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Class II one-peptide bacteriocins target a phylogenetically defined subgroup of mannose phosphotransferase systems on sensitive cells

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Membrane-located proteins (IIC and IID) of the mannose-phosphotransferase system (man-PTS) have previously been shown to serve as target receptors for several bacteriocins. Although many bacteria contain at least one such man-PTS in their genome, most bacteriocins display a narrow inhibitory spectrum, targeting predominantly bacteria closely related to the producers. In the present study we have analysed the receptor spectrum of one-peptide bacteriocins of class II. A phylogenetic analysis of 86 man-PTSs from a wide range of bacterial genera grouped the man-PTSs into three main clusters (groups I–III). Fourteen man-PTSs distributed across the phylogenetic tree were selected for experimental analysis in a heterologous host. Only members of group I could serve as receptors for class IIa bacteriocins, and the receptor efficiencies varied in a pattern directly related to their phylogenetic position. A multiple sequence alignment of IIC and IID proteins revealed three sequence regions (two in IIC and one in IID) that distinguish members of the bacteriocin-susceptible group from those of the other groups, suggesting that these amino acid regions confer the specific bacteriocin receptor function. Moreover, we demonstrated that variation in sensitivity might also exist within the same species due to differential expression levels of the receptor, since three strains of *Lactobacillus sakei* harbouring identical man-PTSs were shown to display different expression levels of a man-PTS gene that corresponded to the variation in bacteriocin sensitivity. Together, the results of our study show that the level of bacteriocin susceptibility for a bacterial cell is primarily determined by differences in its man-PTS proteins, although the expression levels of the corresponding genes also play an important role.

INTRODUCTION

Bacteriocins are ribosomally synthesized antimicrobial peptides produced by a wide range of Gram-positive bacteria, of which lactic acid bacteria (LAB) constitute the major part. Most of these antimicrobial substances direct their activity toward bacteria closely related to the producers, but some also have broader inhibitory spectra, including important pathogens such as the food-borne *Listeria monocytogenes* and pathogenic species of *Enterococcus*. The LAB bacteriocins are classified into two major classes (Nes *et al.*, 2007): the class I lantibiotics, containing post-translationally modified amino acids such as lanthionine, dehydrated amino acids and thioether cross-linked amino acids; and the class II non-lantibiotics, containing only non-modified amino acids. The class II bacteriocins are further categorized into several subgroups. The subclass IIa bacteriocins have a conserved sequence (YGNG[V/L]) and a disulfide bridge in their N-terminal region and are known for their strong anti-listerial activity (Nissen-Meyer *et al.*, 2009); these peptides are normally referred to as pediocin-like bacteriocins after pediocin PA-1, the first member characterized from this subclass (Henderson *et al.*, 1992; Nieto Lozano *et al.*, 1992). Subclass IIb contains bacteriocins whose full activity is dependent on the complementary action of two different peptides (e.g. lactococcin G and plantaricin EF and JK), and subclass IId contains bacteriocins without leader sequences (e.g. enterocin L50 and lacticin Q). Finally, the bacteriocins that do not belong to any of the above subgroups (e.g. lactococcin A and B) are placed in class IIc (Nes *et al.*, 1996, 2007).

Most bacteriocins are believed to kill sensitive cells by disrupting the integrity of target membranes, which leads

Abbreviations: aLRT, approximate likelihood-ratio test; BU, bacteriocin unit; 2-DG, 2-deoxy-D-glucose; man-PTS, mannose-phosphotransferase system.

A supplementary figure, showing a multiple sequence alignment of all 86 man-PTSs used in the phylogenetic analysis, and a supplementary table, listing man-PTSs used in the phylogenetic analysis, are available with the online version of this paper.

to dissipation of the proton motive force, depletion of intracellular solutes, and eventually cell death (Drider *et al.*, 2006). The fact that each bacteriocin displays a defined inhibitory spectrum strongly suggests that the individual bacteriocins recognize specific receptor molecules on target cells. In fact, it has been shown that several of the class I lantibiotics as well as the non-lantibiotic lactococcin 972 employ lipid II, a precursor in cell wall synthesis, as a docking molecule, and interaction between a bacteriocin and lipid II leads to inhibition of cell wall synthesis and/or pore formation, depending on the structure of the bacteriocin and its concentration (Brotz *et al.*, 1998; Martinez *et al.*, 2008; Wiedemann *et al.*, 2001). Similarly, some studies have shown that the mannose-phosphotransferase system (man-PTS) might serve as a receptor for some pediocin-like bacteriocins, based on the observations that resistant mutants have an altered expression pattern of man-PTS and that heterologous expression of cloned man-PTS genes renders resistant cells sensitive (Dalet *et al.*, 2001; Gravesen *et al.*, 2002; Héchard *et al.*, 2001; Ramnath *et al.*, 2000, 2004). More recently, it has been conclusively demonstrated for several bacteriocins from subclass IIa and some from subclass IIc that the membrane components (ManM/PtnC and ManN/PtnD) of the man-PTS are directly involved as receptors, and that in bacteriocin-producing cells, a cognate immunity protein tightly binds the receptor in a bacteriocin-dependent manner, to prevent killing by the bacteriocin (Diep *et al.*, 2007).

The man-PTS transporter family is responsible for the concomitant import and phosphorylation of carbohydrates such as mannose and glucose in bacteria (Postma *et al.*, 1993). A PTS transporter normally consists of three major components: enzyme I (EI), HPr and enzyme II (EII). The first two are cytoplasmic proteins involved in the transfer of a phosphoryl group to EII, which in turn relays the phosphoryl group to imported sugar molecules. EI and HPr serve as common phosphoryl group suppliers for different EIIs, while the individual EIIs are specific for each PTS family and are responsible for the sugar specificity. EII in man-PTS consists of four subunits, IIA, IIB, IIC and IID, in which the first two appear as one single (IIAB) or two separate (IIA and IIB) proteins, and the last two (IIC and IID) normally are separate proteins. Subunits IIA and IIB are located in the cytoplasm, while IIC and IID together form a membrane-located complex through which the sugar entities enter the cell (Postma *et al.*, 1993). Expression of the genes encoding these four subunits is coordinated, as they are commonly clustered in one operon (Deutscher *et al.*, 2006).

Hitherto, a few reports have linked sensitivity to class IIa bacteriocins to specific man-PTSs. For instance, the *mpt* operons in *Enterococcus faecalis* V583 and *Li. monocytogenes* EGD-e have been shown to be required for sensitivity to mesentericin Y105 (Dalet *et al.*, 2001; Héchard *et al.*, 2001). Another man-PTS of *Li. monocytogenes* EGD-e, encoded by the *mpo* operon, has also been shown to be involved in sensitivity to class IIa bacteriocins via regulation of the *mpt* operon (Arous *et al.*, 2004). Furthermore, in a recent study of bacteriocin sensitivity and immunity in our laboratory (Diep *et al.*, 2007), it was shown that the *man* operon of *Lactobacillus sakei* 23K confers sensitivity to a number of class IIa bacteriocins, while the *ptn* operon of *Lactococcus lactis* IL1403 confers sensitivity to the class IIc bacteriocins lactococcin A and lactococcin B. However, for several bacteria of both bacteriocin-sensitive and non-sensitive species, a large number of homologous putative man-PTS transporter genes are found in the genomes (Zúñiga *et al.*, 2005) and whether these homologous man-PTS proteins can serve as receptors for bacteriocins has not been evaluated. Starting from a bioinformatics approach, we systematically selected a number of man-PTSs from different genera, and showed that only a defined phylogenetic group of the man-PTSs confers sensitivity to class IIa bacteriocins and, more importantly, that variation in bacteriocin sensitivity can, to a large extent, be predicted on the basis of man-PTS phylogenetic positions.

