Applied and Environmental Microbiology, July 2007, p. 4677–4680 Vol. 73, No. 14
0099-2240/07/$08.00+0 doi:10.1128/AEM.02351-06

Insertional Mutagenesis To Generate Lantibiotic Resistance in *Lactococcus lactis*▽

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Received 5 October 2006/Accepted 12 May 2007

While the potential emergence of food spoilage and pathogenic bacteria with resistance to lantibiotics is a concern, the creation of derivatives of starter cultures and adjuncts that can grow in the presence of these antimicrobials may have applications in food fermentations. Here a bank of *Lactococcus lactis* IL1403 mutants was created and screened, and a number of novel genetic loci involved in lantibiotic resistance were identified.

The development of resistance to the lantibiotic nisin has been widely investigated, and it is well documented that mutants can be readily created in a laboratory setting (14). The phenotypic and physiological consequences of this resistance have been largely elucidated (23, 30), but the underlying genetic alterations that are responsible for these phenotypic changes have been the focus of relatively less attention. In recent years, some efforts have been made in this regard. First, a number of genes (*dltA*, *pbp2229*, *hk1021*, and *lisK*) have been associated with either enhanced sensitivity or resistance of *Listeria monocytogenes* to nisin (1, 9, 13, 15), and second, transcriptomic analysis of a *Lactococcus lactis* IL1403 nisin-resistant mutant enabled researchers to speculate as to how the altered expression of certain genes could contribute to nisin resistance (18).

Lacticin 3147, produced by *L. lactis* DPC3147, is a lantibiotic which differs from nisin in that it is active via the synergistic activity of two peptides (22). Like nisin, it exhibits a number of traits that are highly desirable in a food biopreservative (16). Despite being one of the most extensively characterized lantibiotics, relatively few studies have investigated the development of resistance to this antimicrobial. Studies so far have established that low-level resistance can occur at low frequencies after a single exposure to low concentrations of lacticin 3147 (7, 17, 26) and that mutants can become moderately more resistant following repeated exposure to increasing lantibiotic concentrations (17, 27). To date, the identity of the genes that contribute to lacticin 3147 resistance is unknown. Here we endeavor to identify genetic loci involved in lantibiotic resistance development. *L. lactis* IL1403 was chosen as a target organism because it is highly sensitive to lacticin 3147 (17) and its genome has been fully sequenced (4), making the identification of plasmid insertion sites relatively straightforward. In addition, the data generated could be used by the dairy industry to determine the likelihood and mechanism of resistance development among undesirable spoilage bacteria during food fermentations. Importantly, however, using the corollary logic, this study could also reveal how starter and desirable nonstarter lactococci could be altered in a targeted way to facilitate their survival in the presence of specific lantibiotics that may be added to fermented foods to improve food quality. A mechanism of creating stable food-grade starters or starter adjuncts that remain resistant to lantibiotics could be an important tool in the control of microorganisms in food fermentations (for a review, see reference 16).

Isolation of lacticin 3147-resistant mutants. A bank of pORI19 integration mutants of *L. lactis* IL1403 was created as described previously (19). Briefly, IL1403 genomic DNA was digested with Sau3A, and fragments were ligated with BamHI-digested pORI19 (RepA$^-$, erythromycin resistant) (19). The plasmid bank was then electroporated into *Escherichia coli* EC101 cells, which can support the replication of the pORI19 plasmid from a chromosomal copy of RepA. The resultant transformants (approximately 30,000) were pooled, and the plasmids were isolated and electroporated into *L. lactis* IL1403 containing the helper plasmid pVE6007 (RepA$^+$, temperature sensitive, chloramphenicol resistant). To select for integration of the pORI19 derivatives into the IL1403 genome by homologous recombination, the bank of mutants was cultured at 37°C (in the presence of 5 μg/ml erythromycin) to induce loss of the temperature-sensitive pVE6007 plasmid. Concentrated lacticin 3147 was obtained from the overproducer strain MG1363 carrying pMRC01 and pOM02 as described previously (11). The activity of the preparation was assessed by assaying against the lacticin 3147-sensitive indicator *L. lactis* HP by agar well diffusion assay (24), and activity was expressed as arbitrary units per ml (AU/ml) (25). Mutants with increased resistance to lacticin 3147 were screened by plating on GM17 plates containing 110 to 250 AU/ml of lacticin 3147 for 48 h at 30°C (17). In order to identify the site of insertion, the pVE6007 plasmid (RepA$^+$) was reintroduced by electroporation to rescue the inserted pORI19 containing the original cloned fragment. The inserts were amplified from five mutants, designated BRM1 to BRM5 (BRM for bacteriocin-resistant mutant), using primers M13F and M13R (Table 1). Sequence analysis revealed the identity of the segment of strain IL1403 DNA in the respective pORI19 plasmids. All mutants were

