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Application and Evaluation of the Phage Resistance- and Bacteriocin-Encoding Plasmid pMRC01 for the Improvement of Dairy Starter Cultures

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The conjugative 63-kb lactococcal plasmid pMRC01 encodes bacteriophage resistance and production of and immunity to a novel broad-spectrum bacteriocin, designated lacticin 3147 (M. P. Ryan, M. C. Rea, C. Hill, and R. P. Ross, Appl. Environ. Microbiol. 62:612–619, 1996). The phage resistance is an abortive infection mechanism which targets the phage-lytic cycle at a point after phage DNA replication. By using the genetic determinants for bacteriocin immunity encoded on the plasmid as a selectable marker, pMRC01 was transferred into a variety of lactococcal starter cultures to improve their phage resistance properties. Selection of resulting transconjugants was performed directly on solid media containing the bacteriocin. Since the starters exhibited no spontaneous resistance to the bacteriocin as a selective agent, this allowed the assessment of the transfer of the naturally occurring plasmid into a range of dairy starter cultures. Results demonstrate that efficient transfer of the plasmid was dependent on the particular recipient strain chosen, and while high-frequency transfer (10^{-3} per donor) of the entire plasmid to some strains was observed, the plasmid could not be conjugated into a number of starters. In this study, transconjugants for a number of lactococcal starter cultures which are phage resistant and bacteriocin producing have been generated. This bacteriocin-producing phenotype allows for control of nonstarter flora in food fermentations, and the phage resistance property protects the starter cultures in industry. The 63-kb plasmid was also successfully transferred into *Lactococcus lactis* MG1614 cells via electroporation.

The susceptibility of lactococcal starter cultures to infection by bacteriophage remains a major problem facing the dairy fermentation industry worldwide, resulting in production and economic losses (15). In response, recent years have seen an intensive research effort focusing on the molecular nature of infecting bacteriophage and mechanisms of phage resistance, phage adsorption inhibition, phage DNA injection inhibition, restriction and modification of phage DNA, and abortive infection (5, 18). At present, the conjugal transfer of naturally occurring plasmids is the only generally accepted approach for the genetic improvement of starter cultures destined for consumer products (7), although food-grade cloning systems, such as that described by Platteeuw et al. (28), may be introduced in the future.

A prerequisite to the conjugal transfer of naturally occurring plasmids is that they possess a suitable selectable marker. The phage resistance phenotype has been used in numerous conjugations to date, with transconjugants being selected for on the basis of improved resistance to a homologous phage (9, 12, 34). With this type of selection, it is necessary that the phage resistance plasmid being transferred should provide adequate resistance, as partially protected strains would be less likely to survive exposure to high-titer phage in the selection. In addition, bacteriophage-insensitive mutants, which are phenotypically indistinguishable from putative transconjugants, can develop at high frequencies during this type of selection. The presence of lactose-fermenting determinants on a plasmid harboring phage resistance genes (e.g., pTR1040 [17]) offers an alternative naturally occurring selectable marker. In such a system, a lactose-deficient derivative of the cheese-making recipient has to be generated prior to introduction of such a phage resistance plasmid (35). However, interference with the natural lactose system of industrial starters is not favored (34), diminishing the usefulness of this approach for strain improvement.

In lactococci, the genetic linkage of phage resistance and bacteriocin production and immunity determinants on pMRC01 may offer a useful alternative to starter strain development. Such a strategy was previously used (29, 30) in experiments in which the bacteriocin dricin was used in broth medium as a selectable marker. Phage-resistant transconjugants were identified by their ability to ferment lactose and their resistance to the bacteriocin. Phage resistance has also been associated with the sucrose-nisin transposon in lactococci (8). The nisin resistance (*nsr*) gene has been used as a selectable marker for the improvement of a commercial starter culture (9) and also as the basis for the construction of a food grade vector system (3).

In this communication we describe the transfer of the phage resistance plasmid pMRC01 into a selection of lactococcal starter cultures by using bacteriocin production and immunity encoded by the plasmid as a selection. This 63-kb plasmid encodes production of and immunity to a novel broad-spectrum bacteriocin, lacticin 3147. Since lactococci exhibit negligible resistance to this bacteriocin, the genetic determinant(s) governing its immunity provides a valuable selectable marker for use in lactococci. The bacteriocin could be incorporated into solid media, eliminating the need for preenrichment in broth prior to plating. Because of the efficiency of the selection, it was possible to evaluate and compare transfer of the plasmid to a variety of starter strains. By this approach, transconjugants derived from a variety of lactococcal starters

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TABLE 1. Bacterial strains used in this study

