Thermosensitive Plasmid Replication, Temperature-Sensitive Host Growth, and Chromosomal Plasmid Integration Conferred by *Lactococcus lactis* subsp. *cremoris* Lactose Plasmids in *Lactococcus lactis* subsp. *lactis*†

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Evidence is presented that lactose-fermenting ability (Lac$^+$) in *Lactococcus lactis* subsp. *cremoris* AM1, SK11, and ML1 is associated with plasmid DNA, even though these strains are difficult to cure of Lac plasmids. When the Lac plasmids from these strains were introduced into *L. lactis* subsp. *lactis* LM0230, they appeared to replicate in a thermosensitive manner; inheritance of the plasmid was less efficient at 32 to 40°C than at 22°C. The stability of the *L. lactis* subsp. *cremoris* Lac plasmids in lactococci appeared to be a combination of both host and plasmid functions. Stabilized variants were isolated by growing the cultures at 32 to 40°C; these variants contained the Lac plasmids integrated into the *L. lactis* subsp. *lactis* LM0230 chromosome. In addition, the presence of the *L. lactis* subsp. *cremoris* Lac plasmids in *L. lactis* subsp. *lactis* resulted in a temperature-sensitive growth response; growth of *L. lactis* subsp. *lactis* transformants was significantly inhibited at 38 to 40°C, thereby resembling some *L. lactis* subsp. *cremoris* strains with respect to temperature sensitivity of growth.

In many *Lactococcus lactis* subsp. *lactis* and citrate-fermenting *L. lactis* subsp. *lactis* strains, the instability of lactose utilization is due to loss of plasmid DNA (13, 21, 24, 32, 34). However, several investigators, using a variety of techniques, failed to establish a link between plasmids and the ability of most strains of *L. lactis* subsp. *cremoris* to utilize lactose (Lac$^+$) (10, 25). Therefore, these investigators suggested that the stability of Lac was either due to a Lac plasmid resistant to plasmid-curing techniques or due to chromosomal linkage of the *lac* genes. However, other reports indicated that Lac in some *L. lactis* subsp. *cremoris* strains is not always stable (1, 19, 36, 39). Furthermore, Snook and McKay (40) and Okamoto et al. (35) were able to transfer plasmids that encode Lac from some *L. lactis* subsp. *cremoris* strains to *L. lactis* subsp. *lactis* by using conjugal matings and protoplast fusions, respectively. Okamoto et al. (35) therefore suggest that there may be two types of *L. lactis* subsp. *cremoris* strains with respect to the genetic stability of lactose utilization.

In this study we provide physical and genetic evidence that lactose-fermenting ability in three strains of *L. lactis* subsp. *cremoris* (AM1, SK11, and ML1) is associated with plasmid DNA. The Lac plasmids from these strains exhibited thermosensitive replication when present in *L. lactis* subsp. *lactis*. These *L. lactis* subsp. *lactis* strains yielded variants that were stable for the Lac phenotype via integration of the Lac plasmid into the chromosome. In addition, the *L. lactis* subsp. *cremoris* Lac plasmids in *L. lactis* subsp. *lactis* caused a temperature-sensitive growth (Tsg) response;

growth of Lac$^+$ *L. lactis* subsp. *lactis* strains was significantly inhibited at 38 to 40°C. These unique properties conferred by the Lac plasmids of *L. lactis* subsp. *cremoris* could greatly enhance the study of plasmid and chromosomal biology of lactococci.

MATERIALS AND METHODS

Bacterial strains. All lactococcal strains used in this study (Table 1) were maintained by biweekly transfers at 22 or 30°C for 16 h in M17 broth (43) containing either 0.5% glucose or lactose. Lac$^-$ variants of Lac$^+$ transformants were isolated by single passage of the transformant in M17-glucose broth at 30°C, followed by plating on bromocresol purple (BCP)-lactose indicator agar (33). In other studies, the medium described by Elliker et al. (14) was used.

DNA isolation, restriction endonuclease digestions, and agarose gel electrophoresis. Plasmid DNA was isolated by the method of Anderson and McKay (2). Plasmids used for transformation and restriction endonuclease analysis were further purified by cesium chloride-ethidium bromide density gradient centrifugation (22). DNA was desalted by five washes with TE (10 mM Tris, 1 mM EDTA [pH 8.0]) in Centricon microconcentrators (Amicon Corp., Danvers, Mass.) as described by the manufacturer. Genomic DNA was isolated by the method of Pitcher et al. (38). It was further extracted with TE-saturated phenol-chloroform-isoamyl alcohol (25:24:1) followed by chloroform-isoamyl alcohol (24:1) before being precipitated. Gel electrophoresis was performed as described by Maniatis et al. (30) through 0.6% agarose gels in 40 mM Tris acetate–2 mM EDTA buffer (pH 8.1), except as noted for Southern hybridizations.

Transformation and electroporation protocols. Protoplast transformation was performed as described by Kondo and

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TABLE 1. Descriptions of strains and plasmids

