DAIRY FOODS

Analysis of the Physical and Functional Characteristics of Cell Clumping in Lactose-Positive Transconjugants of *Lactococcus lactis* ssp. *lactis* ML3[1]

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ABSTRACT

Transconjugants of *Lactococcus lactis* ssp. *lactis* ML3 that acquire the ability to utilize lactose often exhibit self-aggregation and become able to transfer the ability to utilize lactose at frequencies 10^2 to 10^5 times higher than strain ML3. Our laboratory investigated the physical and functional characteristics of cell aggregation in ML3 transconjugants. Results showed that donor cell autoaggregation was dissociated when cells were suspended in buffers that contained EDTA or when they were briefly exposed to proteinase K or α-chymotrypsin. Conjugation studies revealed that disruption of donor cell aggregation substantially decreased the efficiency of lactose plasmid transfer. Dissociation by EDTA or proteolytic enzyme treatments was reversible, and recovery of high frequency lactose plasmid transfer ability accompanied the restoration of donor self-aggregation. Analysis of cell-surface proteins isolated from lactose-positive, aggregation-positive and from lactose-positive, aggregation-negative transconjugants of ML3 by PAGE indicated that aggregation-positive cells produced a unique protein of approximately 125 kDa. The results suggested that cell aggregation was essential for high frequency lactose plasmid transfer in ML3 transconjugants and that at least one large protein was involved in aggregation.

(**Key words**: *Lactococcus* sp., conjugation, cell clumping)

Abbreviation key: **Agg** = lactococcal aggregation substance, **BCP** = bromcresol purple, **Clu** = the ability (+) or inability (–) to self-aggregate or lactococcal clumping substance, **DPC** = direct plate conjugation, **EGTA** = ethylene glycol-bis(β-aminoethyl ether) N,N,N′,N′-tetraacetic acid, **Lac** = ability (+) or inability (–) to ferment lactose.

INTRODUCTION

Lactococci are commonly used as starter cultures by the dairy industry to manufacture cheese and fermented milks. Because of their important role in the dairy industry, these organisms have long been a target for genetic strain improvements. One gene transfer mechanism in lactococci that has already been used to improve commercial lactococcal starter cultures is conjugation (24), a process in bacteria that requires physical contact between viable donor and recipient cells. Gene transfer studies in lactic acid bacteria have revealed that these organisms may exchange a variety of important phenotypic traits by conjugation (9, 20), but information on conjugal mechanisms in these bacteria remains limited. An improved understanding of these mechanisms in lactic acid bacteria would likely provide new strategies for commercial strain improvement.

Although knowledge of conjugal mechanisms in lactic acid bacteria is limited, sequential models for the physical events in conjugation are available from studies of fertility

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plasmid transfer in *Escherichia coli* (12, 35). The first step in this process involves physical contact between donor and recipient cells to establish a stable mating pair. As a result, conditions that impede pair formation diminish conjugal efficiency, and those that promote cell-cell contact may be expected to enhance the frequency of conjugation. Development of stable cell-cell contact between Gram-negative bacteria requires sex pili, which are produced by the donor cell (12). Donor and recipient pair formation between Gram-positive cells, however, must involve distinct mechanisms because these organisms do not produce pili. Analysis of hemolysin plasmid conjugation in *Enterococcus faecalis* has suggested that stable pair formation in this genus is initiated by recipient synthesis of sex pheromones (6). These pheromones are small peptides that trigger protein synthesis from specific conjugative plasmids in donor cells. One of the proteins produced by the donor in response to the pheromone is called aggregation substance. This protein associates tightly with a component on the recipient cell surface, termed binding substance, in a reaction that is thought to facilitate stable pair formation (5, 6). Although pheromone production has not been detected in lactococci, evidence suggests that at least one conjugal mechanism in these organisms may involve proteins, the functions of which are analogous to aggregation and binding substances in *E. faecalis* (31).

