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Novel Activator of Mannose-Specific Phosphotransferase System Permease Expression in *Listeria innocua*, Identified by Screening for Pediocin AcH Resistance

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To identify genes that are important for class IIa bacteriocin interaction and resistance in *Listeria* species, transposon Tn*917* knockout libraries were constructed for *Listeria innocua* strain Lin11 and screened for mutants that are resistant to pediocin AcH. A highly resistant mutant (G7) (MIC > 20 μg/ml; 1,000-fold less susceptible than the wild type), in which the transposon integrated into the putative promoter of the lin0142 gene, was isolated. lin0142 is located immediately upstream of the *mpt* operon (*mptA*/*mptC*/*mptD*) that encodes the mannose-specific phosphoenolpyruvate-dependent phosphotransferase system permease EII_t^{Man}, which serves as a docking protein for class IIa bacteriocins. The transcription of the *mpt* operon is known to be positively controlled by σ^{54} factor and ManR (a σ^{54}-associated activator). Transcripts for lin0142 and *mpt* were undetectable in the G7 mutant, based on quantitative real-time reverse transcriptase PCR analysis. When the wild-type lin0142 gene was expressed at a 7.9-fold-elevated level in the mutant via a multicopy-number plasmid, the level of *mpt* mRNA became 70% higher than that in the wild-type strain. In addition, the complementation strain reverted back to the pediocin AcH-susceptible phenotype. The levels of *manR* and *rpoN* (σ^{54}) mRNAs were not directly influenced by the level of lin0142 transcription. lin0142 is the only one of the three *mpt* regulatory genes whose transcription is induced, albeit slightly (1.2-fold), by glucose. The combined results show that the lin0142 gene encodes a novel activator of the *mpt* operon. The Lin0142 protein contains a winged-helix DNA-binding motif and is distantly related to the Crp-Fnr family of transcription regulators.

Bacteriocins are small, cationic, antimicrobial peptides produced by lactic acid bacteria (12, 16, 27, 28). These peptides are grouped into three major classes: lantibiotics (class I); small, heat-stable peptides functioning alone (class IIa) or in pairs (class IIb); and large, heat-labile proteins (class III) (28). Pediocin AcH (also known as pediocin PA-1) is a 44-amino-acid class IIa bacteriocin, produced primarily by *Pediococcus* species (40). The peptide is active against many types of lactic acid bacteria and has the highest activity against *Listeria* species of any class IIa bacteriocin (15). Largely because of their antilisterial activities, class IIa bacteriocins have provoked considerable interest for use as biopreservatives (12, 16, 40).

Class IIa bacteriocins (also known as YGNGV bacteriocins) have a common secondary structure (19, 48, 51) and mechanism of action (16). Initially, these peptides are thought to bind to negatively charged molecules, such as teichoic acids in the cell wall (7). Subsequently, they transfer to the cytoplasmic membrane, where they interact with anionic phospholipids (9) and a phosphoenolpyruvate-dependent phosphotransferase system (PTS) permease of the mannose structural family (EII_t^{Man}) (38). Once they are bound to cell membranes, class IIa bacteriocins fold into a βα conformation (19, 48, 51) in which the nonpolar α-helical region is inserted into the phospholipid bilayer (32). Ultimately, ion conductance pores are formed, resulting in cell death due to the dissipation of the membrane electrochemical gradient (10).

One concern regarding the use of bacteriocins as antimicrobial agents is the development of resistance in target bacterial strains (13, 34). In *Listeria* species, low-level resistance (two- to fourfold) to class IIa bacteriocins is caused by alterations in membrane lipid composition (13, 49, 50). High-level resistance (1,000-fold) in *Listeria monocytogenes* and *Enterococcus faecalis* results primarily from the loss of EII_t^{Man}, which is encoded by the *mpt* (mannose permease two) operon (14, 26, 38). High-level resistance in *L. monocytogenes* is also caused by the loss of σ^{54} factor (encoded by *rpoN)* (43) and the σ^{54}-associated activator ManR (14), which together positively regulate the *mpt* operon. ManR activity also may be regulated by reversible phosphorylation of its two PTS regulation domains (14, 46).

Listeria uses both low-affinity proton motive force-dependent and high-affinity PTS transporters for glucose uptake (11). This genus appears to lack transporters of the glucose PTS structural family (ListiList database [http://genolist.pasteur.fr/ListiList]) (21). The high-affinity glucose transporter in *Listeria* probably is the EII_t^{Man} permease, whose expression is induced by glucose (14). It is not unusual that mannose PTS permeases participate in glucose transport, as they often display relatively broad substrate specificities (37). The interaction of class IIa bacteriocins with *L. monocytogenes* is highly dependent on EII_t^{Man}, which contains three subunits ($IIAB^{Man}$, IIC^{Man}, and IID^{Man}).

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Deletion analysis indicates that the IIC^{Man} subunit encoded by the *mpt* operon is needed for permease-peptide recognition (39). Inactivation of the *mpo* (mannose permease one) operon, which encodes a permease (EII_o^{Man}) with four subunits (IIA^{Man}, IIB^{Man}, IIC^{Man}, and IID^{Man}), causes moderate resistance to class IIa peptides in *L. monocytogenes* due to a slight reduction in *mpt* expression (3). One common finding is that a β-glucoside-specific PTS (EII^{Bgl}) is overexpressed in strains that are highly resistant to class IIa peptides (23, 24). This outcome may result from the absence of EII_t^{Man} and consequent relief of catabolite control protein (CcpA)-mediated repression of the transcription of the EII^{Bgl} operon (2, 24, 42).

