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Recombinant Plasmid Associated with Cell Aggregation and High-Frequency Conjugation of *Streptococcus lactis* ML3†

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Lactose-positive (Lac^+) transconjugants resulting from matings between *Streptococcus lactis* ML3 and *S. lactis* LM2301 possess a single plasmid of approximately 60 megadaltons (Mdal) which is nearly twice the size of the lactose plasmid of the donor. The majority of these Lac^+ transconjugants aggregated in broth and were able to transfer lactose-fermenting ability at a frequency higher than 10^{-1} per donor on milk agar plates or in broth. Lac^+ transconjugants which did not clump conjugated at a much lower frequency. Lactose-negative derivatives of Lac^+ clumping transconjugants did not aggregate in broth and were missing the 60-Mdal plasmid. The ability to aggregate in broth was very unstable. Strains could lose the ability to clump but retain lactose-fermenting ability. The majority of these Lac^+ nonclumping derivatives of clumping transconjugants contained a plasmid of approximately 33 Mdal, the size of the lactose plasmid of the original donor ML3. These strains transferred lactose-fermenting ability at a frequency of approximately 10^{-6} per donor, resulting in both Lac^+ clumping transconjugants which contained a 60-Mdal plasmid and Lac^+ nonclumping transconjugants which possessed a 33-Mdal plasmid. Our results suggest that the genes responsible for cell aggregation and high-frequency conjugation are on the segment of deoxyribonucleic acid which recombined with the 33-Mdal lactose plasmid in *S. lactis* ML3.

The conjugal transfer of plasmid DNA in group N streptococci was recently reported. In our laboratory (8, 13), it was shown that *Streptococcus lactis* subsp. *diacetylactis* strains 18-16, DRC3, 11007, and WM_4 and *S. lactis* ML3 and C_2O are capable of low-frequency conjugal transmission of plasmid DNA (10^{-6} to 10^{-9} lactose-positive [Lac^+] transconjugants per donor). Gasson and Davies (5) have described the transfer of *Streptococcus faecalis* plasmid pAMβ to strains of *S. lactis*. These authors more recently reported high-frequency conjugation in *S. lactis* associated with donor cell aggregation, although no change in the plasmid profile of the recipient was detected (6).

In our earlier report (13), Lac^+ transconjugants resulting from matings between *S. lactis* ML3 and a streptomycin-resistant (Str^r) derivative of *S. lactis* LM0230 (herein designated as LM2301) were shown to contain a single plasmid of approximately 60 megadaltons (Mdal). The lactose plasmid of the donor is 33 Mdal (11); the recipient is cured of plasmid DNA (9, 12). In this communication, the ML3 conjugation system is further characterized. We found that some of the ML3 × LM2301 transconjugants transfer the ability to ferment lactose at a frequency higher than 10^{-1} per donor. We further observed that these transconjugants formed aggregates in broth that did not disperse even after vigorous blending in a Vortex mixer. Other transconjugants which did not clump mated at a much lower frequency. Our results suggest that donor cell aggregation and high-frequency conjugation are associated with a 60-Mdal plasmid.

MATERIALS AND METHODS

Organisms. The microorganisms used in this study are maintained in our stock culture collection through biweekly transfer at 21°C in M17 broth (15) containing 0.5% glucose or lactose. The *Escherichia coli* strains were propagated in brain heart infusion broth, and resident plasmids in these strains were used as mobility reference plasmids for agarose gel electrophoresis. Strains of *S. lactis* and *Streptococcus cremoris* used in this study are described in Table 1.

Isolation of mutants. Str^r mutants were isolated as previously described (13). Erythromycin-resistant (Ery^r) mutants were isolated by transferring a culture into fresh broth containing 0.5 μg of erythromycin per ml and incubating the culture at 32°C until growth

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TABLE 1. *Strains of S. lactis and S. cremoris used in this study*

