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pAMβ1-Associated Mobilization of Proteinase Plasmids from *Lactococcus lactis* subsp. *lactis* UC317 and *L. lactis* subsp. *cremoris* UC205

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A combination of plasmid curing and DNA-DNA hybridization data facilitated the identification of proteinase plasmids of 75 (pCI301) and 35 kilobases (pCI203) in the multi-plasmid-containing strains *Lactococcus lactis* subsp. *lactis* UC317 and *L. lactis* subsp. *cremoris* UC205, respectively. Both plasmids were transferred by conjugation to a plasmid-free background only after introduction of the conjugative streptococcal plasmid, pAMβ1. All Prt$^+$ transconjugants from matings involving either donor contained enlarged recombinant Prt plasmids. UC317-derived transconjugants were separable into different classes based on the presence of differently sized cointegrate plasmids and on segregation of the pCI301-derived Lac and Prt markers. All UC205-derived transconjugants harbored a single enlarged plasmid that was a cointegrate between pCI203 and pAMβ1. The identification of *prt* genes on pCI301 and pCI203 derivatives was achieved by a combination of restriction enzyme and hybridization analyses.

The proteinase activity (Prt phenotype) of *Lactococcus* spp. is essential for adequate growth of these bacteria in milk and for their contribution to proteolysis and flavor development during cheese ripening. Consequently, the genetics and biochemistry of this important but unstable trait have been the subject of intensive studies (18, 39). The linkage of Prt to specific plasmids, however, has frequently depended solely on the results of plasmid curing experiments, the interpretation of which may be complicated by the simultaneous loss of cryptic plasmids unrelated to Prt (18). It is therefore desirable to demonstrate cotransfer of Prt and the suspected plasmid. Detection of Prt transfer by transduction from *Lactococcus lactis* subsp. *lactis* C2 and 712 (9, 27, 28) and by conjugation from strain 712 (11) was facilitated only through the fortuitous linkage of this marker with the more readily selectable lactose-fermenting (Lac) phenotype. The development of a broadly based conjugative mobilization system for nonconjugative Prt and other plasmids of *Lactococcus* spp. is therefore of considerable interest. The pVA797-pVA838 homologous vector delivery system, initially developed by Smith and Clewell for use with *Streptococcus faecalis* (36), has been adapted to the lactococci (34), and pVA797 has also been employed in the conjugative mobilization of a nonhomologous Lac Prt plasmid in *L. lactis* subsp. *lactis* Z270 (31).

A further homology-independent approach designed to mobilize nontransferable plasmids of the lactococci and which was adopted in this study involves the use of the *S. faecalis* plasmid pAMβ1 (4) as the mobilizing agent. pAMβ1 (26.5 kilobases [kb]) is a broad-host-range plasmid encoding macrolide-lincosamide-streptogramin B resistance, which has been transferred by conjugation to at least nine streptococcal species, members of the lactococci (10), and a wide range of other gram-positive genera. pAMβ1-associated mobilization of *Bacillus subtilis* chromosomal markers (19), of native *Bacillus thuringiensis* plasmids (21), and of a nonconjugative tetracycline resistance plasmid from *S. faecalis* (20) has been reported; pAMβ1-mediated transfer of the bacteriophage resistance and Prt plasmids of *L. lactis* subsp. *cremoris* SK11 (6) and of the Lac plasmid of *L. lactis* subsp. *lactis* ML3 (2) has also been observed. In addition, homology-dependent pAMβ1 transfer of a nonconjugative in vitro recombinant plasmid from *B. subtilis* to *Clostridium acetobutylicum* has been achieved (32).

The present study focuses on an examination of the interactions between pAMβ1 and the nonconjugative Prt plasmids pCI301 and pCI203, from *L. lactis* subsp. *lactis* UC317 and *L. lactis* subsp. *cremoris* UC205, respectively, during mobilization. Analysis of novel in vivo recombinant plasmids identified in Prt$^+$ Em^r transconjugants revealed that pCI301 and pCI203 can undergo cointegration with the mobilizing plasmid. In the case of pCI301, cointegrates were also formed with an undetermined segment of DNA at a lower frequency. After their introduction into a plasmid-free background, the localization of UC317- and UC205-derived *prt* genes was readily facilitated by restriction enzyme comparison and hybridization analyses and revealed that both genes exhibited similarities with other characterized *prt* genes.