To identify additional genes that are important for class IIa bacteriocin-*Listeria* interaction and to learn more about basic mechanisms of resistance to these peptides, we have used Tn*917* mutagenesis to isolate mutants resistant to pediocin AcH in *Listeria innocua* strain Lin11. *L. innocua* was used as a model system because *mpt*-based resistance mechanisms are thought to be operative in this species (24). In one mutant designated G7, transcription of the lin0142 gene is eliminated. The present study shows that the inactivation of lin0142 is responsible for pediocin AcH resistance in G7 and that lin0142 is required for the transcription of the *mpt* operon.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Tn*917* libraries were constructed in *L. innocua* strain Lin11 (Jean Richard, Institut National de la Recherche Agronomique, Paris, France). *Escherichia coli* strain DH5 (*supE44 hsd17 recA1 endA1 gyrA96 thi1 relA1*) was used in cloning procedures (25). *L. innocua* strains were grown in brain heart infusion (BHI) (Difco, Sparks, Md.) or Luria-Bertani (LB) medium at 30°C with shaking. *E. coli* strains were grown in LB medium at 37°C with shaking.

Isolation of Tn*917* insertion mutants. Gene knockout libraries were constructed using plasmid pLTV3 to introduce Tn*917* into *L. innocua* Lin11 (8). To identify mutants, ~10,000 cells from a transposon library were screened at ~100 cells per well in 96-well microtiter plates containing pediocin AcH. Pediocin AcH was purified by cell adsorption and desorption and by reversed-phase high-pressure liquid chromatography steps (51, 53). The screening medium was purple base broth (PBB) (Difco) with 1% lactose, 1 μg of erythromycin/ml, 25 μg of lincomycin/ml, and 0.05 μg of pediocin AcH/ml. Wells with resistant cells were identified based on a change in color from purple to yellow due to lactose metabolism after incubating 48 to 72 h at 30°C. Mutants were isolated by streaking on antibiotic plates and cultured through five or more passages to confirm the stability of the resistance phenotype.

Identification of the Tn*917* insertion site in the G7 mutant. DNA flanking the Tn*917* insertion site in a mutant designated G7 was cloned by digesting genomic DNA with KpnI restriction enzyme, self-ligating the DNA (which contains a portion of the original pLTV3 vector with its ColE1 *E. coli* replication origin and an insert derived from chromosomal DNA), and transforming the ligation reaction into *E. coli* DH5. The insert in the resulting plasmid designated pG7 was sized by restriction enzyme analysis, and 262 bp of the chromosomal DNA adjacent to the transposon was sequenced by use of a primer (5′-AACTCACA ATAGAGAGATGTCACCG-3′) complementary to the *lacZ* gene of pLTV3. All primers used in this study were obtained from Integrated DNA Technologies, Inc. (Coralville, Iowa). The *L. innocua* CLIP 11262 and *L. monocytogenes* EGD-e databases available at the Institut Pasteur website were used to conduct BLAST-N searches to locate the Tn*917* insertion site in G7 (ListiList) (21).

Southern blot analysis. Genomic DNA was isolated from the *L. innocua* G7 mutant by using standard procedures (18). A DNA-shearing step was added to improve the migration of genomic DNA in agarose gels. Plasmid DNA was purified from *E. coli* DH5/pG7 as a control by using the QIAfilter plasmid midi kit (QIAGEN, Valencia, Calif.). Plasmid and genomic DNAs were digested with KpnI restriction enzyme and analyzed on a 0.6% agarose gel. After electrophoresis, DNA was transferred to Zeta-Probe GT blotting membranes (Bio-Rad, Hercules, Calif.), and the DNA was fixed to the membranes by baking at 80°C for 30 min in a vacuum oven. Hybridization was performed at 43°C using probes complementary to the pLTV3 *cat* and *ble* genes at probe concentrations of 10 ng/ml. The sequence of the *cat* probe was 5′-CTAACTCTCCGTCGCTATTGT AACC-3′, and the sequence of the *ble* probe was 5′-GCACAGATAGCGTGG TCCGGCC-3′. Probes were labeled at the 3′ ends with fluorescein-dUTP. Labeling, hybridization, and washing were performed by following the instructions in the Gene Images 3′-oligolabeling module kit (Amersham Biosciences, Piscataway, N.J.). Membranes were first washed at a low stringency in 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and 0.1% sodium dodecyl sulfate at room temperature and then at a high stringency in 1× SSC and 0.1% sodium dodecyl sulfate at 52°C. DNA bands were visualized with the Gene Images ECL detection kit (Amersham Biosciences). X-ray films were exposed for 1 h when the *cat* probe was used and for 2 h when the *ble* probe was used.

Complementation testing. The lin0142 gene was amplified by PCR using *L. innocua* Lin11 genomic DNA as a template and cloned into the shuttle vector, pAM401 (52). BamHI and SalI restriction sites were incorporated into the forward and reverse PCR primers for cloning purposes. The forward primer sequence was 5′-CGGGATCCATTGAAAGAATAGTTTCGG-3′, and the reverse primer sequence was 5′-ACGCGTCGACTTAATTTCATATTAACCC-3′. BamHI and SalI restriction sites are underlined. The BamHI-SalI fragment was ligated into the corresponding sites of the gram-negative tetracycline resistance gene in pAM401, and the construct was transformed into *E. coli* DH5. The gram-negative and gram-positive chloramphenicol resistance genes are preserved in the recombinant plasmid, which is named pJX0142. The sequence of the lin0142 gene was determined by DNA sequencing.

pJX0142 was isolated from *E. coli* DH5/pJX0142 grown in LB medium containing 20 μg of chloramphenicol/ml and transformed into *L. innocua* Lin11 and G7 by electroporation (33). Transformation mixtures were plated on BHI plates containing 30 μg of chloramphenicol/ml at 30°C. pAM401 also was transformed into the strains for controls. Plasmid isolation and restriction enzyme digestion were performed to confirm that all transformations were successful.