Strain or plasmid	Relevant phenotype[a]	Phenotype stability[b]	Transformant derivation		Plasmid content (kbp)	Reference or source
			Recipient strain	Plasmid source		
Parental strain						
L. lactis subsp. cremoris AM1	Lac$^+$ Tsg$^+$	+			72, 48, 27, 12, 9.6	9
L. lactis subsp. cremoris SK11	Lac$^+$ Tsg$^+$	+			72, 48, 15, 9.6	9
L. lactis subsp. cremoris ML1	Lac$^+$ Tsg$^+$	+			59, 44, 27, 14, 6, 3.3, 2.7	25
Recipient strain						
L. lactis subsp. lactis LM0230	Lac$^-$ Tsg$^-$ Rec$^+$ Em^s					Plasmid-cured L. lactis subsp. lactis C2 (13)
L. lactis subsp. lactis MG1363	Lac$^-$ Tsg$^-$ Rec$^+$					Plasmid-cured L. lactis subsp. lactis 712 (18)
L. lactis subsp. lactis MMS368	Lac$^-$ Tsg$^-$ Rec$^-$					Plasmid-cured L. lactis subsp. lactis MMS367 (41)
L. lactis subsp. lactis LM0220	Lac$^-$ Tsg$^-$ Rec$^+$				27, 18, 8.3, 3.0, 1.5	Lac$^-$ variant of L. lactis subsp. lactis C2 (13)
L. lactis subsp. lactis KR5-1a	Lac$^-$ Tsg$^+$ Rec$^+$				60, 35, 32, 25, 16, 12, 2.3	Lac$^-$ variant of L. lactis subsp. lactis KR5 (15)
L. lactis subsp. cremoris EB5	Lac$^-$ Tsg$^+$ Rec$^+$					Plasmid-cured L. lactis subsp. cremoris KR1 (2a)
Transformants and variants						
L. lactis subsp. lactis JF2128	Lac$^+$ Tsg$^+$	–	LM0230	AM1	48	This study
L. lactis subsp. lactis JF2129	Lac$^-$ Tsg$^-$					Lac$^-$ variant of JF2128
L. lactis subsp. lactis JF3216	Lac$^+$ Tsg$^+$	–	LM0230	SK11	48	This study
L. lactis subsp. lactis JF3217	Lac$^-$ Tsg$^-$					Lac$^-$ variant of JF3216
L. lactis subsp. lactis JF3206	Lac$^+$ Tsg$^+$	+				Stable variant of JF3216
L. lactis subsp. lactis JF3101	Lac$^+$ Tsg$^+$	–	LM0230	ML1	59, 14	This study
L. lactis subsp. lactis JF3100	Lac$^+$ Tsg$^+$	+				Stable variant of JF3101
L. lactis subsp. lactis JF3436	Lac$^+$ Tsg$^+$	–	LM0220	SK11	48, 27, 18, 8.3, 3.3	This study
L. lactis subsp. lactis JF3512	Lac$^+$ Tsg$^+$	–	MG1363	SK11	48	This study
L. lactis subsp. lactis JF3630	Lac$^+$ Tsg$^+$ Rec$^-$	–	MMS368	SK11	48	This study
L. lactis subsp. lactis KMP1	Lac$^-$ Tsg$^-$ Em^r	–	LM0230	pKMP1	21	20a
L. lactis subsp. lactis KMP1-S3	Lac$^-$ Tsg$^-$ Em^r	+				Stable variant of KMP1 (20a)
Plasmids						
pGB301	Em^r				9.8	3
pAMβ1	Em^r				26	5
pAM1	Lac$^+$ Tsg$^+$	±^{c}			48	This study
pSK11	Lac$^+$ Tsg$^+$	±			48	This study
pML1	Lac$^+$ Tsg$^+$	±			59	This study
pKMP1	Lac$^-$ Tsg$^-$ Em^r	±			21	pSK11 subclone (see text) (20a)

[a] Em, erythromycin; Lac, lactose utilization; Rec, homologous recombination; Tsg, temperature-sensitive growth. For each strain, only the phenotypes germane to the use of that strain are listed.

[b] Phenotype stability is applicable only to the phenotypes associated with L. lactis subsp. cremoris plasmids (Lac, Tsg) or derivatives (Em for pKMP1).

[c] When in L. lactis subsp. lactis LM0230, the plasmid is stable when integrated and unstable when autonomous.

McKay (23) with modifications by Froseth et al. (16). DNA was added in the presence of polyethylene glycol 6000 (Fluka Chemical Corp., Ronkonkoma, N.Y.). Transformants were selected by plating with erythromycin at 2.5 to 5.0 µg/ml and/or by their ability to ferment lactose on the medium described by Kondo and McKay (23).

Additional transformants were obtained by using electroporation as described by McIntyre and Harlander (31), with the following modifications. Cultures were incubated for 16 h at 30°C in 35 ml of RPMI 1640 broth (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 0.24% DL-threonine and 1% glucose, and the cells were harvested, washed twice

with ice-cold deionized distilled water, and suspended in 100 μl of ice-cold water. Purified plasmid DNA was used to transform 50 μl of chilled cell suspension by using a Gene Pulser (Bio-Rad, Richmond, Calif.) (31). Following electroporation, cells were held on ice for 10 min, diluted with 350 μl of M17-glucose broth, and incubated at 30°C for 2 h before being plated on the appropriate medium.

Southern transfer and nonradioactive labeling. The Southern transfer technique as modified by Davis et al. (8) was used. Fragments to be labeled were isolated from agarose gels by using the GENECLEAN kit (Bio 101, La Jolla, Calif.). The Genius DNA labeling and detection kit (Boehringer Mannheim Corp., Indianapolis, Ind.) was used to generate nonradioactive probes. Hybridizations (high stringency) and immunological detection were carried out as described by the manufacturer.

Occurrence of Lac⁻ cells within Lac⁺ colonies. To obtain evidence for the instability of lactose-fermenting ability, Lac⁺ transformants were grown in M17-lactose broth at 30°C for 16 h. The broth culture was diluted and plated on BCP-lactose agar. Plates were incubated at 21, 32, 37, or 40°C for 48 h, after which 4 to 7 Lac⁺ colonies were picked into individual tubes containing 1.0 ml of 0.85% NaCl. The colony suspensions were mixed thoroughly and examined for the presence of Lac⁻ cells by diluting and plating on BCP-lactose agar at 25°C.

Growth experiments. For these studies, growth of the cells at 30, 38, or 40°C was determined by measuring the change in A_{600} or by growing the cultures at the indicated temperatures, serially diluting at hourly intervals, and plating on BCP-lactose agar. Following incubation at 32°C for 48 h, Lac⁺ and Lac⁻ colonies were counted.

RESULTS

Identification of Lac plasmids in _L. lactis_ subsp. _cremoris_ AM1, SK11, and ML1. In initial experiments to isolate the proteinase-encoding plasmid from _L. lactis_ subsp. _cremoris_ AM1, cotransformation of plasmid-free _L. lactis_ subsp. _lactis_ LM0230 with the plasmid pool from AM1 and pGB301 resulted in isolation of Em^r transformants. Although no proteinase-positive transformants were isolated, an Em^r Lac⁺ transformant was obtained. When cured of the Em^r pGB301, this strain (JF2128) retained the Lac⁺ phenotype and possessed a single 48-kbp plasmid (pAM1) (Fig. 1A). To confirm that this plasmid conferred lactose-fermenting ability, we isolated Lac⁻ derivatives following serial transfer in M17-glucose broth at 30°C. The Lac⁻ variants were missing pAM1 (Fig. 1A). Furthermore, when plasmid DNA from JF2128 was used to transform LM0230, Lac⁺ transformants containing the 48-kbp plasmid were obtained (data not shown). These results indicated that the 48-kbp plasmid from AM1 was linked to lactose utilization.