MATERIALS AND METHODS

Bacterial strains, media, and reagents. The bacterial strains used in this study are described in Table 1. All lactococcal strains were routinely subcultured at 30°C in the M17 medium of Terzaghi and Sandine (38) containing lactose (0.5%) or glucose (0.5%; GM17). *Escherichia coli* strains were grown in LB medium (25) at 37°C. For solid media, agar (1.5%, Oxoid no. 3) was added to broth before autoclaving. Antibiotics included in selective media were added at the following concentrations: erythromycin (50 μg/ml) and streptomycin (500 μg/ml) for lactococcal strains and chloramphenicol (20 μg/ml) for *E. coli*. Lactose indicator agar (LIA [30]) and fast-slow differential agar (FSDA [14]) supplemented with glucose (0.5%) and/or tryptone (0.5%) were used to assess the ability of selected strains to ferment lactose and utilize milk proteins. Chemical reagents were

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TABLE 1. Bacterial strains, phenotypes, plasmid content, and derivation

Strain	Relevant phenotype	Plasmid content (kb)	Description
L. lactis subsp. *cremoris*			
UC205	Lac$^+$ Prt$^+$ Sm^s Em^s	45 (pCI201), 43 (pCI202), 35 (pCI203), 4 (pCI204)	UCC culture collection
EC209	Lac$^+$ Prt$^+$ Sm^s Em^r	45, 43, 35, 26.5 (pAMβ1), 4	Transconjugant from MG3020 × UC205 (this work)
L. lactis subsp. *lactis*			
UC317	Lac$^+$ Prt$^+$ Sm^s Em^s	75 (pCI301), 55 (pCI302), 38 (pCI303), 16 (pCI304), 8.7 (pCI305)	UCC[a] culture collection
FH068	Lac$^+$ Prt$^+$ Sm^s Em^r	75, 55, 38, 26.5 (pAMβ1), 16, 8.7	Transconjugant from MG3020 × UC317 (this work)
MG1363Sm	Lac$^-$ Prt$^-$ Sm^r Em^s	None	Plasmid-free, Sm^r derivative of *L. lactis* subsp. *lactis* 712 (8)
MG3020	Lac$^-$ Prt$^-$ Sm^r Em^r	26.5 (pAMβ1)	pAMβ1-containing derivative of MG1363Sm (10)
FH082	Lac$^+$ Prt$^+$ Sm^r Em^r	95 (pCI306), 26.5 (pAMβ1)	Transconjugant from FH068 × MG1363Sm (this work)
FH084	Lac$^+$ Prt$^+$ Sm^r Em^r	115 (pCI307), 26.5 (pAMβ1)	Transconjugant from FH068 × MG1363Sm (this work)
FH130	Lac$^+$ Prt$^+$ Sm^r Em^r	102 (pCI344)	Transconjugant from FH068 × MG1363Sm (this work)
FH138	Lac$^+$ Prt$^-$ Sm^r Em^r	95 (pCI355), 26.5 (pAMβ1)	Transconjugant from FH068 × MG1363Sm (this work)
EC210	Lac$^-$ Prt$^+$ Sm^r Em^r	60 (pCI210)	Transconjugant from EC209 × MG1363Sm (this work)
FH088	Lac$^-$ Prt$^+$ Sm^r Em^s	22 (pCI310)	Derivative of FH082 cured of pAMβ1 and containing a deleted Prt plasmid (this work)
E. coli			
V517		55.9, 7.5, 5.8, 5.3, 4.1, 3.1, 2.8	Source of size reference plasmids (24)
C600(pGD4)	Cm^r	11.6 (pGD4)	pGD4 is the *Bam*HI B fragment of pWV05 cloned in pACYC184 (17)

[a] UCC, University College, Cork.

obtained from Sigma Chemical Co., Poole, Dorset, England. Restriction endonucleases were supplied by the Boehringer Corp. Ltd., Dublin, Ireland, and were used according to the manufacturer's instructions.

Plasmid curing experiments. Proteinase-negative (Prt$^-$) derivatives of *L. lactis* subsp. *lactis* UC317 and *L. lactis* subsp. *cremoris* UC205 were isolated by serial transfer in GM17 broth at 30 or 37°C. Subcultures were diluted, spread on GM17 plates, and incubated at 30°C for 18 h, and approximately 200 isolated colonies were tested for loss of the proteinase phenotype by spotting on FSDA plates. Prt$^-$ mutants were small, colorless colonies against a faint yellow background on FSDA supplemented with glucose in contrast to Prt$^+$ strains, which gave larger, yellow colonies on this medium. Mutant phenotypes were confirmed by testing their ability to reduce the pH of 10% reconstituted skim milk containing glucose (0.5%) with or without tryptone (0.5%) after 18 h of incubation at 21°C.

