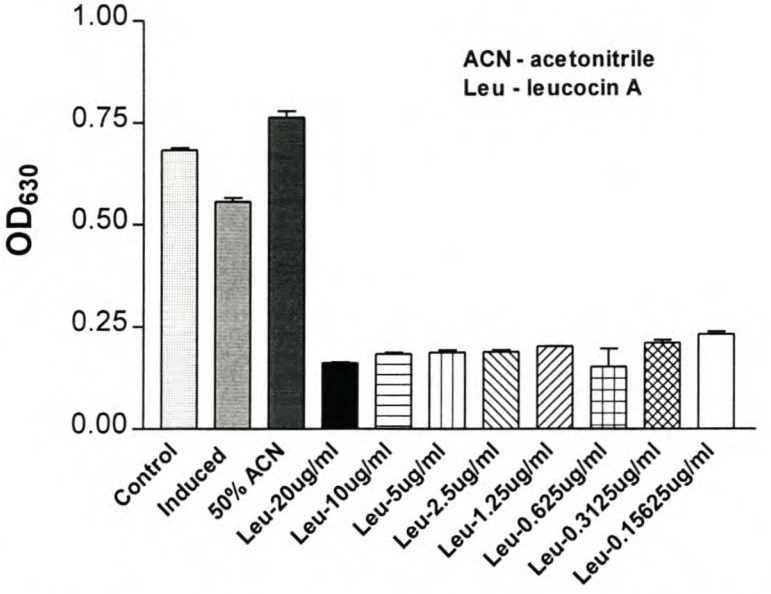


Fig. 3. The effect on *Lactococcus lactis* MG-Mpt (*mpt* operon in NICE expression system) induced with 2.5 ng/ml nisin, after 9 hours of exposure to leucocin A. Error bars represent, standard deviations from the means of quadruplicate experiments.

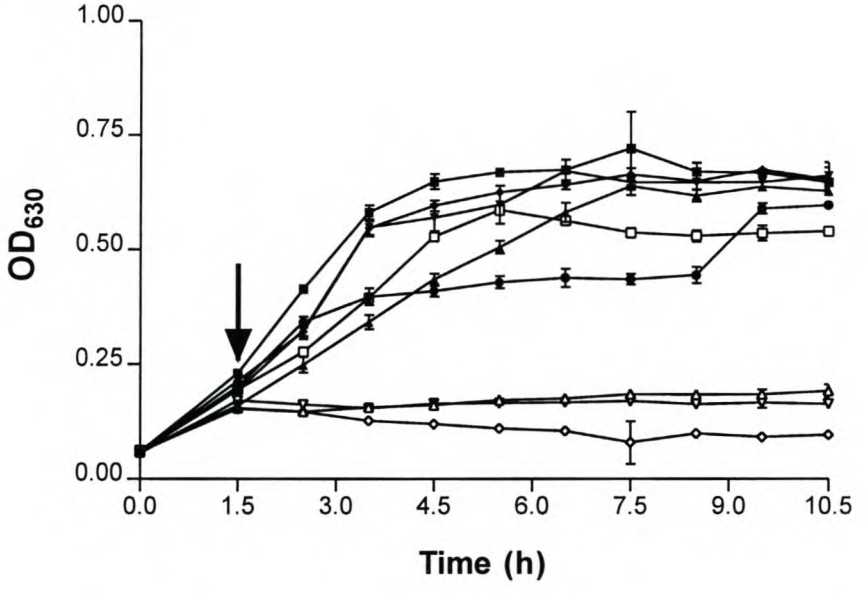


Fig. 4. Effect of pediocin, leucocin A and enterocin on *Lactococcus lactis* MG-Mpt (*mpt* operon cloned into the NICE system) grown in BHI broth at 30°C after induction with 2.5 ng/ml nisin. Approximately 2 000 AU of each bacteriocin was supplemented into each well as follows: uninduced control (■), induced and with no class IIa bacteriocin supplementation (▲), acetonitrile control (▼), leucocin A only (♦), pediocin PA-1 only (●), enterocin A only (□), induced and supplemented with leucocin A (Δ), induced and supplemented with pediocin PA-1 (∇), and induced and supplemented with enterocin (◊). Time zero represents the point of nisin addition with the arrow indicating the point of supplementation of class IIa bacteriocins. Error bars represent standard deviations of the mean of quadruplicate experiments.