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▽ Published ahead of print on 25 May 2007.

TABLE 1. Primers used in this study

Primer[a]	Sequence[b] (5′–3′)
yjjCpORI	GTTCGCTCTTATTCTAAAGG
ymcFpORI	GATTACATATTGAGCAATTCC
pi322pORI	CTCGAACCAGACCCAAC
mleSpORI	ACTCACTTATCTACCCAG
tra981I	ATCGATAGCTGCGCTGACC
yjjCSOEA	TTGGTCTAGATCAAGCCT
yjjCSOEB	CCGTTTTTCTTGATTGCTTG
yjjCSOEC	**CAAGCAATCAAGAAAAACGG**CCACCAACAC TTGAAGATC
yjjjCSOED	CAGAATTCGGCCCCAGA
yjjCSOEE	CCCGTAATTAAAGTCATGG
yjjCSOEZ	ATGTATAAGTTAGTTGAGGTA
ymcFSOEA	TGGAATTCTTTATGCTCAAG
ymcFSOEB	CCCATCCGTTGTATAAGT
ymcFSOEC	**ACTTATACAACGGATGGG**CTTGCAGCTGAT GTGACAG
ymcFSOED	AATCTAGACCAACAGTTCC
ymcFSOEE	CCTTGAAAAGTATAACCAG
ymcFSOEZ	GGTAAAAAGACTTATAAGTAC
mlePR	CAAGTAGGAGAGTTAACCC
M13F	GTTTTCCCAGTCACGAC
M13R	CAGGAAACAGCTATGAC

[a] pORI in primer names indicates that the primers were used to confirm plasmid integration into the chromosome, while SOE in primer names indicates that the primers were used to make deletion mutants through splicing by overlap extension (SOE) PCR.

[b] Underlined sequences are XbaI and EcoRI restriction sites. Sequences in bold type are overhangs that are the reverse complement of the corresponding SOEB primer.

re-created by pORI19 mutagenesis with the recovered plasmid, and the site of integration was confirmed by PCR (Table 1).