L. lactis strain	Details	Source or reference[a]
Parent strains		
HP	Cheese starter	DPC
AM2	Cheese starter	DPC
DPC4268	Cheese starter	DPC
DPC4269	Cheese starter	DPC
DPC4270	Cheese starter	DPC
DPC4271	Cheese starter	DPC
DPC4272	Cheese starter	DPC
DPC4273	Cheese starter	DPC
DPC4274	Cheese starter	DPC
SK11G	Cheese starter	DPC
290P	Cheese starter	DPC
ML8	Cheese starter	DPC
077	Cheese starter	DPC
007	Cheese starter	DPC
057	Cheese starter	DPC
C13B	Cheese starter	DPC
047	Cheese starter	DPC
712	Cheese starter	NCDO
DRC3	pNP40-containing strain	24
DPC220	Lactic butter starter	13
DPC2723	DPC220 transconjugant containing pNP40	9
NCDO496	Nisin producer	NCDO
DPC3147	Lacticin 3147 producer	32
Other strains		
MG1363	Plasmid-free derivative of *L. lactis* 712	6
MG1614	Streptomycin-resistant derivative of MG1363	6
MG1614.pNP40	MG1614 transconjugant containing pNP40	DPC
MG1614.pMRC01	MG1614 transconjugant containing pMRC01	32
MG1363.pMRC01	MG1363 transconjugant containing pMRC01	This study

[a] DPC, National Dairy Products Research Centre, Teagasc, Moorepark, Fermoy, County Cork, Ireland. NCDO, National Collection of Dairy Organisms, Institute of Food Research, Reading, United Kingdom.

have been constructed which are improved with respect to their phage resistance and which are bacteriocin producing (Bac^+).

MATERIALS AND METHODS

Bacterial strains, bacteriophages, and media. The bacterial strains used in this study are listed in Table 1. Bacteriophages homologous to each of these strains either were available from the DPC collection or were isolated from commercial whey samples. Lactococcal strains were routinely propagated at 30°C in M17 medium (Difco Laboratories, Detroit, Mich.) supplemented with 0.5% glucose (GM17) or lactose (LM17) where necessary (36). MRS (Difco), 10% reconstituted skim milk (RSM), KCA (26), and fast/slow differential agar (FSDA) (10% RSM, 1.9% β-glycerophosphate [Sigma Chemical Co., St. Louis, Mo.], 0.004% bromocresol purple, 1.0% agar) were also used during the course of this study. Solid media contained 1.0% (wt/vol) bacteriological agar (Oxoid, Hampshire, England). The antibiotic streptomycin was added to agar at a concentration of 500 μg/ml where required. Tryptone (0.5%) was added to RSM in the case of the proteolytically deficient strain *L. lactis* subsp. *lactis* biovar diacetylactis DPC220 and its derivatives. All strains were stocked in M17 containing 40% glycerol and stored at −80°C. Working cultures were stored at 5°C and transferred periodically. Selective plates containing lacticin 3147 were prepared as follows: an overnight culture (1% inoculum) of *L. lactis* DPC3147 (grown in GM17) was centrifuged at 10,000 × *g* for 10 min and the resulting supernatant (adjusted to pH 7.0 with concentrated NaOH) was filter sterilized with Millipore (Middlesex, England) HVLP filters (0.45 μm pore size) and tempered to 45°C. This bacteriocin was added to an equal volume of double-strength lactose indicator agar (LIA) (24), generating a final lacticin 3147 concentration of 400 arbitrary units (AU)/ml.

Lacticin 3147 production by strains was estimated by an agar diffusion assay as described by Parente and Hill (27) and modified by Ryan et al. (32). Production in the test cultures was compared to production by *L. lactis* DPC3147. The bacteriocin activity is expressed in AU with *L. lactis* subsp. *cremoris* HP as the indicator strain. Bacteriocin production by a culture in broth was assayed by analyzing the filter-sterilized supernatant, whereas production in milk was assayed after heating the sample to 80°C for 10 min.

Phage assays. Plaque assays were performed according to the method of Terzaghi and Sandine (36) with the following modifications. First, 250 μl of an overnight culture, 100 μl of 1 M $CaCl_2$ and 100 μl of the appropriate phage dilution were added to 3 ml of M17 overlay (0.7% agar). The contents were mixed, poured onto M17 agar, and incubated at 30°C for 18 h. The efficiency of plaquing (EOP) of a phage on a particular host was determined by dividing the phage titer on the test strain by the titer on the homologous phage-sensitive host.

Bacteriophage adsorption to host cells was determined by the method of Sanders and Klaenhammer (33). The percentage of phage adsorption was calculated as $[(\text{control titer} - \text{test residual titer})\ (\text{control titer})^{-1}] \times 100$.