Strain	Plasmid composition[a]	Relevant phenotype	Comment
ML3	33, 5.5, 2, 1	Lac$^+$	Parent culture (11)
MA1	33, 5.5, 2, 1	Lac$^+$	UV-induced Mal$^-$ mutant of ML3
LM0230		Lac$^-$	Nitrosoguanidine- and UV-induced mutant of *S. lactis* C2 cured of plasmid DNA (9, 12)
LM2301		Lac$^-$	Spontaneous Str^r mutant of LM0230
LM2302		Lac$^-$	Spontaneous Ery^r mutant of LM2301
PW1	60	Lac$^+$	Nonclumping transconjugant of ML3 × LM2301
PW2	60	Lac$^+$	Clumping transconjugant of ML3 × LM2301
LM0330	5.5, 2, 1	Lac$^-$	Spontaneous mutant of ML3
LM3301	5.5, 2, 1	Lac$^-$	Spontaneous Str^r mutant of LM0330
LM3302	5.5, 2, 1	Lac$^-$	Spontaneous Ery^r mutant of LM0330
LM3303	5.5, 2, 1	Lac$^-$	Spontaneous Str^r mutant of LM3302
BC1		Lac$^-$	Acriflavine-induced mutant of *S. cremoris* B_1 cured of plasmid DNA (2)
BC101		Lac$^-$	Spontaneous Str^r mutant of BC1
BC102		Lac$^-$	Spontaneous Ery^r mutant of BC1
MM12	33, 5.5, 2, 1	Lac$^+$	Nonclumping transconjugant of MA1 × LM3303
MM4	60, 5.5, 2, 1	Lac$^+$	Clumping transconjugant of MA1 × LM3303
MM6	60, 5.5, 2, 1	Lac$^+$	Clumping transconjugant of MA1 × LM3303
MS5	60, 5.5, 2, 1	Lac$^+$	Clumping transconjugant of ML3 × LM3301
MS8	33, 5.5, 2, 1	Lac$^+$	Nonclumping transconjugant of MA1 × LM3301
MS10	60, 5.5, 2, 1	Lac$^+$	Nonclumping transconjugant of MA1 × LM3301
MC2	60	Lac$^+$	Nonclumping transconjugant of ML3 × LM2301
PN210	60	Lac$^+$	Spontaneous nonclumping derivative of PW2
PN221	33	Lac$^+$	Spontaneous nonclumping derivative of PW2
NM218	33, 5.5, 2, 1	Lac$^+$	Nonclumping transconjugant of PN221 × LM3302
NM219	60, 5.5, 2, 1	Lac$^+$	Clumping transconjugant of PN221 × LM3302

[a] Plasmid molecular weight × 10^6.

was evident. Successive transfers were then made into M17-glucose broth containing increasing quantities of erythromycin. The Str^r isolates and Ery^r isolates were maintained in M17-glucose broth containing 500 μg of streptomycin sulfate per ml and 15 μg of erythromycin per ml, respectively.

A maltose-negative (Mal$^-$) mutant of ML3 designated MA1 was isolated by exposing cells to UV light for 40 s. Cells were diluted and plated onto bromocresol purple indicator agar (14) containing 0.5% maltose.

Lac$^-$ derivatives of PW2, MM4, and MM6 were isolated by plating on lactose indicator agar (14). Lac$^-$ colonies which arose spontaneously were recognized by the white color of the colony.

Mating conditions. Solid surface matings on milk plus glucose agar plates were carried out as previously described (13). Broth matings were carried out in M17-glucose. Two percent inocula of overnight broth cultures of the donor and recipient were made into M17-lactose and M17-glucose broth, respectively. After incubation at 32°C for 4.5 h, 0.15 ml of donor cells was combined with 1.5 ml of recipient cells in a total volume of 15 ml. The cells were incubated at 32°C in a shaker water bath with mild agitation. At selected time intervals, samples were removed, diluted, and plated onto lactose indicator agar with and without 15 μg of erythromycin per ml. After 48 h at 32°C, Lac$^+$ Ery^s donors, Lac$^-$ Ery^r recipients, and Lac$^+$ Ery^r transconjugants were counted. Controls included the donor and recipient incubated alone.

Plasmid analysis. Unless noted otherwise, isolation of plasmid DNA and agarose gel electrophoresis were as previously described (9). Because of the poor recovery of the 60-Mdal plasmid, a procedure for the isolation of large plasmids (7) was modified for *S. lactis* and *S. cremoris*. Strains were grown 4 h at 32°C in 80 ml of lysis broth (9) and harvested by centrifugation. The cells were washed in 10 ml of TES buffer (50 mM NaCl, 5 mM disodium [Na_2] EDTA, 30 mM Tris [pH 8.0]) and resuspended in 1 ml of 25% sucrose in 50 mM Tris–1 mM Na_2 EDTA, pH 8.0. Lysozyme (0.25 ml of a solution [10 mg/ml] in 0.25 M Tris, pH 8.0) was added, and the tubes were gently inverted. After incubation at 37°C for 5 to 7 min, 0.4 ml of Na_2 EDTA (0.25 M in 0.05 M Tris, pH 8.0) was added, immediately followed by 0.4 ml of sodium dodecyl sulfate (20%, wt/vol, in 0.05 M Tris and 0.02 M Na_2 EDTA, pH 8.0). The tubes were gently inverted and then subjected to eight cycles of a 55°C heat pulse and mixing. At ambient temperature, 0.13 ml of 3 N NaOH was added, followed by 3 min of gentle inversions. To neutralize the solution, 0.75 ml of distilled water was added, followed by 1.0 ml of Tris (2 M, pH 7.0). Sodium dodecyl sulfate (0.65 ml of 20%) immediately followed by 1.25 ml of NaCl (5 M) was then added. The tubes were gently inverted, chilled in an ice water bath, and stored overnight at 4°C. After centrifugation at 12,000 rpm (4°C, 30 min) in a Beckman JA-20 rotor, the supernatants were poured into chilled conical tubes and concentrated with PEG 6000 (Carbowax 6000, Union Carbide) (7). The PEG pellet was resuspended in 125 μl of cold TES and subjected to agarose gel electrophoresis.