Electroporation of LM0230 with pAMβ1 (Em^r encoding) and the plasmid pool from _L. lactis_ subsp. _cremoris_ SK11 resulted in the isolation of Lac⁻ Em^r and Lac⁺ Em^s transformants. Lac⁺ transformant JF3216 contained a 48-kbp plasmid (pSK11) which appeared identical to pAM1 when compared by digesting the two plasmids with _Hin_dIII, _Cla_I, _Pst_I, _Eco_RI, _Eco_RV, or _Ava_I (data not shown). Even though lactose metabolism in _L. lactis_ subsp. _cremoris_ SK11 has been considered a stable trait (10), the results obtained indicate that lactose-fermenting ability in SK11, as in AM1, was mediated by the 48-kbp plasmid.

When the plasmid pool from ML1 was used to transform LM0230 via electroporation, a Lac⁺ transformant, JF3101,
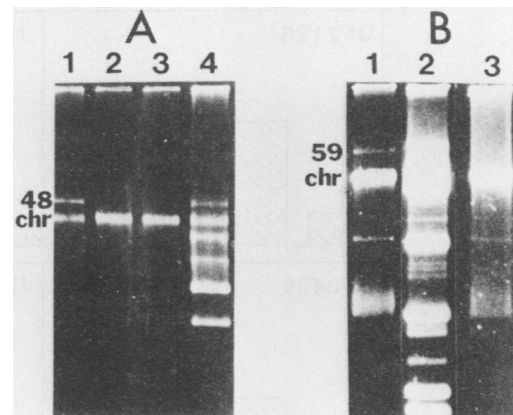

FIG. 1. Agarose gel electrophoresis of plasmids isolated from Lac⁺ transformants of _L. lactis_ subsp. _lactis_ LM0230 containing plasmid DNA from _L. lactis_ subsp. _cremoris_ AM1 (A) or _L. lactis_ subsp. _cremoris_ ML1 (B). In panel A, the lanes are as follows: 1, Lac⁺ transformant _L. lactis_ subsp. _lactis_ JF2128; 2, Lac⁻ derivative of JF2128; 3, plasmid-free _L. lactis_ subsp. _lactis_ LM0230 used as a recipient in transformation trials; 4, plasmid pool of _L. lactis_ subsp. _cremoris_ AM1. In panel B, the lanes are as follows: 1, Lac⁺ transformant _L. lactis_ subsp. _lactis_ JF3101; 2, plasmid pool of _L. lactis_ subsp. _cremoris_ ML1; 3, Lac⁻ derivative of JF3101. The positions of the 48- and 59-kbp Lac plasmids from AM1 and ML1, respectively, are indicated in the margin. chr refers to chromosomal DNA.

was obtained that contained 59- and 14-kbp plasmids (Fig. 1B). The transformant became Lac⁻ upon loss of the 59-kbp plasmid (pML1), suggesting its linkage to Lac.

Stability of the _L. lactis_ subsp. _cremoris_ Lac plasmids in _L. lactis_ subsp. _lactis_ LM0230. When the Lac⁺ transformants JF2128, JF3216, and JF3101 were grown in M17-glucose broth at 30 or 38°C for 10 to 16 h, 16 to 67% of the total population was Lac⁻ when plated on BCP-lactose agar, even after inoculation with a single Lac⁺ colony (data not shown). No Lac⁻ derivatives were detected from _L. lactis_ subsp. _cremoris_ AM1, SK11, or ML1 grown under similar conditions (data not shown). To obtain further evidence for instability of _L. lactis_ subsp. _cremoris_ Lac plasmids in _L. lactis_ subsp. _lactis_ LM0230, we grew Lac⁺ transformants at various temperatures on BCP-lactose agar and examined the colonies for the presence of Lac⁻ cells. The percentage of Lac⁻ cells within a Lac⁺ colony of LM0230 containing pAM1 (JF2128), pSK11 (JF3216), or pML1 (JF3101) generally increased as the temperature of incubation increased from 21 to 40°C (Fig. 2).

Stability of _L. lactis_ subsp. _cremoris_ Lac plasmids in other lactococci. pSK11 and pML1 were transformed into Lac⁻ _L. lactis_ subsp. _lactis_ LM0220, KR5-1a, and MG1363 and Lac⁻ _L. lactis_ subsp. _cremoris_ EB5 to determine whether the temperature-dependent instability of Lac plasmids observed in LM0230 occurred in other hosts. Agarose gel electrophoresis of plasmid DNA isolated from the various Lac⁺ transformants revealed the presence of pSK11 or pML1 (data not shown). Lactose utilization appeared to be stable in _L. lactis_ subsp. _cremoris_ EB5 transformed by pSK11, as no Lac⁻ cells were detected in colonies grown at 21, 32, 37, or 40°C (data not shown). The same result was observed when pSK11 or pML1 was transformed into _L. lactis_ subsp. _lactis_ KR5-1a. Lac⁺ transformants of LM0220 containing pSK11 were unstable for lactose utilization even at 21°C, and the
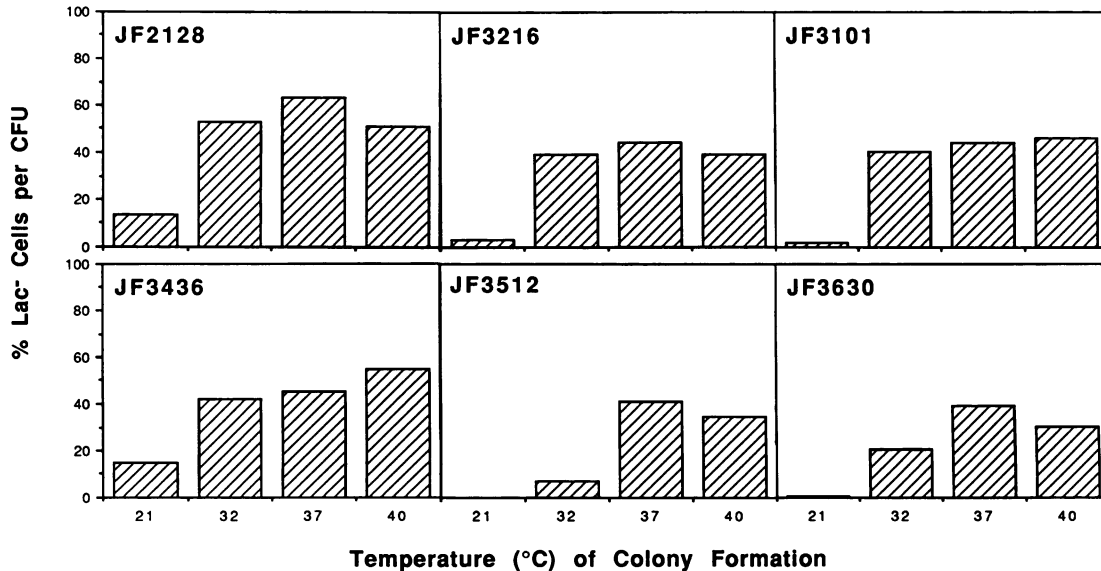
FIG. 2. Percentage of Lac⁻ cells per CFU of *L. lactis* subsp. *lactis* Lac⁺ transformants JF2128, JF3216, and JF3101 containing the Lac plasmids from *L. lactis* subsp. *cremoris* AM1, SK11, or ML1, respectively, during colony formation at 21, 32, 37, and 40°C. JF3436, JF3512, and JF3630 are Lac⁺ transformants of *L. lactis* subsp. *lactis* LM0220, MG1363, and MMS368, respectively, containing the Lac plasmid from *L. lactis* subsp. *cremoris* SK11 and were examined similarly.

