5-1992

Physical and Functional Events Involved in Conjugal Transfer of Lactose Utilization in Lactococcus lactis subsp. lactis

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PHYSICAL AND FUNCTIONAL EVENTS INVOLVED IN CONJUGAL TRANSFER OF LACTOSE UTILIZATION IN *LACTOCOCCUS LACTIS* SUBSP. *LACTIS*

by

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A thesis submitted in partial fulfillment
of the requirements for the degree

of

MASTER OF SCIENCE

in

Nutrition and Food Sciences

Approved:

UTAH STATE UNIVERSITY
Logan, Utah

1992

ACKNOWLEDGMENTS

I would like to express my sincere appreciation to Dr. Jeffery K. Kondo, my major advisor, for his advice and encouragement in my whole research program, his patience in the reviewing of this thesis, and his kind help in preparing my presentation at ADSA. My sincere thanks are also extended to Dr. Dennis L. Welker and Dr. Charles E. Carpenter for serving as members of my committee and for reviewing this thesis.

I would thank the lab colleagues, Kevin Gillies for providing some of the cultures in the research; Jeffery Broadbent for providing cultures, his friendly cooperation and discussions during the whole research; and Yat-Chen Chou for her concern and help when I first came to the United States.

Finally, I give my sincere thanks to my family for their support, love and understanding, especially to my dad, for his special love and support throughout my years in school. I thank the teachers and professors at Fudan University and the Shanghai Middle School for their encouragement and recognition, and for their influence on my attitude in both research and personality.

Hua Wang

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ABSTRACT

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by

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Utah State University, 1992

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The nature of the cell surface components involved in donor cell clumping (Clu^+) and the relationship of Clu^+ to high frequency conjugal transfer of lactose utilization (Lac) in *Lactococcus lactis* subsp. *lactis* ML3 was examined. Lactose positive (Lac^+), Clu^+ transconjugants, containing a novel 104 kilobase Lac plasmid, were obtained by mating ML3 with LM2301. When used as Lac^+ donors in second round matings, these transconjugants transferred Lac at high frequencies ranging from 10^{-2} to 10^{-4} transconjugants per donor CFU. Treatment of donor cells with EDTA and EGTA containing solutions or proteolytic enzymes (proteinase K and chymotrypsin A_4) resulted in a loss of Clu^+. By using a direct plate conjugation technique, these treatments also decreased the capacity fortransferring Lac at high frequency.

Analysis of cell-surface proteins by SDS-PAGE identified a novel protein of approximately 125 kDa which was present in Clu^+ transconjugants, but not in non-clumping transconjugants. These results suggest that Clu^+ is required for high frequency Lac transfer in ML3 transconjugants, and at least one large protein is involved in Clu^+. De novo synthesis requirements of donor cells for conjugal transfer of Lac were tested on direct plate conjugation technique. Results indicate that de novo protein synthesis and RNA synthesis are not required for conjugal transfer of Lac.

(62 pages)

CHAPTER I

INTRODUCTION

Lactic acid bacteria are essential in the fermentation of many food products, such as fermented meat, milk, cereals, and vegetable products. Often used as starter cultures, this nontaxonomic group of bacteria includes the genera of Lactobacillus, Lactococcus, Leuconostoc, Pediococcus, and Streptococcus. They produce acids from fermenting carbohydrates, lower the pH of foods, and inhibit food spoilage microorganisms. Also, lactic fermentations contribute to improved food flavor, texture, aroma, and nutritional values. Because of their importance in food processing and their contribution to human health, study of the metabolism and genetics of lactic acid bacteria has gained considerable scientific attention. Early studies of lactic acid bacteria were mainly focused on the metabolism and basic microbiological characteristics of these organisms. With the recent availability of biotechnological techniques, research has centered on the genetics and molecular biology of lactic acid bacteria. A comprehensive understanding of the genetics of these bacteria is necessary because it would enable the efficient improvement of the performance of industrial starter cultures.

Conjugation is now one of the most important gene

transfer systems in lactococci. As a natural gene transfer mechanism, it has been used to disseminate important functional plasmids to industrial starter culture strains. Further investigation into the mechanisms involved in conjugation is needed.

The purpose of this research is to study the physical and functional characteristics of conjugation in Lactococcus lactis subsp. lactis, focusing on the high frequency, cell aggregation system of L. lactis subsp. lactis ML3.

Chapter II is a literature review of gene transfer systems in lactococci, conjugation models, conjugation in Enterococcus faecalis, the model conjugation system for lactococci, and conjugation in lactococci. Chapter III contains the experimental design and research results which will be submitted to the journal of Applied and Environmental Microbiology. Chapter IV contains conclusions from this research.

CHAPTER II

HISTORICAL REVIEW

Fermentations involving lactic acid bacteria have been used for food preservation and development of new foods since early times (52, 89). During the fermentation process, microorganisms ferment sugar, hydrolyze proteins, and synthesize flavor compounds, which improve the quality (flavor, texture, body, aroma) and nutritional value of food products (38, 70). These microorganisms also decrease oxidation-reduction potential and produce substances such as acidic end products (mainly lactic acid and acetic acid), hydrogen peroxide, diacetyl, and bacteriocins, which all contribute to inhibiting spoilage microorganisms and extending the shelf-life of food (31, 52). Greater control of the wide variation of fermentation process and accelerated fermentation were achieved by deliberately adding starter cultures. The starter cultures were selected to make the fermentation processes more predictable, rather than relying on a natural fermentation with indigenous microflora of the substrate.

The lactic acid bacteria are essential as starter cultures in the manufacture of fermented milk, meat, vegetables, and cereal products. The species of lactic acid bacteria used in the process include the genera

Lactobacillus, Lactococcus, Leuconostoc, Pediococcus, and Streptococcus (52). The dairy industry is a foremost customer of these organisms and is the most advanced in the knowledge of starter culture usage and improvement. Dairy starter culture improvement has recently gained great attention with the discovery of plasmids coding for essential milk-fermenting characteristics such as lactose utilization, proteinase activity, and citrate utilization (56). Along with the increasing knowledge of metabolism, physiology, genetics in lactic acid bacteria, and the development of the genetic methodology, construction of "optimized" lactic acid bacteria by genetic engineering techniques is possible (56, 76).

Among the lactic acid bacteria, the lactococci are the targeted organisms for improvement because they are responsible for the fermentation process in many milk products. Also, compared to other lactic acid bacteria, they are relatively easy to handle in the laboratory. As many metabolic properties essential for the manufacture of fermented dairy products in lactococci are plasmid-mediated (84), it is easier to improve the strain performance using current biotechnological techniques.

GENE TRANSFER SYSTEMS IN LACTOCOCCI

There are several ways of transferring genetic information into target organisms. In lactococci,

transduction, conjugation, transformation, and protoplast fusion are the four well-documented gene transfer systems. These gene transfer techniques are not only essential for genetic analysis of important phenotypic traits coded by plasmid DNA, but also are vital for future dairy starter strain improvement using genetic engineering principles (for review, 50, 56, 76).

Transduction. Transduction is a bacteriophage-mediated gene transfer process during which cartain phages act as vectors and transfer host markers between bacterial cells (29, 50). It was the first gene transfer system reported in lactic acid bacteria, and many important genetic advancements, such as the linkage of lactose utilization and proteinase activity to plasmid DNA, were achieved by transduction (54, 57). Transduction in lactococci was first described by Allen *et al*. (1) and Sandine *et al*. (77) in the early 1960's, using a virulent bacteriophage and chromosomal genetic markers for tryptophan biosynthesis and streptomycin resistance. However, most of the other studies have been achieved with temperate bacteriophages. In 1973, McKay *et al*. first reported the transduction of lactose metabolism using a temperate phage from *L. lactis* C2 (59). Transduction has also been reported in *L. cremoris* C3 (81), *L. lactis* C_2O (58), and *L. lactis* 712 (19). Outside of lactococci, transduction of a number of plasmids in *Streptococcus salivatius* subsp. *thermophilus* has been reported (62).

Among the gene transfer systems operating in the lactococci, transduction is not as relevant as conjugation and transformation, but it has made a significant contribution to the improved understanding of genetics of these bacteria (29, 34, 50).

Conjugation. The gene transfer mechanism of bacterial mating or conjugation is widely described in lactic acid bacteria (20, 29, 35, 36, 42, 58, 66, 74, 90, 95). In some strains, it is a highly efficient process and can support the transfer of large amounts of DNA. So far, conjugation has been the most useful genetic technique in studying lactic acid bacteria, especially lactococci (76). Conjugation will be described later in this paper.

Transformation. Transformation is a process in which a recipient cell acquires phenotypic traits by the uptake of free or naked DNA from the surrounding medium. Transformation has great potential in dairy starter strain improvement because of its high specificity of gene transfer. Also transformation is important because it provides the direct correlation between <u>in vitro</u> analysis of DNA and its <u>in vivo</u> function (50, 61). Transformation can be divided into four mechanisms: natural competence, artificially induced competence, protoplast-dependent systems, and electroporation. Protoplast transformation and electroporation have been the major transformation mechanisms used in lactococci.

Protoplasts are osmotically labile spheroplasts with the cell wall enzymatically removed, leaving the cell surrounded by the cell membrane (50). Protoplast formation and regeneration in L. lactis was first reported by Gasson in 1980 (33) and then Okamoto et al. in 1982 (68). A polyethylene glycol (PEG)-induced protoplast transformation of L. lactis was first reported in lactococci by Kondo and McKay (48, 49). The transfection (uptake of phage DNA) of protoplasts of L. lactis subsp. lactis biovar. diacetylactis was also reported by Geis in 1982 (37). Deletions of the plamid molecules is not generated in this protoplast transformation system (for review, 50). But this protocol is restricted to select strains, as the efficiency of protoplast formation, regeneration, and transformation varied from strain to strain, and the repeatability among laboratories, or operators, is not optimal.