Conjugation among lactococci was first demonstrated in the late 1970s by Gasson and Davies (10) and Kempler and McKay (13), each of whom noted conjugal exchange of lactose-fermenting ability (**Lac$^+$**). Subsequent investigators (11, 33) noted that some Lac$^+$ transconjugants from *Lactococcus lactis* ssp. *lactis* strains 712 or ML3 formed tight aggregates in broth that resisted vortex dispersion. Since then, this phenomenon has also been noted in Lac$^+$ transconjugants of strains 33-4 (7) and C_2O (14). In each of those reports, Lac$^+$ transfer from transconjugants that exhibited self-aggregation (**Clu$^+$**) was detected at frequencies 10^2 to 10^5 times higher than those noted with the parental strain. Analysis of these transconjugants revealed that all Clu$^+$ and some Clu$^-$ transconjugants acquired a large novel plasmid, which was formed by cointegration between the parental lactose plasmid and an independent plasmid (2, 14, 34) or a portion of the bacterial chromosome (9). Further study demonstrated that cointegration was mediated by an insertion sequence, IS*S1S* (23), located on the parental lactose plasmid. Investigators also found that plasmid recombination occurred in more than one orientation (2, 34) and that this feature actually determined whether or not a transconjugant could express Clu$^+$ (2, 9).

Van der Lelie et al. (31) recently demonstrated that Clu$^+$ is actually the result of an interaction between two lactococcal cell surface components designated as Clu and aggregation substance (**Agg**). The Agg is synthesized constitutively by many lactococci, but production of Clu occurs only in cells that contain a cointegrate lactose plasmid in the Clu$^+$ orientation (2, 9). Self-aggregation occurs when both gene products are expressed by the same bacterium. The functional analogies between lactococcal Clu and Agg components and enterococcal aggregation and binding substances suggest that Clu$^+$ in lactococci may be important for stable mating pair formation (9). Despite such an intriguing analogy, lactococcal Clu and Agg have not been identified or characterized. The objectives of this study were to investigate the physical and functional characteristics of Clu$^+$ in Lac$^+$ transconjugants of *L. lactis* ssp. *lactis* ML3. Experiments that reversibly disrupted Clu$^+$ on donor cells confirmed that Clu$^+$ was required for high frequency Lac$^+$ transfer and indicated that donor cell surface proteins were involved in cell aggregation. Analysis of lactococcal cell surface proteins identified a unique 125-kDa protein on Clu$^+$ donors that may be the lactococcal Clu.

MATERIALS AND METHODS

Bacterial Strains and Plasmids

Lactococcal strains used in this study are shown in Table 1. Cultures were stored at 4°C and maintained by biweekly transfers in M17 broth (29) that contained .5% glucose or lactose (M17-G or M17-L) as the sole carbohydrate source. Lactococcal cultures were grown at 30°C. *Escherichia coli* strain V517 (16) was propagated in brain-heart infusion broth (BBL Microbiology Systems, Cockeysville, MD) at 37°C with aeration.

TABLE 1. *Lactococcus lactis* ssp. *lactis* strains used in the study.

Strain	Plasmids	Relevant phenotype[1]	Description	Reference
	(kb)			
ML3	104, 55, 48.4, 8.5, 3.0, 1.5	$Lac^+Clu^-Str^sEry^s$	Parental Lac^+ donor	(15)
LM0230	ND[2]	$Lac^-Str^sEry^s$	Plasmid-cured derivative of C2	(8)
LM2301	ND[2]	$Lac^-Str^rEry^s$	Plasmid-cured, Str^r derivative of strain C2	(18)
LM2306	ND[2]	$Lac^-Ery^rStr^r$	Plasmid-cured, Ery^r derivative of strain C2	(25)
JB0213	4.9	$Lac^-Str^sEry^r$	LM0230 electrotransformed with pGK13	(27); This laboratory
MMS367	2	$Lac^-Str^rEry^sRif^r$	Str^r derivative of MMS336	(26)
HW048	104	$Lac^+Clu^+Str^rEry^s$	Transconjugant of ML3 × LM2301	This study
HW001	104	$Lac^+Clu^-Str^rEry^s$	Transconjugant of ML3 × LM2301	This study
HW05	104, 4.9	$Lac^+Clu^+Ery^rRif^s$	Transconjugant of ML3 × JB0213	This study

[1]Abbreviations: Clu, ability (+) or inability (–) to self-aggregate; Ery, erythromycin-resistant (r) or erythromycin-sensitive (s); Lac, ability (+) or inability (–) to ferment lactose; Rif, rifampicin-resistant (r) or rifampicin-sensitive (s); Str, streptomycin-resistant (r) or streptomycin-sensitive (s).