Characterization of disrupted loci. In mutant strain BRM1, pORI19 inserted near the end of the *mleS* gene (Fig. 1). *mleS* encodes a malate-inducible malolactic enzyme and is followed by *mleP*, which encodes the associated malate permease. This operon has been previously characterized in *L. lactis*, and the two genes are thought to be cotranscribed (2, 3). Therefore, it is likely that the pORI19 insertion has a polar effect on *mleP*. In mutant strain BRM2, the disrupted gene is *yjjC*, which encodes a putative ATP binding domain of an ABC transporter and is followed by a putative permease-encoding gene, *yjjD*. YjjD has 12 hydrophobic domains characteristic of a secondary membrane transporter (28). Interestingly, transcription of *yjjCD* was also found to be reduced in a spontaneous lacticin 3147-resistant mutant (unpublished data). A homologue of *yjjC*, *tnrB2* in *Streptomyces longisporoflavus* (51% identity), has been found to be important in resistance to the polyether ionophore antibiotic tetronasin (20), and bioinformatics analysis revealed a possible role in lantibiotic immunity (www.ncbi.nlm.nih.gov/structure/cdd/cdd.shtml). API 50CH-based carbohydrate utilization analysis (bioMerieux) carried out in line with the manufacturer's instructions revealed that the *yjjC* mutant was impaired compared to the parental control with respect to maltose utilization (after 24 h) and gluconate utilization (after 48 h), indicating that resistance was a consequence of an altered metabolic pathway. In mutant strain BRM3, the pORI19 plasmid has inserted within the coding region of the *ymcF* gene encoding a putative peptidoglycan-bound protein, the C terminus of which shows homology to a cell wall surface anchor family of proteins. *ymcF* is followed by an insertion element (IS*905*) in the same orientation, but the genes were not found to be cotranscribed (not shown). Strain BRM4 had a plasmid insertion in an operon encoding phage-like proteins, and in BRM5, pORI19 disrupts an insertion sequence (IS*981*). Curiously, the IS*981* sequence is found at 10 different locations (*tra*981A [the *tra* gene that is found in copy A of IS*981*] to *tra*981J) throughout the IL1403 genome. Thus, it was necessary to determine which of these loci had been disrupted. A PCR-based strategy established that the plasmid had inserted in *tra*981I (data not shown).

Lantibiotic resistance. The growth of *L. lactis* IL1403 and BRM1 to BRM5 strains in GM17 broth in the presence of various levels of lantibiotics lacticin 3147 and nisin was analyzed (optical density at 600 nm). The majority of the mutants (pORI:19:*yjjC*, pORI:19:*ymcF*, pORI:19:*pi322*, and pORI:19:*mleS*) were not dramatically more resistant to lacticin 3147 but did show statistically significant ($P < 0.05$; log phase) increased

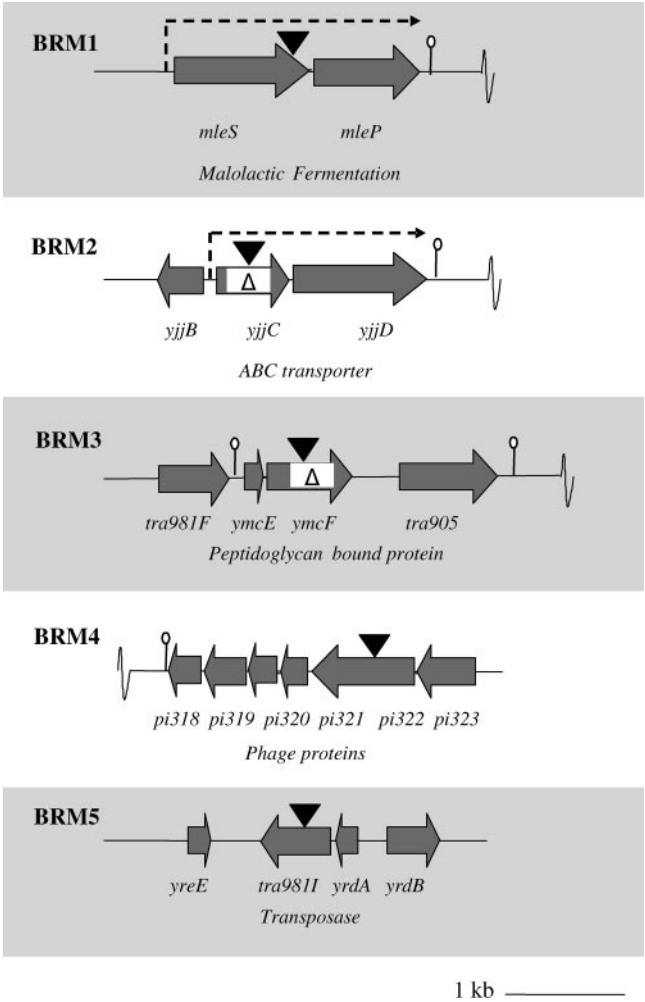

FIG. 1. Genes disrupted in the *L. lactis* IL1403 genome. Black arrowheads point to the sites of pORI19 insertion in IL1403 lacticin 3147-resistant mutants. White regions in BRM2 and BRM3 mutant strains indicate the positions of deletions made through splicing by overlap extension PCR. Putative terminators are indicated by lollipops, and operons proven experimentally are represented by broken-line arrows.