Pediocin AcH susceptibility testing. The MICs of pediocin AcH were measured by use of a microtiter plate assay. Freshly grown cultures in PBB-lactose-antibiotic media were diluted and inoculated at ~100 cells per well into microtiter plate wells containing 200 μl of PBB-lactose broth plus chloramphenicol as needed and a range of 0 to 20 μg of high-pressure liquid chromatography-purified pediocin AcH/ml. Lin11 was grown in PBB–1% lactose without antibiotics, Lin11/pAM401 and Lin11/pJX0142 were grown in PBB–1% lactose with 10 μg of chloramphenicol/ml, G7 was grown in PBB–1% lactose with 5 μg of chloramphenicol/ml, and G7/pAM401 and G7/pJX0142 were grown in PBB–1% lactose with 40 μg of chloramphenicol/ml. Higher chloramphenicol concentrations were used for G7 strains transformed with plasmids because G7 carries a copy of the *cat* gene from pLTV3. Plates were incubated at 30°C, and growth was scored between 48 to 72 h.

Sequencing of lin0142. The BamHI-SalI PCR product described above was used to sequence the lin0142 gene. In addition, a PCR fragment spanning the lin0142 promoter region was sequenced to verify the results obtained with the larger PCR fragment. The forward primer used in amplification of the promoter region was 5′-ACCTGGTAAGCAAAAGACAGCAAC-3′, and the reverse primer was 5′-CGTTCCTCTGGATAAGCCGAC-3′. The PCR products were treated with ExoSAP-IT (U.S. Biochemical Corp., Cleveland, Ohio) to remove primers and nucleotides, and sequencing was then performed using the ABI Prism dRhodamine terminator cycle sequencing ready reaction kit (Applied Biosystems, Foster City, Calif.). The same primers used for PCR amplification were used for the sequencing of both DNA strands. Dye terminators were removed from reactions by use of AGTC gel filtration cartridges (Edge BioSystems, Gaithersburg, Md.). Electrophoresis was performed using an Applied Biosystems model 3100 genetic analyzer.

RNA isolation and transcript analysis by quantitative real-time RT-PCR. Strains were grown at 30°C in BHI or in LB medium supplemented with or without 2 g of D-glucose/liter to optical densities at 600 nm of 0.4 to 0.6. RNA from 3-ml cultures was stabilized using the RNAprotect bacterial reagent (QIAGEN) and was purified with the RNeasy mini kit (QIAGEN). Residual genomic DNA in the RNA samples was eliminated by DNase I (Invitrogen) treatment. cDNA was synthesized from total RNA by use of SuperScript II reverse transcriptase (RT) and random hexamers (both supplied by Invitrogen). To standardize the amount of RNA added to cDNA synthesis reactions performed with different RNA preparations, 16S rRNA was amplified as an endogenous control. Additional controls included a no-RT control, to assess contamination from genomic DNA, and no-template controls for each primer pair to measure interference due to primer dimer formation.

Real-time RT-PCRs were performed in 25-μl mixtures containing 12.5 μl of iQ SYBR green supermix (Bio-Rad), 10 ng of cDNA, and a 0.2 μM concentration of each of the forward and reverse primers (Table 1). Amplification steps were performed with an iCycler iQ real-time PCR detection system and an

TABLE 1. Primers used for real-time RT-PCR

Primer[a]	Sequence[b]
16S rRNA	
F	AAGCAACGCGAAGAACCTTA
R	TGCACCACCTGTCACTTTGT
lin0142	
F	CGGGTCAGAATGGTTTTGAG
R	AGACGGAACATTTTGCGAAC
lin0143	
F	TTTGCCAATGCTGATTGAAG
R	GCGGATACCTTCTTGAGCTG
manR	
F	GCCATCAAAGTCATCCCAAT
R	GGATCTTGAATTCCGACAGC
rpoN	
F	TTCGTCCAAAAGCAACAACA
R	TGCCACTAAATCAGCTGTCG

[a] F, forward primer; R, reverse primer.
[b] Primers were designed using published sequences available at the ListiList server (http://genolist.pasteur.fr/ListiList).

iCycler thermal cycler (Bio-Rad). The thermal cycler was programmed to initially hold at 95°C for 3.5 min and then to complete 40 cycles of 95°C for 15 s, 60°C for 30 s, and 68°C for 1 min. To carry out a melting curve analysis of PCR products, the temperature of samples was raised from 55 to 95°C by increases of 0.5°C per 10 s. The data were analyzed by using the comparative C_T method, where C_T is the cycle number at which the fluorescence emission due to PCR products exceeds the threshold. The threshold was set within the linear range of the PCR (44). The C_T values for PCR products from strains Lin11/pJX0142, G7, and G7/pJX0142 were compared to those for products from strain Lin11 in order to determine differences (*n*-fold) in C_T values. The REST program, which uses the pairwise fixed reallocation randomization test, was used to perform statistical analyses (35). A P value of <0.05 was considered significant.