[2]No detectable plasmid DNA.

Plasmid DNA was isolated from lactococci by the method of Anderson and McKay (1) and from *E. coli* V517 by the alkaline lysis procedure of Maniatis et al. (17). The presence of plasmids in cell lysates was established by electrophoresis in .6% agarose gels at 1.5 V/cm for 14 h with plasmid standards from *E. coli* V517.

Conjugal Matings

Conjugations were performed by either the direct plate conjugation (**DPC**) technique (4) or by the solid surface milk agar method of McKay et al. (18). For DPC, 10 ml of M17 broth were inoculated with .1 ml of an 18-h culture and then incubated at 30°C for 4 h (absorbance at 600 nm = .6; approximately 10^7 to 10^8 cfu/ml). Cells were harvested by centrifugation at 4300 × *g* for 10 min, washed with one volume of .85% saline, and then suspended in .1 volume of saline. Donor and recipient cells were mixed 1:2, and .1 ml of the cell mixture was plated directly on bromcresol purple (**BCP**)-lactose indicator agar (19) that contained 600 U/ml of streptomycin sulfate or 5 μg/ml of erythromycin (Sigma Chemical Co., St. Louis, MO). Lactose-positive transconjugants were verified by phenotypic characterization and plasmid analysis. Transfer frequencies were expressed as the number of transconjugants per colony-forming unit of the donor, and the values reported are the means from at least two separate experiments.

Donor Cell Treatments

Observations in this laboratory indicated that Clu^+ transconjugants of ML3 dissociated when they were suspended in STE buffer (200 m*M* NaCl, 1 m*M* EDTA, 10 m*M* Tris; pH 8.0) or TES buffer (50 m*M* NaCl, 5 m*M* EDTA, 30 m*M* Tris·HCl; pH 8.0). To identify the buffer components responsible for this phenomenon, the influence of various buffers on cell aggregation was investigated. Buffers used in these experiments included: .85% saline (control), TES, STE, .5 *M* EDTA, .5 *M* ethylene glycol-bis(β-aminoethyl ether) N,N,N′,N′-tetraacetic acid (**EGTA**), buffer A (237.5 m*M* NaCl, 20 m*M* Tris; pH 8.0), buffer B (200 m*M* NaCl, .5 m*M* EDTA, 10 m*M* Tris; pH 8.0), buffer C (100 m*M* NaCl, .5 m*M* EDTA, 5 m*M* Tris; pH

8.0), or buffer D (200 m*M* NaCl, .05 m*M* EDTA, 10 m*M* Tris). The Clu^+ cells were grown and harvested as described for DPC and washed with one of the buffers; then the cell suspension was visually examined to determine whether Clu^+ had been disrupted.

To determine whether dissolution of Clu^+ also produced a decrease in the efficiency of Lac^+ conjugation, Clu^+ cells of the high frequency Lac^+ donor HW048 were washed with TES buffer and then used in matings with LM2306. The HW048 cells for these matings were grown, harvested, and washed with saline as described and then washed twice with one volume of TES buffer.

The HW048 cells were also exposed to various enzymes to analyze the biochemical nature of Clu^+ cell surface components. Proteolytic enzyme treatments were performed with α-chymotrypsin or proteinase K (Boehringer Mannheim Biochemicals, Indianapolis, IN). Other enzyme treatments included α-amylase, β-amylase, dextranase, or cellulase (Sigma Chemical Co.). Before use, enzymes were dissolved in .1 *M* Na_2HPO_4 buffer, pH 7.0, and sterilized by passage through a .45-μm filter. The HW048 cells for enzyme treatments were grown, harvested, and washed with saline as described for DPC. The cell pellets were then suspended in 1 ml of enzyme solution, and the recipient LM2306 and control HW048 donor cells were suspended in the .1 *M* Na_2HPO_4 buffer alone. Cells treated with proteinase K (400 U/ml), dextranase (225 U/ml), or cellulase (25 U/ml) were incubated for 15 min at 37°C; those treated with α-chymotrypsin (400 U/ml), β-amylase (200 U/ml), or α-amylase (390 U/ml) were incubated for 15 min at 30°C. After incubation, cell suspensions were examined visually to determine whether enzyme treatment had disrupted Clu^+, and then the cells were used in conjugal matings with LM2306.