Electroporation is a technique of introducing DNA into cells whose biological membranes have been damaged by high electric voltage shock (for review, 73). It is a technique that first found application in the transformation and transfection of many types of eukaryotic cells including mammalian cells, plant protoplasts, and intact yeast cells, and then bacteria protoplast systems (for brief review, 53). Harlander in 1986 (40) first reported electroporation of intact bacterial cells by transforming L. lactis subsp. lactis. Although the frequencies were hard to reproduce, it

proved that whole bacteria cells could be transformed by electroporation (61). Thus it offers a relatively simple, rapid and reliable alternative to protoplast transformation procedures. Recently, electroporation has been proven effective in many other strains, species, and genera (6, 7, 28, 41, 63, 82, 99, 100). The effectiveness and frequency of transformation may still vary considerably, and an optimal voltage and time constant must be found for each cell type and electroporation apparatus.

Protoplast fusion. Protoplast fusion is the genetic exchange mechanism when polyethylene glycol (PEG) induces the fusion or joining together of protoplasts. During this process, extentive recombination of genetic information from the two cells can occur (50). Protoplast fusion has been used in genetic studies and improvement of many different organisms, including animal cells, plant cells, and fungi (for brief review, 39). While the first successful fusions of bacterial protoplasts with PEG were performed within strains of Bacillus subtilis (78) and B. megaterium (30), protoplast fusion in lactococci was first reported by Gasson (33) in L. lactis subsp. lactis 712 derivatives in 1980. In Gasson's study (33), the recombination of the chromosomal markers, maltose utilization (Mal^+) and Str^r, and transfer of the plasmid markers, Lac^+ and Ery^r among L. lactis subsp. lactis 712 derivatives, were demonstrated. Okamoto et al. also described protoplast fusion among L. lactis subsp.

lactis 527 derivatives (67). In lactic acid bacteria, intergeneric transfer of plasmid and chromosomal genes by protoplast fusion has also been reported (17, 94).

Because the procedure of protoplast fusion is simple, the fusion recombinant is random, and both interspecific and intergeneric hybrids can be formed, this technique offers advantages for industrial strain improvement (33). Protoplast fusion also shows potential for genetic studies and gene manipulation at the cellular level (50). It has been reported that hybrid cells can express genes at an increased level (86). However, several problems are still associated with this protoplast related approach. The experiment is time consuming; different strains often require different optimized conditions for protoplast formation and regeneration; the efficiency of fusion recombinants formation appear to be strain specific; and the results are inconsistent.

CONJUGATION MODELS

Conjugation is a gene transfer process in which, when cells come into close physical contact with each other, the donor cell transfers a copy of its genetic information to the recipient cell. Compared to the other forms of bacterial gene transfer, conjugation is the one that involves cell to cell contact.

Usually the conjugal transfer process can be divided

into three stages: Specific donor-recipient pair formation, mobilization (preparation for DNA transfer) and DNA transfer, formation of a functional DNA (plasmid or chromosome) in the recipient (32).

Conjugation in gram-negative bacteria, especially Escherichia coli, has been extensively studied and may involve sex pili to facilitate mating pair formation (43). Conjugal transfer of plasmid DNA, especially the fertility (F) plasmid in E. coli, is the best understood system. Although considerable diversity in genetic organization and properties of plasmid-transfer system is now evident, the basic functions exhibited by F remain typical for gram-negative conjugation systems (11, 43, 98).

The F plasmid is a self-transmissible plasmid, which means it carries genes that determine the effective contact function, and it can prepare its DNA for transfer (32). The unique structure, F pilus, plays an essential role for the necessary cell interactions preceding the conjugal transfer of DNA. It is known that treatments inhibiting donor-recipient pair formation will inhibit conjugation (for review, 96). Studies at the gene level revealed that a 33.3-kb region of F plasmid DNA includes clustered genes and sites essential for plasmid fertility. Isolation and complementation analysis of transfer-defective mutants have identified cistrons required for F transmission and cistrons that confer its surface exclusion properties. These mutants

define tra genes M, J, A, L, E, K, B, V, C, W, U, N, F, H, G, S, T, D, and I. Other analyses also identified the existence of three more tra genes: Y, Q and Z. Most of the tra genes are involved in pili synthesis, while others are required for surface exclusion, pairing, nicking, displacing, and transportation of a single strand of DNA to the recipient (for review, 32, 43).

However, conjugal transfer systems in gram positive organisms do not involve pili. Of the gram positive bacteria, those within the family of Streptococcaceae (Streptococcus, Enterococcus, and Lactococcus) have been studied. The types of conjugation observed in these organisms include: 1) sex pheromone-induced transfer of plasmids in Enterococcus faecalis (16, 22), 2) transfer of plasmids that does not involve pheromones (12, 64) and 3) transfer of conjugative transposons (13). Among these, sex pheromone-induced transfer of plasmids in Enterococcus faecalis has been extensively studied. The mechanism of donor-recipient pair formation in this system is different from that of E. coli.

SEX PHEROMONE-INDUCED TRANSFER OF PLASMIDS IN ENTEROCOCCUS FAECALIS

Antibiotic resistance and production of bacteriocins and hemolysins are commonly found as plasmid-coded traits in Enterococcus faecalis (21). Conjugal-like plasmid-mediated

gene transfer in this species was first demonstrated by Jacob and Hobbs in 1974 (44). Research work in Dr. Clewell's lab also showed that a number of E. faecalis hemolysin plasmids are capable of self transfer, as well as mobilization of other plasmids and chromosomal genes (23, 69). However, the mechanism is different than E. coli.

In 1978, Clewell's research group at the University of Michigan first reported the discovery of a bacterial sex pheromone which induced cell aggregation and mating in E. faecalis (21). Some of the molecular aspects of pheromone-inducible plasmid transfer were worked out in studies of the tetracycline-resistance plasmid, pCF10 (24) and studies of the hemolysin plasmid pAD1 (15). It was demonstrated that donor cells responded to a recipient-produced sex pheromone (CIA) by synthesis of adherent, proteinaceous cell surface substances which facilitated the formation of mating aggregates (10, 14, 16, 21, 46, 65, 91, 92) and surface exclusion of related plasmids (26). In the pCF10 system, the sex pheromone which induces conjugative transfer of pCF10 has been proven to be a seven amino acid peptide (65). The regions of plasmid pCF10 that encodes antibiotic resistance and the pheromone response function have been identified (9), and the genes encoding pheromone-inducible antigens have been cloned and expressed (10). Similar results have been obtained with the hemolysin plasmid, pAD1 (for review, 14,

16).

CONJUGATION SYSTEM IN THE *LACTOCOCCUS LACTIS* SUBSP. *LACTIS*

Lactococcus lactis subsp. *lactis* conjugation systems do not appear to involve sex pheromones. In the lactococci, many important phenotypic traits are encoded by conjugative plasmids, plasmids which may be mobilized by conjugative plasmids, or mobile chromosomal DNA. These traits include carbohydrate fermentation (lactose and sucrose), inhibitor production and immunity (nisin, diplococcin, and bacteriocin), proteinase production, phage resistance, citrate metabolism, antibiotic resistance (streptomycin and erythromycin), and mucoidness (for review, 82). A better understanding of conjugation in *Lactococcus* is needed because it has been an effective strategy for the improvement of dairy starter cultures (5, 25).

Conjugal transfer of lactose catabolism was first described by Kempler and McKay (45) and Gasson and Davies (35, 36). Conjugal transfer of lactose genes has since been reported for a wide variety of *L. lactis* strains (18, 47, 55, 58, 79, 80) and for a lactose plasmid in *Lactobacillus casei* (8). Conjugation systems in *L. lactis* ML3 and 712 have been studied in detail (34).

Recent studies in *L. lactis* subsp. *lactis* ML3 have characterized both low and high frequency conjugal transfer

of plasmid-encoded lactose utilization (Lac^+). Initial studies of L. lactis subsp. lactis ML3 showed that this strain contains a 33 MDa plasmid coding for lactose metabolism (pSK08) and three cryptic plasmids of 5.5, 2 and 1 MDa (51). However, using an improved plasmid isolation procedure, two additional plasmids of approximately 27 MDa (pRS01) and 60 MDa are also observed in ML3 at low copy number (3, 97).

In conjugal matings using ML3 as a donor, transfer of pSK08 to plasmid-free L. lactis LM2301 occurred at low frequency (about 10^{-7} to 10^{-9} transconjugants/donor) and resulted in the majority of transconjugants exhibiting clumping (Clu^+) or cell aggregation when grown in broth. Examination of the plasmid profiles from Lac^+Clu^+ and Lac^+Clu^- transconjugants revealed the presence of a 60 MDa plasmid in the majority of transconjugants but only the 33 MDa Lac^+ plasmid in some. Restriction endonuclease analysis of plasmids isolated from transconjugants indicated that the 60 MDa plasmid was a cointegrate formed from the fusion of pSK08 and pRS01 (3). Subsequent experiments showed that the fusion event was mediated by an insertion sequence located on pSK08 (72). When Lac^+Clu^+ transconjugants were used as donors in second round matings, they were able to transfer lactose metabolism at high frequency (about 10^{-2} to one transconjugants/donor); Lac^+Clu^- transconjugants carrying the 60 MDa plasmid transferred Lac^+ at intermediate frequencies

(about 10^{-4} to 10^{-5} transconjugants/donor); and $Lac^{+}Clu^{-}$ transconjugants containing the 33 MDa plasmid transferred Lac^{+} at low frequency (3, 50, 97). Restriction analysis after insertional inactivation experiments indicated that transfer (Tra^{+}) and Clu^{+} phenotypes were associated with pRS01 (3).