In summary, our study shows a direct correlation between the presence of the EII_t^{man} and the sensitivity to class IIa bacteriocins with no ambiguity associated with downstream genes possible. Based on the current evidence we propose that the entire EII_t^{man} serves as a docking complex for class IIa bacteriocins, which facilitates sensitivity to this subclass of bacteriocins in a dose dependent fashion. The membrane bound permease (IIC-IID) occupies a position at the membrane water interface [30] and could therefore mediate interaction with the bacteriocin.

Reference List

1 **Abee, T.** 1995. Pore-forming bacteriocins of gram-positive bacteria and self-protection mechanisms of producer organisms. FEMS Microbiol. Lett. **129**:1-10.

2 **Anderson, D. G. and L. L. McKay**. 1983. Simple and rapid method for isolating large plasmid DNA from lactic streptococci. Appl. Environ. Microbiol. **46**:549-552.

3 **Aymerich, T., H. Holo, L. S. Havarstein, M. Hugas, M. Garriga, and I. F. Nes**. 1996. Biochemical and genetic characterization of enterocin A from *Enterococcus faecium*, a new antilisterial bacteriocin in the pediocin family of bacteriocins. Appl. Environ. Microbiol. **62**:1676-1682.

4 **Chen, Y., R. D. Ludescher, and T. J. Montville**. 1997. Electrostatic interactions, but not the YGNGV consensus motif, govern the binding of pediocin PA-1 and its fragments to phospholipid vesicles. Appl. Environ. Microbiol. **63**:4770-4777.

5 **Chen, Y., R. D. Ludescher, and T. J. Montville**. 1998. Influence of lipid composition on pediocin PA-1 binding to phospholipid vesicles. Appl. Environ. Microbiol. **64**:3530-3532.

6 **Chen, Y., R. Shapira, M. Eisenstein, and T. J. Montville**. 1997. Functional characterization of pediocin PA-1 binding to liposomes in the absence of a protein receptor and its relationship to a predicted tertiary structure. Appl. Environ. Microbiol. **63**:524-531.

7 **Chikindas, M. L., M. J. Garcia-Garcèra, A. J. Driessen, A. M. Ledeboer, J. Nissen-Meyer, I. F. Nes, T. Abee, W. N. Konings, and G. Venema**. 1993. Pediocin PA-1, a bacteriocin from *Pediococcus acidilactici* PAC1.0, forms hydrophilic pores in the cytoplasmic membrane of target cells. Appl. Environ. Microbiol. **59**:3577-3584.

8 **Cotter, P. D., C. M. Guinane, and C. Hill**. 2002. The LisRK signal transduction system determines the sensitivity of *Listeria monocytogenes* to nisin and cephalosporins. Antimicrob. Agents Chemother. **46**:2784-2790.

9 **Dalet, K., C. Briand, Y. Cenatiempo, and Y. Hèchard**. 2000. The *rpoN* gene of *Enterococcus faecalis* directs sensitivity to subclass IIa bacteriocins. Curr. Microbiol. **41**:441-443.

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

11 **De Ruyter, P. G., O. P. Kuipers, M. M. Beerthuyzen, I. Alen-Boerrigter, and W. M. de Vos**. 1996. Functional analysis of promoters in the nisin gene cluster of *Lactococcus lactis*. J. Bacteriol. **178**:3434-3439.

12 **De Ruyter, P. G., O. P. Kuipers, and W. M. de Vos**. 1996. Controlled gene expression systems for *Lactococcus lactis* with the food-grade inducer nisin. Appl. Environ. Microbiol. **62**:3662-3667.