Database searches and sequence analyses. Searches for promoter locations and prediction of the lin0142 transcription start point were performed with the Neural Network program (http://www.fruitfly.org/seq_tools/promoter.html) (41). *Listeria* genome analyses were performed using the ListiList server (http://genolist.pasteur.fr/ListiList) (21). GenBank database searches were carried out using the National Center for Biotechnology Information BLAST web server (http://www.ncbi.nlm.nih.gov/BLAST) (1). Protein sequences were analyzed for functionally important motifs and domains by use of the InterProScan server (http://www.ebi.ac.uk/InterProScan) (4, 6, 17, 22, 30). Multiple sequence alignments were carried out with the Tcoffee software package (http://igs-server.cnrs-mrs.fr/Tcoffee) (36).

RESULTS

Pediocin AcH resistance in the G7 mutant is caused by loss of lin0142 function. Gene knockout libraries were constructed for *L. innocua* Lin11 by using transposon Tn*917* introduced into the strain on plasmid pLTV3 (8). Altogether, 11 mutants which had various levels of resistance to pediocin AcH were isolated. Mutants were recovered from libraries at frequencies of 1 in 2,000 to 1 in 10,000 cells, depending on the library. One mutant, G7, is 1,000-fold less susceptible to the peptide (MIC $>$ 20 μg/ml) than is the wild type. For the wild-type Lin11 strain, the MIC of pediocin AcH is only 0.02 μg/ml.

To determine where Tn*917* resides in G7, the pG7 plasmid was constructed from genomic DNA and cloned in *E. coli* DH5. DNA sequencing of the pG7 insert showed that the transposon integrated between the lin0141 and lin0142 genes that have been mapped by sequencing the entire *L. innocua* CLIP 11262 genome (ListiList) (21) (Fig. 1). lin0141 encodes a putative cell wall protein with an LPXTG sortase cleavage motif (31). The function of the protein encoded by lin0142 is listed as unknown. lin0142 is just upstream of the *mpt* operon that contains *mptA* ($IIAB^{Man}$), *mptC* (IIC^{Man}), and *mptD* (IID^{Man}) (Fig. 1). A putative *rho*-independent terminator occurs between lin0142 and *mptA* (ListiList) (21), which suggests that lin0142 and the *mpt* operon are not cotranscribed.

Southern blotting was performed with KpnI-digested

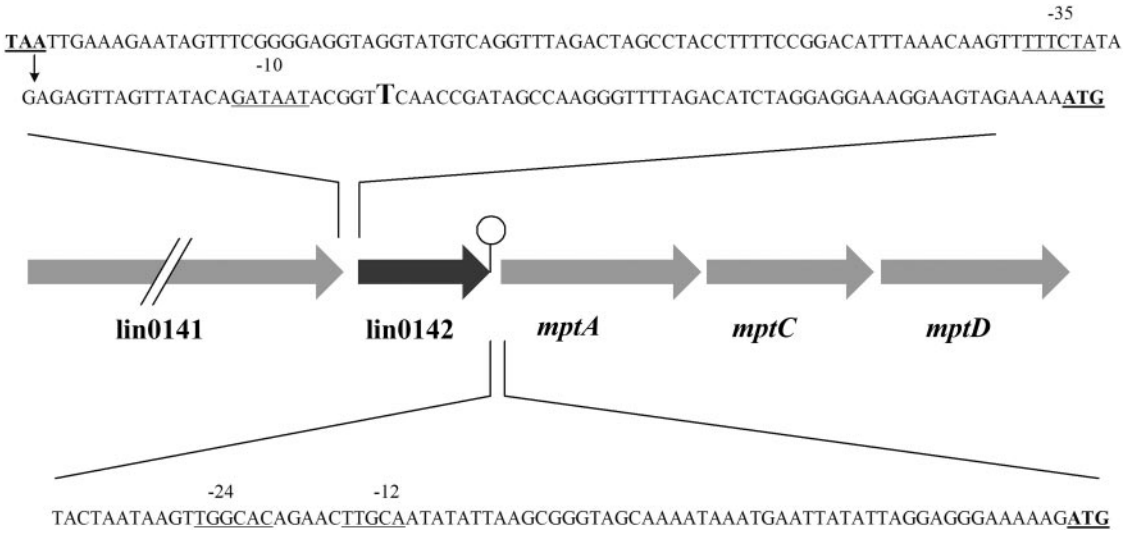

FIG. 1. Physical map of the Tn*917* insertion site in the G7 mutant. The insertion site is shown by an arrow. The gene products are as follows: putative peptidoglycan bound protein with LPXTG motif (from lin0141), a protein of unknown function (from lin0142), EII_t^{Man} component IIAB (from *mptA*), EII_t^{Man} component IIC (from *mptC*), and EII_t^{Man} component IID (from *mptD*). The stem-loop symbol denotes a *rho*-independent terminator (ListiList). The likely −35 and −10 and −24 and −12 sequences of the respective lin0142 and *mpt* operon promoters are underlined. The putative transcription start point for the lin0142 mRNA is shown in larger font. The stop codon of lin0141, the start codon of lin0142, and the start codon of *mptA* also are underlined. Note that only part of the sequence between the lin0142 and *mptA* genes is shown.

genomic DNA to determine the number of Tn*917* insertions in G7. pG7 plasmid DNA was used as a size control, since this plasmid was constructed directly from KpnI-digested genomic DNA. Southern blots obtained by using a probe complementary to the pLTV3 *ble* gene showed only one hybridization band (~19.5 kb) in G7 genomic DNA (data not shown). This band was the same size as that of the pG7 plasmid, and one band of this size also was obtained using a probe complementary to the pLTV3 *cat* gene. The results indicate that the G7 mutant carries a single copy of the Tn*917* transposon. They also eliminate the possibility that the phenotype of G7 is caused instead by insertion of a second copy of the transposon elsewhere in the genome.