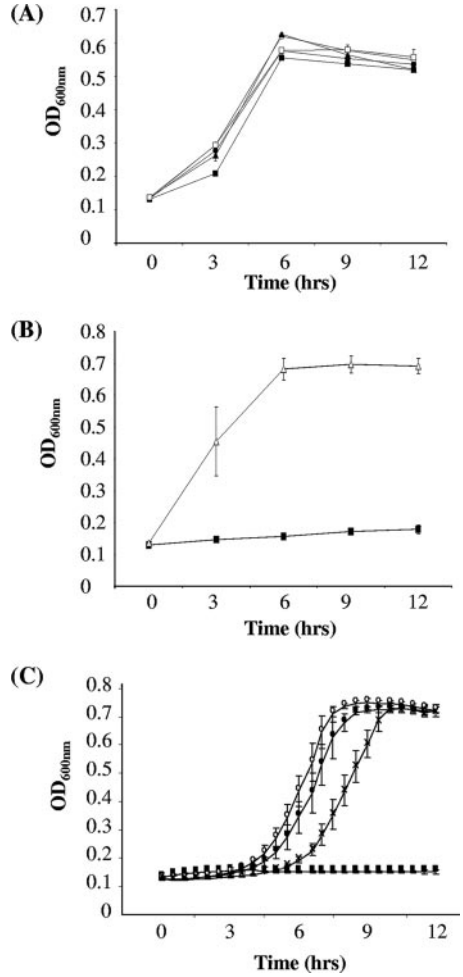

FIG. 2. Growth of *L. lactis* IL1403 pORI19 mutants in the presence of 25 AU/ml of lacticin 3147 (A) and growth of pORI19::*tra*981I in 50 AU/ml of lacticin 3147 (B) at 30°C. Symbols: ■, IL1403; □, pORI19::*mleS*; ●, pORI19::*yjjC*; ○, pORI19::*ymcF*; ▲, pORI19::*pi322*; Δ, pORI19::*tra*981I. (C) Growth of IL1403 (■), IL1403Δ*yjjC* (●), IL1403Δ*ymcF* (○) and IL1403Δ*yjjC*Δ*ymcF* (×) in 4 μg/ml of nisin powder (Sigma).

growth in the presence of low levels of lacticin 3147 (25 AU/ml) (Fig. 2A). pORI::*tra*981I was unique however in that it demonstrated an ability to grow even in the presence of 50 AU/ml lacticin 3147 (Fig. 2B). Analysis of the cultures in the presence of nisin demonstrated that the growth of pORI19::*yjjC* and pORI19::*ymcF* was significantly greater than that of the wild-type IL1403 in the presence of 4 μg nisin powder/ml (Sigma; data not shown). To confirm the association between mutagenesis of *yjjC* and *ymcF* and nisin resistance, additional mutants in which these genes were deleted in a nonpolar food-grade manner were created as described previously (10) (Table 1). It was established that the deletion mutants (i.e., Δ*yjjC* and Δ*ymcF*) behaved identically to their respective pORI19 counterparts, indicating that the phenotypes observed in the corresponding pORI19 mutants were not due to polar effects (Fig. 2C). A double Δ*yjjC* Δ*ymcF* mutant was also generated to assess whether nisin resistance could be further enhanced. It was established that, although this mutant grew at levels of nisin that prevented growth of the parental strain, it was not more tolerant than the corresponding single mutants and, in fact, displayed a relatively extended lag phase. Thus, while under certain circumstances in the future, it may be possible to combine different mutations with a view to enhancing the lantibiotic tolerance of starter strains, this was not the case in this instance. The three remaining mutants did not show significantly increased resistance to nisin as determined by growth analysis (data not shown).