Conjugation experiments. Conjugation experiments were performed by a modification of the method of McKay et al. (29). Briefly, overnight cultures of donor and recipient were diluted twofold in M17 or GM17 as appropriate and incubated for 2 h at 30°C. These were mixed in a 1:1 ratio, and samples (0.2 ml) were spread on glucose-supplemented skim milk agar mating plates (29) and incubated anaerobically with a Gas Generating kit (Oxoid) at 30°C for 18 to 24 h. Cells were harvested from the mating plates in quarter-strength Ringer solution, and Lac$^+$, Lac$^+$ Prt$^+$, and Prt$^+$ transconjugants were selected for on LIA, FSDA, and FSDA-glucose plates, respectively. Plates contained the relevant antibiotics and were incubated anaerobically at 30°C for up to 5 days. In each mating, donor and recipient alone were plated on selective media to score for spontaneous mutation. All Lac$^+$, Prt$^+$, or Em^r transconjugants were screened for cotransfer of the nonselected markers, and phenotypes were confirmed by the ability of the isolates to reduce the pH of 10% reconstituted skim milk as described above.

Plasmid DNA preparation and analysis. Rapid preparation of plasmid DNA from lactococcal strains was by the method of Anderson and McKay (1). Plasmid profiles were analyzed by electrophoresis on 0.8% agarose gels with a vertical gel apparatus operating at 5 V/cm for 3 to 4 h in Tris acetate buffer (0.04 M Tris acetate [pH 7.9], 0.002 M disodium EDTA). Gels were stained in a solution of ethidium bromide (0.5 μg/ml) for 30 min, destained in water, viewed under shortwave UV light, and photographed by using Polaroid type 667 film. Purified plasmid DNA was isolated from *E. coli* C600(pGD4) by the method of Birnboim and Doly (3) and from *L. lactis* subsp. *lactis* as described by Anderson and McKay (1), followed by repeated cycles of CsCl-ethidium bromide density gradient ultracentrifugation at 50,000 rpm at 15°C for at least 20 h in a Beckman type VTi65 rotor. Plasmid DNA was dialyzed extensively against 0.01 M Tris hydrochloride (pH 7.2)–0.001 M disodium EDTA at 4°C before concentration by ethanol precipitation. Restriction endonuclease digestions of plasmid DNA were analyzed by electrophoresis on 0.7%–1.2% horizontal agarose gels containing 0.5 μg of ethidium bromide per ml running at 1.5 to 2.5 V/cm for 12 to 24 h in Tris acetate buffer. Restriction maps were constructed by correlating the results of single, double, and triple digests performed either simultaneously or sequentially.

Preparation of biotinylated DNA probes. Plasmid pGD4 and its 4.3-kb *Bam*HI-*Hin*dIII fragment, isolated from low-melting-temperature agarose or by electrophoresis onto dialysis membranes (25), were used as proteinase probes. pGD4 consists of the *Bam*HI fragment B of pWV05, the proteinase plasmid of *L. lactis* subsp. *cremoris* Wg2, cloned in pACYC184 (17). The 4.3-kb fragment contains no non-proteinase-encoding lactococcal DNA (16). The Em^r plasmid pAMβ1 was also used as a probe. Probe DNA was labeled with biotin-11-dUTP according to specifications supplied with the BRL (Paisley, Scotland) nick translation kit

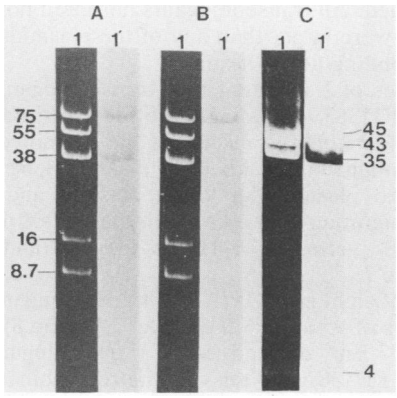

FIG. 1. Hybridization analysis of plasmid profiles of *L. lactis* subsp. *lactis* UC317 and *L. lactis* subsp. *cremoris* UC205. (A) Lanes: 1, plasmid profile of *L. lactis* subsp. *lactis* UC317; 1', color reaction obtained after hybridization with biotin-11-dUTP-labeled pGD4. (B) Lanes: 1, plasmid profile of *L. lactis* subsp. *lactis* UC317; 1', color reaction obtained after hybridization with biotin-11-dUTP-labeled 4.3-kb *Bam*HI-*Hin*dIII fragment of pGD4. (C) Lanes: 1, plasmid profile of *L. lactis* subsp. *cremoris* UC205; 1', color reaction obtained after hybridization with biotin-11-dUTP-labeled 4.3-kb *Bam*HI-*Hin*dIII fragment of pGD4. Numbers on the left and right refer to sizes (kilobases) of plasmids in UC317 and UC205, respectively.