percent Lac⁻ cells within a Lac⁺ colony increased with increasing incubation temperature (Fig. 2, JF3436). When pSK11 was transformed into MG1363 (JF3512), the resultant Lac⁺ phenotype was also unstable at the higher temperatures (Fig. 2). In contrast, when pML1 was present in MG1363, lactose utilization was stable at 21 and 40°C (data not shown).

Association of *L. lactis* subsp. *cremoris* Lac plasmids with temperature-dependent inhibition of growth of *L. lactis* subsp. *lactis* LM0230. Lac⁺ *L. lactis* subsp. *lactis* LM0230 transformants containing the Lac plasmids from *L. lactis* subsp. *cremoris* were observed to grow more slowly than LM0230 did. At 30°C, LM0230 exhibited the growth response (as monitored by optical density) shown in Fig. 3A; however, upon acquiring pAM1, the LM0230 transformant, JF2128, exhibited a longer lag phase. A growth response similar to that of LM0230 was observed upon loss of the Lac plasmid (Fig. 3A, JF2129). At 40°C, this inhibition of host growth by pAM1 was more evident, but again, when the Lac plasmid was cured, growth at 40°C was similar to that of the plasmid-free host (Fig. 3B).

When JF3216 and JF3101 (containing pSK11 and pML1, respectively) were examined by enumerating viable cells, similar results were obtained. When JF3216, LM0230, and JF3217 (a Lac⁻ derivative of JF3216) were grown at 30°C in M17-glucose broth, all three strains possessed similar growth responses (Fig. 3C). However, at 38°C no increase in viable-cell numbers was observed over an 11-h period for JF3216 (Fig. 3D), while JF3217 and LM0230 grew.

When present in LM0230, pML1 also inhibited host growth at 40°C (Fig. 3F) but not at 30°C (Fig. 3E). It therefore appeared that the presence of the Lac plasmid from *L. lactis* subsp. *cremoris*, AM1, SK11, or ML1 in *L. lactis* subsp. *lactis* LM0230 exerted a temperature-sensitive growth (Tsg) response on the host cells.

The Tsg phenomenon was also manifested by the number of cells per colony. When LM0230 carried pSK11 (JF3216;

Fig. 4A), at higher temperatures, there were both fewer Lac⁺ and fewer total cells. Similar observations were made when LM0230 carried pML1 (JF3101; Fig. 4C).

Morphologies and plasmid content of colonies at different temperatures. When broth cultures of *L. lactis* subsp. *lactis* carrying a Lac plasmid from *L. lactis* subsp. *cremoris* were plated on BCP-lactose agar, different colony morphologies were observed depending on the incubation temperature. For example, if a 22°C broth culture of *L. lactis* subsp. *lactis* JF3216 was plated at 22, 32, and 37°C, four colony morphologies were observed (Table 2).

When irregular colonies were picked, grown in Elliker-lactose broth at 32°C, and examined for plasmid content, they contained a 48-kbp plasmid, as did smooth Lac⁺ colonies from plates incubated at 22°C (data not shown). However, the infrequent smooth Lac⁺ colonies from plates at 32 or 37°C yielded no detectable plasmid DNA, which suggested that the Lac plasmid may have integrated into the chromosome (see below). Some fried-egg colonies yielded plasmid DNA, whereas others did not.

When irregular colonies were streaked for isolation on BCP-lactose agar and incubated at 37°C, fried-egg, Lac⁻, and (rarely) smooth Lac⁺ colonies were observed. When fried-egg colonies originally derived from 37°C platings of the 22°C broth culture were streaked and incubated at 37°C, fried-egg, smooth Lac⁺, and Lac⁻ colonies were observed. The proportions of these three colony types varied from one streak plate to another.