Lactococcal cell survival in the presence of phage was assayed as described by Behnke and Malke (2). Surviving cells were enumerated as CFU per milliliter. The percentage of cell death was calculated as [(CFU/ml in culture without phage − CFU/ml in culture with phage)][(CFU/ml in culture without phage)$^{-1}$] × 100.

Burst sizes were measured by a variation of the method of Klaenhammer and Sanozsky (17) at 30°C. A growth curve was set up with the test phage and a susceptible and resistant host. At 5-min intervals, a sample of the phage-host mixture was removed and assayed immediately for phage against a sensitive host (*L. lactis* MG1614). One-step growth curves were generated for φc2, and the latent period and burst sizes were calculated.

Analysis of intracellular phage DNA replication. Intracellular phage c2 DNA replication was monitored by the method of Hill et al. (11). DNA samples were digested with the restriction enzyme *Eco*RI (New England BioLabs) in cuts-all reaction buffer (200 mM Tris-HCl [pH 7.5], 70 mM $MgCl_2$, 20 mM 2-mercaptoethanol) and electrophoresed on a 0.7% agarose gel with ethidium bromide staining.

Strain construction, DNA isolation and manipulations. The initial conjugal mating between *L. lactis* MG1363 and *L. lactis* DPC3147 was performed at a recipient-to-donor ratio of 20:1 as follows. First, 1 ml of freshly grown recipient cells (MG1363) was concentrated 20-fold and mixed with 50 μl of donor cells (DPC3147). This mixture was then spotted onto the center of a nonselective GM17 agar plate and allowed to dry. Following an overnight incubation at 30°C, the mating mix was harvested from the agar plates and resuspended in 500 μl of GM17. Serial dilutions of the mating mix were then plated onto selective media (LIA containing lacticin 3147). Resulting transconjugants were Lac^- and Bac^+.

By using the above transconjugant, *L. lactis* MG1363(pMRC01), as a donor, conjugations were performed with a variety of wild-type lactococcal starter cultures as recipients (Table 1). These matings were performed as described above, but the recipient and donor were mixed at a ratio of 1:1 (with the exception of the AM2 mating which was performed at a recipient-to-donor ratio of 20:1). Resulting transconjugants were clearly identified as Lac^+ colonies against a Lac^- background on plates containing lacticin 3147.

The procedure of Anderson and McKay (1) was used for both large-scale and rapid isolation of plasmid DNA from lactococci. Plasmid profiles were analyzed by overnight electrophoresis on 0.7% vertical agarose (Sigma) gels at 40 V on a Biorad Protean unit to allow adequate separation of plasmids in the size range of 50 to 70 kb. Electrophoresis was performed in TAE buffer (0.04 M Tris-HCl, 0.01 M disodium EDTA, 0.012 M sodium acetate [pH 7.8]). Plasmid sizes were determined by comparing mobilities with the larger plasmids in *L. lactis* subsp. *lactis* biovar diacetylactis DRC3 (78, 65, and 51 kb).

For hybridization analysis, plasmid profiles and restricted DNA were electrophoresed as described above and transferred to Hybond N^+ nylon membrane (Amersham) by the capillary blotting method (21). A DNA probe consisting of a 9-kb *Hin*dIII fragment of pMRC01 was used to detect the presence of pMRC01-homologous DNA in the plasmid profiles. DNA hybridizations were performed at 65°C by using conditions recommended by the supplier of the ECL gene detection system (Amersham). Two other probes were used: pPG01 (AbiF) and pPG23 (AbiE) (4).

Electroporation of lactococcal strains was carried out according to the procedure of Wells et al. (38) with a Bio-Rad gene pulser apparatus (Bio-Rad Corp., Richmond, Calif.). pMRC01 DNA (0.5 μg) was added to electrocompetent MG1614 cells. Samples were plated onto LIA supplemented with lacticin 3147. These plates were incubated at 30°C and observed for Lac^- colonies. These Lac^- colonies were tested for lacticin 3147 production after which plasmid profile analysis was performed.

Starter activity test. Phage susceptibility was determined by propagating the test culture in the presence of lytic phage by using the starter activity test of Harrington and Hill (9) in 10% RSM at 21°C. This activity test was performed for five cycles, and at the conclusion of the cycles 10 μl of whey from cycle 5 was spotted onto M17 agar which had been overlaid with the test culture to detect the presence low levels of phage, which may not have affected the pH value achieved.