(8160 SB) and detected by using the BRL DNA detection system (8239 SA).

Southern hybridization. DNA was transferred from agarose gels to nitrocellulose filters by the method of Southern (37) as modified by Wahl et al. (42). Hybridization was allowed to proceed for 12 h at 65°C in 5× SSC (1× SSC is 0.15 M NaCl–0.015 M trisodium citrate)–0.1% polyvinylpyrrolidone–0.1% Ficoll–0.1% bovine serum albumin–0.1% sodium dodecyl sulfate (SDS)–100 μg of denatured herring sperm DNA per ml. Posthybridization washes consisted of four 15-min washes in 2× SSC–0.1% SDS at 65°C, two 15-min washes in 0.2× SSC–0.1% SDS at 65°C, one 15-min wash in 0.1× SSC–0.1% SDS at 65°C, and then two final washes in 0.1× SSC–0.1% SDS at room temperature.

RESULTS

Identification of proteinase plasmids. Prt^- mutants of *L. lactis* subsp. *lactis* UC317, defined by their appearance on FSDA plates and their poor acid production in 10% reconstituted skim milk, were isolated at a frequency of 28.5% after approximately 30 generations in nonselective medium (GM17) at 37°C. No spontaneous Prt^- variants of this strain were obtained at 30°C. This differed markedly from the easy isolation of Prt^- mutants of strain UC205 at frequencies of 44 and 77% at 30 and 37°C, respectively. Strain UC317 contained five plasmids of 75 (pCI301), 55 (pCI302), 38 (pCI303), 16 (pCI304), and 8.7 (pCI305) kb (Fig. 1A, lane 1). The loss of pCI301 correlated with the loss of the Prt^+ phenotype in this strain. Strain UC205 contained four plasmids of 45 (pCI201), 43 (pCI202), 35 (pCI203), and 4 (pCI204) kb (Fig. 1C, lane 1). All Prt^- variants of this strain lacked pCI203. The loss of pCI301 and pCI203 from their respective hosts was occasionally accompanied by the disappearance of various other components of the strains' plasmid complements. However, no Prt^+ isolates lacking pCI301 or pCI203 were ever observed.

DNA-DNA hybridization experiments were undertaken with both biotinylated pGD4 and the 4.3-kb *Bam*HI-*Hin*dIII fragment of this plasmid as proteinase probes. In the case of strain UC317, both probes hybridized to pCI301, whereas pGD4 also hybridized to pCI303 (Fig. 1A, lane 1'; Fig. 1B, lane 1'). This suggested that hybridization of pGD4 to pCI303 was due to homology with the nonproteinase lactococcal DNA of pGD4. Hybridization of both pGD4 and the 4.3-kb fragment was observed only to the putative proteinase plasmid pCI203 in strain UC205 (Fig. 1C, lane 1').

Conjugation and pAMβ1-associated mobilization of pCI301 and pCI203. In an attempt to introduce pCI301 and pCI203 into a plasmid-free background, agar surface mating experiments involving strains UC317 and UC205 as donors and MG1363Sm as the recipient were undertaken. Since pCI301 encodes Lac in addition to Prt (13), selection for transfer of either the Lac or Lac Prt phenotypes was attempted. A direct selection for the Prt marker was necessary to detect transfer of pCI203 from strain UC205. However, no transconjugants were isolated by employing either donor in repeated mating experiments.