When conjugal transfer of pLP712 in L. lactis was investigated using a donor carrying only the lactose plasmid and a plasmid-free recipient strain, similar observations were made. Enlarged lactose plasmids were detected in progeny which exhibit cell aggregation and elevated conjugation frequency (36). Restriction mapping analysis revealed that related groups of enlarged plasmids had received identical regions of a chromosomal DNA although the amount of this additional DNA varied in size (34). In L. lactis ML3 and 712, high frequencies of conjugal transfer of lactose utilization were consistently correlated with donor cell aggregation (3, 97, 36). This suggested that an enhanced cell-cell contact prior to DNA transfer might be important in high frequency conjugation. Transfer of enlarged lactose plasmids to a wider range of lactococcal strains led to the identification of a new chromosomal factor, agg, which is essential for cell aggregation and subsequent high-frequency conjugal transfer (93). It was proposed that cell aggregation depends on the interaction of two cell surface components (34). One is present only after intermolecular rearrangement of the lactose plasmid and is controlled by a

gene *clu* on plasmid pRS01 in *L. lactis* ML3 or the equivalent chromosomally located sex factor in 712. Another is a constitutively expressed component controlled by a chromosomal factor, *agg*. Some lactococcal strains such as ML3 and 712 contain *agg*, but some others do not (93). Cell aggregation and associated high-frequency transfer thus only occur when both *agg* and *clu* are expressed. Although these genes have been identified, their gene products have not been extensively studied.

CHAPTER III

PHYSICAL AND FUNCTIONAL EVENTS INVOLVED IN CONJUGAL TRANSFER OF LACTOSE UTILIZATION IN *LACTOCOCCUS LACTIS* SUBSP. *LACTIS*

INTRODUCTION

Conjugation has proven very useful in studies of the genetics and plasmid biology of lactococci. Conjugation has also been used to increase bacteriophage resistance in lactococcal strains for the dairy industry. Because it is a natural process, strains which are improved by conjugation have been used in industrial applications (75). Therefore, a detailed understanding of conjugal mechanisms in lactic acid bacteria is needed. Except for the pheromone-induced cell-to-cell adhesion system of *Enterococcus faecalis*, conjugal mechanisms in gram positive bacteria remain poorly understood.

During the 1980's, reports from Dr. Gasson's laboratory in England and Dr. McKay's lab at Minnesota indicated that some Lac^+ transconjugants from strain 712 or ML3 formed tight pellets in broth that resisted vortex dispersion (3, 36, 97). Since then, the phenomenon has been observed in Dr. Dunny's lab on strain 33-4 (25) and in this lab on strain C_2O. These transconjugants were able to transfer lactose utilization at frequencies of 10^{-2} to 10^{-1} transconjugants per donor CFU (3,

25, 36, 97) during second round matings, which is 10^4 to 10^6 times higher than those for parental strains. These transconjugants all contained a novel plasmid which was larger than the original Lac plasmid (for review, 34). The inserted DNA was a sex factor that began as either an independent plasmid (3) or a bacterial chromosomal factor (29). Restriction mapping in ML3 facilitated the identification of the clu and tra regions on the pRS01 that were involved in controlling both high-frequency transfer and cell aggregation. The consistent correlation between Clu^+ and transfer frequency changes in the conjugation systems of L. lactis 712 and ML3 suggests that donor cell aggregation is a prerequisite for high-frequency conjugation.

Recent reports support the hypothesis that another chromosomal factor, agg, may be involved in cell aggregation (93). Conjugal transfer of Clu^+ plasmids into different Lactococcus lactis strains revealed a correlation between donor cell aggregation and high frequency conjugal transfer. It has been observed that when some Clu^+ plasmids were transferred into some L. lactis strains, transconjugants were obtained that do not aggregate. But when these transconjugants were used as donor cells and mated with L. lactis subsp. lactis 712 derivatives, they retained high frequency conjugal transfer ability, and the resulting progeny aggregated. Researchers also observed that when the mating mixtures were being set up, cell aggregation appeared

after certain recipients were mixed with donor cells, and, in these cases, the high frequency conjugation was observed. This led to the discovery of the chromosomal factor, agg, existing in certain L. lactis strains. Cell aggregation appears to depend on the presence of both agg and clu. It also provides positive evidence that cell aggregation is needed for high frequency conjugation, although the mechanism of how cell aggregation, especially donor aggregation, increases the DNA transfer from donor to recipient is unknown.

Using a direct plate conjugation technique (5), we were able to investigate the relationship between the clumping phenotype and the high frequency conjugal transfer of lactose utilization, and the de novo synthesis requirements of this gene transfer. We identified a novel large cell surface protein in clumping transconjugants, which might be involved in facilitating conjugation in this specific system.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains used in this study are described in Table 1. Figure 1 is the agarose gel patterns of plasmid DNA from some of these strains. Lactococcus lactis strains were grown in M17 broth (88) containing lactose (M17-L) or glucose (M17-G) at 30^0C.

Plasmids were isolated by the method of Anderson and McKay (2). Agarose gel electrophoresis was performed using

TABLE 1. Lactococcus strains used in this study (I)

Strain	Plasmid(Kb)	Relevant Phenotype	Derivation/References
LM0230	ND[a]	$Lac^-Str^sEry^sMal^+$	Plasmid-cured derivative of C_2 (27)
LM2301	ND	$Lac^-Str^rEry^sMal^+$	Str^r derivative of LM0230 (80)
LM2306	ND	$Lac^-Ery^rStr^rMal^-$	$Str^rEry^rMal^-$ derivative of LM0230 (83)
ML3	104, 55, 48.4, 8.5, 3.0, 1.5	$Lac^+Clu^-Str^sEry^s$	Parent (51)
HW048	104	$Lac^+Clu^+Str^rEry^s$	Lac^+ transconjugant obtained from ML3 x LM2301 matings (this study)
HW007	104	$Lac^+Clu^+Str^rEry^s$	Lac^+ transconjugant obtained from ML3 x LM2301 matings (this study)
HW001	104	$Lac^+Clu^-Str^rEry^s$	Lac^+ transconjugant obtained from ML3 x LM2301 matings (this study)

[a]No detectable (ND) plasmid DNA.

TABLE 1. Lactococcus strains used in this study (II)

Strain	Plasmid(Kb)	Relevant Phenotype	Derivation/References
HW015	55	$Lac^{+}Clu^{-}Str^{r}Ery^{s}$	Lac^{+} transconjugant obtained from ML3 x LM2301 matings (this study)
JK522	55	$Lac^{+}Clu^{-}Str^{r}Ery^{s}$	pJK550 electroporated into LM2301 (K. Gillies and J. K. Kondo, unpublished data)
MMS367	2	$Lac^{-}Str^{r}Ery^{s}Rif^{r}$	Str^{r} derivative of MMS336 (3), cured of all plasmids except the 2 MDa one (85).
JB0213	4.9	$Lac^{-}Str^{s}Ery^{r}Rif^{s}$	LM0230 carrying pGK13 (J. Broadbent and J.K. Kondo, unpublished data)
HW05	4.9, 104	$Lac^{+}Clu^{+}Ery^{r}Rif^{s}$	Lac^{+} transconjugant from mating ML3 and JB0213 (this study)
HW07	4.9, 104	$Lac^{+}Clu^{+}Ery^{r}Rif^{s}$	Lac^{+} transconjugant from mating ML3 and JB0213 (this study)

FIG. 1. Agarose gel electrophoretic patterns of plasmid DNA isolated from Lactococcus lactis subsp. lactis strain ML3 (lane A), $Lac^{+}Clu^{-}$ transconjugants HW001 (lane B) and HW015 (lane C), $Lac^{+}Clu^{+}$ transconjugants HW048 (lane E) and HW007 (lane G), Ery^{r} recipient JB0213 (lane F), $Lac^{+}Clu^{+}Ery^{r}$ transconjugants HW05 and HW07 (lane H and lane I). Numbers represent the reference plasmid sizes. The **arrow** below indicates that the 104 kb plasmid existed in all Clu^{+} transconjugants and some of the Clu^{-} transconjugants.

a Wide Mini-Sub Cell gel apparatus (Bio-Rad Laboratories, Richmond, CA). Electrophoresis was performed at 80 V for 2-3 h or as conditions dictated.

Conjugal matings. Conjugation was performed using direct plate conjugation (DPC) methods (5) and the conventional milk agar conjugation technique (58). For DPC, ten ml of M17 broth was inoculated with 0.1 ml of an 18 h culture and incubated at 30^0C for about 4 h ($O.D._{600}$ of 0.6; 10^7-10^8 CFU/ml). Cells were harvested by centrifugation at 4300 x g for 10 min, washed with one volume of 0.85% saline, and then suspended in 0.1 volume of saline. Donor and recipient cells were mixed at a ratio of 1:2, and 0.1 ml of the mixture was plated directly on bromo-cresol purple lactose indicator agar plates (BCP-lac) (60) containing 600 U/ml streptomycin sulfate (Sigma Chemical Co., St. Louis, MO) or 5 μg/ml erythromycin (Sigma Chemical Co., St. Louis, MO). Lactose positive (Lac^+) transconjugants were scored, purified by single colony isolation, and grown in M17-L broth.

Washing and enzyme treatment of donor cells. Clumping donor cells were washed with different solutions to identify if any soluble cell surface components were involved in donor cell clumping and high frequency conjugation. The relationship between the changing of the clumping phenotype and the frequency of conjugation was also determined. Donor cells were grown as above for conjugation, harvested by centrifugation at 4300 x g for 10 min, and washed with one

volume of saline (0.85% NaCl). The cell pellets were then washed twice (5 min per wash) with one volume of different washing solutions: saline, TES (50 mM NaCl, 5 mM EDTA, 30 mM Tris-HCl) buffer, STE (200 mM NaCl, 1 mM EDTA, 10 mM Tris) buffer, solution A (237.5 mM NaCl, 20 mM Tris), B (200 mM NaCl, 0.5 mM EDTA, 10 mM Tris), C (100 mM NaCl, 0.5 mM EDTA, 5 mM Tris), D (200 mM NaCl, 0.05 mM EDTA, 10 mM Tris), E (0.5 M EDTA), or F (0.5 M EGTA). Cells were harvested, suspended in 0.1 volume of saline, and then used in conjugal matings.