Isolation of Cell Surface Proteins

Cell surface proteins from Clu^+ and Clu^- transconjugants of ML3 were isolated and compared by SDS-PAGE. Cell surface extracts were prepared by a modification of the procedure described by Tortorello and Dunny (30). One-half liter of fresh M17-L broth was inoculated with 10 ml of an 18-h culture and incubated at 30°C for 24 h. The supernatant was collected after centrifugation at 4300 × *g* for 10 min, and then the protein fraction was concentrated by precipitation with 60% (wt/vol) of ammonium sulfate. The solution was stirred at 4°C for 1 h, and then the precipitate was concentrated by centrifugation at 6000 × *g* for 15 min. The pellet was suspended in 10 ml of 10 m*M* Tris·HCl buffer (pH 7.5), concentrated 20 times by polyethylene glycol (10,000 molecular weight) dialysis (28), and then dialyzed overnight at 4°C against double deionized water to a final volume of 1 to 2 ml.

The SDS-PAGE was performed as described by Ausubel et al. (3) in a New Protean II (Bio-Rad Laboratories, Hercules, CA) vertical electrophoresis system. Proteins were separated at room temperature (23 to 25°C) in 10% polyacrylamide gels (4.5% stacking gel) with a current of 20 mA/mm for 4.5 h and detected with brilliant blue R stain. Bio-Rad low molecular mass protein size standards were included in the gels to estimate the size of lactococcal cell-surface proteins.

Requirement for RNA Synthesis in Donor Cells for Conjugation

To determine whether donor cells required de novo RNA synthesis for Lac^+ conjugation, matings were performed between the Clu^+ Lac^+ donor HW05 and recipient MMS367 on BCP-lactose agar, which contained .5 mg/ml of rifampicin (21).

RESULTS

Effects of Donor Cell Treatments on Clu^+ and High Frequency Lac^+ Transfer

Cells of HW048, a Clu^+ high frequency Lac^+ donor, were washed with various buffers to determine the reagent in these solutions that dissociated Clu^+. As illustrated in Figure 1 and summarized in Table 2, HW048 cells washed with solutions that contained EDTA (≥.5 m*M*) or EGTA dissociated, but those washed with solutions that lacked these reagents remained Clu^+. When the HW048 cells, which had been washed with TES buffer to disrupt Clu^+, were mated with LM2306, Lac^+ transfer efficiency was substantially lower than that of control donor cells washed with saline (Table 3). Simi-

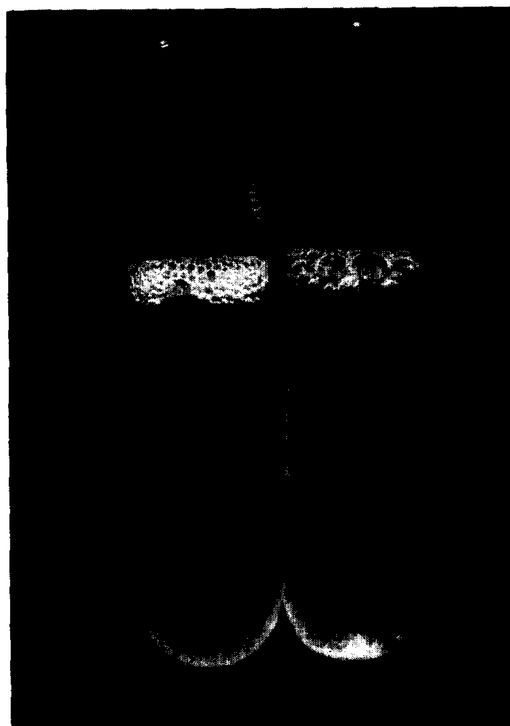

Figure 1. Effect of solutions that contained EDTA on HW048 donor cell clumping. Cells on the left were suspended in TES buffer (50 m*M* NaCl, 5 m*M* EDTA, 30 m*M* Tris; pH 8.0), and those on the right were suspended in .85% saline.

lar experiments performed on solid surface milk agar did not detect any significant change in conjugal frequency (data not shown).