Colonies that formed from cells bearing the Lac plasmid in the autonomous state were subject to at least three temperature-dependent phenomena which determined their final morphologies. These included (i) less efficient inheritance of the Lac plasmid as the temperature increased from 22 to 37°C; (ii) increased frequency of apparent chromosomal integration of the Lac plasmid at higher temperatures; and (iii) increased inhibition of growth of Lac plasmid-bearing cells as the temperature increased. As a consequence of
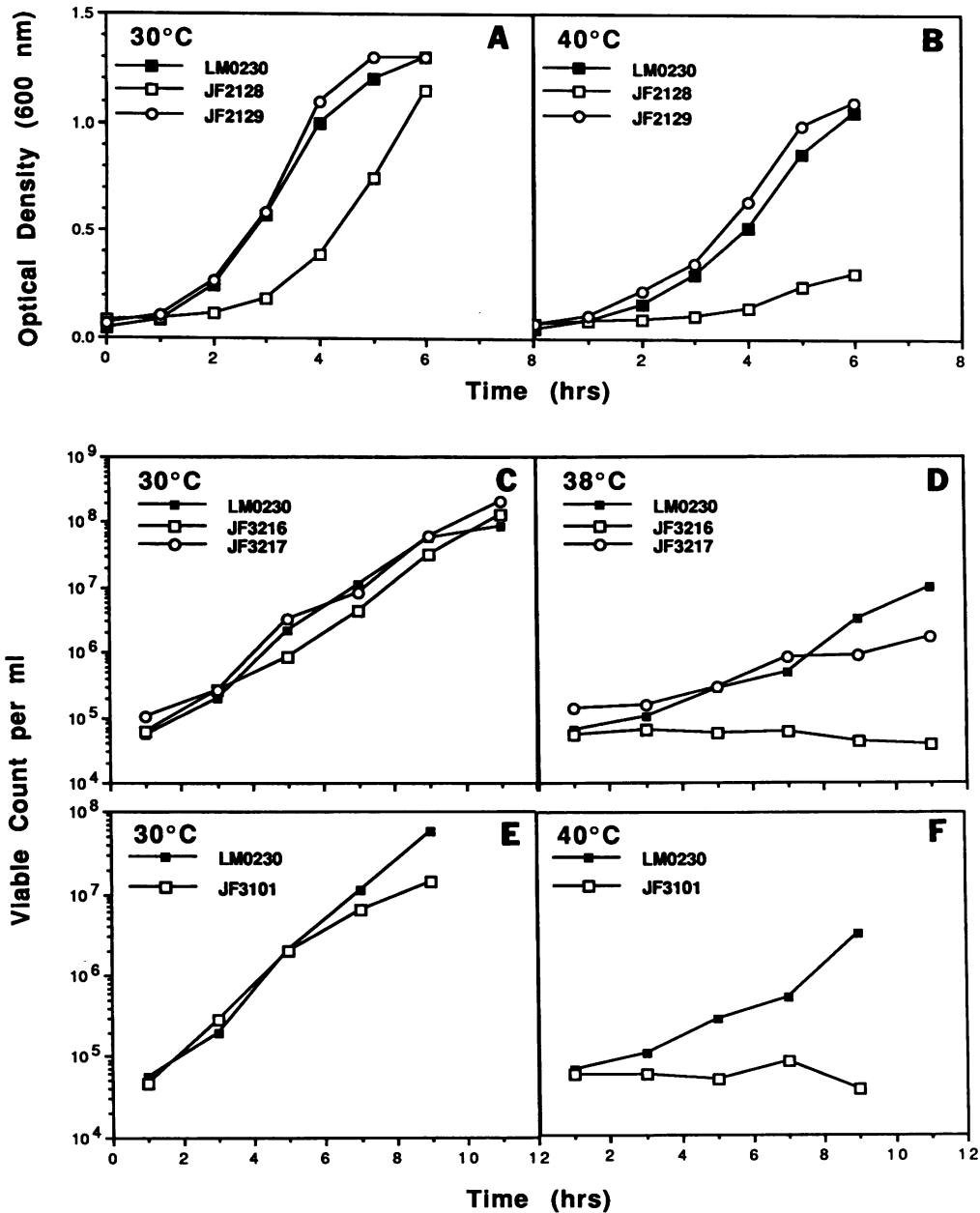
FIG. 3. Effect of temperature on the growth of Lac$^+$ *L. lactis* subsp. *lactis* LM0230 transformants containing the Lac plasmids from *L. lactis* subsp. *cremoris*. (A and B) Growth patterns (optical densities) at 30°C (panel A) and 40°C (panel B) of plasmid-free *L. lactis* subsp. *lactis* LM0230; JF2128, a Lac$^+$ transformant of LM0230 containing the Lac plasmid from *L. lactis* subsp. *cremoris* AM1; and JF2129, a Lac$^-$ derivative of JF2128. (C and D) Growth patterns (viable counts) at 30°C (panel C) and 38°C (panel D) of *L. lactis* subsp. *lactis* LM0230; JF3216, a Lac$^+$ transformant of LM0230 carrying the Lac plasmid from *L. lactis* subsp. *cremoris* SK11; and JF3217, a Lac$^-$ derivative of JF3216. (E and F) Growth patterns (viable counts) at 30°C (panel E) and 40°C (panel F) of *L. lactis* subsp. *lactis* LM0230 and JF3101, a Lac$^+$ transformant of LM0230 carrying the Lac plasmid from *L. lactis* subsp. *cremoris* ML1.

these temperature effects, fried-egg colonies on 37°C plates contained larger areas of Lac$^-$ growth than did irregular colonies on 32°C plates. Likewise, when fried-egg colonies were streaked for isolation and incubated at 37°C, more smooth Lac$^+$ colonies (putative integrants) appeared as compared with streak plates at 37°C of irregular colonies originating from 32°C plates.

Because all three temperature-dependent effects were greatly diminished at 22°C, smooth Lac$^+$ colonies from 22°C

plates that appeared identical to smooth Lac$^+$ colonies at 32 or 37°C differed with respect to the state of the Lac plasmid. The majority of smooth Lac$^+$ colonies at 22°C contained the plasmid in the autonomous state, whereas smooth Lac$^+$ colonies at the higher temperatures did not contain detectable plasmid DNA (data not shown).

Lac$^+$ variants of LM0230 without detectable autonomous plasmid DNA were examined for Lac$^+$ stability by screening colonies formed at various temperatures. JF3206 (Fig. 4B)
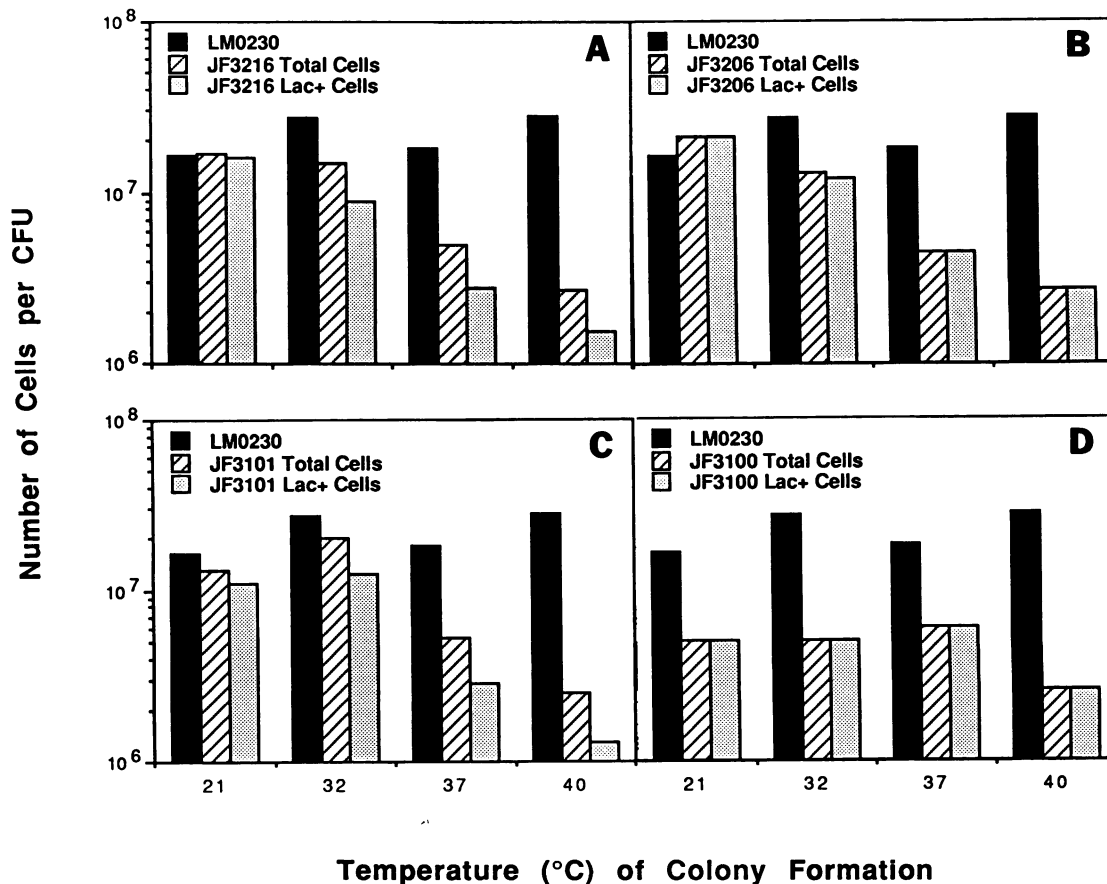
FIG. 4. Effect of temperature on total cells and Lac+ cells per CFU during colony formation at 21, 32, 37, and 40°C of *L. lactis* subsp. *lactis* LM0230 Lac+ transformants containing *L. lactis* subsp. *cremoris* Lac plasmids. (A and B) *L. lactis* subsp. *cremoris* SK11 Lac plasmid in the autonomous (panel A) and integrated (panel B) states. (C and D) *L. lactis* subsp. *cremoris* ML1 Lac plasmid in the autonomous (panel C) and integrated (panel D) states.