Matings were also performed by adding back zinc and calcium cations to washed cells. After washing and suspension of cells in 0.1 volume of saline as above, 10-20 μl of 0.1 M zinc sulfate, or 10 μl of 0.1 M calcium chloride were added to the cell mixture. Cells were used as donor in conjugal matings. For a control the cell mixture was washed once more with saline before being resuspended in 0.1 ml saline, without adding any cations. Cells were then used as donor in conjugal matings.

Clumping donor cells were also treated with different kinds of enzymes to identify the nature of the clumping substance and the effect that the treatment would have on high frequency conjugation. Donor cells were harvested by centrifugation at 4300 x g for 10 minutes and washed once with one volume of saline. The cell pellets were then treated with 0.1 volume of proteinase K (400 U/ml), dextrase (225 U/ml), or cellulase (25 U/ml) at 37^0C, or chymotrypsin

A_4 (400 U/ml), beta-amylase (200 U/ml), alpha-amylase (390 U/ml) at 30^0C for 15 minutes. The enzymes were dissolved in buffer containing 0.1 M Na_2HPO_4 (pH 7.0). The treated cells were then used as donors in conjugal matings. Conjugation frequency changes were compared to an untreated control mating to determine if treatments decreased the conjugation frequency.

Other matings were performed to determine if treated cells could resynthesize cell surface components necessary for high frequency conjugation. Enzyme-treated donor cells were given a short incubation in antibiotic-free M17-L broth to allow resynthesis of cell surface components. The enzyme-treated cells were centrifuged (16,000 x g, 15 s), suspended in 0.1 volume of M17-L broth, and incubated at 30^0C for 0, 5, 10, 15, and 20 min. Cells were centrifuged and cell pellets were then suspended in 0.1 volume of saline and used as donor cells in conjugal matings.

All conjugal matings and cell treatments were performed at least twice, and the values reported represent an average. When treated donor cells were used in matings, frequencies were always compared to an untreated control.

Cell surface antigen isolation. Cell surface proteins from Clu^+ and Clu^- transconjugants were compared to see if any additional cell surface protein(s) existed in Clu^+ strains. Cell surface extracts were prepared by modifying the methods described by Tortorello and Dunny (92) and Tang et al. (87).

Five hundred ml fresh M17 broth was inoculated with 10 ml of an 18 h L. lactis subsp. lactis M17-L broth culture and incubated at 30°C for 24 h. The culture supernatant was collected after centrifugation at 4300 x g for 10 min. Proteins were then concentrated by precipitating with 60% (w/v) ammonium sulfate. The mixture was stirred at 4°C for at least 1 h and then centrifuged at 6000 x g for 15 minutes. The precipitate was suspended in 10 ml of 10 mM Tris-HCl buffer (pH 7.5). The preparations were further concentrated to approximately 0.5 ml by polyethylene glycol (10,000 molecular weight) dialysis (87). The sample preparation was finished by volume-limited overnight dialysis at 4°C against double deionized water to a final volume of 1-2 ml.

Electrophoresis of proteins. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using the Bio-Rad New Protean II vertical electrophoresis system (4). Separating gels were constructed with 10% polyacrylamide in the resolving portion and 4.5% in the stacking portion. Electrophoresis was carried out at room temperature, with a current of 35 mA per 1.5 mm gel for 4.5 h. After electrophoresis the gel was subjected to brilliant blue R staining.

De novo synthesis requirements testing. To determine whether de novo protein synthesis was required for high frequency conjugation, this researcher performed matings on selective media containing the de novo protein synthesis

inhibitor streptomycin and erythromycin. Lactococcus lactis subsp. lactis strain LM 2301 ($Str^{r}Ery^{s}Lac^{-}$) and LM 2306 ($Str^{r}Ery^{r}Lac^{-}$) were used as recipient strains and mated with ML3 and the derivative HW048, respectively.

To determine if RNA synthesis was required in donors, we mated HW05, a $Clu^{+}Lac^{+}Ery^{r}Str^{s}Rif^{s}$ transconjugant from mating ML3 and JB 0213, with MMS367, a $Rif^{r}Str^{r}Rec^{-}$ derivative from ML3, on selective media containing 0.5 mg/ml of the de novo RNA synthesis inhibitor, rifampicin.

RESULTS

Effects of cell washing and enzyme treatment of cells on high frequency conjugation. Several buffer combinations were used to wash cells to determine if cell aggregation could be disrupted by washing. Results in Table 2 indicate that certain amount of EDTA or EGTA was required to dissociate the cell clumps. Clumping cells cannot be dissociated by washing with solutions containing no EDTA or EGTA or even very low concentration of EDTA. Figure 2 shows the phenotypic difference of broth-grown clumping HW048 donor cells in TES buffer (containing EDTA) and saline. When the clumping cells were suspended in saline (right), the colloidal distribution survived even after vortexing. However, when the cells were suspended in TES buffer (left), it gave a homogenous appearance.

Clumping donor cells of HW048, the $Lac^{+}Clu^{+}$

TABLE 2. Effect of cell washing on clumping phenomenon

Solution	Concentration	Effect on clump ($Clu^{+/-}$)
Saline	0.85% NaCl	++[a]
TES	50 mM NaCl, 5 mM EDTA, 30 mM Tris	-
STE	200 mM NaCl, 1 mM EDTA, 10 mM Tris	-
EDTA	0.5 mM EDTA	-
EGTA	0.5 mM EGTA	-
Solution A	237.5 mM NaCl, 20 mM Tris	+
Solution B	200 mM NaCl, 0.5 mM EDTA, 10 mM Tris	-
Solution C	100 mM NaCl, 0.5 mM EDTA, 5 mM Tris	-
Solution D	200 mM NaCl, 0.05 mM EDTA, 10 mM Tris	+

[a]++ strong clumping
+ moderate clumping
- no clumping observed

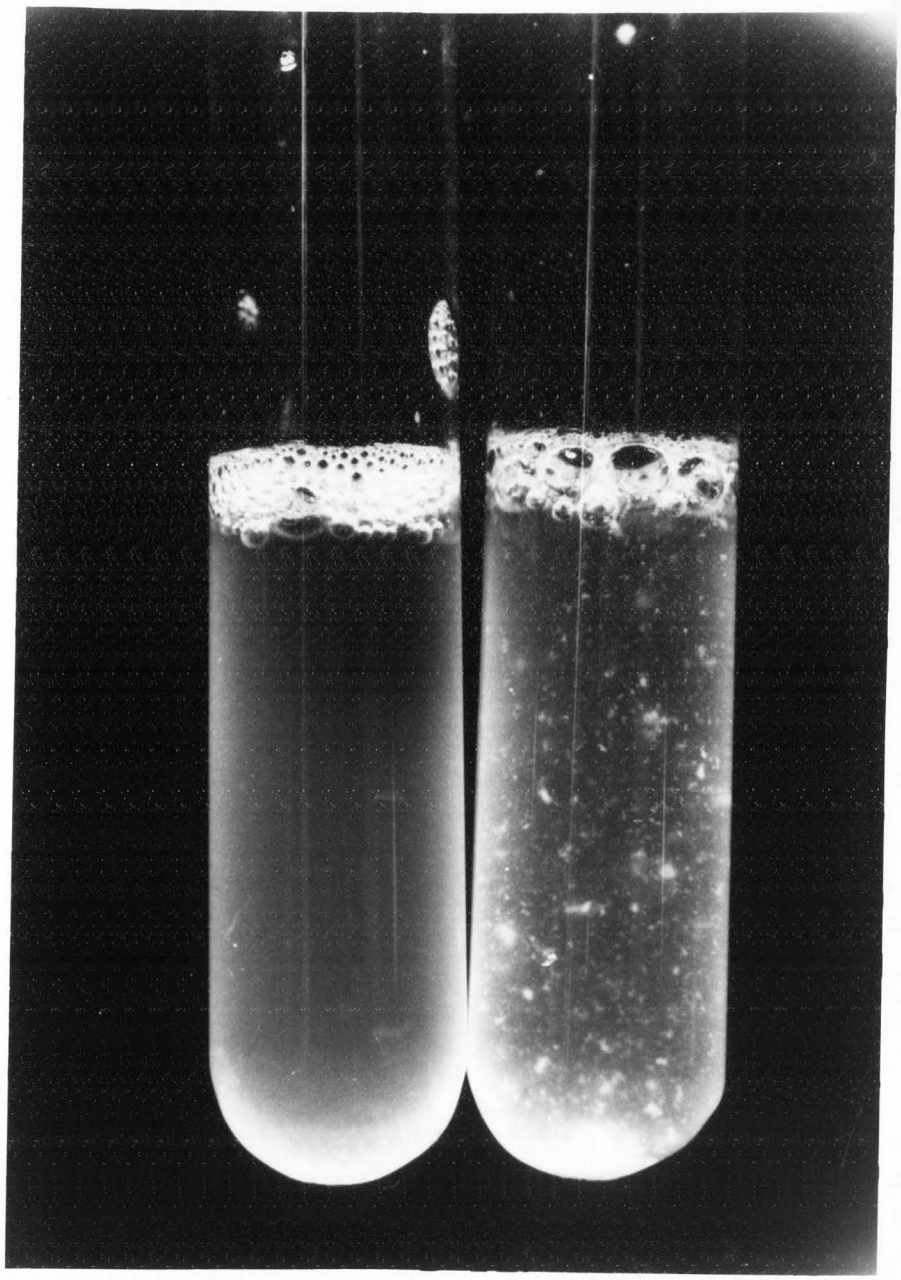


FIG. 2. Comparison of the clumping of $Lac^{+}Clu^{+}$ transconjugant HW048 when resuspended in TES buffer and saline. When washed with TES buffer (left), cells are dissociated.

transconjugant from mating ML3 and LM2301, were then washed with different buffers to determine if disruption of clumping by washing would have an effect on conjugation frequency. HW048 cell clumping was disrupted by washing with EDTA containing buffers STE and TES. The washed cells were then used as donors in DPC matings. Results in Table 3 indicate that washing decreases conjugation frequency from 6.4×10^{-2} to as low as 9.5×10^{-4}. When the same experiment was performed using conventional milk agar matings instead of DPC, no significant changes in frequency were observed as conjugation frequencies for washed cells were approximately 6.0×10^{-2} in all cases.