The potential of pAMβ1 to function as a mobilizing agent in the transfer of pCI301 and pCI203 was investigated. Plasmid pAMβ1 was introduced into UC317 and UC205 from an *L. lactis* subsp. *lactis* MG3020 donor generating approximately 10^{-3} transconjugants per recipient (Table 2). Strains FH068 (Fig. 2A, lane 3) and EC209 (Fig. 3A, lane 3), pAMβ1-containing derivatives of strains UC317 and UC205, respectively, were subsequently used as donors with MG1363Sm as the recipient (Table 2). No selection was made for the mobilizing plasmid. Lac^+ transconjugants from FH068 × MG1363Sm matings were isolated at a frequency of 1.2×10^{-7} transconjugant per recipient. Prt activity cotransferred with the Lac phenotype in approximately 95% of transconjugants tested, suggesting that low-frequency dissociation or rearrangement of the Lac and Prt markers of pCI301 had occurred. After simultaneous selection of the

TABLE 2. Conjugal transfer of Lac, Prt, and Em phenotypes between *L. lactis* subsp. *cremoris* and *L. lactis* subsp. *lactis*

Donor	Recipient	Selected marker(s)	Frequency of marker transfer per recipient[a]
L. lactis subsp. *lactis* MG3020	*L. lactis* subsp. *lactis* UC317	Lac Prt Em	1.4×10^{-3}
L. lactis subsp. *lactis* MG3020	*L. lactis* subsp. *cremoris* UC205	Lac Prt Em	5.0×10^{-3}
L. lactis subsp. *lactis* FH068	*L. lactis* subsp. *lactis* MG1363Sm	Lac Sm	1.2×10^{-7}
		Lac Prt Sm	9.1×10^{-8}
		Em Sm	1.5×10^{-2}
L. lactis subsp. *cremoris* EC209	*L. lactis* subsp. *lactis* MG1363Sm	Prt Sm	5.3×10^{-9}
		Lac Sm	4.1×10^{-8}
		Em Sm	7.7×10^{-3}

[a] Figures represent averages of at least three trials per mating.

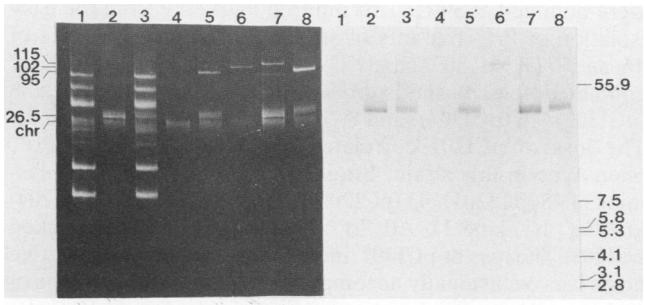

FIG. 2. (A) Plasmid profiles of donors and recipients used and representative transconjugants isolated in mating experiments involving *L. lactis* subsp. *lactis* UC317. Lanes: 1, *L. lactis* subsp. *lactis* UC317; 2, *L. lactis* subsp. *lactis* MG3020; 3, *L. lactis* subsp. *lactis* FH068; 4, *L. lactis* subsp. *lactis* MG1363Sm; 5, *L. lactis* subsp. *lactis* FH082; 6, *L. lactis* subsp. *lactis* FH130; 7, *L. lactis* subsp. *lactis* FH084; 8, *L. lactis* subsp. *lactis* FH138. (B) Color reactions obtained after hybridization of gel in panel A with biotin-11-dUTP-labeled pAMβ1. Numbers on the left refer to sizes (kilobases) of plasmids in FH068-derived transconjugants, whereas numbers on the right refer to sizes of marker plasmids in *E. coli* V517. chr, Chromosomal DNA.

Lac and Prt markers, Lac$^+$ Prt$^+$ transconjugants were obtained at 9.1×10^{-8} transconjugant per recipient. In EC209-derived transconjugants, transfer of the Prt phenotype occurred at a frequency of 5.3×10^{-9} transconjugant per recipient (Table 2).

Even in the absence of selection for pAMβ1-encoded erythromycin resistance, all transconjugants derived from matings involving either FH068 or EC209 as donors expressed this marker. Combined with the failure to detect marker transfer in the absence of pAMβ1, this suggested a role for pAMβ1 in the mobilization of the Prt plasmids pCI301 and pCI203 from FH068 and EC209, respectively.

Transconjugant plasmid analysis. Plasmid complements of transconjugants isolated during mobilization experiments were examined. All transconjugants harbored novel plasmid species that were larger than any of the plasmids present in the corresponding donor strains.

Three types of Lac$^+$ Prt$^+$ Em^r transconjugants from the FH068 × MG1363Sm mating were identified based on the plasmid contents of 60 transconjugants examined (Fig. 2A): (i) 83.3% contained a 102-kb novel plasmid (lane 6); (ii) 15% harbored two plasmids (a 95-kb plasmid and a 26.5-kb plasmid comigrating with pAMβ1 in the FH068 donor) (lane 5); (iii) 1.6% possessed a 115-kb plasmid and a 26.5-kb plasmid (lane 7).