METHODS

Phylogenetic analysis. To screen for man-PTS proteins in databases, the proteins ManM (YP_395063) and ManN (YP_395064) from *Lb. sakei* 23K were used as query sequences; these two proteins are members of the conserved IIC and IID protein families. Amino acid sequences of man-PTS proteins were retrieved from the Entrez Gene database (http://www.ncbi.nlm.nih.gov/sites/entrez?db=gene) using the Basic Local Alignment Search Tool (BLASTP; http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) with standard settings. All PTS transporters selected for further study, as listed in Supplementary Table S1, have been classified as man-PTSs (Zúñiga *et al.*, 2005). Phylogenetic analysis was performed using the web service Phylogeny.fr (http://www.phylogeny.fr; Dereeper *et al.*, 2008): Sequences were aligned using MUSCLE v3.7 (Edgar, 2004) in default mode. The phylogenetic tree was constructed with the maximum-likelihood algorithm implemented in PhyML v3.0 (Guindon & Gascuel, 2003) with the following settings: (i) initial tree: BIONJ; (ii) amino acid substitution model: WAG; (iii) proportion of invariant sites: estimated; (iv) number of substitution rate categories: 4; (v) gamma shape parameter: estimated. The reliability of the branches was assessed using bootstrap analysis with 100 replicates and an approximate likelihood-ratio test (aLRT) (Anisimova & Gascuel, 2006) with the Shimodaira–Hasegawa-like (non-parametric) procedure. The phylogenetic trees were visualized using TreeDyn (Chevenet *et al.*, 2006).

Bacterial strains and growth conditions. The bacterial strains used in this study are listed in Table 1. *Lc. lactis* IL1403 and clones derived therefrom were routinely grown at 30 °C in M17 medium (Oxoid) supplemented with 0.4 % (w/v) glucose or 0.4 % (w/v) galactose, *Lactobacillus plantarum* WCFS1 and *Lactobacillus delbrueckii* ATCC 11842 in MRS medium (Oxoid) at 30 and 37 °C, respectively, *Li. monocytogenes* EGD-e and *Ent. faecalis* V583 in BHI medium (Oxoid) at 37 °C, *Streptococcus thermophilus* LMG 18311 in Todd–Hewitt broth (BD) supplemented with 0.8 % (w/v) glucose and *Escherichia coli* in LB medium (Oxoid) at 37 °C with agitation (250 r.p.m.). When appropriate, erythromycin (5 μg ml^{-1}) and chloramphenicol (5 μg ml^{-1}) were added to the growth medium. Growth analysis was performed using Bioscreen C (Oy Growth Curves); overnight cultures were diluted 100-fold, and OD_{600} was measured at intervals of 20 min.

Table 1. Plasmids and bacterial strains used in this study

Plasmid or strain	Characteristics*†	Reference or source
Plasmids		
pNZ9530	Vector expressing *nisRK* (nisin regulatory genes), EryR	Kleerebezem *et al.* (1997)
pNZ8037	Expression vector in lactococci, nisin-inducible, CamR	de Ruyter *et al.* (1996)
p513	pNZ8037 with man-PTS Lsak (*manL*, *manM*, *manN*) of *Lb. sakei* LMGT2313	This study
p515	pNZ8037 with man-PTS Lsak (*manL*, *manM*, *manN*) of *Lb. sakei* 23K	Diep *et al.* (2007)
p517	pNZ8037 with man-PTS Lsak (*manL*, *manM*, *manN*) of *Lb. sakei* Lb790	This study
p423	pNZ8037 with man-PTS Llac (*ptnAB*, *ptnC*, *ptnD*) of *Lc. lactis* IL1403	Diep *et al.* (2007)
pEcol1	pNZ8037 with man-PTS Ecol1 (*agaB*, *agaC*, *agaD*) of *E. coli* BL21	This study
pEcol2	pNZ8037 with man-PTS Ecol2 (*manX*, *manY*, *manZ*) of *E. coli* BL21	This study
pEfae1	pNZ8037 with man-PTS Efae1 (*EF0020*, *EF0021*, *EF0022*) of *Ent. faecalis* V583	This study
pEfae12	pNZ8037 with man-PTS Efae12 (*EF3136*, *EF3137*, *EF3138*, *EF3139*) of *Ent. faecalis* V583	This study
pLdel	pNZ8037 with man-PTS Ldel (*Ldb1799*, *Ldb1800*, *Ldb1801*) of *Lb. delbrueckii* ATCC 11842	This study
pLmon1	pNZ8037 with man-PTS Lmon1 (*lmo0781*, *lmo0782*, *lmo0783*, *lmo0784*) of *Li. monocytogenes* EGD-e	This study
pLmon2	pNZ8037 with man-PTS Lmon2 (*lmo0021*, *lmo0022*, *lmo0023*, *lmo0024*) of *Li. monocytogenes* EGD-e	This study
pLmon3	pNZ8037 with man-PTS Lmon3 (*lmo1997*, *lmo2000*, *lmo2001*, *lmo2003*) of *Li. monocytogenes* EGD-e	This study
pLmon4	pNZ8037 with man-PTS Lmon4 (*lmo0096*, *lmo0097*, *lmo0098*) of *Li. monocytogenes* EGD-e	This study
pLpla1	pNZ8037 with man-PTS Lpla2 (*pts9A*, *pts9B*, *pts9C*, *pts9D*) of *Lb. plantarum* WCFS1	This study
pLpla2	pNZ8037 with man-PTS Lpla2 (*pts19A*, *pts19D*, *pts19C*, *pts19B*) of *Lb. plantarum* WCFS1	This study
pSthe	pNZ8037 with man-PTS Sthe (*manL*, *manM*, *manN*) of *S. thermophilus* LMG18311	This study
Strains		
Ent. faecalis V583	Source of man-PTSs	Sahm *et al.* (1989)
Enterococcus faecium P13	Enterocin P producer	LMGT strain collection‡
E. coli BL21	Source of man-PTS	Invitrogen
Lb. delbrueckii ATCC 11842	Source of man-PTS	S. Orla-Jensen (unpublished)
Lb. plantarum WCFS1	Source of man-PTS	Kleerebezem *et al.* (2003)
Lb. sakei		
B316	*Lb. sakei* Lb790 clone producing penocin A	Diep *et al.* (2006)
B317	*Lb. sakei* Lb790 clone producing sakacin A	Diep *et al.* (2006)
LTH673	Sakacin P producer	Tichaczek *et al.* (1992)
Lb790	Source of man-PTS	Schillinger & Lucke (1989)
23K	Source of man-PTS	Lauret *et al.* (1996)
LMGT 2313	Source of man-PTS	LMGT strain collection
Lc. lactis		
IL1403	Indicator strain for lactococcin A	Chopin *et al.* (1984)
B488	*ptn* deletion mutant of *Lc. lactis* IL1403 carrying pNZ9530 with *nisRK*, EryR	Diep *et al.* (2007)
B515	B488 with p423, CamR, EryR	Diep *et al.* (2007)
B520	B488 with pNZ8037, CamR, EryR	Diep *et al.* (2007)
B628	B488 with p513, CamR, EryR	This study
B630	B488 with p515, CamR, EryR	Diep *et al.* (2007)
B632	B488 with p517, CamR, EryR	This study
M127	B488 with pLpla1, CamR, EryR	This study
M128	B488 with pLpla2, CamR, EryR	This study
M129	B488 with pEcol1, CamR, EryR	This study
M130	B488 with pEcol2, CamR, EryR	This study
M148	B488 with pLmon1, CamR, EryR	This study
M149	B488 with pLmon2, CamR, EryR	This study

Table 1. cont.