Phage susceptibilities of the strains were also determined by propagating the test culture in the presence of a homologous phage and a mixture of industrially isolated phage and whey cocktails, which date back over 10 years. These assays

TABLE 2. Results of successful conjugal matings between *L. lactis* MG1363(pMRC01) and a selection of lactococcal starter cultures

Recipient	Mating frequency per donor	Lacticin 3147 production (AU/ml)	Improved phage resistance	Transfer of pMRC01 from transconjugants to MG1614
DPC4268	2.3×10^{-3}	160	−	−
DPC4272	1.1×10^{-5}	320	−	+
DPC4273	1.0×10^{-4}	160	−	−
DPC4274	4.0×10^{-5}	320	+	+
DPC220	5.4×10^{-4}	160	+	+
HP	6.0×10^{-6}	640	+	+
712	1.0×10^{-5}	640	+	+
ML8	5.5×10^{-4}	640	+	+
077	1.1×10^{-5}	320	−	+
007	1.3×10^{-7}	640	ND[a]	+
MG1363	2.0×10^{-3}	640	+	

[a] ND, not determined.

were carried out according to the method of Heap and Lawrence (10), in which the strains were successively exposed to phage and whey over a period of 10 days.

Plasmid stability test. The stability of pMRC01 in a selection of transconjugants was investigated by growing the culture for 75 generations in 10% RSM at 21°C, after which individual colonies were tested for lacticin 3147 production.

Diacetyl production, citrate utilization, and acetolactate production. Diacetyl production (31, 37), citrate utilization (22) and acetolactate production (14, 39) assays were conducted on cells grown in 10% RSM plus 0.5% tryptone at 21°C for 18 h.

RESULTS

Construction of a food grade donor strain for pMRC01. In order to construct a suitable donor strain for pMRC01, it was necessary to generate a lactose-deficient (Lac^-) strain containing the plasmid, which could be easily distinguished from the Lac^+ starter recipients in subsequent conjugations. Initially, several attempts to isolate a Lac^- derivative of DPC3147 (by temperature curing at 39°C), which retained its bacteriocin phenotype were unsuccessful. In an alternative strategy to obtain a pMRC01-containing Lac^- strain, the plasmid was conjugally transferred from the DPC3147 donor into *L. lactis* MG1363, an antibiotic-sensitive plasmid-free strain (6). Putative Lac^- Bac^+ transconjugants were isolated at a frequency of 2.0×10^{-3} per donor. Analysis of the plasmid DNA complements of a selection of these isolates confirmed the presence of the 63-kb plasmid.

Transfer of pMRC01 to lactococcal starter cultures. By using *L. lactis* MG1363(pMRC01) as a donor, conjugations were performed with a variety of wild-type lactococcal starter cultures as recipients (Table 1). These transconjugants were obtained at frequencies of between 10^{-3} and 10^{-7} per donor, depending on the particular recipient strain. Importantly, no spontaneous resistance to lacticin 3147 was observed during the selection, with the exception of DPC220 and its derivative DPC2723, both of which exhibited low levels of spontaneous resistance (less than 10^{-8} per donor). Of the 22 starters tested, putative transconjugants were successfully isolated for 10 of the strains (Table 2). All transconjugants were found to produce lacticin 3147, but at levels varying between 160 AU/ml [DPC220(pMRC01)] and 640 AU/ml [ML8(pMRC01)]. In contrast, none of the parent strains produced bacteriocin.

Plasmid profile analyses were performed for all of the putative transconjugants and their respective parent strains. In some cases, for example, *L. lactis* subsp. *cremoris* HP (Fig. 1), the presence of pMRC01 was evident by the appearance of a 63-kb band in the transconjugant's plasmid profile. However, for many of the putative transconjugants, the presence of pMRC01 was masked by similarly sized resident plasmids. For

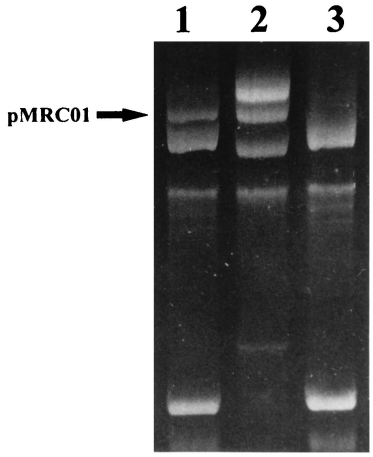

FIG. 1. Agarose gel electrophoresis of plasmid DNA from *L. lactis* HP (pMRC01) (lane 1) and *L. lactis* HP (lane 3). The plasmid profile of *L. lactis* DRC3 is included (lane 2) as a source of molecular size markers (78, 65, and 58 kb). The 63-kb plasmid pMRC01 is evident in the putative transconjugant (lane 1).

these, acquisition of pMRC01 was confirmed by DNA/DNA hybridization with a 9-kb *Hin*dIII DNA fragment of pMRC01, e.g., the result obtained following hybridization of the probe to a 63-kb plasmid in the profile of the ML8 transconjugant as shown in Fig. 2. A more efficient approach to confirming that these strains were true transconjugants involved the conjugal mating of the plasmid back out of the transconjugants by using MG1614 ($Strep^r$) as the recipient and bacteriocin immunity and streptomycin resistance as selectable markers. Transfer of pMRC01 from the transconjugants to MG1614 was confirmed by plasmid profile analysis and/or bacteriocin production phenotypes.