exhibited 2.4, 1.2, 0.9, and 0.6% Lac⁻ cells at 21, 32, 37, and 40°C, respectively, whereas at the same temperatures, JF3216 (containing autonomous pSK11 [Fig. 4A]) possessed 3, 40, 44, and 40% Lac⁻ cells, respectively. No Lac⁻ cells were detected during colony formation at any of the temperatures with JF3100 (Fig. 4D), yet JF3101 (containing autonomous pML1 [Fig. 4C]) possessed 1.7, 41, 45, and 47% Lac⁻ cells at 21, 32, 37, and 40°C, respectively. The increased stability of Lac in JF3206 and JF3100 in contrast to JF3216 and JF3101 supports the concept that the Lac plasmids had integrated into the chromosome.

Examination of putative integrants by Southern hybridization analysis. To determine whether chromosomal integration was responsible for the stabilization of Lac in strains devoid of detectable autonomous plasmid, Southern hybridizations of genomic DNA were performed with *L. lactis* subsp. *lactis* LM0230 transformed with pSK11 and derivative plasmids. pSK11 in its entirety was unsuitable as a probe because of its large size and because of extensive hybridization between the plasmid-free host (LM0230) and pSK11 (data not shown). However, a 14-kbp *Pvu*II fragment of pSK11 had been cloned into a vector that does not replicate in lactococci. This clone, pKMP1, carried the origin of replication and an area involved in plasmid stabilization (putatively, integration) derived from pSK11 (20a). This subclone did not encode Lac, but the vector encoded

Emʳ. Like pSK11, pKMP1 replicated in a thermosensitive manner but could be stabilized, putatively by integration. In strains stabilized for Emʳ, autonomous pKMP1 could not be detected.

Figure 5A and B shows the results of hybridizations with an internal 1.3-kb *Eco*RV-*Ava*I fragment of the vector portion of pKMP1 as the probe. When DNA preparations were digested with *Pvu*I (which does not restrict pKMP1) and probed, there was hybridization to genomic preparations of strain KMP1 (autonomous pKMP1 [Fig. 5A, lane 1]) in the region of the lane where the monomeric covalently closed circular form of pKMP1 migrated, as well as higher in the lane where what appeared to be oligomeric forms of the plasmid were located. However, when a putative integrant (KMP1-S3 [Fig. 5A, lane 2]) was probed, there was little or no hybridization in the oligomeric region but a strong signal was observed in the area corresponding to large chromosomal fragments. One possible explanation for the broadness of the signal is that the integrated plasmid had undergone amplification. Because *Pvu*I does not restrict the plasmid, the large chromosomal fragment carrying the amplified plasmid would be randomly sheared during preparation of the genomic DNA.

Figure 5B shows digestions with *Sph*I or *Nru*I, both of which restrict pKMP1 once (within the 1.3-kbp *Eco*RV-*Ava*I fragment of the vector portion). Restrictions of pKMP1

TABLE 2. Effect of temperature on proportions of colony
morphology types when a culture of L. lactis subsp. lactis
carrying the pSK11 Lac plasmid in the autonomous
state was plated on BCP-lactose agar[a]

Colony morphology[b]	% Colony type of a broth culture grown at 22°C and plated at:		
	22°C (n = 2,663)[c]	32°C (n = 2,374)	37°C (n = 2,195)
Lac⁺ smooth	82.3	0.2	1.5
Irregular	13.1	95.6	0
Fried-egg	0	0	95.1
Lac⁻	4.4	4.3	3.4

[a] Strain JF3216 was inoculated (0.25%) into 200 ml of Elliker broth with 1% lactose as the sole added carbohydrate, incubated for 17 h at 22°C, and plated on BCP-lactose agar. After incubation at the indicated temperature for 3 days, colonies were examined under a dissecting microscope.

[b] Lac⁺ smooth: yellow, circular. Irregular: lobate margins with areas of Lac⁺ and Lac⁻ growth. Fried-egg: umbonate, with yellow, opaque centers surrounded by translucent Lac⁻ growth. Lac⁻: white, translucent, circular.
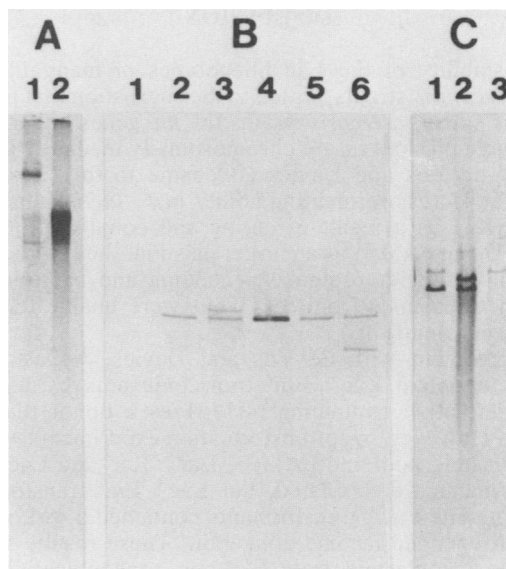
[c] n, Total number of colonies examined.