Further analysis of this phenomenon revealed that, if 10 μl of .1 *M* $CaCl_2$ or .1 *M* $ZnSO_4$ were added (final concentration approximately 1 m*M*) to TES-treated cells of HW048, Clu^+ was immediately restored. In each case, HW048 donor cells that regained Clu^+ also recovered high frequency, Lac^+ transfer capability in DPC matings (Table 3). Similar levels of Mn^{2+} or Mg^{2+} also produced reaggregation in EDTA-dissociated HW048 cells. Yagi et al. (37) discovered that divalent ions such as Ca^{2+}, Mg^{2+}, Mn^{2+}, or Co^{2+} were required for *E. faecalis* cell aggregation, and others have shown that Zn^{2+} is involved in mating pair formation in *E. coli* (22).

Effects of Donor Cell Enzyme Treatment

To investigate the biochemistry of Clu^+, HW048 cells were briefly exposed to various enzymes, and then conjugations were performed. When HW048 was incubated with proteinase K or α-chymotrypsin, Clu^+ disappeared, and Lac^+ transfer efficiency decreased approximately 100-fold. In contrast, other enzyme treatments to HW048 did not affect Clu^+ or Lac^+ transfer efficiency (Table 3). When proteinase K was used to treat HW048 cells in similar experiments on solid surface milk agar, no changes in Lac^+ transfer frequency were detected (data not shown).

Additional matings were designed to determine whether proteinase K-treated HW048 cells could regain Clu^+ and high frequency Lac^+ transfer ability. After enzyme treatment, the cells were centrifuged (16,000 × *g* for 15 s), suspended in .1 volume of M17-L broth,

TABLE 2. Effect of cell wash treatments[1] on self-aggregation substance (Clu^+) in HW048.

Wash solution	Composition	Clu
Saline	.85% NaCl	+
TES	50 m*M* NaCl, 5 m*M* EDTA, 30 m*M* Tris	−
STE	200 m*M* NaCl, 1 m*M* EDTA, 10 m*M* Tris	−
.5 m*M* EDTA		−
.5 m*M* EGTA		−
Buffer A	237.5 m*M* NaCl, 20 m*M* Tris	+
Buffer B	200 m*M* NaCl, .5 m*M* EDTA, 10 m*M* Tris	−
Buffer C	100 m*M* NaCl, .5 m*M* EDTA, 5 m*M* Tris	−
Buffer D	200 m*M* NaCl, .05 m*M* EDTA, 10 m*M* Tris	+

[1]Cells were prepared as described for direct plate conjugation, harvested and washed with one of the solutions listed, and then visually examined for disruption of Clu^+.

TABLE 3. Effects of donor cell treatments on self-aggregation (Clu$^+$) and the frequency of lactose plasmid transfer.[1]

Treatment	Clu	Transfer frequency[2]
Donor cell washes[3]		
Saline (control)	+	7.8×10^{-2}
TES	–	2.4×10^{-3}
TES and then add $CaCl_2$[4]	+	4.4×10^{-2}
TES and then add $ZnSO_4$[4]	+	2.1×10^{-2}
Enzyme treatments[5]		
Control[6]	+	3.2×10^{-2}
α-Chymotrypsin	–	7.0×10^{-4}
Proteinase K	–	1.9×10^{-4}
α-Amylase	+	1.1×10^{-2}
β-Amylase	+	2.1×10^{-2}
Cellulase	+	4.7×10^{-2}
Dextranase	+	2.1×10^{-2}

[1]HW048 × LM2306 by direct plate conjugation (DPC) method.

[2]Lactose-fermenting transconjugants per colony-forming unit of the donor.

[3]Cells prepared as described for DPC, harvested, washed with one of the solutions listed, and then used in DPC.

[4]Final divalent ion concentration in the cell mixture approximately 1 m*M*.

[5]Cells prepared as described for DPC, harvested, and then suspended with enzyme for 15 min before DPC was performed.