Plasmid or strain	Characteristics*†	Reference or source
M150	B488 with pLmon3, CamR, EryR	This study
M151	B488 with pLmon4, CamR, EryR	This study
M158	B488 with pSthe, CamR, EryR	This study
M159	B488 with pLdel, CamR, EryR	This study
M160	B488 with pEfae1, CamR, EryR	This study
M161	B488 with pEfae12, CamR, EryR	This study
Li. innocua LMGT 2785	Indicator strain for class IIa bacteriocins	LMGT strain collection
Li. monocytogenes EGD-e	Source of man-PTSs	Glaser *et al.* (2001)
Pediococcus acidilactici PAC 1.0	Pediocin PA-1 producer	Henderson *et al.* (1992)
S. thermophilus LMG 18311	Source of man-PTS	Bolotin *et al.* (2004)

*EryR, erythromycin resistance; CamR, chloramphenicol resistance.
†Locus tags of the man-PTS genes are given in parentheses. Protein accession numbers are given in Supplementary Table S1.
‡Laboratory of Microbial Gene Technology, Ås, Norway.

Genetic cloning. The constructs used in this study are listed in Table 1. Man-PTS genes from different strains were amplified by PCR, using the primers listed in Table 2. Total genomic DNA from the respective strains was used as template in all PCRs. The PCR products were cleaved with SphI and *Xho*I, except Lmon3, which was cleaved with *Sph*I and *Sal*I (all restriction endonucleases from New England Biolabs), and ligated into the nisin-regulated pNZ8037-derivative plasmid p519 (Diep *et al.*, 2007) between the *Sph*I and *Xho*I sites, resulting in the plasmids listed in Table 1. All cloned genes were confirmed by sequencing. Transformation into the man-PTS-free strain *Lc. lactis* B488 (Diep *et al.*, 2007) was performed using the electroporation protocol of Holo & Nes (1989).

RNA isolation, cDNA synthesis and RT-PCR. RT-PCR was performed on three strains of *Lb. sakei* (23K, Lb790 and LMGT 2313). Cells in exponential growth (OD_{600} ~0.5) were harvested by centrifugation and stored at −80 °C. The cell pellet was dissolved in 700 µl buffer RTL (Qiagen) supplemented with 0.1 % (v/v) β-mercaptoethanol (Sigma), and the suspension was transferred to 2 ml screw-capped FastPrep tubes (MP Biomedicals) containing 0.5 g acid-washed glass beads (<106 µm, Sigma), 300 µl phenol and 300 µl chloroform. Cells were lysed using Fp120 Fastprep (Bio 101) for three times 25 s at 4 m s^{-1} at 4 °C. Following a short centrifugation (10 000 ***g*** for 1 min), the water phase was transferred to a fresh Eppendorf tube containing 500 µl ethanol. The lysate was then applied to RNeasy spin columns (Qiagen), and RNA was purified as described by the manufacturer (Qiagen). To remove remnants of DNA, RNA was treated with RNase-free DNase I (Qiagen). cDNA was synthesized using the Superscript III Reverse Transcriptase set (Invitrogen), and RNA was then removed by treatment with RNase H (Takara Bio). RT-PCR was carried out using primers mk86 (5′-CCATGTCTTAT-CTTAGGCGG-3′) and mk87 (5′-ACCATCTGTTAACCATACTGG-3′) for *manM* (encoding the IIC subunit), and mk96 (5′-CGTG-CTTCCATACCTTCAAC-3′) and mk97 (5′-ACCTCAAGTTGC-TTACCGTG-3′) for the housekeeping gene *fusA* (encoding translation elongation factor EF-G). Primers were designed based on the annotated genome sequence of *Lb. sakei* 23K (Chaillou *et al.*, 2005).

Bacteriocin assays. Bacteriocins (pediocin PA-1, enterocin P, sakacin P, penocin A and lactococcin A) were concentrated from supernatants of overnight cultures by ammonium sulphate precipitation as described previously (Diep *et al.*, 2007). The bacteriocin activity in the concentrated supernatants was determined using *Listeria innocua* LMGT 2785 and *Lc. lactis* IL1403 as indicator strains for class IIa bacteriocins and lactococcin A, respectively. Bacteriocin sensitivity of the different *Lc. lactis* clones was determined using a microtitre plate assay. Overnight cultures were diluted 100-fold in a medium containing 0.1 ng nisin ml^{-1} to induce expression of the cloned genes, and growth inhibition was measured spectrophotometrically at 600 nm after 15–18 h. One bacteriocin unit (BU) was defined as the amount of bacteriocin required to produce 50 % growth inhibition in a 200 µl culture of the indicator strain. MIC was defined as the minimum bacteriocin concentration needed to produce at least 50 % growth inhibition of tested clones.

RESULTS AND DISCUSSION

Bioinformatic analysis of man-PTS family proteins

A large number of homologous man-PTS genes are found in the genome sequences of different bacterial species (Zúñiga *et al.*, 2005). To get an overview of different members of the man-PTS family in the context of being a reservoir of potential bacteriocin receptors, we first analysed these proteins with a phylogenetic approach. We chose to focus only on the membrane-located subunits (IIC and IID), because expression of these genes (without IIAB) has previously been shown to be sufficient to confer sensitivity to several class IIa bacteriocins and class IIc lactococcins A and B (Diep *et al.*, 2007). By using the proteins ManM (IIC) and ManN (IID) from *Lb. sakei* 23K as query sequences, a large number of homologous man-PTS IIC and IID protein sequences were retrieved from the Entrez Gene database (sequence identity ranging from 84 to 22 %) using BLAST. Members of the man-PTS family were found predominantly in species belonging to two separate phylogenetic taxa, the Gram-positive Firmicutes and the Gram-negative Proteobacteria. This observation is in line with the evolutionary analysis of man-PTS transporters of Zúñiga *et al.* (2005), who propose that these transporters have been distributed to species of Firmicutes and Proteobacteria by horizontal gene transfer. The *in silico* analysis of published genomes revealed that