To test for the effect of any EDTA remaining in washed cells on matings, we added divalent cations, Ca^{2+} and Zn^{2+}, to the cell solutions. An immediate cell aggregation was then observed. Further observation indicated that the cells can also reaggregate if washed with saline again between buffer washing and saline resuspension. These reaggregated cells all recovered high frequency conjugal transfer capability in DPC matings (Table 4).

To determine the nature of clumping materials and their effects on high frequency conjugation, we treated clumping donor cells with different enzymes, and the conjugation frequencies in subsequent matings were compared. When cells of HW048 were treated with proteolytic enzymes proteinase K and chymotrypsin A_4, the cell clumping was destroyed and the

TABLE 3. Effect of cell washing on conjugation frequency[a]

Solution	Frequency of Conjugation	Standard Deviation
Saline	6.4×10^{-2}	3.0×10^{-2}
TES	1.3×10^{-3}	5.5×10^{-4}
STE	9.5×10^{-4}	1.6×10^{-4}
Solution A	7.9×10^{-3}	4.2×10^{-3}

[a] Conjugation was performed using DPC method.

TABLE 4. Effects of various treatments of HW048 cells on conjugation frequency[a]

Treatment	Clumping(+/-)	Conjugation Frequency
A. Saline wash	+	9.1×10^{-2}
B. TES wash	-	3.5×10^{-3}
C.		
1. TES wash	-	
2.* add Ca^{2+}	+	4.4×10^{-2}
D.		
1. TES wash	-	
2.** add Zn^{2+}	+	2.1×10^{-2}
E.		
1. TES wash	-	
2. Saline wash	+	6.3×10^{-2}

[a] Conjugation was performed using DPC technique.
* Calcium chloride solution with final concentration of 1 mM Ca^{2+} in the cell mixture.
** Zinc sulfate solution with final concentration of 1 to 2 mM Zn^{2+} in the cell mixture.

conjugation frequency was decreased at least 10-fold, while enzymes working on various kinds of polysaccharide bonds did not have the same effect (Table 5). This suggested that cell surface protein(s) may be responsible for cell aggregation and high frequency conjugation. When the same experiment was repeated using conventional conjugation techniques which allow for an 18 h mating incubation on milk agar plates before plating on selective media, a much higher conjugation frequency (1.2×10^{-2} transconjugants/donor) was observed for proteinase K treated cells. Significant decreases of conjugation frequency were not detected when compared with the control.

Because the decrease in conjugation frequency was not observed in conventional matings after treatment of donor cells with proteolytic enzymes, proteinase K treated HW048 cells were given an incubation time in protein synthesis inhibitor free broth, M17-L, for various periods of time before DPC matings. Results in Table 6 indicate that enzyme treatment decreases conjugation frequencies from 2.5×10^{-2} to 3.2×10^{-4}. However, conjugation frequencies gradually recover as incubation time in broth prior to DPC matings increases. After 20 min incubation in broth, high frequencies of conjugation (1.3×10^{-2} transconjugants/donor) are again observed suggesting that cells have resynthesized the proteins necessary for high frequency conjugation.

TABLE 5. Effect of enzyme treatment on conjugation frequency[a]

Enzyme	Transfer Frequency	Standard Deviation
buffer[b]	7.5×10^{-3}	4.9×10^{-3}
Chymotrypsin A_4	7.0×10^{-4}	8.5×10^{-5}
Proteinase K	1.9×10^{-4}	1.5×10^{-4}
alpha-amylase	1.1×10^{-2}	5.4×10^{-3}
buffer[c]	2.4×10^{-2}	1.8×10^{-2}
cellulase	4.7×10^{-2}	4.5×10^{-2}
dextrase	2.1×10^{-2}	9.2×10^{-3}
beta-amylase	2.1×10^{-2}	7.1×10^{-4}

[a]conjugation was performed using DPC method.

[b]Buffer contains 0.1M $Na2HPO_3$, pH 7.0. Used to dissolve Chymotrypsin A_4, Proteinase K, and alpha-amylase.

[c]Buffer contains 0.1M Na_2HPO_3, pH 7.0. Used to dissolve cellulase, dextrase, and beta-amylase.

TABLE 6. Effect of broth incubation time after enzyme treatment on recovery of high frequency conjugation ability[a]

Donor Treatment	Incubation Time Before Plating (min)	Conjugation Frequency (transconjugant/donor)	Standard Deviation
none	0	2.5×10^{-2}	2.4×10^{-2}
proteinase K	0	3.2×10^{-4}	2.1×10^{-4}
Proteinase K	5	1.7×10^{-3}	1.2×10^{-4}
Proteinase K	10	2.0×10^{-3}	2.0×10^{-4}
Proteinase K	15	4.7×10^{-3}	4.5×10^{-4}
Proteinase K	20	1.3×10^{-2}	7.9×10^{-3}

[a]Conjugation was performed using DPC method.

Comparison of cell surface proteins of Clu^+ and Clu^- transconjugants. Cell surface extracts from Lac^+Clu^+ transconjugants containing the 104 kb cointegrate plasmid, Lac^+Clu^- transconjugants containing the 104 kb plasmid, transconjugants harboring the 55 kb plasmid (lac^+Clu^-), and the recipient strain LM2301 were compared on SDS-PAGE gels. Figure 3 shows that a specific protein with a molecular weight of approximately 125,000 was present in Clu^+ transconjugants but not in Clu^- transconjugants or in the recipient, LM2301. This suggests that at least one large protein is involved in cell clumping in Clu^+Lac^+ transconjugants. The protein extracts were very quickly degraded even at -20^0C (the protein eas degraded after over night storage in -20^0C freezer).

Determination of <u>de novo</u> synthesis requirements of donor cells for conjugal transfer of Lac^+. Conjugal transfer of lactose utilization was detected on selective media containing <u>de novo</u> protein synthesis inhibitors, erythromycin (HW048 x LM2306) and streptomycin (ML3 x LM2301), indicating that <u>de novo</u> protein synthesis is not required in donor cells for conjugal transfer of Lac^+.

High frequency conjugation (10^{-1} to 1 transconjugant per donor CFU, HW05 x MMS367) was detected on selective media containing 0.5 mg/ml rifampicin. This indicated that RNA synthesis was not required within donor cells.

FIG. 3. Profiles of cell wall extracts of Lactococcus lactis subsp. lactis strains. The extracts were subjected to SDS-PAGE and brilliant-blue staining. Lane B, Lane E, and lane I are the molecular mass standards; Lane A is the recipient strain LM2301; Lane C and lane H are the $Lac^{+}Clu^{-}$ transconjugants HW001 and HW015; Lane D and Lane G are the $Lac^{+}Clu^{+}$ transconjugants HW048 and HW007; Lane F is the broth control. **Arrow** below indicates the specific protein harbored only by Clu^{+} transconjugants.

DISCUSSION

Conjugation in bacteria involves three critical steps: formation of a stable donor-recipient pair; DNA mobilization and transfer; and DNA replication and mating pair resolution in the new host (22, 32, 96). Forming an efficient donor-recipient pair must be the preliminary step for an effective gene transfer. In the system of E. coli, treatments which block the formation of an effective donor-recipient pair can dramatically decrease the gene transfer capability (71, 96). But still little is known about this natural gene transfer mechanism in gram positive bacteria. An enhanced study of conjugation in these organisms is needed.

Early studies in Lactococcus lactis subsp. lactis ML3 and 712 system and a similar gram positive system Enterococcus faecalis suggested that there is a direct correlation between donor cell clumping and high frequency conjugation (3, 21, 36, 97). In E. faecalis, it has been demonstrated that the recipient cell produces a sex pheromone which induces donor cells to synthesize new cell surface proteins. One of the new cell surface proteins is responsible for cell aggregation in conjugal matings and may enhance conjugal transfer by increasing donor-recipient contact (10).

Recent research work in lactococci indicated that cell

aggregation and the correlated high frequency conjugation depends on the presence of both Agg and Clu factor. Either Agg or Clu itself cannot cause cell aggregation and high frequency conjugation. When the two factors are present, cell clumping occurs and high frequency transfer of Lac^+ is detected. Agg and Clu may be analogous to the binding substance and aggregation substance that are involved in the hemolysin plasmid transfer system of E. faecalis (93).

The major objectives of this study were to investigate the relationship between the clumping phenotype and high frequency conjugal transfer of Lac^+, and to identify the possible cell surface component(s) involved in the Clu^+ phenotype.

Two prior developments facilitated our investigation. First, observations in this laboratory indicated that EDTA containing solutions dissociated clumping donor cells, which allowed us to examine the effect of aggregation on conjugation frequency. Second, use of the DPC conjugation technique allowed us to disrupt or destroy donor cell clumping and examine the consequences that this had on conjugation frequency when cells were plated directly on selective media containing the de novo protein synthesis inhibitors, streptomycin or erythromycin. While using a conventional solid surface milk agar mating technique, we were not able to investigate conjugation frequency changes when the donor cell clumping was disrupted or destroyed

because the cell mixture was first given 18 hours incubation on non-selective milk agar, and during this period of time the disrupted cell surface components could be resynthesized.