13 **Eichenbaum, Z., M. J. Federle, D. Marra, W. M. de Vos, O. P. Kuipers, M. Kleerebezem, and J. R. Scott**. 1998. Use of the lactococcal *nisA* promoter to regulate gene expression in gram-positive bacteria: comparison of induction level and promoter strength. Appl. Environ. Microbiol. **64**:2763-2769.

14 **Ennahar, S., T. Sashihara, K. Sonomoto, and A. Ishizaki**. 2000. Class IIa bacteriocins: biosynthesis, structure and activity. FEMS Microbiol. Rev. **24**:85-106.

15 **Glaser, P., L. Frangeul, C. Buchrieser, C. Rusniok, A. Amend, F. Baquero, P. Berche, H. Bloecker, P. Brandt, T. Chakraborty, A. Charbit, F. Chetouani, E. Couve, A. de Daruvar, P. Dehoux, E. Domann, G. Dominguez-Bernal, E. Duchaud, L. Durant, O. Dussurget, K. D. Entian, H. Fsihi, F. G. Portillo, P. Garrido, L. Gautier, W. Goebel, N. Gomez-Lopez, T. Hain, J. Hauf, D. Jackson, L. M. Jones, U. Kaerst, J. Kreft, M. Kuhn, F. Kunst, G. Kurapkat, E. Madueno, A. Maitournam, J. M. Vicente, E. Ng, H. Nedjari, G. Nordsiek, S. Novella, B. de Pablos, J. C. Perez-Diaz, R. Purcell, B. Remmel, M. Rose, T. Schlueter, N. Simoes, A. Tierrez, J. A. Vazquez-Boland, H. Voss, J. Wehland, and P. Cossart**. 2001. Comparative genomics of *Listeria* species. Science **294**:849-852.

16 **Gravesen, A., M. Ramnath, K. B. Rechinger, N. Andersen, L. Jänsch, Y. Hèchard, J. W. Hastings, and S. Knøchel**. 2002. High-level resistance to class IIa bacteriocins is associated with one general mechanism in *Listeria monocytogenes*. Microbiology **148**:2361-2369.

17 **Guyonnet, D., C. Fremaux, Y. Cenatiempo, and J. M. Berjeaud**. 2000. Method for rapid purification of class IIa bacteriocins and comparison of their activities. Appl. Environ. Microbiol. **66**:1744-1748.

18 **Hèchard, Y., J. M. Berjeaud, and Y. Cenatiempo**. 1999. Characterization of the *mesB* gene and expression of bacteriocins by *Leuconostoc mesenteroides* Y105. Curr. Microbiol. **39**:265-269.

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

20 **Hèchard, Y. and H. -G. Sahl**. 2002. Mode of action of modified and unmodified bacteriocins from Gram-positive bacteria. Biochimie **84**:545-557.

21 **Henderson, J. T., A. L. Chopko, and P. D. van Wassenaar**. 1992. Purification and primary structure of pediocin PA-1 produced by *Pediococcus acidilactici* PAC-1.0. Arch. Biochem. Biophys. **295**:5-12.

22 **Jack, R. W., J. R. Tagg, and B. Ray**. 1995. Bacteriocins of gram-positive bacteria. Microbiol. Rev. **59**:171-200.

23 **Kaiser, A. L. and T. J. Montville**. 1996. Purification of the bacteriocin bavaricin MN and characterization of its mode of action against *Listeria monocytogenes* Scott A cells and lipid vesicles. Appl. Environ. Microbiol. **62**:4529-4535.

24 **Klaenhammer, T. R.** 1993. Genetics of bacteriocins produced by lactic acid bacteria. FEMS Microbiol. Rev. **12**:39-85.

25 **Kleerebezem, M., M. M. Beerthuyzen, E. E. Vaughan, W. M. de Vos, and O. P. Kuipers**. 1997. Controlled gene expression systems for lactic acid bacteria: transferable nisin-inducible expression cassettes for *Lactococcus*, *Leuconostoc*, and *Lactobacillus* spp. Appl. Environ. Microbiol. **63**:4581-4584.