Table 2. Primers used in this study

Amplified product	Primer	Sequence (5′→3′); restriction site*
Lpla1	mk54	AGCTGCATGCCACTGTAACGAAAAATAGGAGG; *Sph*I
	mk46	ATCGCTCGAGCCCAATATTATGTTAACAATAGC; *Xho*I
Lpla2	mk53	ACGTGCATGCCGTAGGTTAATTACTATCCGC; *Sph*I
	mk48	ATGCCTCGAGCAACAGATAACTTGTTAATCGG; *Xho*I
Ecol1	mk49	ACGTGCATGCAGTGCCTTAATGGAAAAGGAG; *Sph*I
	mk50	ATCGCTCGAGCACCAGACGCAGTGC; *Xho*I
Ecol2	mk51	ACGTGCATGCGACGATTCAAAAATACATCTGG; *Sph*I
	mk52	ATCGCTCGAGGCCAAAAGGCCCCGGTAG; *Xho*I
Lmon1	mk56	ACGTGCATGCCTTATCCACGCTTAAGGAGG; *Sph*I
	mk57	ATGCCTCGAGAGAACCGGAATTTTAATCCCG; *Xho*I
Lmon2	mk58	ACGTGCATGCAACCCTATGAATATATTGAAAGC; *Sph*I
	mk59	ATCGCTCGAGACCCATTTTGTCTATTCCTCC; *Xho*I
Lmon3†	mk62	ACGTGCATGCAGTTGATTTTAACTAATACTAAGG; *Sph*I
	mk63	GTACAAACAAAAATTGCTTTCATTCTTATTTTAATAAACCTGTGAACG
	mk60	ACGTGTCGACAAAGACAGCCGCAGCTGTC; *Sal*I
	mk61	CGTTCACAGGTTTATTAAAATAAGAATGAAAGCAATTTTTGTTTGTAC
Lmon4	mk64	ACGTGCATGCGCAATAAATATAGCGGGTAGC; *Sph*I
	mk65	ATCGCTCGAGTCGGTGAATATTGCACCAGC; *Xho*I
Sthe	mk72	ACGTGCATGCAAGAAGCAATATATAAAAGGAGG; *Sph*I
	mk73	ATGCCTCGAGTCAGCTTTGCTAAACTCTTGC; *Xho*I
Ldel	mk74	ACGTGCATGCTACCGAAGTTTTTGAGGAGG; *Sph*I
	mk75	ATGCCTCGAGACGGTCTTATTCATTGATTGAG; *Xho*I
Efae1	mk76	AGCTGCATGCAAAAACATCAATTATACAGGAGG; *Sph*I
	mk77	ATGCCTCGAGTTGATTAGAAGTAATAAACTTACC; *Xho*I
Efae12	mk80	AGCTGCATGCCCAAAACCAAGGATAAGAAGG; *Sph*I
	mk81	ATGCCTCGAGTCCCCATTTCATTGCTTTCAC; *Xho*I
Lsak	pr205	CATTATAATTGCATGCGTTTTTATCAGTGTG; *Sph*I
	pr206	AGTTAATCCTCGAGATATAAACACCTGC; *Xho*I

*Restriction sites are underlined in the sequences.
†In Lmon3, the gene encoding the IIA subunit is separated from the other genes by 2112 bp. To remove this sequence, Lmon3 was amplified using a two-step PCR approach (Higuchi, 1990), with mk60 and mk62 as outer primers and mk61 and mk63 as inner primers.

the number of different man-PTSs within a strain is highly variable between different genera, e.g. only one is found in *Lc. lactis*, *Lb. sakei* and *S. thermophilus*, four in *Li. monocytogenes* and a total of 13 in *Ent. faecalis* V583. Some of the man-PTS operons are incomplete, i.e. missing one or two of the four subunits (e.g. the *agaBCD* operon in *E. coli*, encoding IIB, IIC and IID, but no IIA subunit). Based on an initial phylogenetic clustering of all collected sequences, 86 man-PTSs from the Entrez Gene database were selected for further phylogenetic analysis, and the man-PTSs were designated using the following four-letter system: the first letter was derived from the first letter of the genus name, the next three from the first three letters in the species name, and a digit when more than one man-PTS was present in the bacterial species, e.g. Lmon1 for one of the man-PTSs from *Li. monocytogenes* (an overview of the proteins used in the phylogenetic analysis can be found in Supplementary Table S1). The phylogenetic trees of protein families IIC and IID (Fig. 1) show that their members are clustered into three distinct groups in both families, named groups I, II and III, and this grouping is supported by significant branch support values (bootstrap value >0.85, aLRT value >0.9). Group III is the largest and most diverse. In all cases, the phylogenetic position of a IIC protein and its cognate IID protein (from the same man-PTS) correspond well with each other, i.e. both occupy a similar position in their respective trees, suggesting that the pairwise IIC and IID subunits have evolved in parallel. Notably, we observed that the man-PTSs previously suggested to be involved as receptors for class IIa bacteriocins Efae1 (*mpt* operon from *Ent. faecalis*; Héchard *et al.*, 2001), Lmon4 (*mpt* operon from *Li. monocytogenes*; Dalet *et al.*, 2001) and Lsak (*man* operon from *Lb. sakei*; Diep *et al.*, 2007) are clustered in group I.

Class IIa and IIc bacteriocins target only a phylogenetically defined subset of man-PTSs

To evaluate which man-PTSs from different loci or different bacteria act as receptors for bacteriocins of class

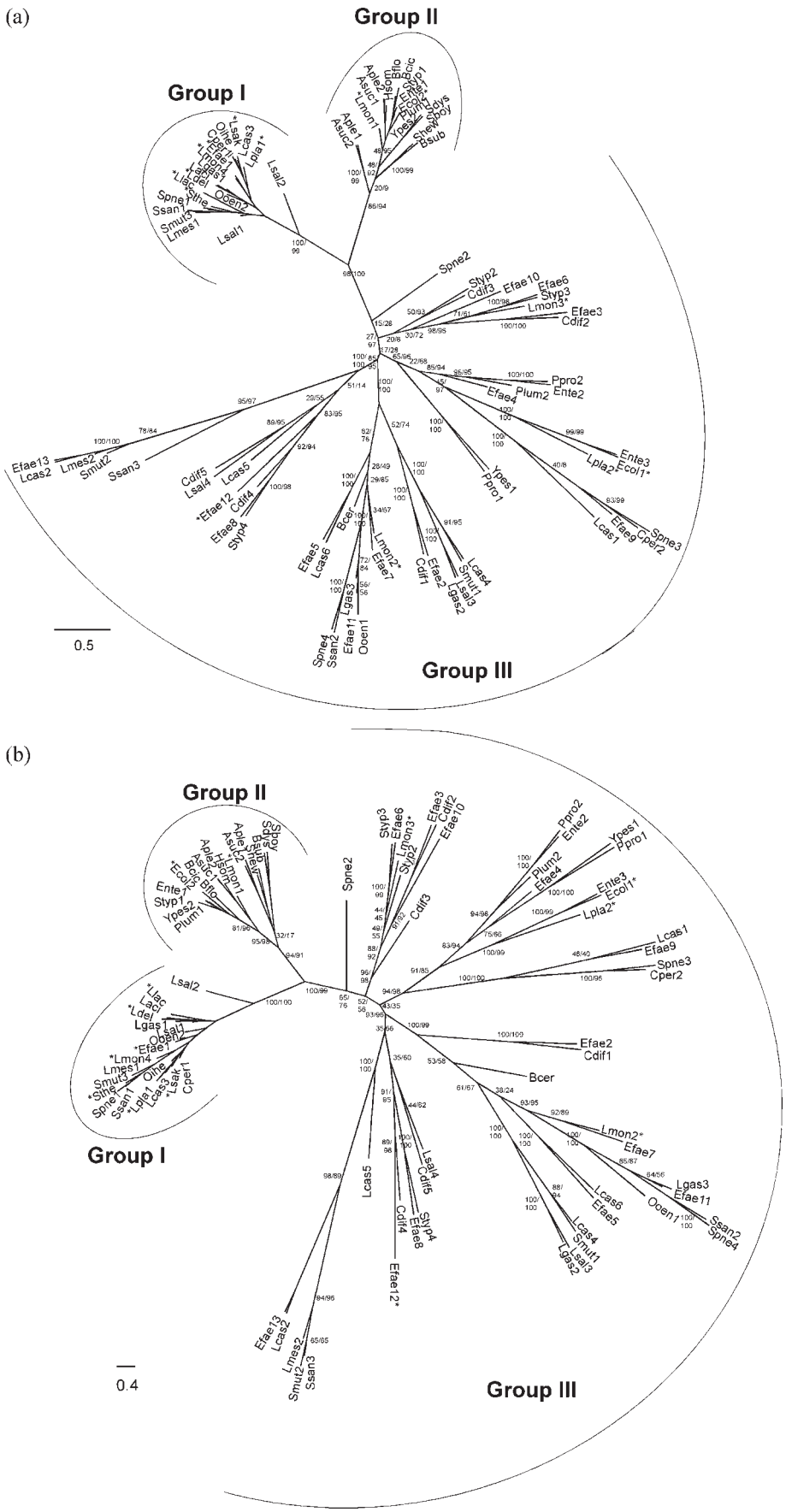
(a)
Group I
Group II
Group III
0.5
(b)
Group I
Group II
Group III
0.4