[6]Donor cells suspended in .1 *M* Na_2HPO_3 (pH 7.0) without enzyme for 15 min before DPC was performed.

and incubated at 30°C for 0, 5, 10, 15, and 20 min. The cells were collected by centrifugation, suspended in .1 volume of saline, and used as donors in DPC matings. As shown in Table 4, Lac$^+$ transfer efficiency from cells treated with proteinase K gradually improved with time, and high frequency transfer was restored in cells that had been incubated for 20 min. No significant change in Lac$^+$ transfer frequency was detected when untreated control cells were harvested and incubated in fresh M17-L broth for 10 or 20 min at 30°C (data not shown).

Comparison of Cell Surface Proteins of Clu$^+$ and Clu$^-$ Transconjugants

Cell surface proteins were isolated from Lac$^+$Clu$^+$ and Lac$^+$Clu$^-$ transconjugants that contained 104-kb cointegrate plasmids (2), from Lac$^+$Clu$^-$ transconjugants that harbored the 55-kb Lac plasmid only (2), and from the plasmid-free recipient, LM2301 (18). As shown in Figure 2, SDS-PAGE gels revealed that Clu$^+$ transconjugants expressed a unique cell surface protein of approximately 125,000 Da, which was absent in Clu$^-$ transconjugants that contained a 104-kb cointegrate lactose plasmid. This protein was also not detected on the recipient LM2301 or on Lac$^+$Clu$^-$ transconjugants that harbored only the 55-kb Lac plasmid (data not shown).

TABLE 4. Effect of broth incubation time after enzyme treatment on high frequency lactose plasmid transfer.[1]

Donor treatment	Incubation time before mating	Transfer frequency[2]
	(min)	
None	0	2.5×10^{-2}
Proteinase K	0	3.2×10^{-4}
Proteinase K	5	1.7×10^{-3}
Proteinase K	10	2.0×10^{-3}
Proteinase K	15	4.7×10^{-3}
Proteinase K	20	1.3×10^{-2}

[1]HW048 × LM2306 by direct plate conjugation method.

[2]Transconjugants with the ability to ferment lactose (Lac$^+$) per colony-forming unit of the donor.

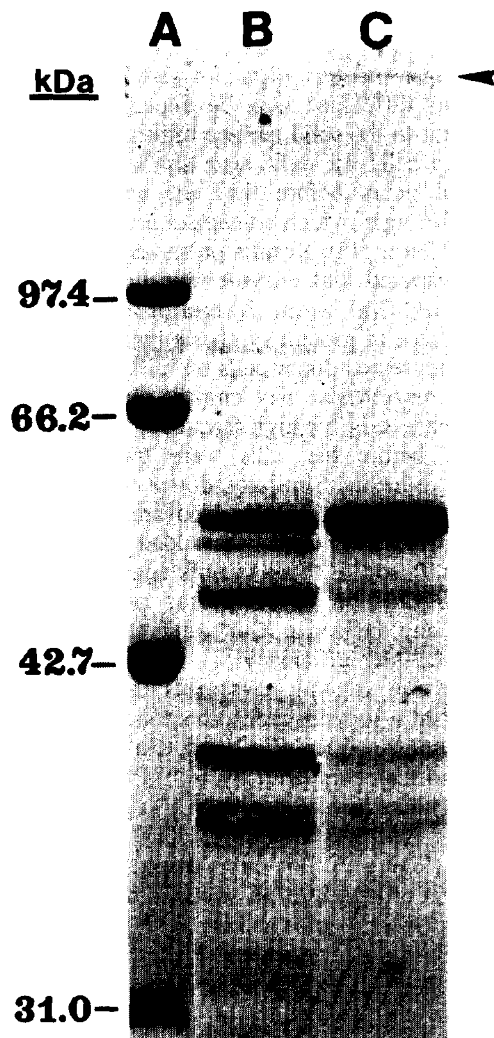

Figure 2. The SDS-PAGE separation of cell-surface proteins isolated from lactose-positive, aggregation-positive (Lac^+Clu^+) and lactose-positive, aggregation-negative (Lac^+Clu^-) transconjugants of ML3. Included in the gel were protein molecular weight standards (A), the Lac^+Clu^- transconjugant HW001 (B), and the Lac^+Clu^+ transconjugant HW048 (C). Molecular mass values for protein standards are provided in kilodaltons.