To determine if the pediocin AcH-resistant phenotype of G7 is caused by disruption of lin0142 transcription per se or is due to a polar effect on *mpt* transcription, complementation testing was performed using plasmid pJX0142. This plasmid contains lin0142 and its putative promoter and terminator and none of the *mpt* operon or promoter sequences. As noted above, the MIC of pediocin AcH for strain G7 (>20 μg/ml) is 1,000-fold greater than the MIC (0.02 μg/ml) for the Lin11 wild-type strain. When the pJX0142 plasmid was introduced into G7, the MIC (0.02 μg/ml) decreased to the level of the wild-type strain. However, the MIC (>20 μg/ml) for the G7/pAM401 vector-only control was not reduced. This result indicates that the wild-type lin0142 gene complements the pediocin AcH-resistant phenotype of G7 in *trans*. It also suggests that the loss of lin0142 transcription causes resistance, as only this gene is required for complementation. Further support for this conclusion was obtained via real-time RT-PCR analysis of lin0142 transcription in G7 and the G7/pJX0142 complementation strain (see below).

Promoter analysis performed with the Neural Network program (41) indicates that the Tn*917* insertion site may reside within the lin0142 promoter (Fig. 1). The insertion point (between coordinates 11065 and 11066 relative to the *L. innocua* CLIP 11262 sequence) is 77 bp upstream of the lin0142 start codon and between the −35 and −10 sequences of a promoter predicted by the Neural Network algorithm (probability score, 0.94). The predicted initiation site for the lin0142 mRNA is a T residue at coordinate 11092. Real-time RT-PCR analysis of lin0142 mRNA level in G7 indicates that the Tn*917* insertion may indeed have inactivated the lin0142 promoter.

lin0142 regulates *mpt* transcription. Previous work has shown that 1,000-fold-increased resistance to class IIa bacteriocins in *L. monocytogenes* and *E. faecalis* results from the loss of *mpt* expression (14, 24, 26, 38). However, this fact has not yet been demonstrated directly for *L. innocua*. Because lin0142 is closely linked to the *mpt* operon, we tested whether lin0142 is involved in *mpt* control. We also tested whether lin0142 is needed for the transcription of the *rpoN* and *manR* genes, which positively regulate *mpt* transcription in *L. monocytogenes*. In this regard, searches conducted at the ListiList website (21) indicate that orthologs of all genes known to be important for class IIa bacteriocin resistance in *L. monocytogenes* are present in *L. innocua*. In both species, *manR* (lin0778/lmo0785) is located immediately upstream of the *mpo* operon. *rpoN* genes (*sigL*) also are present in both species. In addition, *L. monocytogenes* contains a lin0142 homolog (lmo0095) located immediately upstream of the *mpt* operon, which is composed of the *mptA*, *mptC*, and *mptD* genes.

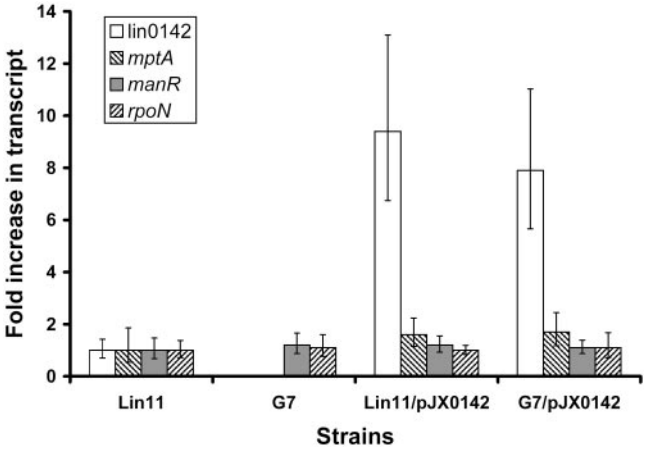

FIG. 2. Real-time RT-PCR analysis of the relative levels of the lin0142, *mptA*, *manR*, and *rpoN* mRNAs in Lin11, G7, and both strains transformed with pJX0142. The averages of results obtained from three independent RNA preparations are shown. All transcript levels were measured in triplicate for each RNA preparation. Error bars represent standard deviations from the means. All strains were grown in BHI medium.

To study the effects of lin0142 inactivation on gene expression, real-time RT-PCR was performed using the Lin11, G7, Lin11/pJX0142, and G7/pJX0142 strains grown in BHI medium. Real-time RT-PCR results indicate that the transcription of lin0142 is abolished in the G7 mutant (Fig. 2). Furthermore, lin0142 inactivation decreased *mptA* mRNA to an undetectable level. In contrast, the 7.9-fold overexpression of lin0142 from plasmid pJX0142 led to an increase in the *mpt* mRNA level in the G7/pJX0142 complementation strain (1.7-fold higher, $P < 0.05$). *rpoN* and *manR* transcript levels were not significantly affected ($P > 0.05$) by differences in lin0142 mRNA levels in the four strains grown in BHI medium.