Analysis of eight Lac$^+$ Prt$^-$ Em^r transconjugants revealed two plasmids of 95 and 26.5 kb (Fig. 2A, lane 8).

Lac$^-$ Prt$^-$ Em^r derivatives of transconjugants FH082, FH084, and FH138 were missing the recombinant plasmids pCI306, pCI307, and pCI355, respectively, yet retained the 26.5-kb species. Lac$^-$ Prt$^-$ derivatives of strain FH130 were also Em^s and were plasmid free.

All of 40 Lac$^-$ Prt$^+$ Em^r transconjugants of the EC209 × MG1363Sm mating that were analyzed harbored a single 60-kb plasmid (Fig. 3A, lane 5). There was no evidence of a distinct 26.5-kb plasmid in these transconjugants, which suggested that pCI203 may have formed part of a cointegrate plasmid with pAMβ1 in these strains. This was supported by the result of curing studies, where simultaneous loss of the Prt and Em markers was always observed and correlated with the concomitant loss of the recombinant plasmid pCI210.

These results suggested that pCI301 and pCI203 were capable of cointegrate formation with pAMβ1, leading to the appearance of recombinant plasmids such as pCI344 (Fig. 2A, lane 6), in which the Lac and Prt markers of pCI301 and the pAMβ1-derived Em markers were linked, and pCI210 (Fig. 3A, lane 5), in which the Prt and Em markers of pCI203 and pAMβ1, respectively, had become associated. In addition, pCI301 also formed distinct non-pAMβ1 cointegrates such as pCI306 (Fig. 2A, lane 5), pCI307 (lane 7), and pCI355 (lane 8).

pAMβ1 hybridization analysis of cointegrate plasmids. Hybridization of transconjugant plasmid profiles with a pAMβ1 probe confirmed the presence of pAMβ1 in strains FH082, FH084, and FH138 (Fig. 2B), whereas Lac$^+$ Prt$^+$ Em^s derivatives of FH082 and FH084 and Lac$^+$ Prt$^-$ Em^s derivatives of FH138, all of which were lacking the 26.5-kb band, displayed no hybridization to the probe (data not shown). In FH130, hybridization of pAMβ1 was observed only to the 102-kb Lac Prt Em plasmid pCI344 (Fig. 2B, lane 6′). Evidence for the presence of pAMβ1 as part of the novel plasmids in EC209-derived Lac$^-$ Prt$^+$ Em^r transconjugants was provided by the results of hybridization experiments in which pAMβ1 hybridized to pCI210 in EC210 (Fig. 3B, lane 5′), thus confirming the formation of pCI203::pAMβ1 cointegrates during matings involving strain EC209 as donor.

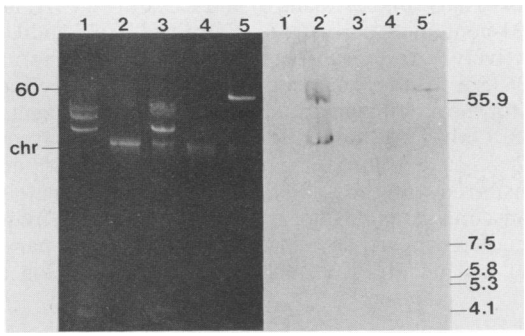

FIG. 3. (A) Plasmid profiles of donors and recipients used and representative transconjugants isolated in mating experiments involving *L. lactis* subsp. *cremoris* UC205. Lanes: 1, *L. lactis* subsp. *cremoris* UC205; 2, *L. lactis* subsp. *lactis* MG3020; 3, *L. lactis* subsp. *cremoris* EC209; 4, *L. lactis* subsp. *lactis* MG1363Sm; 5, *L. lactis* subsp. *lactis* EC210. (B) Color reaction obtained after hybridization of gel in panel A with biotin-11-dUTP-labeled pAMβ1. The number on the left refers to the size (kilobases) of the plasmid in *L. lactis* subsp. *lactis* EC210, whereas the numbers on the right refer to the sizes of marker plasmids in *E. coli* V517. chr, Chromosomal DNA. To facilitate resolution of plasmid bands in strains EC205 and EC209, DNA equivalent to one-quarter of that loaded in other lanes was applied to lanes 1 and 3, resulting in only a faint signal to pAMβ1 in lane 3′.