RNA Synthesis in Donor Cells During Conjugation

Matings designed to investigate whether donor cells required de novo RNA synthesis to conjugate revealed that Lac^+ transfer was unimpeded on BCP-lactose agar that contained .5 mg/ml of rifampicin 10^{-1} transconjugants/donor CFU.

DISCUSSION

The objectives of this study were to investigate physical and functional characteristics of Clu^+ in Lac^+ transconjugants of *L. lactis* ssp. *lactis* ML3. Van der Lelie et al. (31) recently reported that Clu^+ in lactococci results from an association between Clu and Agg cell surface components. These components appear analogous to the binding and aggregation substances produced during hemolysin plasmid transfer in *E. faecalis* (6), which supports the hypothesis that Clu^+ promotes high frequency Lac^+ transfer in lactococci via enhanced mating pair formation (9). Although apparently similar in function, the two systems are regulated quite differently. Stable pair formation in *E. faecalis*, for example, is initiated by recipient sex pheromones that induce protein synthesis from specific conjugative plasmids in donor cells (6). One of the proteins produced by the donor in response to the pheromone is an aggregation protein that associates tightly with binding substance located on recipient cell surfaces (5, 6). Because enterococcal binding substance is constitutively expressed in donors and recipients, induction of aggregation protein also results in donor self-aggregation (6). In similar fashion, lactococcal Clu^+ results from an interaction between two cell surface components, Clu and Agg (31), but pheromone synthesis has not been detected in these bacteria. Instead, expression of Clu becomes active after molecular rearrangement of the parental lactose plasmid, and self-aggregation is observed only with donor cells that also produce Agg (31). In spite of these regulatory differences, the functional similarities between the two systems are intriguing. Unfortunately, efforts to ascertain fully the homology between these two mechanisms for cell aggregation have been frustrated because lactococcal Clu and Agg substances have not been identified or characterized, and knowledge of lactococcal conjugation generally remains poor. A better understanding of conjugation in lactococci would be useful because this form of gene transfer has been effective for the improvement of dairy starter cultures (24).

This laboratory previously described the DPC conjugation method and reported that Lac^+ transfer between ML3 and LM2306 was unimpeded on agar that contained the protein

synthesis inhibitor erythromycin (4). In this study, high frequency transfer of Lac^+ (10^{-1} transconjugants per colony-forming unit from the donor) between HW05 and MMS367 was also detected on BCP-lactose agar that contained rifampicin. This result indicated that lactococcal donor cells do not require de novo RNA synthesis for conjugation and supported previous suggestions that lactococcal genes for conjugation are expressed constitutively (4, 31).

Results also demonstrated that Clu^+ cells dissociated when they were washed with solutions that contained the chelating agents EDTA or EGTA. As shown in Table 3, dissolution of Clu^+ with TES buffer resulted in a concomitant decrease in Lac^+ transfer efficiency, but when Ca^{2+} or Zn^{2+} ions were added to TES-treated cells, cell aggregation and high frequency conjugation ability were restored. Subsequent analysis showed that Mn^{2+} or Mg^{2+} also produced reaggregation among EDTA-dissociated cells of HW048. Yagi et al. (37) investigated cell aggregation in *E. faecalis* and found that EDTA dissociated donor cell aggregates because aggregation in that system required divalent cations such as Ca^{2+}, Mg^{2+}, Mn^{2+}, or Co^{2+}. Results from this study indicate a similar requirement in *L. lactis* ssp. *lactis*. Detailed analysis of conjugal pair formation in *E. coli* has shown that initial contact among donor and recipient cells occurs between the tips of donor pili and Zn^{2+} ions on recipient receptors (22). Although Gram-positive bacteria do not produce pili, the observations of Yagi et al. (37) and results from this study suggest that divalent ions may also be important for stable mating pair formation in Gram-positive species such as *E. faecalis* and *L. lactis* ssp. *lactis*.

Proteolytic enzyme treatment of Clu^+ donor cells also resulted in the loss of Clu^+ and dramatically reduced conjugation frequency (Table 3), suggesting that the enzymes had degraded proteinaceous cell surface components required for Clu^+ and high frequency Lac^+ transfer. This conclusion was supported by data demonstrating that Clu^+ and high frequency Lac^+ transfer were restored when cells were incubated briefly in media without protein synthesis inhibitors (Table 4).