Regulation of gene expression in Lin11 and G7 by glucose. Experiments were performed to investigate the role of glucose in the regulation of genes involved in *mpt* expression in the Lin11 and G7 strains. First, we determined whether transcription of the *mpt* operon is induced in the presence of glucose in *L. innocua*, as it is in *L. monocytogenes* (14). Second, the effects of glucose supplementation on *manR*, *rpoN*, and lin0142 mRNA levels were studied to evaluate whether glucose controls the transcription of these genes. Because BHI medium contains glucose, these experiments were performed in LB medium (14).

To determine whether expression of the *mpt* operon is regulated by glucose in *L. innocua*, real-time RT-PCR was performed using RNA isolated from Lin11 grown in LB medium with or without 2 g of glucose/liter. As shown in Fig. 3, the expression of the *mptA* gene was induced 2.4-fold ($P < 0.01$) by glucose in Lin11. Because transcription of *mpt* was elevated by glucose, it can be concluded that this operon also plays a specific role in glucose transport in *L. innocua* (14, 37).

The effects of glucose on *rpoN*, *manR*, and lin0142 mRNA levels also were studied by real-time RT-PCR analysis (Fig. 3). *rpoN* transcript levels were found to be the same in all media

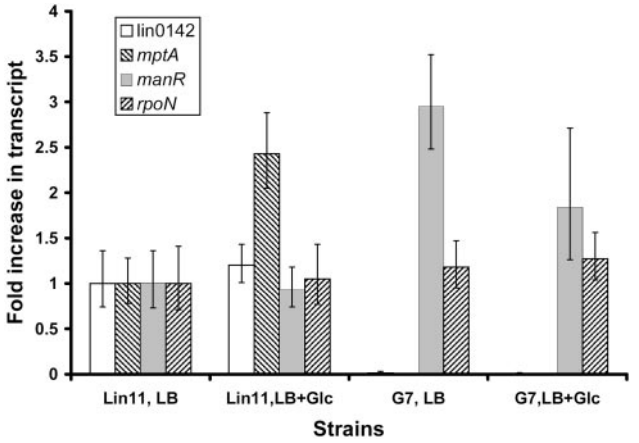

FIG. 3. Real-time RT-PCR analysis of the relative levels of the lin0142, *mptA*, *manR*, and *rpoN* mRNAs in Lin11 and G7 grown in LB medium with or without glucose (Glc). Averages of results obtained from four independent RNA preparations are shown. All transcript levels were measured in triplicate for each RNA preparation. Error bars represent standard deviations from the means.

tested. *manR* mRNA levels in Lin11 were unchanged by the addition of glucose to the media. However, *manR* transcripts in G7 increased 2.7-fold ($P < 0.01$) compared to those in Lin11 when the strains were grown without glucose. Interestingly, the addition of glucose to the media reduced the level of *manR* mRNA in G7 by 40% ($P < 0.01$). Finally, lin0142 mRNA levels were increased 1.2-fold ($P < 0.05$) in Lin11 when glucose was added to the media. Transcription of lin0142 was undetectable in G7, regardless of whether glucose was present. In summary, lin0142 is the only one of the three genes for which expression is modulated by glucose, albeit slightly, in the wild-type strain.

Sequence alignment of Lin0142 homologs. To gain insight into the function of the Lin0142 protein, its amino acid sequence was used as a query in BLAST-P searches conducted with the National Center for Biotechnology Information website and ListiList databases. It should be noted that the sequences of the lin0142 promoter, coding region, and terminator in strain Lin11 were determined and were found to be identical to the corresponding regions of the lin0142 gene present in *L. innocua* CLIP 11262.

Lin0142 was found to be weakly similar to the Crp-Fnr family of transcription regulators (29). The Crp-Fnr family member from other bacterial genera that shows the highest percent identity (27%) to Lin0142 is a protein of unknown function from *Staphylococcus epidermidis* ATCC 12228 (GenBank accession no. NP765769) (Fig. 4). The most similar proteins come from *Listeria* species. For example, the lin0142 ortholog, lmo0095, displays 59% identity, and the lin0131 protein and its ortholog, lmo0085, show 37 and 32% identity, respectively. In no case is the regulatory function of any of these proteins apparent from BLAST search information.

Crp-Fnr regulators have two main sequence features: an N-terminal cyclic nucleotide-binding domain and a C-terminal helix-turn-helix DNA-binding domain (29). An InterProScan search was conducted to look for these and other sequence elements in Lin0142 and its homologs. This server uses SUPERFAMILY, PFAM, SMART, PROFILE, and other algorithms to identify potential signature sequences within a queried sequence. Lin0142 and its homologs were all found to contain C-terminal winged-helix DNA-binding motifs (Table 2) (20). In contrast, only some Lin0142 homologs, and not Lin0142 itself, contain cyclic nucleotide-binding domains. Although the E values for some of the matches are poor, the fact that these sequences were often detected by several algorithms adds to the significance of the findings.

Previously, only one of these proteins (Lmo2132) was classified by phylogenetic analysis as a Crp-Fnr protein (within the D cluster of the family) (29). Lin0142 and the other proteins shown in Fig. 4 may have escaped classification due to the lack of cyclic nucleotide-binding domains or due to weak similarity of their DNA-binding domains to those in more typical Crp-Fnr proteins (29).