In the case of the strains DPC4268 and DPC4273, pMRC01 could not be mobilized from the putative transconjugants. Analysis of four transconjugants of DPC4268 (Fig. 3) revealed that the level of bacteriocin produced corresponded to the formation of a 75-kb plasmid with concomitant loss of a resident 63-kb plasmid in the transconjugants. One such transconjugant, DPC4275 (lane 1), produced twofold more bacteriocin than the others that were analyzed (Fig. 3, lanes 2 to 4). The Bac^+ transconjugants in lanes 2, 3, and 4 still contain the 63-kb plasmid, and the 75-kb plasmid is very faint in lanes 2 and 3 and is not detectable in lane 4, probably due to low copy number. In DPC4275, the 63-kb plasmid was absent and there was a corresponding increase in the copy number of the 75-kb

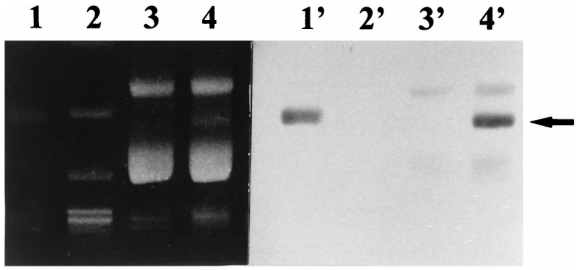

FIG. 2. DNA hybridization analysis of plasmid DNA from *L. lactis* MG1363 (pMRC01) (lane 1), DRC3 marker (lane 2), ML8 (lane 3), and ML8(pMRC01) (lane 4). The 9-kb *Hin*dIII pMRC01 probe hybridized to the 63-kb fragment (indicated by the arrow) in MG1363(pMRC01) (lane 1′) and ML8(pMRC01) (lane 4′). No hybridization to the parent ML8 (lane 3′) was observed.

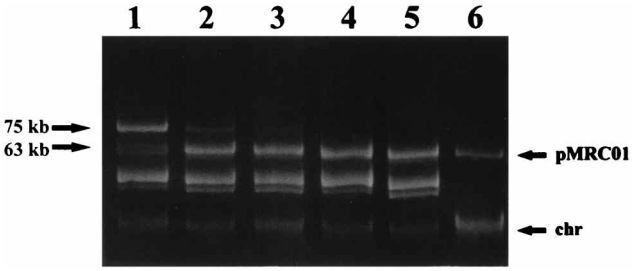

FIG. 3. Plasmid DNA from the donor strain MG1614(pMRC01) (lane 6) and the recipient DPC4268 (lane 5) and four putative transconjugants in lanes 1 to 4. A 75-kb plasmid is evident in lanes 1, 2, and 3 which was not present in either the donor or recipient strain. This plasmid possibly represents a cointegrate between pMRC01 and a resident plasmid of DPC4268.

plasmid. The newly formed 75-kb plasmid was not conjugative, nor did it afford increased phage resistance to the parent strain (DPC4268). Interestingly, DNA/DNA hybridization analysis demonstrated that both the resident 63-kb plasmid and the new 75-kb plasmid share homology with pMRC01 (data not shown).

Electroporation was also used to transfer pMRC01 to *L. lactis* MG1614. Transformants were isolated at a frequency of $8.5 \times 10^3/\mu g$ of DNA on media containing lacticin 3147 and streptomycin. Genetic analysis of a number of these transformants confirmed the presence of a 63-kb plasmid. These strains were also bacteriocin producing.

Analysis of transconjugants for retention of industrially important traits. Improvement in the phage resistance of the modified starters due to the acquisition of pMRC01 was first assessed by comparing the ability of homologous phage to form plaques on the parent and transconjugant strains. Phage were available (or could be isolated) for all but one strain, *L. lactis* 007, whose transconjugant could not be assessed for phage susceptibility for that reason. Increased phage resistance associated with the plasmid was observed for the transconjugants of strains DPC220, 712, ML8, HP, and DPC4274. In the cases of the DPC220, 712, ML8, and HP transconjugants, individual plaque formation was not observed even where relatively high ($>10^9$ PFU/ml) titers of phage were used. With the DPC4274 transconjugant, the plaque size was dramatically reduced and the plaques were turbid in appearance. In contrast, Bac^+ transconjugants of strains DPC4268, DPC4272, DPC4273, and 077 were not protected from attack by homologous phage. The level of plaque formation on these strains was identical to that on the parent strain.