Of the five pORI19 mutants, disruption of the *tra*981I gene resulted in the greatest resistance to lacticin 3147. It is not apparent why integration of the vector into 1 of the 10 IS*981* sites results in this phenotype. A study on IS elements in *L. lactis* IL1403 reported a high frequency of transposition of IS*981* under various laboratory conditions, which increased in stressful environments (12). It may be possible that plasmid insertion into *tra*981I in this study resulted in altered transposition events, leading to increased fitness of the cell. It is also worth noting that *ymcF* is also followed by a transposase, *tra*905. However, as IS*905* has not previously been reported to undergo transposition (12), its positioning is unlikely to be of relevance to the phenotype of this mutant. The role of the YmcF protein will need to be established in order to determine the precise mechanism via which it contributes to nisin resistance. Based on its impaired ability to utilize maltose and gluconate, the enhanced resistance due to mutation of *yjjC* is most likely to be a result of an alteration in the metabolism of the strain. A role for bioenergetics in determining nisin resistance has already been established (6). The *mleS* gene encodes the inducible malolactic enzyme in *L. lactis*. It was confirmed, by reverse transcription-PCR (primers mleSpORI and mlePR [Table 1]) using cells grown to log phase in GM17 broth, that *mleS* was transcribed in the wild-type strain under the conditions used (data not shown), and antibiotic disk assays on GM17 agar demonstrated that the pORI19::*mleS* mutant displayed enhanced neomycin resistance (data not shown). A relationship between lacticin 3147 resistance, neomycin resistance, and acid sensitivity has been previously documented (17), while a relationship between altered acid sensitivity and lantibiotic resistance in *L. monocytogenes* mutants has also been reported (5, 8, 9, 23, 29). As lacticin 3147 has increased activity against energized cells and the presence of a proton motive force promotes the interaction of the bacteriocin with the cell membrane (21), a reduction in the proton motive force of the mutant strain may explain its enhanced lacticin 3147 resistance.

In conclusion, this study has identified novel genes with a role in resistance to lantibiotics. Notably, the majority of the mutants isolated (i.e., all bar the more resistant *tra*981I mutant) exhibited resistance only in the presence of relatively low levels of lacticin 3147, a trend which is in accordance with earlier investigations of spontaneously arising lacticin 3147-resistant *L. lactis* IL1403 derivatives (17). It is particularly interesting that two of the mutants that show a small increase in resistance to lacticin 3147 display a more dramatic increased resistance to nisin. This correlates with an observed greater frequency and level of spontaneous resistance to nisin than to lacticin 3147 (data not shown). As mentioned above, the ability to create stable lantibiotic-resistant starter cultures has relevance in the food industry. This could potentially give control

over the biota in food fermentations with the addition of lantibiotics for food safety purposes. This principle was previously investigated using a spontaneously arising lacticin 3147-resistant variant of *Lactobacillus paracasei* in conjunction with a lacticin 3147-producing starter and resulted in increased control of the nonstarter lactic acid bacteria in cheese manufacture (27). However, as the stability of a spontaneously generated mutant can be variable, the creation of stable lantibiotic-resistant starters and adjuncts could be greatly beneficial. In this study, two mutants are created by nonpolar deletions which result in stable food-grade strains with increased resistance to both lacticin 3147 and nisin. This is potentially a strategy that could be employed by the food industry to increase food safety, through the addition of lantibiotics, without impacting adversely on food quality.

We acknowledge the funding received from the Irish Government under the National Development Plan 2000–2006 and through funding of the Alimentary Pharmabiotic Centre by the Science Foundation of Ireland Centres for Science Engineering and Technology (CSET) scheme.

