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

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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 10³-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^{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,

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

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 over-expressed 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^{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 μ g 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. 10⁷ 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-ATTTCCGCC-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 *EcoRI* 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 μ g 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.

<i>L. monocytogenes</i>	Leucocin A MIC ($\mu\text{g ml}^{-1}$)	Description	Reference or source*
Wild-type isolates			
412	0.98	Wild-type from raw, salted pork	Gravesen <i>et al.</i> (2000)
B73	0.98	Wild-type from meat	Dykes & Hastings (1998)
EGDe	1.95	Clinical	Glaser <i>et al.</i> (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	$>4 \times 10^3$	Mutant of 412 isolated on pediocin PA-1	Gravesen <i>et al.</i> (2000)
412L-A1	$>4 \times 10^3$	Mutant of 412 isolated on leu A	This work
412L2	$>4 \times 10^3$	Mutant of 412 isolated on leu 4010	This work
412C2	$>4 \times 10^3$	Mutant of 412 isolated on carn A9b	This work
B73-MR1	$>4 \times 10^3$	Mutant of B73 resistant to leucocin A	Ramnath <i>et al.</i> (2000)
EGDeP4	$>4 \times 10^3$	Mutant of EGDe isolated on ped PA-1	This work
DMRICC 4053	$>4 \times 10^3$	Mutant of 386 isolated from a meat model containing leu 4010	Anette Granly Koch, DMRI
3.33A	$>4 \times 10^3$	Mutant of O57 isolated from salmon juice containing carn A9b	Lilian Nilsson, DIFRES
Defined mutants			
AG115	0.98	Insertional inactivation of <i>lmo00319</i> in strain 412	This work
AG117	$>4 \times 10^3$	Insertional inactivation of <i>lmo00319</i> in strain 412P	This work
AG119	$>4 \times 10^3$	Insertional inactivation of <i>lmo00027</i> in strain 412P	This work
AG122	0.98	Insertional inactivation of <i>lmo00027</i> in strain 412	This work
EGK51	$>4 \times 10^3$	Insertional inactivation of <i>manR</i> in strain EGDe	Dalet <i>et al.</i> (2001)
EGK54	$>4 \times 10^3$	Insertional inactivation of <i>mptA</i> in strain EGDe	Dalet <i>et al.</i> (2001)
EGY2	$>4 \times 10^3$	84 bp in-frame deletion in <i>mptD</i> in strain EGDe	Dalet <i>et al.</i> (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 (OD₆₀₀ 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 -³²P]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 (OD₆₀₀ 0.45–0.5) in BHI, pH 7.2, 37 °C. Chloramphenicol (Sigma) was added at a final concentration of 20 $\mu\text{g ml}^{-1}$ 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 Ila 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 Ila 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* EGDc (a) and EGY2 (b). An extended-run zoom gel of *L. monocytogenes* EGDc (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* EGDc 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}. 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 divercin 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 (Hécharde *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.

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