FIG. 5. Southern hybridization analyses of L. lactis subsp. lactis strains carrying pKMP1 in the autonomous or integrated state. Electrophoresis was conducted through 0.65% FastLane agarose (FMC BioProducts, Rockland, Maine) at 2.1 V/cm for 16.5 to 17 h; the buffer, transfer, hybridization, detection, and probes are described in the text. (A) (PvuI digests) EcoRV-AvaI fragment of the vector portion of pKMP1 was used as the probe. Lanes: 1, strain KMP1 (autonomous pKMP1); 2, strain KMP1-S3 (integrated pKMP1). (B) NruI digests (lanes 1 to 4) and SphI digests (lanes 5 and 6). Same probe as in panel A. Lanes: 1, strain LM0230; 2, strain KMP1; 3, strain KMP1-S3; 4, pKMP1 DNA; 5, strain KMP1; 6, strain KMP1-S3. (C) NruI digests. The 14-kb PvuII fragment of pSK11 cloned in pKMP1 was used as the probe. Lanes: 1, strain KMP1; 2, strain KMP1-S3; 3, strain LM0230.

plasmid preparations (NruI [Fig. 5B, lane 4]) or genomic preparations of a strain (KMP1) with pKMP1 in the autonomous state (NruI [Fig. 5B, lane 2] and SphI [Fig. 5B, lane 5]) yielded, as expected, one band that hybridized to the EcoRV-AvaI probe. Genomic preparations of a putative integrant (KMP1-S3) had two hybridizing bands (NruI [Fig. 5B, lane 3] and SphI [Fig. 5B, lane 6]). One band corresponded to linear pKMP1, but the other band was not observed in KMP1 (autonomous pKMP1). The unique band in the putative integrant could be a chromosome-plasmid junction fragment, whereas the other band could result from amplification of the integrated plasmid.

A third Southern hybridization analysis used the 14-kb PvuII fragment of pSK11 cloned in pKMP1 as the probe. (This PvuII fragment was further restricted with HindIII to facilitate labeling and hybridization.) Preparations of genomic DNA from LM0230 (plasmid-free host [Fig. 5C, lane 3]), KMP1 (autonomous pKMP1 [Fig. 5C, lane 1]), and KMP1-S3 (putative pKMP1 integrant [Fig. 5C, lane 2]) were restricted with NruI and fractionated by electrohoresis. Following hybridization with the probe, a single hybridizing band was present in LM0230. In KMP1, this band, as well as a second band (linearized pKMP1), hybridized to the probe. The putative integrant (KMP1-S3) had two hybridizing bands. The first corresponded to linearized pKMP1 and could represent amplified copies of the integrated plasmid. The second band was not seen in KMP1 or LM0230, and thus represented a junction fragment. In addition, the hybridizing band present in both LM0230 and KMP1 was absent in KMP1-S3, indicating that this chromosomal fragment of the host was the site of integration by pKMP1.

These results strongly suggest that in Emʳ-stabilized strains devoid of detectable autonomous plasmid, pKMP1 had been integrated into the chromosome. We also conclude that the parental plasmid, pSK11 (as well as the other Lac plasmids), was capable of integration. Lac⁺ transformants without detectable autonomous pSK11 also yielded a junction fragment when hybridized with the 14-kb PvuII fragment of pKMP1 (data not shown).

Transformation of a host-mediated homologous recombination-deficient (Rec⁻) host with L. lactis subsp. cremoris Lac plasmids. To determine the possible mechanism of integration, we transformed pSK11 or pML1 into Rec⁻ plasmid-

free L. lactis subsp. lactis MMS368. Plasmid profiles of the Lac⁺ Rec⁻ transformants examined showed the presence of either a 48- or a 59-kbp plasmid (data not shown). When pSK11 was present in MMS368 (JF3630), the Lac⁺ phenotype was unstable (Fig. 2). In contrast, when pML1 was present in MMS368, lactose utilization was stable at 21, 32, 37, and 40°C (data not shown). No plasmid-free Lac⁺ variants of either strain were detected.

Lac plasmid stability and Tsg are separate genetic entities. JF3206 (integrated pSK11) was compared with JF3216 (autonomous pSK11) with respect to growth and stability at various temperatures. In contrast to LM0230, the total number of cells per CFU in JF3216 decreased with increasing incubation temperature, as did the proportion of Lac⁺ cells (Fig. 4A). When pSK11 was chromosomally integrated as in JF3206, lactose metabolism was stabilized but the Tsg response was retained, as indicated by the decrease in total number of cells per CFU (Fig. 4B). Similar results were observed with pML1. With pML1 in the autonomous state (strain JF3101), the total number of cells per CFU decreased with increasing incubation temperature, as did the proportion of Lac⁺ cells (Fig. 4C). Upon chromosomal integration of pML1 (JF3100), there was no loss of the Lac⁺ phenotype but the Tsg response was retained (Fig. 4D). Furthermore, pKMP1 (the subclone of pSK11) retained the thermosensitive mode of replication but did not confer Tsg (20a).

DISCUSSION

The stability of the Lac phenotypes of many *L. lactis* subsp. *cremoris* strains has led to the suggestion that in some *L. lactis* subsp. *cremoris* strains the *lac* genes either reside on a stable plasmid or are chromosomally mediated (10, 25, 34, 35). de Vos and Davies (10) came to this conclusion about the lactose-fermenting ability in *L. lactis* subsp. *cremoris* SK11 as a result of curing and conjugation experiments. Using pAMβ1 as a marker plasmid, they were able to conjugally transfer proteinase-encoding and bacteriophage resistance plasmids from SK11 but were unable to obtain Lac⁺ transconjugants.