Clumping experiments. To test for clumping induction, clumping strains PW2 and MM4 were grown to mid-log phase in M17-lactose, Todd-Hewitt broth (BBL Microbiology Systems) or Elliker broth (Difco Laboratories). The cells were pelleted, and the supernatant was filtered through a 0.45-μm membrane filter (Millipore Corp.). Induction tubes consisted of 5 ml of filtrate, 4 ml of fresh broth, and 1 ml of a late-log-phase culture of a nonclumping strain. Each strain was tested with filtrate from two clumping strains in each of the three media. The cultures were incubated at 32°C and monitored over a period of 24 h. Controls of PW2 and MM4 were also monitored.

To test for mating enhancement, an overnight cul-

ture of PW1 was inoculated (0.2 ml) into 5 ml of PW2 filtrate plus 5 ml of fresh M17-lactose broth. After incubation at 32°C for 4.5 h, PW1 was mated with LM3302.

To demonstrate instability of cell aggregation, PW2 was repetitively transferred in M17-lactose broth at 32°C at 8- and 16-h intervals. After indicated transfers, the cells were diluted and plated onto lactose indicator agar. Lac^+ colonies were picked into 3 ml of M17-lactose broth. After 24 h at 32°C, the cultures were vigorously blended in a Vortex mixer and examined for the presence of aggregates. Transconjugants were also screened for clumping by picking Lac^+ colonies into 3 ml of M17-lactose broth.

RESULTS

Appearance of clumping strains. We observed that the majority of the Lac^+ transconjugants resulting from matings in which *S. lactis* ML3 was the donor did not grow as a uniform suspension of cells in M17 broth. Instead, the cells formed clumps or aggregates of cells that did not completely disperse even after vigorous blending in a Vortex mixer (Fig. 1). Neither ML3 nor any of the recipients used in this study exhibited this growth behavior. The appearance of the cell aggregates was similar to that described by Dunny et al. (3) for *S. faecalis* mating mixtures.

Comparison with cell aggregation in *S. faecalis*. Dunny et al. (3, 4) described the clumping of mating mixtures of *S. faecalis*. They concluded that recipient strains excrete a substance which causes certain donor strains to aggregate. Cell-free recipient filtrates were able to induce donor cells to clump and to mate at high frequencies. The clumping phenomenon of *S. lactis* ML3 transconjugants appeared to be different. In the ML3 system, the Lac^+ transconjugants alone, but not the original parental mating mixtures, formed aggregates when grown in broth. *S. lactis* ML3, nonclumping transconjugants PW1 and MM12, and Lac^- recipients LM2301 and LM3302 did not clump when grown in the presence of cell-free filtrates of the two clumping strains, PW2 and MM4. The filtrate of PW2 also did not increase the mating efficiency of PW1.

Transfer frequency and cell aggregation. We found a direct correlation between donor cell aggregation and conjugation frequency (Table 2). Strains, such as PW2 and MS5, which aggregated in broth transferred lactose-fermenting ability at a frequency higher than 10^{-1} per donor. All nonclumping transconjugants as well as the original parent ML3 transferred at a much lower frequency. When PW2 was successively transferred in M17-lactose broth, the culture rapidly lost the ability to form clumps. After 5 transfers, only 49% of the isolated colonies examined formed clumps when inoculated into M17-lactose broth. By 15 transfers, the percentage of clumping isolates dropped to 4.7. A concurrent decrease in conjugation frequency was also observed. After 15 transfers, the conjugation frequency dropped to 2.3×10^{-2} Lac^+ transconjugants per donor, and it dropped to 3.2×10^{-3} after 20 transfers.