Restriction enzyme and hybridization analyses of Prt plasmids pCI310 and pCI210. pCI310 is a substantially deleted, spontaneous derivative of the structurally unstable pCI306 (Fig. 2A, lane 5) and confers a Lac$^-$ Prt$^+$ phenotype. Restriction endonuclease maps of this plasmid and of the structurally stable pCI210 plasmid (Fig. 3, lane 5) were constructed (Fig. 4). Localization of *prt* on pCI310 and pCI210 was achieved by a combination of restriction enzyme analysis and by using the 4.3-kb *prt* probe (Fig. 4). A remarkably high degree of restriction site homology was evident in the proximity of *prt*, in agreement with the observations of de Vos (5) and Kok and Venema (18).

Restriction enzyme and hybridization analyses of pCI210

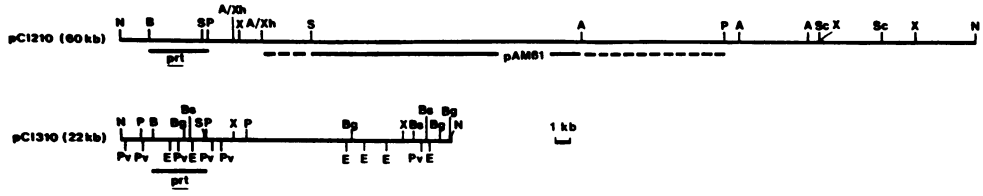

FIG. 4. Restriction endonuclease maps of Prt plasmids pCI210 and pCI310 linearized at unique *Nru*I sites. The regions of homology with the 4.3-kb *prt* probe are indicated. The approximate position of pAMβ1 within pCI210 is shown. A, *Ava*I; B, *Bam*HI; Bg, *Bgl*II; Bs, *Bst*EII; E, *Eco*RI; N, *Nru*I; P, *Pst*I; Pv, *Pvu*II; S, *Sal*I; Sc, *Sac*I; X, *Xba*I; Xh, *Xho*I.

with pAMβ1 revealed that the site of cointegration on pAMβ1 with pCI203 in the formation of pCI210 was within overlapping 5.0-kb *Eco*RI, 8.5-kb *Pvu*II, and 1.45-kb *Hin*dIII fragments of pAMβ1 (data not shown). The site of recombination is therefore to the right of the pAMβ1 replication region (*rep*) and between *rep* and the region implicated in pAMβ1 conjugal transfer, as the restriction map of pAMβ1 is conventionally drawn (20). This allowed an approximate localization of pAMβ1 within pCI210 (Fig. 4).

DISCUSSION

In this study, the ability of pAMβ1 to mobilize lactococcal plasmid DNA was assessed by using Prt plasmids pCI301 and pCI203 in a model system. Positive identification of these Prt plasmids in the β-casein-degrading strains *L. lactis* subsp. *lactis* UC317 and *L. lactis* subsp. *cremoris* UC205, used in Cheddar cheese manufacture in Ireland, was achieved through a combination of plasmid curing and DNA-DNA hybridization data. The usefulness of hybridization techniques in assigning phenotypes to specific plasmids in multi-plasmid-containing lactococcal strains was demonstrated by using plasmid pGD4 (17) as a proteinase probe (Fig. 1). However, the importance of probe specificity in obtaining unambiguous results was highlighted and, in the case of strain UC317, necessitated the use of a more specific probe which contained no non-proteinase-encoding DNA. Such probes have also been employed in the identification of Prt plasmids in other commercial *L. lactis* subsp. *lactis* and *cremoris* strains (18; unpublished data) and were further used in this study in the localization of proteinase-encoding regions on pCI301 and pCI203 derivatives. Similarly, the use of a phospho-β-D-galactosidase probe in the identification of Lac plasmids has been reported (41), whereas a synthetic 19-base-pair oligonucleotide specific for the phage insensitivity plasmid pTR2030 has been employed in monitoring the conjugal transfer of this plasmid to wild-type lactococcal strains (35).

The difficulty in introducing Prt plasmids into plasmid-free derivatives has been ascribed either to their non-self-transmissible nature or to inadequate selection procedures (26). Here, the use of a milk-based agar differential medium, FSDA-glucose (14), in the detection of transfer of Prt plasmid pCI203 was used to overcome the latter problem. However, in our experience, when Prt is linked to a more readily selectable marker such as Lac, as is the case with pCI301, the latter is preferable for initial selection.