The data obtained from cell washing experiments (Table 3) provided evidence that donor clumping is positively correlated to high frequency conjugation using the DPC technique. When cells were washed with TES or STE buffer, cell clumping was disrupted, and the conjugation frequency was lower (Table 3). We propose that the disruption of cell clumping prevented formation of donor-recipient pairs as efficiently as nondisrupted donor cells, thus the transfer frequency is lower.

When the EDTA residue in the cell mixture was washed away with saline, cell aggregation and high frequency conjugation recovered (Table 4). This indicated that components necessary for cell aggregation and high frequency conjugation had not been physically removed. In fact, our finding that adding Ca^{2+} and Zn^{2+} to the EDTA-dissociated clumping mixture caused cell re-aggregation and recovery of high frequency conjugation suggested that EDTA action involves an ionic interaction. It is possible that divalent cations, or at least positively charged residues, which tightly bind to the cell surface, or even specific cell surface protein(s), are necessary for cell aggregation and high frequency conjugation. EDTA may bind to these positive residues at sites on the cell surface and dissociates cell

aggregation. Zn^{2+} and Ca^{2+} must act as competitive agents and bind to EDTA residues so that more chelating agent (EDTA) can be removed from sites on the cell surface.

Proteolytic enzyme treatment of clumping donor cells resulted in a loss of cell aggregation and decreased conjugation frequency (Table 5). This indicated that cell surface protein(s) involved in high frequency conjugation could be degraded by proteolytic treatment.

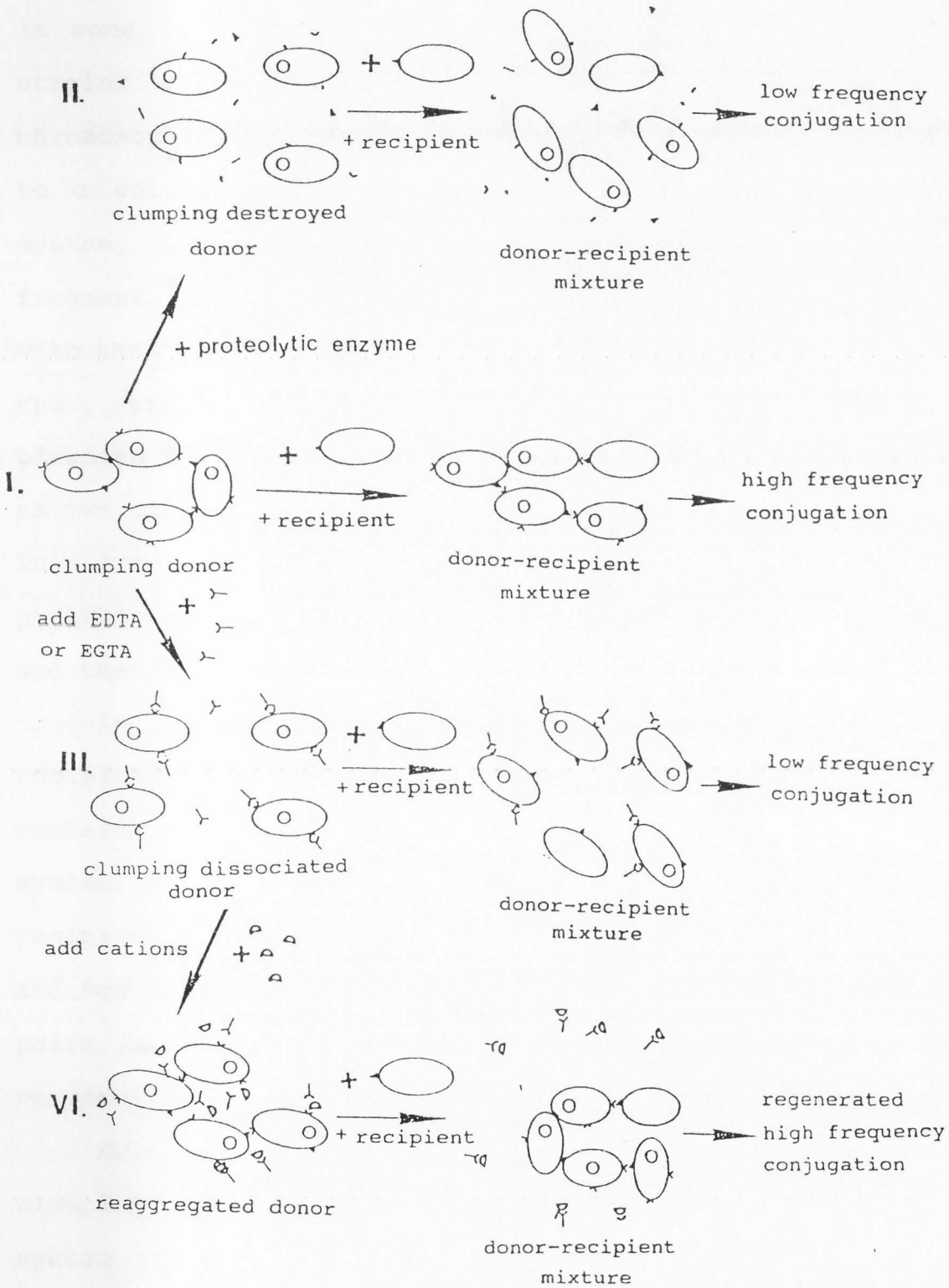
When the proteinase K-treated donor cells were given a short incubation in broth, free of de novo protein synthesis inhibitors, the cells recovered high frequency conjugation ability (Table 6). This supported our hypothesis that conventional milk agar techniques allow for resynthesis of cell surface components necessary for high frequency conjugation while DPC does not allow time for resynthesis of required cell surface components.

The detection of a specific cell surface protein with molecular mass of approximately 125 KDa, which only existed in Clu^+ transconjugants (Figure 3), strongly supported our hypothesis that at least one large protein is involved in cell clumping and the enhanced conjugal transfer of Lac^+.

In summation, we propose a model here to explain how donor cell aggregation correlated to the high frequency conjugation in Lactococcus lactis subsp. lactis ML3 and the related 712 system (Figure 4).

It was proposed that agg is a chromosomal factor present

FIG. 4. Model for the role of cell-clumping on high-frequency lactose plasmid transfer in Lactococcus lactis subsp. lactis ML3. Cell aggregation is a consequence of a specific binding reaction between two lactococcal cell surface proteins: aggregation substance (Agg) and binding substance (Clu). (I) Donors can self-aggregate because they produce both Clu and Agg. When recipient cells which express Agg are added, they also are able to form tight cell-cell interactions with donor cells. Cell aggregation promotes specific pair formation thus conjugal transfer frequency is dramatically increased. (II) Treatment of clumping donor cells with proteolytic enzymes degrades Clu (and perhaps Agg), so donor cells can no longer bind to Agg on the recipient. As a result, the efficiency of stable mating pair formation is reduced and conjugation becomes inefficient. (III) Chelating agents such as EDTA or EGTA are also able to associate tightly with Clu in an unknown manner and sterically inhibit binding between Clu and Agg. Without this interaction, donors and recipients again cannot form tight aggregates and conjugal frequency is dramatically reduced. (IV) When cations are added to this system, they competitively bind chelator, which frees Clu and permits the cells to resume aggregation and high frequency conjugation.


II.
+ recipient
low frequency conjugation
clumping destroyed donor
donor-recipient mixture
+ proteolytic enzyme
I.
+ recipient
high frequency conjugation
clumping donor
donor-recipient mixture
add EDTA or EGTA
III.
+ recipient
low frequency conjugation
clumping dissociated donor
donor-recipient mixture
add cations
VI.
+ recipient
regenerated high frequency conjugation
reaggregated donor
donor-recipient mixture

in some (e.g., ML3, 712, and C2 derivatives) but not all strains of lactococci (93), and *clu* is the plasmid or chromosomal located sex factor (34) whose expression related to a cointegrate plasmid formation process. In the ML3 system, the orientation of an invertible 4.3-kb *Kpn*I-*Pvu*II fragment on pRS01 was identified to be directly correlated with the clumping phenotype. Analysis of the pRS01 fragments that were interrupted in the formation of cointegrate plasmids confirmed the involvement of this fragment as well as two other contiguous fragments in the expression of Clu. This total region was defined as *clu* region. Also it was postulated that Clu expression depends on the *clu* gene dosage and the replicon upon which the *clu* gene resided (3).

In our research system, nonclumping donor cells and recipient cells both contain Agg, while clumping donor cells contain both Agg and Clu. In the clumping conjugation system, not only donor cells aggregate, but the donor and recipient cells because of the interaction of Clu in donor and Agg in recipient. Thus the formation of donor-recipient pairs is more efficient than in a nonclumping system, and respectively, the conjugal transfer frequency is much higher.

EDTA containing solutions can dissociate donor cell clumping and decrease the conjugation frequency in this system (Table 3), but it will not influence the transfer tendency in the nonclumping conjugation system (data not presented). Because Clu existed or expressed more in the

clumping system, but not in the nonclumping system, and Agg existed in every L. lactis subsp. lactis strains we studied, we expect that EDTA binds to the Clu with an ionic interaction. Both the Clu and Agg interaction between the donor-donor and donor-recipient are decreased; thus, donor cell aggregation is dissociated, and we have a lowered conjugation frequency. When we treated our donor cells with proteolytic enzymes, Clu or Agg was destroyed, and the ability of forming donor-recipient pair via the Clu-Agg interaction was presumably lowered.