Broth matings. Because of the high fre-

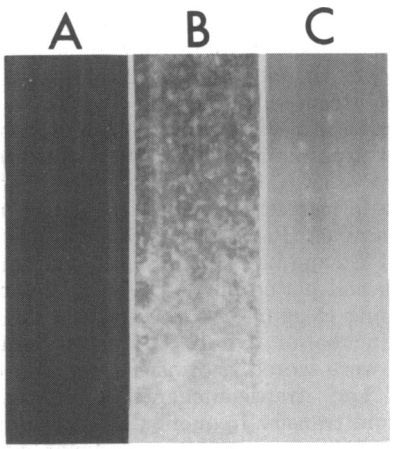

FIG. 1. *Appearance of clumping and nonclumping strains. (A) Uninoculated M17-lactose broth. (B) Overnight culture of the clumping transconjugant PW2 diluted 1:50 into fresh M17-lactose broth and incubated 4.5 h at 32°C. (C) Similar culture of nonclumping donor ML3. After incubation the cultures were blended vigorously in a Vortex mixer in test tubes (16 by 150 mm) and immediately photographed.*

TABLE 2. *Transfer frequencies of ML3 and its transconjugants*

Mating pair		Donor		Transconjugants per donor[c]
Donor	Recipient	Plasmid content[a]	Clumping behavior[b]	
ML3	LM2301	33, 5.5, 2, 1	NC	3.0×10^{-6}
ML3	LM3302	33, 5.5, 2, 1	NC	2.77×10^{-6}
ML3	BC101	33, 5.5, 2, 1	NC	8.4×10^{-7}
PW1	LM3302	60	NC	1.5×10^{-7}
PW1	BC102	60	NC	$<10^{-8}$
PW2	LM3302	60	C1	1.7
PW2	BC102	60	C1	5.3×10^{-1}
MS5	LM2302	60, 5.5, 2, 1	C1	1.7
MS8	LM2302	33, 5.5, 2, 1	NC	6.9×10^{-7}
MS10	LM2302	60, 5.5, 2, 1	NC	$<10^{-8}$
MC2	LM3302	60	NC	2.0×10^{-5}
PN210	LM3302	60	NC	7.9×10^{-3}
PN221	LM3302	33	NC	2.7×10^{-6}

[a] Plasmid molecular weight $\times 10^6$.

[b] NC, Does not aggregate in broth; C1, cells aggregate in broth.

[c] Average of at least two separate mating experiments.

quency of transfer on milk agar plates, broth matings of the clumping transconjugant PW2 and of the original parent ML3 were attempted. No Lac^+ transconjugants resulted from a 1-h broth mating of ML3 × LM2302, although over 10^5 Lac^+ transconjugants per ml resulted from a similar PW2 × LM3302 mating experiment. The kinetics of transfer of lactose-fermenting ability was examined (Fig. 2). Immediately upon combining PW2 and LM3302, a sample was removed, diluted, and plated onto lactose indicator agar containing 15 μg of erythromycin per ml. After 48 h of incubation, over 10^3 Lac^+ transconjugants per ml were scored, a transfer frequency of 5.8 × 10^{-4} Lac^+ transconjugants per donor. The maximum transfer frequency of approximately 10^{-1} Lac^+ transconjugants per donor was obtained after 4 h of incubation. It is possible that the data do not represent the kinetics of lactose plasmid transfer but rather the kinetics of mating pair formation, with subsequent transfer occurring on the agar plates. This would be supported by the high number of Lac^+ transconjugants at zero time.

Plasmid analysis. The plasmid analysis of the transconjugants was not straightforward. We found that the lysis procedure of Klaenhammer et al. (9), although excellent for the parent strains, did not give reproducible results for the transconjugants. For this reason we adapted the large plasmid isolation procedure of Hansen and Olsen (7) for use with *S. lactis*. The 60-Mdal plasmid was easily recovered from transconjugants by this procedure. Parent strains were also examined by the modified Hansen and Olsen procedure. The 60-Mdal plamid was not detected in these strains by either plasmid isolation procedure.