In agreement with de Vos and Davies, we also were unable to obtain Lac⁺ Emʳ transconjugants by using an SK11 derivative containing pAMβ1 as a donor (data not shown). However, in cotransformation experiments with the SK11 plasmid pool and pAMβ1, Lac⁻ Emʳ and Lac⁺ Emˢ transformants were isolated, but Lac⁺ Emʳ transformants were not. The Lac⁺ transformants contained a 48-kbp plasmid that encoded lactose utilization. These results suggest that the Lac plasmid from SK11 is nonconjugative or is incompatible with pAMβ1 in *L. lactis* subsp. *lactis* LM0230. Since SK11 is a phage-resistant mutant of AM1 (9), it was expected that AM1 would also contain a 48-kbp Lac plasmid. Restriction analysis revealed that the 48-kbp plasmids from AM1 and SK11 were identical. Genetic and physical evidence also revealed that a 59-kbp plasmid in *L. lactis* subsp. *cremoris* ML1 coded for Lac⁺. Thus, three strains of *L. lactis* subsp. *cremoris* (AM1, SK11, and ML1) were shown to possess plasmids that coded for Lac⁺ when present in *L. lactis* subsp. *lactis* LM0230.

The three *L. lactis* subsp. *cremoris* Lac plasmids had a thermosensitive mode of replication in LM0230. This thermosensitive replication was also observed with some of the Lac plasmids in other strains of *L. lactis* subsp. *lactis* (LM0220, MG1363, and MMS368). However, when *L. lactis* subsp. *cremoris* EB5 or *L. lactis* subsp. *lactis* KR5-1a was transformed with pSK11 or pML1, no Lac⁻ variants were detected. These results suggest that the inheritance of the Lac plasmids from *L. lactis* subsp. *cremoris* may be dependent on the host harboring them. KR5-1a is a temperature-sensitive *L. lactis* subsp. *lactis* strain (like *L. lactis* subsp. *cremoris*, it will not grow at 40°C), and its classification as an *L. lactis* subsp. *lactis* strain was probably due to its ability to hydrolyze arginine. Crow and Thomas (6) have shown that arginine hydrolysis is only a relative property in separating *L. lactis* subsp. *lactis* from *L. lactis* subsp. *cremoris*, because some strains of the latter can also hydrolyze arginine. The stability of the two *L. lactis* subsp. *cremoris* Lac plasmids in KR5-1a suggests that this strain could actually be an *L. lactis* subsp. *cremoris* strain. It is interesting that the Lac plasmid from *L. lactis* subsp. *lactis* KR5 demonstrates properties similar to those seen with *L. lactis* subsp. *cremoris* Lac plasmids (i.e., Tsg and instability [data not shown]). When introduced into *L. lactis* subsp. *lactis* MG1363, pSK11 exhibited instability whereas pML1 was stable. Therefore, these results indicate that there are also plasmid-linked characteristics that influence the stability of Lac plasmids within host cells.

Stabilization of the Lac plasmids appeared to occur via chromosomal integration. This was supported by the absence of autonomous plasmid in stabilized strains as well as by Southern hybridizations that showed the appearance of a new band (junction fragment) in stabilized strains and the disappearance of the hybridizing fragment that represented the target of plasmid integration in the host. The inability to detect two junction fragments obscures the mechanism of integrative recombination. In their studies of the integration of pE194 in *Bacillus subtilis*, Hofemeister et al. (20) reported some Southern hybridizations that yielded only one junction fragment. They concluded that integration occurred by a *recE*-independent Campbell-like reciprocal recombination.

Although *L. lactis* subsp. *lactis* ML3, C2, and 712 are related (7), the behavior of the Lac plasmids in LM0230 (C2 derivative) was different from that observed in MMS368 and MG1363 (ML3 and 712 Lac⁻ derivatives, respectively). When pSK11 or pML1 was introduced into Rec⁻ MMS368, integration of these plasmids was not observed. These results suggest that the integration event may depend upon the homologous recombination system of the host. On the other hand, it was also noted that pSK11 was unstable in MMS368 and MG1363, whereas pML1 was stable in these two strains at all temperatures examined. Unlike MG1363 and MMS368, LM0230 was isolated following UV irradiation and chemical mutagenesis; therefore, the integration event in LM0230 may be unique to this strain and the absence of observed integration in MMS368 may be unrelated to the Rec⁻ phenotype.

Integration of other plasmids into the lactococcal chromosome has been reported (4, 26, 27). Chopin et al. (4) constructed integrative plasmids by cloning bacteriophage DNA into a plasmid that does not replicate in the lactococci. The cloned DNA provided homologous regions for integrative recombination with prophage sequences. Leenhouts et al. (26, 27) reported integration of recombinant plasmids that contained fragments cloned from the lactococcal chromosome, thereby facilitating recombination and integration. The integrative plasmids in the present report differ from these other plasmids in that they are naturally occurring plasmids that do not carry antibiotic resistance determinants. These characteristics make these plasmids more amenable for the development of food-grade integrative vectors for the construction of industrially useful starter strains.

L. lactis subsp. *lactis* and *L. lactis* subsp. *cremoris* are differentiated from one another primarily on the basis of phenotypic properties such as the ability of *L. lactis* subsp. *lactis*, and the inability of *L. lactis* subsp. *cremoris*, to grow at 40°C (17). The results presented in this study indicate that *L. lactis* subsp. *lactis* LM0230 acquired a Tsg determinant when Lac plasmids from *L. lactis* subsp. *cremoris* were introduced into this host. In practice, *L. lactis* subsp. *lactis* strains tend to produce bitterness in Cheddar cheese. This is because these strains grow at the cooking temperature (38 to 40°C) used in manufacture of Cheddar cheese (29); this leads to high total proteinase activity, which in turn can contribute to the development of bitterness (28). Thus, strains whose growth is repressed at the cooking temperature (such as *L. lactis* subsp. *cremoris*) are usually preferred in Cheddar cheese manufacture. By constructing *L. lactis* subsp. *lactis* strains containing a Lac plasmid from *L. lactis* subsp. *cremoris*, it may be possible to develop temperature-sensitive *L. lactis* subsp. *lactis* strains, thereby increasing the number of suitable strains available for cheese manufacture.

This is the first report that some plasmids may code for the Tsg response in lactococci. The Tsg response appears similar to that reported for the plasmid R*ts*1 of the family *Enterobacteriaceae*, which confers many unusual characteristics including inhibition of growth at elevated temperatures (i.e., Tsg), thermosensitive replication of the plasmid, and/or thermosensitive formation of non-covalently closed circular

(i.e., linear) forms of R*ts*1 (11, 12, 37, 42, 44, 45). In the present study, some preparations of pSK11 and pKMP1 contained linear forms of these plasmids (data not shown). The unusual properties exhibited by the L. lactis subsp. cremoris Lac plasmids in L. lactis subsp. lactis are of significance in studying host-plasmid interactions in these industrially important bacteria.

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