Fig. 1. Phylogenetic analysis of the man-PTS protein families IIC (a) and IID (b). Altogether, 86 protein pairs were analysed. The trees were constructed using the maximum-likelihood algorithm implemented in PhyML. Bootstrap values from 100 replicates (first value) and the aLRT branch support values (second value) are shown for most branches (some of the values are not shown due to space limitations). Asterisks indicate man-PTSs selected for the experimental analysis.

IIa and the class IIc bacteriocin lactococcin A, 14 different man-PTSs, distributed all over the phylogenetic tree, were selected for experimental analysis. These included all four man-PTSs from *Li. monocytogenes* (Lmon1–4), two (of 13) from *Ent. faecalis* (Efae1 and Efae12), two (of two) from *Lb. plantarum* (Lpla1–2), two (of two) from *E. coli* (Ecol1–2), and the single man-PTSs found in *Lb. sakei* (Lsak), *Lc. lactis* (Llac), *Lb. delbrueckii* (Ldel) and *S. thermophilus* (Sthe). The nisin-controlled expression (NICE) system (Kuipers *et al.*, 1998) was used to express the individual man-PTS operons in an *Lc. lactis* mutant clone (B488) in which the single indigenous man-PTS locus in the genome has been deleted (Diep *et al.*, 2007), thus containing no background man-PTS that might interfere with our bacteriocin receptor assays. The different homologous man-PTSs were first assessed for their ability to transport glucose in *Lc. lactis* by adding the non-metabolizable glucose analogue 2-deoxy-D-glucose (2-DG) to the growth medium (Thompson & Chassy, 1982); growth inhibition with 2-DG is an indication of a functional man-PTS transporter. The results from the growth analysis, as summarized in Table 3, showed that all the man-PTSs of group I (Llac, Lsak, Efae1, Lmon4, Ldel, Sthe and Lpla1), in addition to Ecol2 (group II), can take up 2-DG when expressed in the heterologous host *Lc. lactis*. On the other hand, none of the remaining man-PTSs of group III (Lmon2, Lmon3, Ecol1, Lpla2 and Efae12) or Lmon1 (group II) was functional as a glucose transporter in *Lc. lactis*, which suggests that these homologous transporters fulfil roles in the cells other than glucose transport (e.g. uptake of other sugars) or are not functional at all. Alternatively, these man-PTSs could be dependent on species-specific factors to be functional. It should be noted that a functional man-PTS in terms of sugar uptake is not a prerequisite for being a bacteriocin receptor, as we have previously shown that expression of the man-PTS IIC and IID genes in the absence of the man-PTS IIAB gene can provide a functional receptor (Diep *et al.*, 2007).

To examine whether the expressed man-PTS genes could serve as bacteriocin receptors, all 14 *Lc. lactis* clones were challenged with four different class IIa bacteriocins, pediocin PA-1 (Marugg *et al.*, 1992), enterocin P (Cintas *et al.*, 2000), sakacin P (Tichaczek *et al.*, 1994) and penocin A, (Diep *et al.*, 2006) and one class IIc bacteriocin, lactococcin A (Holo *et al.*, 1991). The MIC values determined by microtitre plate assays are given in Table 3. In group I, six out of the seven selected man-PTSs were able to confer sensitivity to the class IIa bacteriocins but not to lactococcin A, while the last man-PTS from this

Table 3. Experimental analysis of selected man-PTS family members

Group	man-PTS (clone)	MIC value* (BU ml^{-1}) for bacteriocin					2-DG inhibition†	Genetic org.‡
		Ped PA-1	EntP	SakP	PenA	LcnA		
I	Lsak (B630)	25	10	10	35	NI	+	AB-C-D
	Lpla1 (M127)	35	25	140	30	NI	+	AB-C-D
	Lmon4 (M151)	20	5	<0.5	10	NI	+	AB-C-D
	Efae1 (M160)	20	5	5	5	NI	+	AB-C-D
	Ldel (M159)	35	10	35	35	NI	+	AB-C-D
	Sthe (M158)	95	95	25	50	NI	+	AB-C-D
	Llac (B515)	NI	NI	NI	NI	190	+	AB-C-D
II	Lmon1 (B148)	NI	NI	NI	NI	NI	−	A-B-C-D
	Ecol2 (M130)	NI	NI	NI	NI	NI	+	AB-C-D
III	Lmon2 (M149)	NI	NI	NI	NI	NI	−	A-B-C-D
	Lmon3 (M150)	NI	NI	NI	NI	NI	−	A-i-B-C-D
	Ecol1 (M129)	NI	NI	NI	NI	NI	−	B-C-D
	Lpla2 (M128)	NI	NI	NI	NI	NI	−	B-C-D-A
	Efae12 (M161)	NI	NI	95	190	NI	−	C-D-B-A

*The numbers indicate the minimum concentrations (in BU ml^{-1}) required to produce at least 50 % growth inhibition. MIC values were determined at least twice with virtually the same results. NI, No inhibition observed at the highest bacteriocin concentration tested (400 BU ml^{-1}).
†+, Growth of the *Lc. lactis* clone was inhibited by 2-DG; −, no inhibition.
‡Organization of the genes encoding the man-PTS subunits. Separated genes are indicated with hyphens. In Lmon3, an inserted gene (i) is found between the man-PTS IIA and IIB genes.