Twelve ML3 × LM2301 transconjugants examined were found to contain a plasmid of approximately 60 Mdal, although the lactose plasmid in ML3 is only 33 Mdal. Three of the 13 ML3 × LM3302 transconjugants examined contained a 33-Mdal plasmid, and the others contained a 60-Mdal plasmid. Since transconjugants which contained a 33-Mdal plasmid had the same plasmid profile as the donor, the possibility of mutation of ML3 to antibiotic resistance existed. To lessen this possibility, multiple chromosomal markers were used. A maltose-negative (Mal^-) derivative of ML3 designated MA1 was mated with the Str^r Ery^r recipient LM3303. Only Lac^+ Ery^r Str^r transconjugants able to ferment maltose were scored. One of the seven examined still contained a 33-Mdal plasmid. Using PW1 and PW2 as donors in matings with LM3302, we found that the 60-Mdal plasmid was transferred intact.

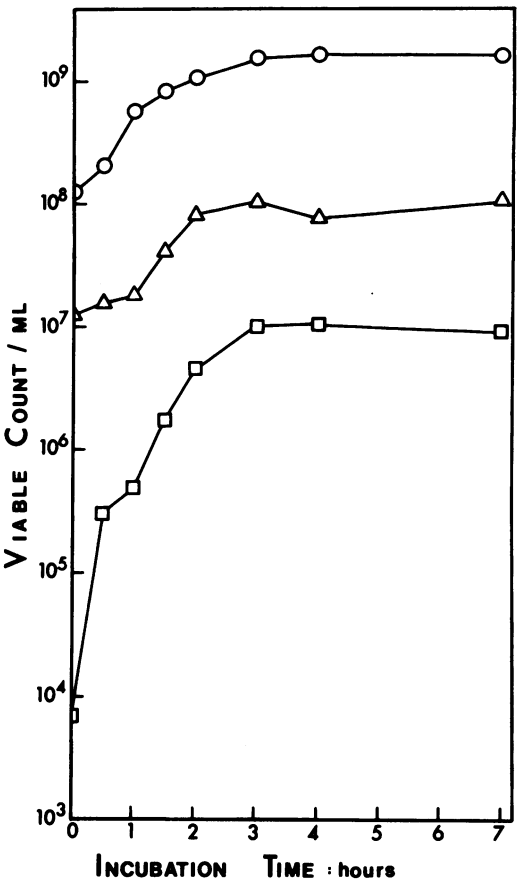

FIG. 2. *Kinetics of transfer of lactose-fermenting ability from PW2 to LM3302. Broth cultures of donor PW2 and recipient LM3302 were mixed and incubated as described in the text. At the times indicated, samples were withdrawn, diluted, and plated to determine the viable count per milliliter of the donor PW2 (△), the recipient LM3302 (○), and Lac^+ transconjugants (□).*

Relationship between cell aggregation and the 60-Mdal plasmid. All 25 of the Lac^+ clumping transconjugants examined contained a plasmid of approximately 60 Mdal; however, some transconjugants which contained a plasmid of apparently the same molecular weight as that in a clumper did not aggregate in broth (Fig. 3). Three of the 12 ML3 × LM2301 transconjugants isolated did not clump, although all three appeared to contain a plasmid of approximately 60 Mdal. In an ML3 × LM3303 mating experiment, we found that 3 of the 10 nonclumping Lac^+ transconjugants studied contained a plasmid of apparently the same molecular weight as that in a clumper. Small differences in molecular weight, however, would not be resolved in the gel system used. All 11 of the Lac^+ transconjugants examined which contained a 33-

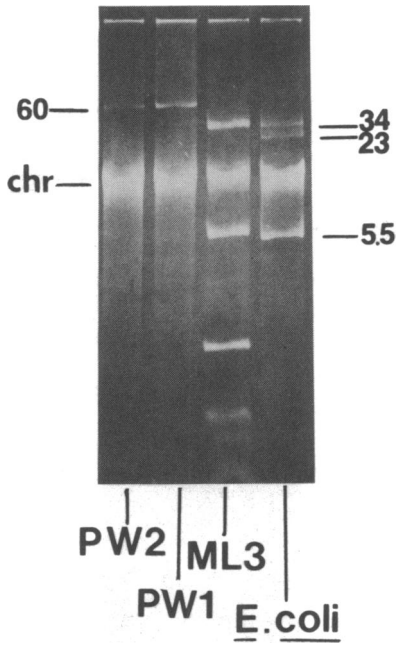

FIG. 3. *Agarose gel electrophoresis of ethanol-precipitated DNA from S. lactis ML3, the clumping transconjugant PW2, and the nonclumping transconjugant PW1. ML3 possessed plasmids of molecular weights 33 × 10⁶, 5.5 × 10⁶, 2 × 10⁶, and 1 × 10⁶. The transconjugants contained a single plasmid of approximately 60 Mdal. E. coli reference plasmids RP4 (34 Mdal), Sa (23 Mdal), and RSF1010 (5.5 Mdal) were used to determine molecular weights as previously described (9).*