26 **Kunji, E. R., D. J. Slotboom, and B. Poolman**. 2003. *Lactococcus lactis* as host for overproduction of functional membrane proteins. Biochim. Biophys. Acta **1610**:97-108.

27 **Mengaud, J., C. Geoffroy, and P. Cossart**. 1991. Identification of a new operon involved in *Listeria monocytogenes* virulence: its first gene encodes a protein homologous to bacterial metalloproteases. Infect. Immun. **59**:1043-1049.

28 **Montville, T. J. and Y. Chen**. 1998. Mechanistic action of pediocin and nisin: recent progress and unresolved questions. Appl. Microbiol. Biotechnol. **50**:511-519.

29 **Pavan, S., P. Hols, J. Delcour, M. C. Geoffroy, C. Grangette, M. Kleerebezem, and A. Mercenier**. 2000. Adaptation of the nisin-controlled expression system in *Lactobacillus plantarum*: a tool to study in vivo biological effects. Appl. Environ. Microbiol. **66**:4427-4432.

30 **Postma, P. W., J. W. Lengeler, and G. R. Jacobson**. 1993. Phosphoenolpyruvate:carbohydrate phosphotransferase systems of bacteria. Microbiol. Rev. **57**:543-594.

31 **Ramnath, M., M. Beukes, K. Tamura, and J. W. Hastings**. 2000. Absence of a putative mannose-specific phosphotransferase system enzyme IIAB component in a leucocin A-resistant strain of *Listeria monocytogenes*, as shown by two-dimensional sodium dodecyl sulfate- polyacrylamide gel electrophoresis. Appl. Environ. Microbiol. **66**:3098-3101.

32 **Vadyvaloo, V., J. W. Hastings, M. J. van der Merwe, and M. Rautenbach**. 2002. Membranes of class IIa bacteriocin-resistant *Listeria monocytogenes* cells contain increased levels of desaturated and short-acyl-chain phosphatidylglycerols. Appl. Environ. Microbiol. **68**:5223-5230.

33 **Yan, L. Z., A. C. Gibbs, M. E. Stiles, D. S. Wishart, and J. C. Vederas**. 2000. Analogues of bacteriocins: antimicrobial specificity and interactions of leucocin A with its enantiomer, carnobacteriocin B2, and truncated derivatives. J. Med. Chem. **43**:4579-4581.

Chapter 6

General conclusions

The development of bacterial resistance is a universal problem complicating the use of chemotherapeutic agents [6]. Resistance to class IIa bacteriocins in *L. monocytogenes* is also a potential problem. The purpose of this study was to enhance our understanding of the molecular mechanisms of high-level resistance to class IIa bacteriocins, and in turn further elucidate the mode action of these antimicrobial peptides.

Proteomic analysis in *L. monocytogenes* has been extensively used to study a number of stress responses [5, 8, 13, 14, 15]. We have optimised a reproducible protein extraction method and 2-D electrophoresis protocol to study proteins extracted from *L. monocytogenes* strains. This protocol may be useful to researchers that are investigating protein expression changes in *L. monocytogenes*. The partial 2-D map generated for the *L. monocytogenes* EGDe strain could be a valuable tool for the identification of predominant spots in food-isolated strains of *L. monocytogenes*.

Prior to our study several seemingly unrelated molecular changes associated with class IIa resistance development had been reported [3, 7, 16]. We have studied the molecular bases of high-level class IIa bacteriocin development by studying several strains that were highly resistant to four diverse class IIa bacteriocins that were isolated after five independent wild type strains were exposed to the bacteriocins. The prevention of synthesis of the mannose phosphotransferase system (PTS) (encoding EII_t^{man}) was established as the basis of high-level resistance. Increased expression of the β-glucoside PTS and a phospho-β-glucosidase among all the resistant strains was possibly a regulatory consequence of the abolishment of EII_t^{man} expression. A general mechanism of resistance was therefore identified regardless of the wild type strain, class IIa bacteriocin, or the environmental condition. In addition, we found that resistant strains generated in food systems also acquired resistance using the same general mechanism.