Phage susceptibilities of a selection of the transconjugants were also determined by propagation of each in 10% RSM at 21°C in the presence of lytic phage according to the modified starter activity test (9). Homologous phage (10^7 PFU/ml) were added to the test culture at the start of each cycle. All the parent strains failed on the first cycle, as did the DPC4268 transconjugant, which was previously shown to be unprotected. In contrast, both the *L. lactis* HP and 712 transconjugants grew normally after five cycles of exposure. The 220 and ML8 transconjugants survived two and three cycle exposures, respectively. Thus, the level of protection afforded by pMRC01 against homologous phage varied considerably depending on the particular strain or phage tested. A more rigorous test involved the use of whey cocktails to replace the homologous phage in a Heap-Lawrence test which was performed over 10 cycles. This cocktail represented a series of phage-containing wheys collected from cheddar cheese factories over a number of years. The homologous phage for each test strain was also included in the assay. The transconjugants for strains HP, 712, and DPC220 survived exposure to the whey cocktail for the duration of the test, and the parent strain 712 also survived. The DPC4274 and ML8 transconjugants did not survive exposure to the whey, failing on the first and second cycles.

The transconjugants produced acid at rates approaching those of the parent strains, which is sufficient for commercial cheese manufacture (Fig. 4). To confirm that pMRC01-containing starters were suitable as starters for food fermentations, cheddar cheese was manufactured by using the *L. lactis* HP transconjugant at pilot scale (450-liter vats). Under commercial conditions, the transconjugant succeeded in reducing the pH of the milk to 5.2 at a rate similar to that of the control strain, HP.

pMRC01 was also conjugally mobilized into *L. lactis* subsp. *lactis* biovar diacetylactis DPC220 (425A), which forms part of a mixed strain starter designated 4/25, used to manufacture lactic butter. pNP40 (23) had been previously introduced into DPC220, yielding the phage-resistant strain DPC2723 (9). In the present study both DPC220 and DPC2723 were tested as recipients for pMRC01. Uptake of the plasmid by DPC220 occurred at a frequency of 10^{-4} per donor, whereas the conjugation was not successful for DPC2723. The DPC220 transconjugants were Cit^+ Bac^+ and had increased phage resistance. Assays comparing the commercial production abilities of DPC220, DPC2723, and DPC220(pMRC01) were performed on cells grown in 10% RSM plus 0.5% tryptone at 21°C for 18 h. All of the cultures utilized 100% of the citrate in RSM. Diacetyl was produced by DPC220, DPC2723, and DPC220(pMRC01) at levels of 0.34, 0.37, and 0.42 mM, respectively. Acetolactate was produced at levels of 2.66, 2.71, and 2.76 mM for DPC220, DPC2723, and DPC220(pMRC01), respectively. Thus, neither pMRC01 nor pNP40 has a negative effect on the ability of DPC220 to produce acetolactate.

The stability of selected pMRC01 transconjugants was also investigated by growing the cultures in 10% RSM at 21°C. The HP and 712 transconjugants were grown for 75 generations, and individual colonies were screened for lacticin 3147 production. In both cases, all of the resultant colonies were lacticin

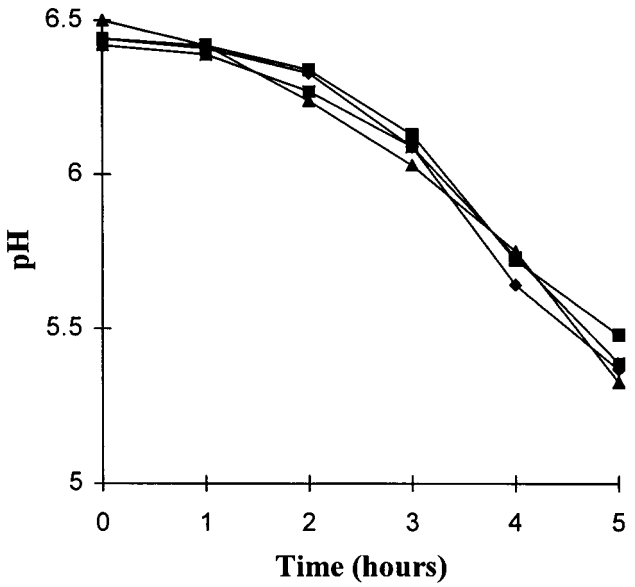

FIG. 4. Acid production in 10% RSM over the cheese temperature profile by *L. lactis* DPC4272 (▲), *L. lactis* DPC4272(pMRC01) (■), *L. lactis* DPC4273 (◆), and *L. lactis* DPC4273(pMRC01) (−).