As the large protein we isolated only exists in Clu^+ strains but not Clu^- strains, and the L. lactis subsp. lactis ML3 and C2 derivatives constantly express Agg (93), the protein most likely is the Clu protein (3).

This lactococcal model appears to be similar to the proposed E. faecalis model (22). The difference is that in the E. faecalis system, the necessary factors for generating the clump protein, the synthesis system and the trigger sex pheromone, reside separately in donor and recipient strains. In the L. lactis system no trigger is needed. The Clu and Agg expressed constantly, and, when mixed, can cause cell aggregation.

As we know that there are other gene(s) (such as tra region) involved in this conjugal transfer system (3), we propose that cell aggregation is one of the preliminary requirements for this enhanced conjugal transfer system.

More evidence is needed to prove our hypothesis about the Agg-Clu interaction. In future work, making antibodies to the possible Clu protein and Agg protein and testing their biological effect on the system can definitely enhance our understanding of this high frequency conjugation mechanism. Also, using competitive inhibitors of cations or positive residues other than EDTA will allow us to study, in more detail, the features of the sites involved in Agg-Clu interactions.

In conclusion, this work demonstrated that a direct relationship exists between Clu^+ and high frequency conjugal transfer of Lac^+ in Lactococcus lactis subsp. lactis ML3. We identified a large cell surface protein that was present only on Clu^+ transconjugants. Furthermore, de novo protein synthesis and RNA synthesis in donor cells are not required for conjugative plasmid transfer. An improved understanding of conjugal mechanisms can be expected as certain aspects of conjugation can now be examined through manipulation of the DPC technique.

CHAPTER IV

SUMMARY AND CONCLUSIONS

Lactic acid bacteria are important microorganisms in the fermentation of dairy, meat, vegetable, and cereal products. Among these genera, lactococci attract special attention in the study of starter culture improvement because of their responsibility for the fermentation process in many milk products, their easy handling in the laboratory, and their many plasmid-linked properties. Conjugation in lactococci receives considerable attention because it has been a "food-grade" strategy for genetic improvement of dairy starter cultures.

In this study, the physical and functional events involved in high frequency conjugal transfer of Lac^+ in L. lactis subsp. lactis ML3 was examined. Transconjugants from mating ML3 and LM2301 with the phenotype, Lac^+Clu^+, were isolated, and the relationship between donor cell aggregation and high frequency conjugation was determined. Donor cell aggregation was disrupted by washing with solutions containing EDTA or destroyed by treating with proteolytic enzymes. The treated donor cells had a decreased ability to conjugally transfer Lac^+ when using the DPC conjugation technique. Analysis of cell-surface proteins by SDS-PAGE identified a novel protein of approximately 125 kDa which was

present only in $Lac^{+}Clu^{+}$ transconjugants. De novo synthesis requirements of donor cells for conjugal transfer of Lac have been tested, and the results indicated that de novo protein and RNA synthesis are not required by donor cells.

The results of this study suggested that donor cell aggregation is a prerequisite of high frequency conjugal transfer, and a cell surface protein is involved in donor cell aggregation. Direct plate conjugation is not only a simplified conjugation technique, but can help detect conjugation events which cannot be seen using the traditional techniques. An improved understanding of conjugal mechanisms can be expected through manipulation of the DPC technique.

Literature Cited

1 **Allen, L.K., W.E. Sandine, and P.R. Elliker.** 1962. Transduction in Streptococcus lactis. J. Dairy Res. **30**:351-357.

2 **Anderson, D.G., and L.L. McKay.** 1983. A simple and rapid method for isolating large plasmid DNA from lactic streptococci. Appl. Environ. Microbiol. **46**:549-552.

3 **Anderson, D.G., and L.L. McKay.** 1984. Genetic and physical characterization of recombinant plasmids associated with cell aggregation and high-frequency conjugal transfer in Streptococcus lactis ML3. J. Bacteriol. **158**: 954-962.

4 **Ausubel, F.M., R. Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith, and K. Struhl.** 1987. Current protocals in molecular biology. Vol. 2. John Wiley & Sons, New York.

5 **Broadbent, J.R., and J.K. Kondo.** 1991. Genetic construction of nisin-producing Lactococcus lactis subsp. cremoris and analysis of a rapid method for conjugation. Appl. Environ. Microbiol. **57**:517-524.

6 **Chassy, B.M.** 1987. Prospects for the genetic manipulation of lactobacilli. FEMS Microbiol. Rev. **46**:297-312.

7 **Chassy, B.M., and J.L. Flickinger.** 1987. Transformation of Lactobacillus casei by electroporation. FEMS Microbiol. Lett. 44:173-177.

8 **Chassy, B.M., and E. Rokaw.** 1981. Conjugal transfer of plasmid-associated lactose metabolism in Lactobacillus casei ssp. casei. p. 590. In S.B. Levy, R.C. Clowes, and E.L. Koenig (eds.), Molecular biology, pathogenesis and ecology of bacterial plasmids. Plenum Publishing Corp., New York.

9 **Christie, P.J., and G.M. Dunny.** 1986. Identification of regions of the Streptococcus faecalis plasmid pCF-10 that encode antibiotic resistance and pheromone response functions. Plasmid 15:230-241.

10 **Christie, P.J., S.M. Kao, J.C. Adsit, and G.M. Dunny.** 1988. Cloning and expression of genes encoding pheromone-inducible antigens of Enterococcus (Streptococcus) faecalis. J. Bacteriol. 162:131-137.

11 **Clark, A.J., and G.J. Warren.** 1979. Conjugal transmission of plasmids. Ann. Rev. Genet. 13:99-125.

12 **Clewell, D.B.** 1981. Plasmids, drug resistance, and gene transfer in the genus Streptococcus. Microbiol. Rev. 45: 409-436.

13 **Clewell, D.B., and M.C. Gawron-Burke.** 1986. Conjugative transposons and the dissemination of antibiotic resistance in streptococci. Ann. Rev. Microbiol. 40: 635-659.

14 **Clewell, D.B., L.T. Pontius, K.E. Weaver, F.Y. An, Y. Ike, A. Suzuki, and J. Nakayama.** 1990. Enterococcus faecalis hemolysin/bacteriocin plasmid pAD1: Regulation of the pheromone response. p. 3-8. In G.M. Dunny, P.P. Cleary, and L.L. McKay (ed.), Genetics and Molecular Biology of Streptococci, Lactococci, and Enterococci. American Society for Microbiology, Washington, D.C.

15 **Clewell, D.B., P.K. Tomich, M.C. Gawron-Burke, A.E. Franke, Y. Yagi, and F.A. AN.** 1982. Mapping of Streptococcus faecalis plasmids pAD1 and pAD2 and studies relating to transposition of Tn917. J. Bacteriol. **152**:1220-1230.

16 **Clewell, D.B., and K.E. Weaver.** 1989. Sex pheromone and plasmid transfer in Enterococcus faecalis. Plasmid **21**:175-184.

17 **Cocconcelli, P.S., L. Morelli, M. Vescovo, and V. Bottazi.** 1986. Intergeneric protoplast fusion in lactic acid bacteria. FEMS Microbiol. Lett. **35**:211-214.

18 **Davey, G.P., V.L. Crow, and L.E. Pearce.** 1984. Enzyme analysis of Lac^+ transconjugants of Streptococcus cremoris. N. Z. J. Dairy Sci. Technol. **19**:185-188.

19 **Davies, F.L., H.M. Underwood, and M.J. Gasson.** 1981. The value of plasmid profiles for strain identification in lactic streptococci and the relationship between Streptococcus lactis 712, ML3, and C2. J. Appl.

Bacteriol. 51:325-337.

20 **Dessart, S.R., and L.R. Streenson.** 1991. High frequency intergeneric and intrageneric conjugal transfer of drug resistance plasmids in Leuconostoc mesenteroides ssp. cremoris. J. Dairy Sci. 74:2912-2919.

21 **Dunny, G.M., B.L. Brown, and D.B. Clewell.** 1978. Induced cell aggregation and mating in Streptococcus faecalis: evidence for a bacterial sex pheromone. Proc. Natl. Acad. Sci. USA 75: 3479-3483.

22 **Dunny, G.M., J.W. Chung, J.C. Gallo, S.M. Kao, K.M. Trotter, R.Z. Korman, S.B. Olmsted, R. Ruhffl, O.R. Torres, and S.A. Zahler.** 1990. Cell-cell interactions and conjugal gene transfer events mediated by the pheromone-inducible plasmid transfer system and the conjugative transposon encoded by Enterococcus faecalis plasmid pCF10. p. 9-15. In G.M. Dunny, P.P. Cleary, and L.L. McKay (ed.), Genetics and Molecular Biology of Streptococci, Lactococci, and Enterococci. American Society for Microbiology, Washington, D. C.

23 **Dunny, G.M., and D.B. Clewell.** 1975. Transmissible toxin (hemolysin) plasmid in Streptococcus faecalis and its mobilization of a noninfectious drug resistance plasmid. J. Bacteriol. 124:784-790.

24 **Dunny, G., C. Funk, and J. Adsit.** 1981. Direct stimulation of the transfer of antibiotic resistance by

sex pheromone in Streptococcus faecalis. Plasmid 6:270-278.

25 **Dunny, G.M., D.A. Krug, C.L. Pan, and R.A. Ledford.** 1988. Identification of cell wall antigens associated with a large conjugation plasmid encoding phage resistance and lactose fermentation ability in lactic streptococci. Biochimie 70:443-450.

26 **Dunny, G.M., D.L. Zimmerman, and M.L. Tortorello.** 1985. Induction of surface exclusion (entry exclusion) by Streptococcus faecalis sex pheromone: Use of monoclonal antibodies to identify an inducible surface antigen involved in the exclusion process. Proc. Natl. Acad. Sci. USA 82:8582-8586.