REFERENCES

1. **Abachin, E., C. Poyart, E. Pellegrini, E. Milohanic, F. Fiedler, P. Berche, and P. Trieu-Cuot.** 2002. Formation of D-alanyl-lipoteichoic acid is required for adhesion and virulence of *Listeria monocytogenes*. Mol. Microbiol. **43:**1–14.
2. **Ansanay, V., S. Dequin, B. Blondin, and P. Barre.** 1993. Cloning, sequence and expression of the gene encoding the malolactic enzyme from *Lactococcus lactis*. FEBS Lett. **332:**74–80.
3. **Bandell, M., V. Ansanay, N. Rachidi, S. Dequin, and J. S. Lolkema.** 1997. Membrane potential-generating malate (MleP) and citrate (CitP) transporters of lactic acid bacteria are homologous proteins. Substrate specificity of the 2-hydroxycarboxylate transporter family. J. Biol. Chem. **272:**18140–18146.
4. **Bolotin, A., P. Wincjer, S. Mauger, O. Jaillon, K. Malarme, J. Weissenbach, S. D. Ehrlich, and A. Sorokin.** 2001. The complete genome sequence of the lactic acid bacterium *Lactococcus lactis* ssp. *lactis* IL1403. Genome Res. **11:**731–753.
5. **Bonnet, M., and T. J. Montville.** 2005. Acid-tolerant *Listeria monocytogenes* persist in a model food system fermented with nisin-producing bacteria. Lett. Appl. Microbiol. **40:**237–242.
6. **Bonnet, M., M. M. Rafi, M. L. Chikindas, and T. J. Montville.** 2006. Bioenergetic mechanism for nisin resistance, induced by the acid tolerance response of *Listeria monocytogenes*. Appl. Environ. Microbiol. **72:**2556–2663.
7. **Coakley, M., G. F. Fitzgerald, and R. P. Ross.** 1997. Application and evaluation of the phage resistance- and bacteriocin-encoding plasmid pMRC01 for the improvement of dairy starter cultures. Appl. Environ. Microbiol. **63:**1434–1440.
8. **Cotter, P. D., N. Emerson, C. G. Gahan, and C. Hill.** 1999. Identification and disruption of *lisRK*, a genetic locus encoding a two-component signal transduction system involved in stress tolerance and virulence in *Listeria monocytogenes*. J. Bacteriol. **181:**6840–6843.
9. **Cotter, P. D., C. M. Guinane, and C. Hill.** 2002. The LisRK signal transduction system determines the sensitivity of *Listeria monocytogenes* to nisin and cephalosporins. Antimicrob. Agents Chemother. **46:**2784–2790.
10. **Cotter, P. D., C. Hill, and R. P. Ross.** 2003. A food-grade approach for functional analysis and modification of native plasmids in *Lactococcus lactis*. Appl. Environ. Microbiol. **69:**702–706.
11. **Cotter, P. D., L. A. Draper, E. M. Lawton, O. McAuliffe, C. Hill, and R. P. Ross.** 2006. Overproduction of wild-type and bioengineered derivatives of the lantibiotic lacticin 3147. Appl. Environ. Microbiol. **72:**4492–4496.
12. **de Visser, J. A., A. D. Akkermans, R. F. Hoekstra, and W. M. de Vos.** 2004. Insertion-sequence-mediated mutations isolated during adaptation to growth and starvation in *Lactococcus lactis*. Genetics **168:**1145–1157.
13. **Gravesen, A., K. Sorensen, F. M. Aarestrup, and S. Knochel.** 2001. Spontaneous nisin-resistant *Listeria monocytogenes* mutants with increased expression of a putative penicillin-binding protein and their sensitivity to various antibiotics. Microb. Drug Resist. **7:**127–135.
14. **Gravesen, A., A. M. Jydegaard Axelsen, J. Mendes da Silva, T. B. Hansen, and S. Knochel.** 2002. Frequency of bacteriocin resistance development and associated fitness costs in *Listeria monocytogenes*. Appl. Environ. Microbiol. **68:**756–764.