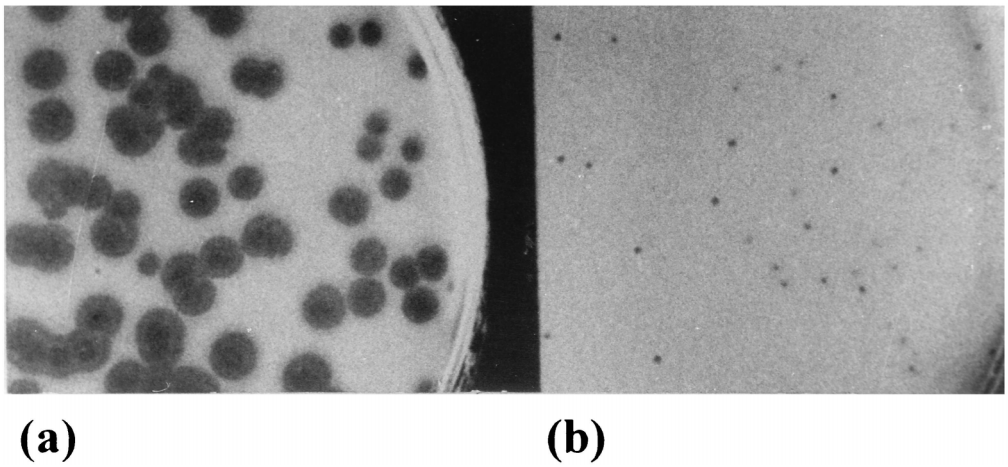

FIG. 5. Plaque assays of phage c2 on *L. lactis* MG1363 (a) and *L. lactis* MG1363(pMRC01) (b). The EOP is identical in both strains, but the plaque size of the phage is sixfold smaller on MG1363(pMRC01).

3417 producing, indicating that the plasmid was stably maintained.

Phage resistance encoded by pMRC01. The phage resistance encoded by pMRC01 was analyzed in the plasmid-free background of *L. lactis* subsp. *lactis* MG1363. The small isometric-headed phage 712 failed to form plaques on MG1363 (pMRC01). In contrast, plaques were obtained with the prolate-headed phage c2 by using this host. These plaques were approximately sixfold smaller than those observed on the MG1363 host itself (Fig. 5). Interestingly, the EOP was not reduced with the plasmid-containing strain, suggesting that the phage resistance does not involve a restriction modification system. Bacteriophage adsorption assays revealed that 90 to 94% of φc2 particles adsorbed to MG1363 and MG1363 (pMRC01) hosts, indicating that adsorption blocking could not account for the phage protection afforded by the plasmid. Based on one-step growth curve experiments (data not shown), the burst size of φc2 on MG1363 was shown to be reduced 10-fold by the presence of pMRC01. In addition, there was a 5-min extension in the latent period of the phage (from 15 to 20 min). Analysis of intracellular phage c2 DNA replication (Fig. 6) revealed the increase in φc2 DNA content of cells following infection was delayed by 5 min in the presence of pMRC01. Although intracellular phage DNA replication was not totally inhibited in these cells, some inhibition was apparent, compared with the control (Fig. 5). From cell survival experiments it was seen that a high proportion (99%) of both MG1363 and MG1363(pMRC01) cells died following φc2 infection. Analysis of the phage assays indicates that the phage resistance mechanism encoded by pMRC01 is an abortive infection system, which appears to target the phage lytic cycle at a point following phage DNA replication. DNA/DNA hybridization has confirmed that this abortive infection system is not AbiE or AbiF from the phage resistance plasmid pNP40 (data not shown).

DISCUSSION

The 63-kb plasmid pMRC01 encodes an abortive infection-type phage resistance mechanism and also production of and immunity to the novel, broad-host-range bacteriocin lacticin 3147. The genetic linkage of these phage resistance and bacteriocin determinants on a conjugative plasmid offers an efficient approach to lactococcal starter strain improvement. In this study it was possible to improve the phage resistance of a number of lactococcal starters, including those that are currently used in Ireland for cheese and lactic butter production, by the introduction of pMRC01. In addition, all of the generated transconjugants produce lacticin 3147, which may be advantageous for many applications.