Mdal plasmid did not clump. To determine the relationship between the 60-Mdal plasmid, lactose metabolism, and cell aggregation, Lac⁻ derivatives of clumping transconjugants PW2, MM4, and MM6 were isolated. These isolates no longer clumped and were missing the 60-Mdal plasmid, although the majority of Lac⁺ colonies isolated in the same way clumped. This suggests that the genes responsible for cell aggregation and lactose-fermenting ability are linked to a 60-Mdal plasmid. In the donor cell aggregation system of *S. lactis* 712 described by Gasson and Davies (6), the genes responsible for cell clumping, which they designated *lax*, are also linked to the lactose genes.

As described above, the ability to clump was an unstable trait. We found that a cell could lose the ability to form aggregates in broth but still maintain lactose-fermenting ability. The plasmid profiles of Lac⁺ nonclumping isolates of PW2, MM4, and MM6 were examined. Two of the three examined for PW2, all four examined for MM4, and three of the five examined for MM6 contained a 33-Mdal plasmid instead of the 60-Mdal plasmid present in the original clumping strains (Fig. 4). The other Lac⁺ nonclumping isolates examined contained a 60-Mdal plasmid, although plasmid recovery in these strains was very poor. These results suggest that the genes responsible for cell aggregation are on the segment of DNA which recombined with the 33-Mdal lactose plasmid. It is not understood, however, why some Lac⁺ nonclumping isolates still contained a 60-Mdal plasmid or why Lac⁺ transconjugants such as PW1 and MC2, which were never observed to clump, contained a 60-Mdal plasmid.

To further study this phenomenon, nonclumping strains were used as donors in mating experiments, and their transconjugants were examined for the ability to clump. Based on the

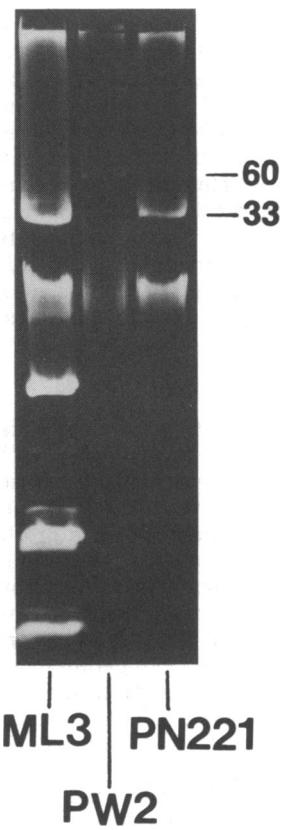

FIG. 4. *Agarose gel electrophoresis of DNA isolated by a modified version of the large plasmid isolation procedure of Hansen and Olsen (7). ML3 possessed plasmids of molecular weights 33 × 10⁶, 5.5 × 10⁶, 2 × 10⁶, and 1 × 10⁶. The Lac⁺ clumping transconjugant PW2 contained a single plasmid of about 60 Mdal. PN221, the Lac⁺ nonclumping isolate of PW2, possessed a single plasmid of approximately 33 Mdal.*

observation that the majority of the transconjugants of ML3, a nonclumping strain, formed aggregates, we hypothesized that if the genes responsible for clumping were still part of the genetic material of a nonclumping strain, clumping transconjugants would result from mating experiments in which a nonclumping strain was the donor. PW1 and MC2, Lac^+ transconjugants which contained a 60-Mdal plasmid but did not clump, were mated with LM3302, and their transconjugants were screened for clumping. None of the 150 PW1 or 100 MC2 Lac^+ transconjugants tested formed aggregates in broth. PN210, a Lac^+ nonclumping isolate of the clumping transconjugant PW2, contained a 60-Mdal plasmid and transferred lactose-fermenting ability to LM3302 at a frequency of 10^{-3} per donor. All 100 of its Lac^+ transconjugants tested were nonclumping. These results suggest that the genes responsible for cell aggregation are not present in these strains or are altered. PN221, a Lac^+ nonclumping isolate of PW2 which contained a 33-Mdal plasmid, transferred lactose metabolism to LM3302 at a frequency of 10^{-6}. Of 106 Lac^+ transconjugants studied, 73 clumped. Four clumping transconjugants examined contained a 60-Mdal plasmid, and the four nonclumping Lac^+ transconjugants examined contained a 33-Mdal plasmid (Fig. 5). This suggests that although PN221 is a nonclumping strain, it still possesses the genes responsible for cell aggregation.