Sub-lethal challenge using leucocin A did not result in any observable proteomic changes in *L. monocytogenes* strains that would provide clues as to the sequence of events that leads to the shut down of the *mpt* operon associated with resistance. The exposure to sub-lethal levels of bacteriocin was found not to down regulate the mannose PTS at the transcriptional level. Identification of physical parameters which influence the shut down of the operon would vastly improve the efficiency of class IIa bacteriocins in the food industry.

Previous studies have implicated the mannose PTS in the sensitivity toward class IIa bacteriocins [2, 9]. However, in all the studies the regulation of other genes by the *mpt* operon could not be excluded. The positive heterologous expression of the entire *mpt* operon in the bacteriocin insensitive *Lactococcus lactis* MG 1363 resulted in induction of bacteriocin sensitivity in this strain. The recombinant *L. lactis* showed a similar level of susceptibility to class IIa bacteriocins as the sensitive *L. monocytogenes* strains. This was the first direct evidence that the EII_t^{man} was required for sensitivity to class IIa bacteriocins.

Our findings therefore, support the recently proposed model for class IIa bacteriocin mode of action [10], where it was proposed that EII_t^{man} was the docking complex for class IIa bacteriocins [10]. Previous studies of defined mutants with knock-outs of the distal gene of the *mpt* operon and a mutant with a deletion of the extra domain found on $EIID^{man}$, showed that these strains were insensitive to class IIa bacteriocins [2]. Moreover, our studies on the same mutants showed lack of expression of the $EIIAB^{man}$ indicating that the entire operon was shut down. Therefore, it remains to be determined if $EIID^{man}$ facilitates the interaction between EII_t^{man} and the bacteriocin [10, 2]. The knockout of the mannose PTS in *Enterococcus faecalis* also resulted in the insensitivity to class IIa bacteriocins [9]. In combination with the induction of sensitivity by heterologous expression of EII_t^{man} in the bacteriocin insensitive *L. lactis* MG 1363 it may be hypothesised that EII_t^{man} is the universal receptor for all class IIa bacteriocins in some different bacterial strains. Even though we have identified a complex that facilitates the activity of class IIa bacteriocins, the physical interaction between the bacteriocin and the possible docking complex remains to be elucidated. Furthermore, the procedure by which EII_t^{man} facilities membrane permeability is unknown. It would be interesting to investigate whether class IIa bacteriocins also

makes use of this putative docking molecule as an anchor for pore formation to form part of a pore as reported for nisin and Lipid II[1].

The evidence thus far indicates that the presence of the EII_t^{man} and its level of expression correlated with sensitivity to class IIa bacteriocins [2, 9]. However, there was increased expression level of $EIIAB^{man}$ in the intermediately resistant strain, *L. monocytogenes* 412C. Another study also showed that, intermediately resistant strains generated from a single wild type strain had, an increase in the positive charge of the cell wall, possible increased cell membrane fluidity, and also reduced expression of the mannose PTS [Vadyvaloo et al., submitted]. Hence, further investigation is required to determine other factors influencing sensitivity in *L. monocytogenes* 412C.

The requirement of a single receptor may explain the narrow spectrum of activity of class IIa bacteriocins [4, 11, 12]. The use of an antimicrobial agent that has an absolute requirement of a single docking molecule that is not critical for cellular activity may not be very successful. This may count against the use of class IIa bacteriocins in the food industry for the control of *L. monocytogenes* strains because of the stability of the resistance phenotype although missing EII_t^{man}. It maybe more feasible to use class IIa bacteriocins in combination with other bacteriocins having different target molecules. It is less likely that stable resistant populations with modifications to multiple target molecules may occur.

In conclusion our studies indicate an unambiguous relationship between high-level class IIa bacteriocin resistance, and EII_t^{man} expression. It is also clear that EII_t^{man} facilitates the activity of class IIa bacteriocins. A direct physical interaction between the EII_t^{man} and class IIa bacteriocins however, needs to be determined to verify the role of EII_t^{man} as a docking complex for class IIa bacteriocins.