Although conjugation is the method of gene transfer most investigated in the lactococci, plasmids of interest can frequently be nonconjugative, as proved for Prt plasmids pCI301 and pCI203. The need for a widely applicable mobilization system within the lactococci becomes apparent. To this end, the potential of pAMβ1 to mobilize pCI301 and pCI203 was examined. pAMβ1 was chosen because of its highly conjugative nature to and within the lactococci (10) and because it has previously been successfully used in the mobilization of lactococcal plasmid DNA (2, 6). In this study, pAMβ1 was readily introduced into strains UC317 and UC205 and with equal ease into any other *L. lactis* subsp. *lactis* and *cremoris* strain used in this laboratory, results which are consistent with those of Gasson and Davies (10). Mobilization of pCI301 and pCI203 from pAMβ1-containing donor strains was subsequently achieved at frequencies approximately 10^5 to 10^6 lower than transfer of pAMβ1 itself (Table 2). Although this may indicate that a basal, high level of pAMβ1 transfer is required for effective plasmid mobilization, the efficiency of mobilization is also likely to be a function of the frequency of cointegrate formation between the two plasmids. No selection was necessary for the mobilizing plasmid, which is also easily curable at elevated temperatures from transconjugants in which it does not form part of a recombinant plasmid. The high frequency of pAMβ1 conjugal transfer, coupled with an ability to mobilize even large plasmids, indicates that this strategy may be suitable for mobilization and the subsequent analysis of both nonhomologous selectable and nonselectable plasmids and cloning vectors. Indeed, comobilization of nonconjugative cryptic plasmids in addition to Prt plasmids was observed in the course of this study (data not shown). Therefore, although the nature of pAMβ1-associated plasmid mobilization is unclear, this plasmid may prove well suited to the task of acting as a general plasmid-mobilizing agent within the lactococci.

The appearance of both pAMβ1 and non-pAMβ1 cointegrate plasmids after mobilization of pCI301 and pCI203 further underlines the frequency of in vivo plasmid recombination during conjugation in the lactococci (7). Speculation that such events reflect the presence of transposable elements within the lactococci (2, 12) has been substantiated by the identification of insertion sequence elements on pSK08, the Lac plasmid of strain ML3 (33), and on pUCL22, the Lac Prt plasmid of strain Z270 (31); both of these plasmids are involved in cointegrate formation during conjugation. The possibility that the formation of recombinant plasmids during pCI301 and pCI203 mobilization is similarly mediated by transposable elements is supported by the observation that pCI301 can integrate into and excise from the *L. lactis* subsp. *lactis* chromosome (unpublished data). The elucidation of the origin of the extra DNA(s) involved in the generation of those pCI301 cointegrates of which pAMβ1 is not part (Fig. 2A, lanes 5, 7, and 8) and the further characterization of both these and pCI301::pAMβ1 cointegrates (Fig. 2A, lane 6) will reveal much with regard to the intermolecular recombination events occurring during pCI301 mobilization from strain UC317.

The region of pAMβ1 involved in the formation of the

pCI203::pAMβ1 chimera pCI210 (Fig. 3A, lane 5) is the same as the target site for transposition of Tn*4430* from *B. thuringiensis* native plasmids to pAMβ1 during conjugation (21, 22) and to that involved in the generation of a pAMβ1 deletion derivative isolated after conjugal transfer of pAMβ1 between *L. lactis* subsp. *lactis* and *Lactobacillus casei* (15). It differs, however, from regions of pAMβ1 implicated in the formation of other pAMβ1 deletion derivatives (20, 23, 40). Characterization of a greater number of pCI210-like plasmids will provide further information on the nature of the pCI203::pAMβ1 recombination event. Significantly, the formation of in vivo pCI203::pAMβ1 fusion plasmids, which retain the conjugative transfer functions of the pAMβ1 moiety, has allowed intrageneric and intergeneric conjugal transfer of *L. lactis* subsp. *cremoris* UC205 *prt* genes (unpublished data) and will create a basis for analyzing lactococcal gene expression in nonlactococcal and/or nontransformable hosts.

The localization of *prt* on pCI301 and pCI203 derivatives was readily achieved by restriction enzyme and hybridization analyses (Fig. 4). Comparison of *prt* on pCI310 and pCI210 with cloned *prt* genes (5, 18) indicated extensive conservation of restriction enzyme sites over a region at least 7.5 kb in size, further emphasizing the relatedness of proteinase genes isolated from lactococcal cultures of diverse origins. Preliminary cloning experiments, however, indicated that an 8.0-kb *Xba*I-*Nru*I fragment of pCI310 that completely spanned the region of probe homology and that extended at least 2.0 kb on either side of the homologous region (Fig. 4) did not confer proteolytic activity on *L. lactis* subsp. *lactis* MG1363Sm (unpublished data).

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