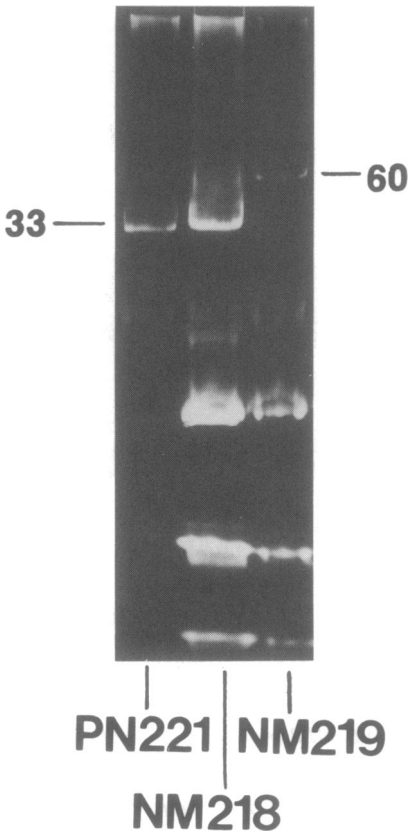

FIG. 5. *Agarose gel electrophoresis of DNA isolated by the large plasmid isolation procedure described in the text. PN221, a nonclumping isolate of the clumping transconjugant PW2, contained a single plasmid of approximately 33 Mdal. NM218, a nonclumping transconjugant of PN221 × LM3302, possessed plasmids of molecular weights 33 × 10^6, 5.5 × 10^6, 2 × 10^6, and 1 × 10^6. The recipient strain LM3302 possessed plasmids of molecular weights 5.5 × 10^6, 2 × 10^6, and 1 × 10^6 (data not shown). NM219, a Lac^+ clumping transconjugant of PW2 × LM3302, possessed plasmids of molecular weights 60 × 10^6, 5.5 × 10^6, 2 × 10^6, and 1 × 10^6.*

DISCUSSION

ML3 × LM2301 transconjugants contain a single plasmid of approximately 60 Mdal which is nearly twice the size of the lactose plasmid of the donor (13). We found that some of these transconjugants aggregated in broth and transferred lactose metabolism at a frequency higher than 10^{-1} per donor. Cell aggregation associated with high-frequency conjugation was previously reported for *S. faecalis* (3) and *S. lactis* (6). It is presumed that cell aggregation increases conjugation frequency by facilitating the formation of mating aggregates (4). This is reasonable, especially in light of the visualization of cell-cell contact during conjugal transfer in *S. faecalis* recently reported by Krogstad et al. (10).

In the *S. faecalis* system, recipient strains excrete a soluble, heat-stable, trypsin-sensitive substance which causes certain donor strains to aggregate and increases the frequency of plasmid transfer (3, 4). We found that the *S. lactis* ML3 system involved a different phenomenon. In our system, the transconjugants alone, but not the original parental mating mixture, formed aggregates when grown in broth. A soluble, extracellular substance was apparently not responsible for the clumping of certain ML3 transconjugants.

Gasson and Davies (6) recently described a system of high-frequency conjugation associated with donor cell aggregation in *S. lactis* 712. This system is similar to what we observed for *S. lactis* ML3 in that the original mating results in two types of transconjugants, those which aggregate and those which do not. The transconjugants which aggregate are more efficient donors than both the original parent and the nonclumping transconjugants. In the system of Gasson and Davies (6), cell aggregation is linked to the lactose genes, but they were unable to identify

the lactose plasmid in either the donor or the transconjugants.

Our results indicated that lactose metabolism, cell aggregation, and high-frequency conjugation in *S. lactis* ML3 transconjugants were linked to a 60-Mdal plasmid. Only Lac^+ transconjugants which contained a 60-Mdal plasmid clumped, and all Lac^+ clumping transconjugants were more efficient donors than ML3 or nonclumping transconjugants. All of the Lac^- derivatives of Lac^+ clumping transconjugants studied no longer clumped and were missing the 60-Mdal plasmid. We found, however, that strains could lose the ability to clump but maintain the Lac^+ phenotype. The majority of these Lac^+ nonclumping derivatives of clumping transconjugants contained a plasmid of approximately 33 Mdal, the size of the lactose plasmid of the original donor ML3. A few of these derivatives still contained a plasmid of apparently 60 Mdal. A small deletion, however, would not be resolved in the gel system used.