group, the *ptn* operon from *Lc. lactis* (Llac), conferred sensitivity to lactococcin A but not to any of the class IIa bacteriocins. Of these, the man-PTSs Lsak (*man* operon), Lmon4 (*mpt*) and Efae1 (*mpt*) from *Lb. sakei*, *Li. monocytogenes* and *Ent. faecalis*, respectively, have previously been shown, in a direct or indirect manner, to be associated with sensitivity to bacteriocins (Dalet *et al.*, 2001; Diep *et al.*, 2007; Héchard *et al.*, 2001), and we finally confirmed here that these man-PTSs indeed act as receptors for several class IIa bacteriocins. In addition, the group I man-PTS systems Lpla1 (*pts9*) from *Lb. plantarum*, Ldel from *Lb. delbrueckii* and Sthe (*man*) from *S. thermophilus* were shown for the first time to function as receptors for bacteriocins. Remarkably, all selected members of groups II and III were unable to confer sensitivity to the different bacteriocins, except for Efae12 (from *Ent. faecalis*), which seemed to cause some sensitivity when the resulting clone was exposed to relatively high concentrations of sakacin P and penocin A (20- to 40-fold higher concentrations than for Efae1, the other enterococcal man-PTS tested). At present we cannot explain why Efae12 has this function, but as both Efae1 and Efae12 are derived from the same host (*Ent. faecalis* V583), the biological function of Efae12 as a bacteriocin receptor is probably insignificant, as it will be overshadowed by Efae1.

Out of the 14 man-PTSs selected, only the lactococcal Llac of group I could function as a receptor for the class IIc bacteriocin lactococcin A. This lactococcal species-specificity, noticed more than 15 years ago (Henderson *et al.*, 1992; Holo *et al.*, 1991; Kok *et al.*, 1993), that lactococcins A and B only target lactococcal species, while pediocin-like bacteriocins do not target these species, can now be ascribed to differences in the amino acid sequences of the IIC and IID subunits of their man-PTSs.

Notably, the selected bacteriocins seemed to differ greatly from each other in their potency in targeting the various man-PTSs. While pediocin PA-1 appeared to target all non-lactococcal receptors (from group I) with a similar range of efficiency (i.e. having only a fivefold difference between the highest and the lowest), a significantly larger variation was seen for the other bacteriocins; the difference between the highest and the lowest was about 19-fold for enterocin P, 280-fold for sakacin P and 10-fold for penocin A. Two of the man-PTSs, Lmon4 and Efae1, were the most active receptors for all four bacteriocins, and not surprisingly, these two systems are most closely related to each other according to the phylogenetic trees (Fig. 1). This correlation became even more evident in a separate phylogenetic analysis of concatenated IIC and IID proteins from 31 group I man-PTSs (including 12 man-PTSs that were left out of the first phylogenetic analysis due to space limitations), in which receptors conferring high, medium and low sensitivity appeared to be organized into distinct domains (Fig. 2). For instance, Lsak and Lpla1, both conferring medium sensitivity, are located next to each other within a domain, and both are well separated from the domain that contains the highly efficient receptors Lmon4 and Efae1. Similarly, Sthe, which confers low sensitivity, is defined within another domain. These results are in line with previous comparative analyses of the inhibitory spectra of class IIa bacteriocins: (i) species of *Enterococcus*, *Listeria* and *Carnobacterium* are highly sensitive to pediocin-like bacteriocins (low MIC values); (ii) species of *Lactobacillus*, *Pediococcus* and *Clostridium* are also frequently inhibited by these bacteriocins, although they are often less sensitive (higher MIC values); and (iii) strains of *Streptococcus* and *Leuconostoc* are occasionally reported to be sensitive to class IIa bacteriocins at a low level (very high MIC values) (Diep *et al.*, 2006; Eijsink *et al.*, 1998).

It is also worth noting that the *in silico* analysis of man-PTSs also revealed an interesting link between man-PTS receptors and bacteriocin producers. From the available sequence information, all bacterial species producing class IIa bacteriocins (Drider *et al.*, 2006; Fimland *et al.*, 2005) seem to have genes encoding a man-PTS belonging to phylogenetic group I. For example, *Bacillus coagulans* is the only *Bacillus* species known to produce a pediocin-like bacteriocin (Le Marrec *et al.*, 2000), and *B. coagulans* is also the only *Bacillus* species harbouring a group I man-PTS. This notion implies that there is an evolutionary link between the production of class IIa bacteriocins and the presence of a group I man-PTS in the same cell, although the biological significance of this observation has yet to be understood.

Group I receptors contain three distinct regions in their sequences

Given that the receptor function of a man-PTS is related to its phylogenetic position (Figs 1 and 2), we looked for important sequence differences between the different man-PTSs that might be responsible for the observed variation in receptor potency. Multiple sequence alignments of the 14 man-PTSs used in the experimental analysis revealed at least three regions in the IIC and IID proteins, termed region-α, region-β and region-γ, that clearly distinguish IIC and IID proteins with good receptor function from those with poor or no receptor function (Fig. 3 and Supplementary Fig. S1). Region-α, which is located in the N-terminal half of IIC of group I, is characterized by several conserved residues and an additional sequence stretch (3–4 aa); this region contains the conserved sequence GGQGXXG in the man-PTSs belonging to the high- and medium-sensitivity group, while a related motif (GG[D/K]FXXXG) is found in the man-PTSs with low sensitivity. Interestingly, Llac, which confers sensitivity only to the class IIc lactococcin A but not to class IIa bacteriocins, lacks such a sequence, suggesting that the motif in region-α is important for interaction with class IIa bacteriocins only. Region-β, which is located at the C-terminal end of the IIC proteins, is characterized for group I man-PTSs by an enrichment in glycine residues and the presence of a conserved sequence DP[I/L/V]GDI[I/L][D/E/