DISCUSSION

We have demonstrated that the lin0142 gene clearly is needed for the sensitivity of *L. innocua* to pediocin AcH. When lin0142 is not expressed, the bacterium becomes highly resistant to pediocin AcH. After lin0142 is reintroduced into the mutant, the bacterium is once again sensitive. We also have shown that lin0142 is required for transcription of the *mpt* operon, which encodes the EII_t^{Man} permease. This transporter is thought to be a docking protein for class IIa bacteriocins in *L. monocytogenes* and *E. faecalis* (14, 26, 38, 39). The level of *mpt* mRNA was very low in the G7 mutant and increased to greater than wild-type levels in the G7/pJX0142 strain due to the elevated transcription of lin0142 caused by the gene dosage effect of the multicopy-number plasmid. These data show for the first time that lin0142 controls the expression of the EII_t^{Man} permease and is needed for sensitivity of *L. innocua* to a class IIa bacteriocin.

Work performed here and bioinformatics analyses indicate that the basic aspects of EII_t^{Man} control are similar in *L. monocytogenes* and *L. innocua*. First, all genes implicated in the regulation of *mpt* in *L. monocytogenes* are present in *L. innocua*. Second, the expression of the *mpt* operon is induced by glucose in both species. Third, the *L. innocua mpt* operon contains an upstream sequence that precisely matches the -24 and -12 σ^{54}-controlled promoter consensus sequence, YTGGCACGrNNNTTGCW (Fig. 1) (5, 45), and therefore is likely to be controlled by σ^{54} and ManR. Fourth, *L. monocytogenes* contains a lin0142 homolog (lmo0095) immediately upstream of its *mpt* operon. In addition, EII^{Bgl} mRNA levels were found to be down-regulated when Lin11 was grown in LB medium supplemented with glucose (data not shown). This result is most likely caused by elevation of EII_t^{Man} levels in the presence of glucose (47).

Because the expression of the *mpt* operon is dependent on *rpoN* and *manR* in *L. monocytogenes* (2, 14, 43), we investigated the role played by lin0142 in the transcription of these two genes. lin0142 was found to be unnecessary for the transcription of either gene. The levels of *rpoN* and *manR* transcripts remained the same and increased, respectively, when the G7 mutant was grown in LB medium. The second of these observations might seem to indicate that lin0142 is a repressor of the *manR* gene. However, it is more likely that *manR* mRNA levels are elevated in the mutant due to the loss of *mpt*

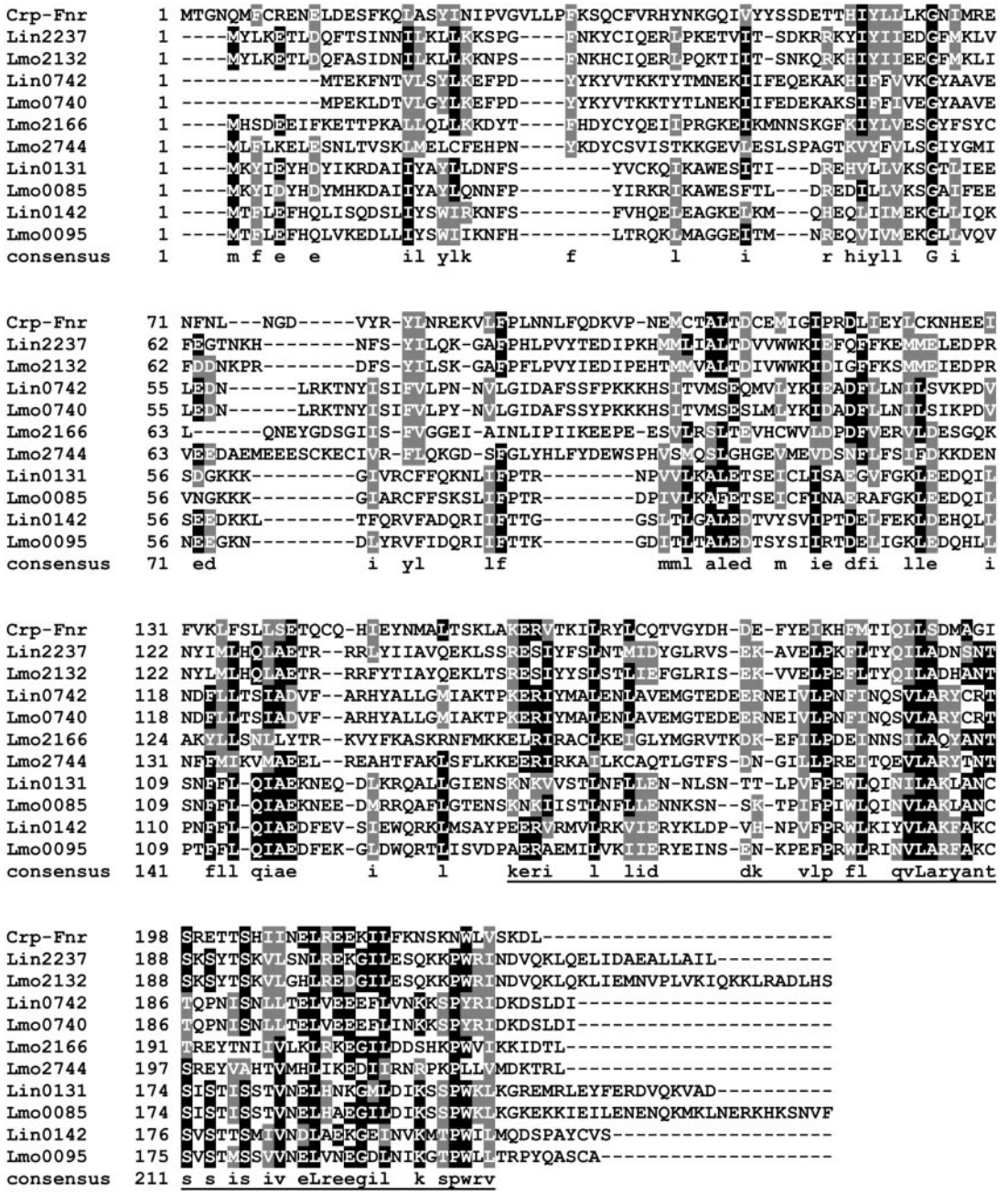

FIG. 4. Alignment of the amino acid sequences of Lin0142 and its homologs and orthologs in *L. innocua* and *L. monocytogenes*. The Crp-Fnr family protein from *S. epidermidis* ATCC 12228 (NP 765769) (labeled Crp-Fnr) also was included in the alignment. Black shading, amino acids that are identical in at least 50% of the sequences; grey shading, amino acids that are similar in at least 50% of the sequences. The putative winged-helix DNA-binding region is underlined.