The origin of the 60-Mdal plasmid is unknown. The possibility exists that this plasmid is a dimer of the lactose plasmid and that cell aggregation and high-frequency conjugation are a result of a gene dosage effect. A study of proteinase activity and phospho-β-galactosidase activity, which are linked to the 33-Mdal lactose plasmid in ML3, however, indicated no significant difference between ML3 and its transconjugants (unpublished data). We propose that a segment of DNA of either plasmid or chromosomal origin, containing the genes responsible for cell aggregation and high-frequency conjugation, recombined with the 33-Mdal lactose plasmid. In our discussion, we refer to this segment of DNA as a transfer factor.

The change from a 60-Mdal plasmid present in the clumping transconjugant PW2 to a 33-Mdal plasmid in the nonclumping isolate PN221 with the subsequent appearance of a 60-Mdal plasmid in PN221 clumping trasnconjugants suggests a chromosomal origin for this transfer factor since no other plasmid was detectable in these strains. It is possible, however, that all of the plasmids present were not resolved in the gel system used or that low copy number plasmids were not detected. We previously reported the conjugal transfer of the chromosome of the plasmid-cured strain *S. lactis* LM0230 (13), and we have observed this event in other strains (unpublished data). This supports the speculation that a transfer factor is located on the chromosome of some strains of *S. lactis*, although the possibility that an unidentified plasmid is involved cannot yet be ruled out. We currently favor the hypothesis that a transfer factor is located on the chromosome, and we propose that when this transfer factor is positioned in the chromosome, lactose plasmid transfer is rare, as demonstrated by the low transfer frequency (10^{-5} to 10^{-9}) observed with ML3, MS8, and PN221. Conjugation selects for the translocation of this factor onto the lactose plasmid. This results in transconjugants such as PW2, which contained a 60-Mdal plasmid, clumped, and were high-frequency conjugators.

The ML3 transconjugants, such as PW1, which possessed a 60-Mdal plasmid but did not clump could result from insertion of the transfer factor involving recombination at different sites, an undetected deletion in a gene essential for the full expression of transfer functions, or insertion in an orientation that prevents expression. Nonclumping Lac^+ isolates of clumping transconjugants such as PN221, which contained a 33-Mdal plasmid and had a transfer frequency similar to the original donor ML3, could represent the translocation of the transfer factor back to the chromosome. It was apparently not lost from the strain since clumping transconjugants containing a 60-Mdal plasmid were isolated.

We suggest that cell aggregation is not solely responsible for the high-frequency conjugation observed for some ML3 transconjugants. We found that cell aggregation was a very unstable trait. It might be lost by the translocation of genes back to the chromosome (change from 60- to 33-Mdal plasmid) or by a small deletion with no apparent change in plasmid molecular weight such that the clumping phenotype is not recovered in transconjugants. Our study of PN210, a nonclumping derivative of PW2, suggested that more than one gene was involved in cell aggregation and high-frequency conjugation. We found that a strain such as PN210 could lose the ability to clump but still conjugate at a frequency higher than that of the original donor and nonclumping transconjugants, although still less than that of a clumping transconjugant. This suggested that although cell aggregation enhanced conjugation frequency, it was not solely responsible for high-frequency conjugation. PN210 still contained a 60-Mdal plasmid, suggesting that other genes which enhance mating besides those responsible for cell aggregation are present on the proposed transfer factor. In *E. coli*, conjugal transfer of the F plasmid depends upon more than 10 genes (1). The existence of a transfer factor containing genes responsible for high-frequency conjugation in the absence of genes for cell aggregation is further supported by the study in our laboratory (J. Kondo, personal communication) of *S. lactis* C_2O Lac^+ transconjugants. The majority of these Lac^+ transconjugants contained a plasmid of approximately 60 Mdal, although the lactose

plasmid of C_2O is 29 Mdal (13). These transconjugants did not clump, although they transferred lactose metabolism at a frequency of approximately 10^{-2} per donor. The relationship between the 60-Mdal plasmid present in C_2O transconjugants and that in ML3 transconjugants is unknown but is under investigation. It is likely that they possess similar transfer factors. The genes responsible for cell aggregation may not be present in all strains which possess transfer factors.

In conclusion, we propose that some strains of *S. lactis* possess a transfer factor that exists in the chromosome. Translocation of this factor onto the lactose plasmid greatly increases the transfer frequency of this plasmid, and in the ML3 system, it results in cell aggregation. We are investigating the molecular relationship between the 60-Mdal plasmid and the genetic material of *S. lactis* ML3 by means of restriction enzyme analysis and nucleic acid hybridizations.

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