Reference List

1 **Breukink, E., H. E. Van Heusden, P. J. Vollmerhaus, E. Swiezewska, L. Brunner, S. Walker, A. J. Heck, and B. De Kruijff**. 2003. Lipid II is an intrinsic component of the pore induced by nisin in bacterial membranes. J. Biol. Chem. e-published ahead of print.

2 **Dalet, K., Y. Cenatiempo, P. Cossart, and Y. Héchard**. 2001. A sigma[54]-dependent PTS permease of the mannose family is responsible for sensitivity of *Listeria monocytogenes* to mesentericin Y105. Microbiology **147**:3263-3269.

3 **Duffes, F., P. Jenoe, and P. Boyaval**. 2000. Use of two-dimensional electrophoresis to study differential protein expression in Divercin V41-Resistant and Wild-Type Strains of *Listeria monocytogenes*. Appl. Environ. Microbiol. **66**:4318-4324.

4 **Ennahar, S., T. Sashihara, K. Sonomoto, and A. Ishizaki**. 2000. Class IIa bacteriocins: biosynthesis, structure and activity. FEMS Microbiol. Rev. **24**:85-106.

5 **Esvan, H., J. Minet, C. Laclie, and M. Cormier**. 2000. Proteins variations in *Listeria monocytogenes* exposed to high salinities. Int. J. Food Microbiol. **55**:151-155.

6 **Finch, R.** 2002. Bacterial resistance: the clinical challenge. Clin. Microbiol. Infect. **8 Suppl 3**:21-32.

7 **Gravesen, A., P. Warthoe, S. Knøchel, and K. Thirstrup**. 2000. Restriction fragment differential display of pediocin-resistant *Listeria monocytogenes* 412 mutants shows consistent overexpression of a putative beta-glucoside-specific PTS system. Microbiology **146**:1381-1389.

8 **Hébraud, M. and J. Guzzo**. 2000. The main cold shock protein of *Listeria monocytogenes* belongs to the family of ferritin-like proteins. FEMS Microbiol. Lett. **190**:29-34.

9 **Héchard, Y., C. Pelletier, Y. Cenatiempo, and J. Frere**. 2001. Analysis of sigma[54]-dependent genes in *Enterococcus faecalis:* a mannose PTS permease EII^{Man} is involved in sensitivity to a bacteriocin, mesentericin Y105. Microbiology **147**:1575-1580.

10 **Héchard, Y. and H. G. Sahl**. 2002. Mode of action of modified and unmodified bacteriocins from Gram-positive bacteria. Biochimie **84**:545-557.

11 **Jack, R. W., J. R. Tagg, and B. Ray**. 1995. Bacteriocins of gram-positive bacteria. Microbiol. Rev. **59**:171-200.

12 **Klaenhammer, T. R.** 1993. Genetics of bacteriocins produced by lactic acid bacteria. FEMS Microbiol. Rev. **12**:39-85.

13 **O'Driscoll, B., C. G. Gahan, and C. Hill**. 1997. Two-dimensional polyacrylamide gel electrophoresis analysis of the acid tolerance response in *Listeria monocytogenes* LO28. Appl. Environ. Microbiol. **63**:2679-2685.

14 **Phan-Thanh, L. and T. Gormon**. 1997. Stress proteins in *Listeria monocytogenes*. Electrophoresis **18**:1464-1471.

15 **Phan-Thanh, L. and F. Mahouin**. 1999. A proteomic approach to study the acid response in Listeria monocytogenes. Electrophoresis **20**:2214-2224.

16 **Ramnath, M., M. Beukes, K. Tamura, and J. W. Hastings**. 2000. Absence of a putative mannose-specific phosphotransferase system enzyme IIAB component in a leucocin A-resistant strain of *Listeria monocytogenes*, as shown by two-dimensional sodium dodecyl sulfate- polyacrylamide gel electrophoresis. Appl. Environ. Microbiol. **66**:3098-3101.