15. **Gravesen, A., B. Kallipolitis, K. Holmstrom, P. E. Hoiby, M. Ramnath, and S. Knochel.** 2004. *pbp2229*-mediated nisin resistance mechanism in *Listeria monocytogenes* confers cross-protection to class IIa bacteriocins and affects virulence gene expression. Appl. Environ. Microbiol. **70:**1669–1679.
16. **Guinane, C. M., P. D. Cotter, C. Hill, and R. P. Ross.** 2005. Microbial solutions to microbial problems: lactococcal bacteriocins in the control of undesirable biota in foods. J. Appl. Microbiol. **98:**1316–1325.
17. **Guinane, C. M., P. D. Cotter, R. P. Ross, and C. Hill.** 2006. Spontaneous resistance in *Lactococcus lactis* IL1403 to the lantibiotic lacticin 3147. FEMS Microbiol. Lett. **260:**77–83.
18. **Kramer, N. E., A. F. T. van Hijum, J. Knol, J. Kok, and O. P. Kuipers.** 2006. Transcriptome analysis reveals mechanisms by which *Lactococcus lactis* acquires nisin resistance. Antimicrob. Agents Chemother. **50:**1753–1761.
19. **Law, J., G. Buist, A. Haandrikman, J. Kok, G. Venema, and K. Leenhouts.** 1995. A system to generate chromosomal mutations in *Lactococcus lactis* which allows fast analysis of targeted genes. J. Bacteriol. **177:**7011–7018.
20. **Linton, K. J., H. N. Cooper, I. S. Hunter, and P. F. Leadlay.** 1994. An ABC-transporter from Streptomyces longisporoflavus confers resistance to the polyether-ionophore antibiotic tetronasin. Mol. Microbiol. **11:**777–785.
21. **McAuliffe, O., M. P. Ryan, R. P. Ross, C. Hill, P. Breeuwer, and T. Abee.** 1998. Lacticin 3147, a broad-spectrum bacteriocin which selectively dissipates the membrane potential. Appl. Environ. Microbiol. **64:**439–445.
22. **McAuliffe, O., C. Hill, and R. P. Ross.** 2000. Each peptide of the two-component lantibiotic lacticin 3147 requires a separate modification enzyme for activity. Microbiology **146:**2147–2154.
23. **McEntire, J. C., G. M. Carman, and T. Montville.** 2004. Increased ATPase activity is responsible for acid sensitivity of nisin-resistant *Listeria monocytogenes* ATCC 700302. Appl. Environ. Microbiol. **70:**2717–2721.
24. **Parente, E., and C. Hill.** 1992. A comparison of factors affecting the production of two bacteriocins from lactic acid bacteria. J. Appl. Bacteriol. **73:**290–298.
25. **Ryan, M. P., M. C. Rea, C. Hill, and R. P. Ross.** 1996. An application in cheddar cheese manufacture for a strain of *Lactococcus lactis* producing a novel broad-spectrum bacteriocin, lacticin 3147. Appl. Environ. Microbiol. **62:**612–619.
26. **Ryan, M. P., W. J. Meaney, R. P. Ross, and C. Hill.** 1998. Evaluation of lacticin 3147 and a teat seal containing this bacteriocin for inhibition of mastitis pathogens. Appl. Environ. Microbiol. **64:**2287–2290.
27. **Ryan, M. P., R. P. Ross, and C. Hill.** 2001. Strategy for manipulation of cheese flora using combinations of lacticin 3147-producing and -resistant cultures. Appl. Environ. Microbiol. **67:**2699–2704.
28. **Saier, M. H., Jr.** 2003. Tracing pathways of transport protein evolution. Mol. Microbiol. **48:**1145–1156.
29. **van Schaik, W., G. C. Gahan, and C. Hill.** 1999. Acid-adapted *Listeria monocytogenes* displays enhanced tolerance against the lantibiotics nisin and lacticin 3147. J. Food Prot. **62:**536–539.
30. **Verheul, A., N. J. Russell, R. Van't Hof, F. M. Rombouts, and T. Abee.** 1997. Modifications of membrane phospholipid composition in nisin-resistant *Listeria monocytogenes* Scott A. Appl. Environ. Microbiol. **63:**3451–3457.