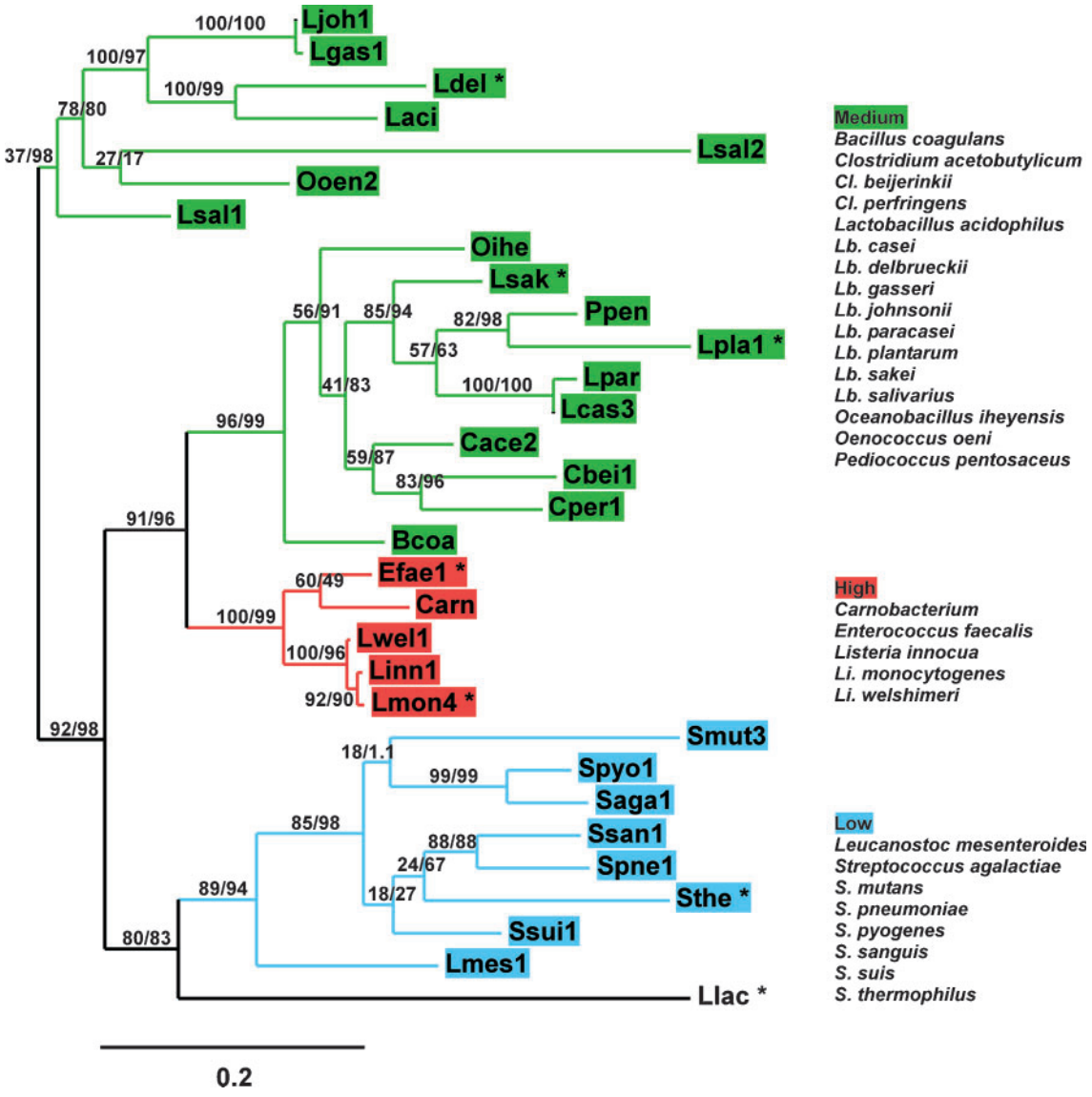

Fig. 2. Phylogenetic clustering of concatenated man-PTS IIC and IID proteins from group I using the maximum-likelihood algorithm in PhyML. Bootstrap support values from 100 replicates (first value) and aLRT branch support values (second value) are shown. The man-PTSs are separated into high- (red), medium- (green) and low-sensitivity (blue) groups based on the receptor potency. Asterisks indicate man-PTSs included in the experimental analysis. The bacterial species carrying these proteins are listed.

N]XY, in which P, I and Y are unique to group I man-PTS. Finally, region-γ designates a location in the IID proteins in which members of phylogenetic group I contain an additional sequence of 35–40 aa which is absent in their counterparts from groups II and III. Interestingly, transmembrane prediction of the IIC and IID proteins using the TMHMM Server 2.0 (Krogh *et al.*, 2001) suggested that all these three regions discussed here are located on the extracellular side of the membrane, and thus might serve as binding sites for bacteriocins on the surface of sensitive cells.

The fact that two of these distinct regions are located in IIC and the last one in IID suggests that the bacteriocin binding site(s) on the receptor are composed of sequences from different proteins. This notion is in line with our recent study (Diep *et al.*, 2007), which showed that expression of individual IIC or IID genes derived from a potent man-PTS (such as Lsak or Llac) could not confer sensitivity. Furthermore, we have also found that, while the pairwise IIC and IID from Lsak and Llac are very potent receptors for pediocin-like bacteriocins and lactococcin A, respectively, the combination of one component from one system

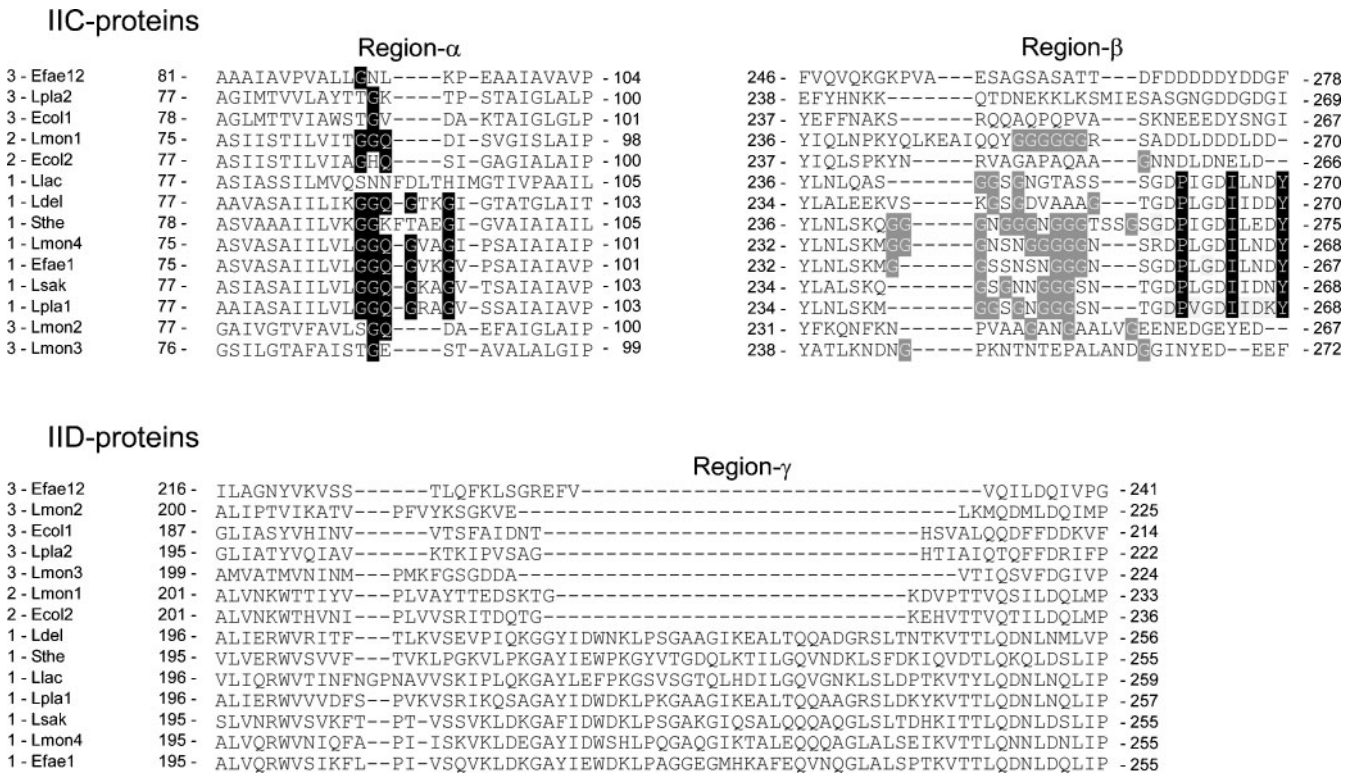

Fig. 3. Multiple sequence alignments of the IIC and IID proteins from the 14 man-PTSs used in the experimental analysis. Shown here are three regions which clearly separate group I from group II and III sequences: region-α in the N-terminal half of IIC (residues in the putative GGQGXXG motif are highlighted with a black background), region-β in the C-terminal end of IIC (the glycine-rich region is indicated by a grey background, and residues that are conserved only in group I man-PTSs are shown with a black background) and region-γ in the C-terminal half of IID. The number in front of the man-PTS names indicates the phylogenetic group (1, 2 or 3), and the numbers next to the sequences indicate amino acid positions in each protein. Alignments of region-α, region-β and region-γ, including all 86 IIC and IID proteins used in the phylogenetic analysis, can be found in Supplementary Fig. S1. The alignment was constructed using MUSCLE.