expression. In this regard, the level of *manR* mRNA remains unchanged when lin0142 mRNA is overexpressed from pJX0142 in both the wild-type and mutant strains grown in BHI medium. It therefore appears that lin0142 is not normally involved in regulating the transcription of *rpoN* and *manR*.

We also examined whether glucose regulates the transcription of the lin0142, *rpoN*, and *manR* genes. Based on the finding that the levels of *rpoN* and *manR* mRNAs were not affected by glucose in the wild-type strain, it appears that these genes are not normally controlled by glucose. While *manR* transcript levels were found to decrease in response to glucose in the G7 mutant, this outcome could be an abnormal regulatory consequence caused by the loss of *mpt* expression. In the case of lin0142, it was found that glucose stimulated the transcription of this gene in the wild-type strain. We speculate that an increase in the Lin0142 protein level may contribute to the induction of the *mpt* operon in glucose media. However, the phosphorylation state and associated activation state of ManR

TABLE 2. BLAST-P results from ListiList and InterProScan searches

Protein[a]	Domain type	Algorithm	Location[b]	E value
Lin0142	Winged-helix DNA binding	SUPERFAMILY	136–196	1.1E−4
Lmo0095	Winged-helix DNA binding	SUPERFAMILY	133–196	6.7E−6
Lin0131	Winged-helix DNA binding	SUPERFAMILY	133–202	6.3E−5
Lmo0085	Winged-helix DNA binding	SUPERFAMILY	133–202	2.3E−6
Lin2237	Cyclic nucleotide binding	SMART	16–130	1.2
	Cyclic nucleotide binding	PROFILE	36–115	9.361
	Cyclic nucleotide binding	SUPERFAMILY	3–126	1.5E−13
	Winged-helix DNA binding	SUPERFAMILY	146–224	9.5E−7
Lmo2166	Cyclic nucleotide binding	PFAM	29–122	0.5
	Cyclic nucleotide binding	SMART	7–132	1.8
	Cyclic nucleotide binding	PROFILE	7–134	10.835
	Cyclic nucleotide binding	SUPERFAMILY	28–132	9.8E−13
	Winged-helix DNA binding	SUPERFAMILY	152–223	9.3E−6
Lin0742	Cyclic nucleotide binding	SUPERFAMILY	14–133	3.0E−10
	Winged-helix DNA binding	SUPERFAMILY	142–212	3.0E−6
Lmo0740	Cyclic nucleotide binding	SUPERFAMILY	1–117	1.6E−10
	Winged-helix DNA binding	SUPERFAMILY	142–212	3.4E−6
Lmo2132	Cyclic nucleotide binding	PROFILE	38–115	8.41
	Cyclic nucleotide binding	SUPERFAMILY	3–126	2.0E−12
	Winged-helix DNA binding	SUPERFAMILY	151–224	2.0E−6
Lmo2744	Cyclic nucleotide binding	PFAM	29–129	0.4
	Cyclic nucleotide binding	PROFILE	29–96	8.507
	Cyclic nucleotide binding	SUPERFAMILY	28–146	2.3E−14
	Winged-helix DNA binding	SUPERFAMILY	157–225	9.5E−11

[a] Proteins are named according to the ListiList server (http://genolist.pasteur.fr/ListiList). The GenBank accession number for *Listeria* sequences is NC003212. Proteins are listed in decreasing order of identity to Lin0142.
[b] Locations of amino acid sequences containing the identified domains.

are likely to be controlled by glucose, and this also may play an important role in the induction of the operon by glucose (14, 46).

Although Lin0142 is not strongly homologous to Crp-Fnr transcription factors (29), it is distantly related to this family of proteins. In addition, it contains a winged-helix DNA-binding motif (20). The winged-helix motif is a compact α/β structure that contains two wings (large loops), three α-helices, and three β-strands. The third α-helix typically participates in DNA binding, and winged-helix proteins often form complexes with other transcription factors (20). Sequence alignments of Lin0142 homologs indicate that the C-terminal region, where the winged-helix motif is located, is more conserved than the N-terminal region of these proteins. With regard to *mpt* promoter activation, Lin0142 is not a canonical σ^{54}-associated activator, as it does not contain a σ^{54} interaction module. It also lacks PTS regulation domains, which are common in PTS regulators (46). At this stage of the work, it is unknown whether the Lin0142 protein plays a direct or indirect role in activation of the *mpt* operon. However, the data suggest that Lin0142 may bind to DNA while carrying out its regulatory functions.

In conclusion, the results show that the lin0142 gene is a novel activator of *mpt* transcription in *L. innocua*. It is possible that additional regulators of EII_t^{Man} permease expression may be discovered by further screening for resistance to class IIa bacteriocins.

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