In this study, lacticin 3147 proved as convenient a selectable marker for use in conjugal matings and transformations as a conventional antibiotic. With the exception of DPC220, for which a low level of resistance was recorded, no spontaneous resistance to lacticin 3147 was observed. In contrast, nisin-resistant mutants can be readily selected from nisin-sensitive populations after exposure to high concentrations of the bacteriocin (16). The efficiency of the selection allowed assessment of the ability of a number of strains, which included industrial starters, to receive the plasmid. Bacteriocins have previously been used as selective agents in conjugations (8, 9, 30), but in these cases they were usually incorporated into broth medium, as a preenrichment step, prior to plating on solid medium. In contrast, lacticin 3147 can be directly incorporated into solid media for selection. This selection did not necessitate the purification of the bacteriocin, because a pH-neutralized supernatant, obtained from a producing strain, could be used as a source. Another advantage of using the bacteriocin genes as selectable markers is that plasmid maintenance should be assured because cells losing the plasmid would also lose the associated bacteriocin immunity and would subsequently be killed by lacticin 3147-producing strains. Work is currently in progress with a view to exploiting the genetic determinants for lacticin 3147 immunity as the basis of a selectable marker system for lactococci.

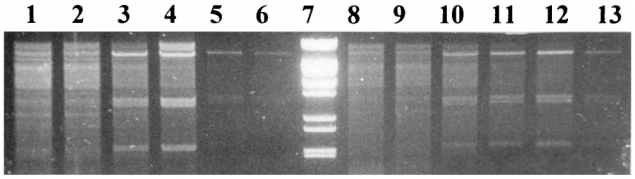

FIG. 6. Intracellular phage DNA content of *L. lactis* MG1363 and *L. lactis* MG1363(pMRC01) following infection with φc2. Lanes 1 to 6 and 8 to 13 show the *Eco*RI-digested total DNA isolated at 0, 10, 20, 30, 40, and 50 min from MG1363 and MG1363(pMRC01), respectively. Lane 7 is λ DNA digested with *Eco*RI and *Hin*dIII. Absence of DNA after 40 min is due to cell death and lysis.

A major concern in the genetic modification of valuable industrial starters is that their industrial properties should not be negatively affected. Since nisin-producing lactococci generally perform poorly as cheese starters with slow acid production, reduced proteolytic activity, and phage sensitivity (19, 20), it was important to assess the performance of the pMRC01-containing transconjugants given that they are also bacteriocin producing. In general, the transconjugants in this study produced acid at rates approaching those of the parent strains, which are sufficient for commercial cheese manufacture. Moreover, acquisition of the plasmid, while improving phage insensitivity, did not affect the ability of strain DPC220 to produce acetolactate or diacetyl in the commercial production of lactic butter. The transconjugants of strains DPC4268 and DPC4273 exhibited bacteriocin production and immunity but did not have improved phage insensitivity. It is possible that the bacteriocin phenotype is associated with the acquisition of a 75-kb nonconjugative plasmid in the DPC4268 transconjugants and that the genes encoding 3147 production and immunity alone had integrated with the 63-kb resident plasmid in that strain. The resultant 75-kb plasmid thus may not possess the functional genetic determinants for pMRC01 transfer and phage resistance.

During this study, pMRC01 could not be transferred to some lactococcal starters for a number of different reasons. This plasmid may be incompatible with a resident plasmid or may not be able to replicate efficiently in the recipient. Alternatively, such nontransfer may be explained in some instances by the sensitivity of particular lactococci to lacticin 3147. It has been observed that lactococci can have various sensitivities to lacticin 3147, with some strains being substantially more sensitive to the bacteriocin (25). Strains AM2, DPC4269, and DPC4270 appeared highly sensitive to the bacteriocin with the result that the bacteriocin selection process employed in the matings may have been too severe. Failure to transfer pMRC01 to *L. lactis* NCDO496 was thought to be due to the fact that this recipient is a nisin-producing strain and thus inhibits the pMRC01 donor strain.

It is important to note that when introducing a bacteriocin-producing (Bac^+) transconjugant to an industrial process which uses a mixed starter system, other components of the starter also require immunity to lacticin 3147. This can be conveniently achieved by introducing pMRC01 to these other starter components by conjugation. There are advantages for the cheese manufacturer, of using a bacteriocin-producing starter. Lacticin 3147 has been shown to be produced at adequate levels and is sufficiently active during cheese manufacture and ripening to affect the developing microflora (32). In particular, such starters can be used to inhibit and/or retard the proliferation of nonstarter lactic acid bacteria (principally lactobacilli) in cheese. Since lacticin 3147 also effectively inhibits *Staphylococcus* and *Listeria*, foods which contain the bacteriocin may have an associated food safety advantage.

The 63-kb conjugative plasmid pMRC01 was also successfully transferred to a plasmid-free strain by electroporation. To our knowledge, this is the first description of the transformation of a large naturally occurring plasmid in lactococci and attests to the utilization of lacticin 3147 as a selectable marker.

Even though this study has generated a number of starter strains with improved phage resistance and bacteriocin production, their potential will not be fully established until they have undergone prolonged use in industry.

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