Although the influence of EDTA and protease on Clu^+ and high frequency Lac^+ transfer were detected by the DPC method, similar results were not obtained with solid surface milk agar matings (data not shown). Reasons for this difference may be due to conditions inherent to the solid surface milk agar method. Mating cells on milk agar are incubated for several hours before they are transferred to selective agar, which contains a protein synthesis inhibitor (18). Results presented in Table 4 demonstrated that enzyme-treated donor cells recovered Clu^+ within 20 min of incubation in media free of protein synthesis inhibitor. Thus, enzyme-treated donor cells on milk agar would likely resynthesize any enzyme-degraded proteins necessary for high frequency Lac^+ conjugation before the cells were transferred to selective media. Similarly, donor cells that had been washed in EDTA recovered Clu^+ immediately when Ca^{2+} was added to the cells (Table 3), so Ca^{2+} ions in milk agar would also restore Clu^+ on EDTA-treated cells and obscure any influence Clu^+ disruption had on Lac^+ transfer efficiency.

Because enzyme treatments indicated that Clu^+ had a proteinaceous component, cell surface proteins from Clu^+ and Clu^- cells were isolated and compared by SDS-PAGE. These experiments revealed a 125-kDa cell surface protein that was unique to Clu^+ transconjugants (Figure 2). For comparison, aggregation proteins produced by *E. faecalis* donors range in size from approximately 130 to 150 kDa (36). Because *L. lactis* ssp. *lactis* ML3 and C2 derivatives all express Agg (31) and because the protein isolated from Clu^+ donors was similar in size to enterococcal aggregation proteins, the 125-kDa protein isolated from Clu^+, but not Clu^-, cells may be the lactococcal Clu substance.

CONCLUSIONS

Early investigators (32, 34) of Lac^+ conjugation among lactococci noted a correlation between Lac^+ transconjugants that exhibited Clu^+ and ≥10,000-fold increases in the efficiency of Lac^+ transfer. Van der Lelie et al. (31) found that Clu^+ results from an interaction between two cell surface components, Clu and Agg. In *L. lactis*, Clu is present only after intermolecular rearrangement of the lactose plasmid, but Agg is expressed constitutively by some, but not all, lactococci. These sub-

stances may be analogous in function to aggregation and binding substances that are integral components of the hemolysin plasmid transfer system in *E. faecalis* (6). Although lactococcal Clu and Agg substances appear to be essential for high frequency Lac^+ transfer, the biochemistry and identity of both components remain unknown.

This study examined the biochemistry of Clu^+ and the effect of Clu^+ loss and restoration on Lac^+ transfer efficiency in Lac^+Clu^+ transconjugants of ML3. Two developments that facilitated this investigation were the DPC technique (4) and the observation that solutions containing EDTA disrupted cell aggregation. Because streptomycin in DPC plates inhibited de novo protein synthesis in donor cells during conjugation, cell surface components of the donor could be enzymatically degraded, and the impact of those treatments on Lac^+ transfer efficiency could be evaluated. Similarly, treatment of donor cells with solutions that contained EDTA allowed reversible disruption of donor cell aggregation and subsequent evaluation of Clu^+ loss or restoration on Lac^+ transfer efficiency.

Conjugation among bacteria must always begin with formation of a stable contact between donor and recipient cells. In *E. coli,* treatments that impede effective pair formation dramatically decrease the efficiency of gene transfer (22, 32). The correlation between Clu^+ and high frequency Lac^+ transfer in lactococci suggests that formation of donor-recipient pairs may be the rate-limiting step for conjugation among these bacteria. A better understanding of conjugation in lactococci would be useful because this form of gene transfer has been effective for the improvement of dairy starter cultures (24). This study demonstrated a direct relationship between Clu^+ and high frequency transfer of Lac^+ in transconjugants of *L. lactis* ssp. *lactis* ML3. Enzyme treatments suggested that Clu^+ included a proteinaceous component, and SDS-PAGE gels identified a large cell surface protein on Clu^+ donor cells. Because this protein was not detected on Clu^- cells, it may be the lactococcal Clu substance.

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