with a non-cognate partner from another system leads to little or no sensitivity to either type of bacteriocin (data not shown). As the pairwise IIC and IID proteins seem to have evolved in a parallel manner (Fig. 1), creation of such hybrids from two phylogenetically divergent subunits probably forms a complex that has become defective for bacteriocin binding. However, we cannot exclude the possibility that the bacteriocin binding site is located in one subunit but that its functionality needs to be stabilized by the presence of the cognate partner. It should also be mentioned that region-γ in IID has previously been associated with sensitivity to class IIa bacteriocins, because an in-frame deletion of this region in *mptD* renders *Li. monocytogenes* EGD-e cells resistant to mesentericin Y105 (Dalet *et al.*, 2001). However, it is not known whether region-γ is directly involved in the interaction with the bacteriocin, or whether this deletion perturbs the structure of MptD. Intragenic and intergenic chimeric receptors designed to address the nature of the bacteriocin receptor-specificity, and the role of region-α, region-β and region-γ in this context, are currently under investigation.

Differential expression of man-PTS causes variation in bacteriocin sensitivity

It has been frequently observed that different strains of the same bacterial species can vary greatly in sensitivity to a given bacteriocin (Eijsink *et al.*, 1998; Katla *et al.*, 2003). In most cases, the reason for this variation is not known. For instance, *Lb. sakei* strains 23K, Lb790 and LMGT 2313 display a great variation in sensitivity toward pediocin PA-1, with LMGT 2313 being the most sensitive (Fig. 4a). By DNA sequencing, all three strains were found to harbour almost identical man-PTS genes in their genomes (except for a few silent mutations in the DNA sequences that have no effect on the amino acid sequences). Furthermore, subcloning of the man-PTSs of *Lb. sakei* 23K, Lb790 and LMGT 2313 in the man-PTS-free strain *Lc. lactis* B488 showed that all three clones display more or less the same degree of sensitivity to pediocin PA-1 (Fig. 4b), illustrating that these silent mutations do not account for the observed variation in sensitivity.

To examine whether the variation in sensitivity among these strains could be due to differential gene expression,

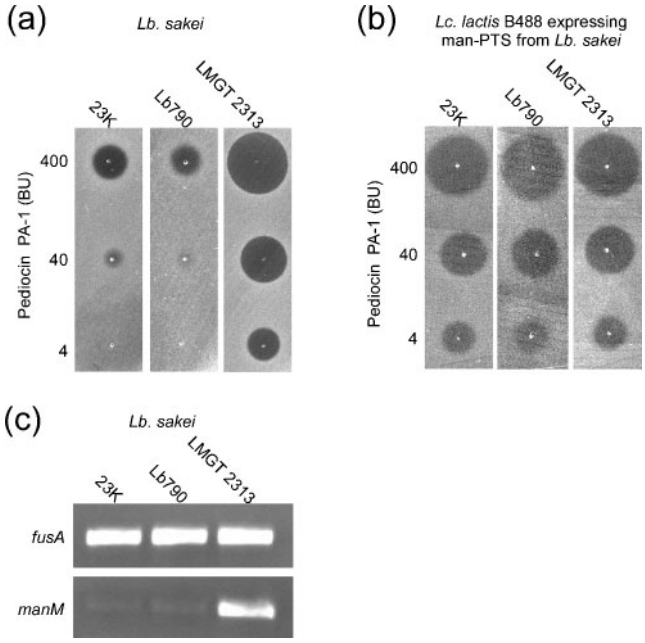

Fig. 4. Variation in bacteriocin sensitivity is due to differential expression levels of the man-PTS. Soft agar overlay assay with the three *Lb. sakei* strains 23K, Lb790 and LMGT 2313 (a), and with the three *Lc. lactis* clones expressing the man-PTSs derived from the *Lb. sakei* strains (b). Cells were challenged with 5 μl of three different concentrations of pediocin PA-1 (400, 40 and 4 BU) and inhibition is seen as clear zones. (c) RT-PCR analysis of *manM* (encoding the man-PTS IIC component) in the *Lb. sakei* strains. The housekeeping gene *fusA* (encoding the translation elongation factor EF-G) was included as a control.

the relative transcription levels of *manM* (encoding the receptor IIC subunit) in the three *Lb. sakei* strains were determined by RT-PCR (Fig. 4c). These results clearly indicate that the *manM* expression levels are higher in the more sensitive strain LMGT 2313 than in 23K and Lb790. Thus, the three *Lb. sakei* strains harbouring the same man-PTSs displayed highly variable sensitivity to pediocin PA-1 because of differential expression levels of their man-PTS genes. This is in line with previous observations, which show that genes involved in the regulation of man-PTS expression, such as *rpoN* (encoding a σ^{54} subunit of RNA polymerase) and *mptR*/*manR* (encoding σ^{54}-associated transcription factors), influence sensitivity to the pediocin-like bacteriocin mesentericin Y105 in *Ent. faecalis* and *Li. monocytogenes* (Dalet *et al.*, 2000, 2001; Héchard *et al.*, 2001). In addition, it should also be kept in mind that other strain-specific factors, including cell-surface differences (Vadyvaloo *et al.*, 2002, 2004) and relics of bacteriocin loci containing immunity genes (Moretro *et al.*, 2005), can be responsible for the observed variability in bacteriocin sensitivity between strains from the same bacterial species.

Concluding remarks

Class IIa bacteriocins are considered promising antimicrobial agents for use in medicine and food preservation. Most studies performed hitherto have been conducted by assaying the susceptibility of randomly selected bacterial strains to various bacteriocins, and these results are often difficult to compare due to variations in growth conditions (of both the bacteriocin producer and the indicator strain), bacteriocin assays and choice of indicator strains. The present work provides a systematic overview of the functionality of receptors for pediocin-like bacteriocins, showing that only one subgroup of man-PTS proteins are efficient receptors. The results highlight the great potential of these bacteriocins to improve therapeutic strategies, in particular against enterococcal and listerial infections. Variation in receptor sequences seems to account for much of the variation in bacteriocin susceptibility between different bacterial species, and we have identified three sequence regions that clearly distinguish man-PTSs that could serve as bacteriocin receptors from those that could not. Whether these three sequence regions are directly involved in the bacteriocin binding sites awaits further investigation. In addition, other factors such as gene expression level also seem to be of high importance in determining the degree of sensitivity of a strain.

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