27 **Efstathiou, J.D., and L.L. McKay.** 1977. Inorganic salts resistance associated with a lactose fermenting plasmid in Streptococcus lactis. J. Bacteriol. 130:257-265.

28 **Fiedler S., and R. Wirth.** 1988. Transformation of bacteria with plasmid DNA by electroporation. Anal. Biochem. 170:38-44.

29 **Fitzgerald, G.F., and M.J. Gasson.** 1988. In vivo gene transfer systems and transposons. Biochimie 70:489-502.

30 **Fodor, K., and L. Alfoldi.** 1976. Fusion of protoplasts of Bacillus megaterium. Proc. Natl. Acad. USA 73:2147-2150.

31 **Foster, E.M.** 1989. A half century of food microbiology. Food Technol. 43:208-216.

32 **Freifelder, D.** 1987. Plasmids. p. 247-280. In Microbial genetics. Jones and Bartlett Publishers, Inc., Boston.

33 **Gasson, M.J.** 1980. Production, regeneration and fusion of protoplasts of lactic streptococci. FEMS Microbiol. Lett. **9**:99-102.

34 **Gasson, M.J.** 1990. In vivo genetic systems in lactic acid bacteria. FEMS Microbiol. Rev. 87:43-60.

35 **Gasson, M.J., and F.L. Davies.** 1979. Conjugal transfer of lactose genes in group N streptococci. Soc. Gen. Microbiol. Quart. 6:87.

36 **Gasson, M.J., and F.L. Davies.** 1980. High-frequency conjugation associated with Streptococcus lactis donor cell aggregation. J. Bacteriol. **143**:1260-1264.

37 **Geis, A.** 1982. Transfection of protoplasts of Streptococcus lactis subsp. diacetylactis. FEMS Microbiol. Lett. **15**:119-122.

38 **Gilliland, S.E.** 1990. Health and nutritional benefits from lactic acid bacteria. FEMS Microbiol. Rev. **87**:175-188.

39 **Gotz, F., S. Ahrne, and M. Lindberg.** 1981. Plasmid transfer and genetic recombination by protoplast fusion in Staphylococci. J. Bacteriol. **145**:74-81.

40 **Harlander, S.K.** 1986. Transformation of Streptococcus lactis by electroporation. p. 229-233. In: J.J. Ferretti and R. Curtiss III, (ed.), Streptococcal

genetics. American Society for Microbiology, Washington, D.C.

41 **Hashiba, H., R. Takiguchi, S. Ishii, and K. Aoyama.** 1990. Transformation of Lactobacillus helveticus subsp. jugurti with plasmid pLHP by electroporation. Agric. Biol. Chem. **54:**1537-1541.

42 **Hill, C., C. Dale, and G.F. Fitzgerald.** 1985. Conjugative transfer of the transposon Tn919 to lactic acid bacteria. FEMS Microbiol. Lett. **30:**115-119.

43 **Ippen-Ihler, K.A., and E.G. Minkley, Jr.** 1986. The conjugation system of F, the fertility factor of Escherichia coli. Ann. Rev. Genet. **20:**593-624.

44 **Jacob, A.E., and S.J. Hobbs.** 1974. Conjugal transfer of plasmid-borne multiple antibiotic resistance in Streptococcus faecalis var. zymogenes. J. Bacteriol. **117:**360-372.

45 **Kempler, G.M., and L.L. McKay.** 1979. Genetic evidence for plasmid-linked lactose metabolism in Streptococcus lactis subsp. diacetylactis. Appl. Environ. Microbiol. **37:**1041-1043.

46 **Kessler, R.E., and Y. Yagi.** 1983. Identification and partial characterization of a pheromone-induced adhesive surface antigen of Streptococcus faecalis. J. Bacteriol. **155:**714-721.

47 **Klaenhammer, T.R., and R.B. Sanozky.** 1985. Conjugal transfer from Streptococcus lactis ME2 of plasmids

encoding phage resistance, nisin resistance and lactose-fermenting ability: evidence for a high-frequency conjugative plasmid responsible for abortive infection of virulent bacteriophage. J. Gen. Microbiol. 131:1531-1541.

48 **Kondo, J.K., and L.L. McKay.** 1982. Transformation of Streptococcus lactis protoplasts by plasmid DNA. Appl. Environ. Microbiol. 43:1213-1215.

49 **Kondo, J.K., and L.L. McKay.** 1984. Plasmid transformation of Streptococcus lactis protoplasts: optimization and use in molecular cloning. Appl. Environ. Microbiol. 48:252-259.

50 **Kondo, J.K., and L.L. McKay.** 1985. Gene transfer systems and molecular cloning in group N streptococci: a review. J. Dairy Sci. 68:2143-2159.

51 **Kuhl, S.A., L.D. Larsen, and L.L. McKay.** 1979. Plasmid profiles of lactose negative and proteinase deficient mutants of Streptococcus lactis C10, ML3, and M18. Appl. Environ. Microbiol. 37:1193-1195.

52 **Lindgren, S.E., and W.J. Dobrogosz.** 1990. Antagonistic activities of lactic acid bacteria in food and feed fermentations. FEMS Microbiol. Rev. 87:149-164.

53 **McIntype, D.A., and S.K. Harlander.** 1989. Genetic transformation of intact Lactococcus lactis subsp. lactis by high-voltage electroporation. Appl. Environ. Microbiol. 55:604-610.

54 **McKay, L.L., and K.A. Baldwin.** 1974. Simulanteous loss of proteinase- and lactose-utilizing enzyme activities in Streptococcus lactis and reversal of loss by transduction. Appl. Microbiol. 28:342-346.

55 **McKay, L.L., and K.A. Baldwin.** 1984. Conjugative 40-megadalton plasmid in Streptococcus lactis subsp. diacetylactis DRC3 is associated with resistance to nisin and bacteriophage. Appl. Environ. Microbiol. 47:68-74.

56 **McKay, L.L., and K.A. Baldwin.** 1990. Applications for biotechnology: present and future improvements in lactic acid bacteria. FEMS Microbiol. Rev. 87:3-14.

57 **McKay, L.L., K.A. Baldwin, and J.D. Efstathiou.** 1976. Transductional evidence for plasmid linkage of lactose metabolism in Streptococcus lactis C2. Appl. Environ. Microbiol. 32:45-52.

58 **McKAy, L.L., K.A. Baldwin, and P.M. Walsh.** 1980. Conjugal transfer of genetic information in group N streptococci. Appl. Environ. Microbiol. 40:84-91.

59 **McKay, L.L., B.R. Cords, and K.A. Baldwin.** 1973. Transduction of lactose metabolism in Streptococcus lactis C2. J. Bacteriol. 115:810-815.

60 **McKay, L.L., A. Miller III, W.E. Sandine, and P.R. Elliker.** 1970. Mechanisms of lactose utilization by lactic acid streptococci: enzymatic and genetic analysis. J. Bacteriol. 102:804-809.

61 **Mercenier, A., and B.M. Chassy.** 1988. Strategies for the development of bacterial transformation systems. Biochimie 70:503-517.

62 **Mercenier, A., P. Slos, M. Fralen, and J.P. Lecocq.** 1988. Plasmid transduction in Streptococcus thermophilus. Mol. Gen. Genet. 212:386-389.

63 **Miller, J.F., W.J. Dower, and L.S. Tompkins.** 1988. High-voltage electroporation of bacteria: Genetic transformation of Campylobacter jejuni with plasmid DNA. Proc. Natl. Acad. Sci. USA 85:856-860.

64 **Morelli, L., P.G. Sarra, and V. Bottazzi.** 1988. In vivo transfer of pAMβ1 from Lactobacillus reuteri to Enterococcus faecalis. J. Appl. Bacteriol. 65:371-375.

65 **Mori, M., Y. Sakagami, Y. Ishii, A. Isogai, C. Kitada, M. Fujino, J.C. Adsit, G.M. Dunny, and A. Suzuki.** 1988. Structure of cCF10, a peptide sex pheromone which induces conjugative transfer of the Streptococcus faecalis tetracycline resistance plasmid, pCF10. J. of Biol. Chem. 268:14574-14578.

66 **Neve, H., A. Geis, and M. Teuber.** 1987. Conjugation, a common plasmid transfer mechanism in lactic acid streptococci of dairy starter cultures. System. Appl. Microbiol. 9:151-157.

67 **Okamoto, T., Y. Fujita, and R. Irie.** 1983. Fusion of protoplasts of Streptococcus lactis. Agric. Biol. Chem. 47:2675-2676.

68 **Okamoto, T., F. Yasuhito, and I. Ryozaburo.** 1982. Protoplast formation and regeneration of Streptococcus lactis cells. Agric. Biol. Chem. **47**:259-263.

69 **Oliver, D.R., B.L. Brown, and D.B. Clewell.** 1977. Characterization of plasmids determining hemolysin and bacteriocin production in Streptococcus faecalis 5952. J. Bacteriol. **130**:948-950.

70 **Olson, N.F.** 1990. The impact of lactic acid bacteria on cheese flavor. FEMS Microbiol. Rev. **87**:131-148.

71 **Ou, T.J., and R. Reim.** 1976. Effect of 1,10-phenanthroline on bacterial conjugation in Escherichia coli K-12: inhibition of maturation from preliminary mates into effective mates. J. Bacteriol. **128**:363-371.

72 **Polzin, K.M., and M.S. Kadota.** 1987. Identification of a new insertion element, similar to gram-negative IS26, on the lactose plasmid of Streptococcus lactis ML3. J. Bacteriol. **169**:5481-5488.

73 **Potter, H.** 1988. Electroporation in biology: methods, applications and instrumentation. Anal. Biochem. **174**:361-373.

74 **Pucci, M.J., M.E. Monteschio, and E.R. Vedamuthu.** 1987. Conjugal transfer in Leuconostoc spp: intergeneric and intrageneric transfer of plasmid-encoded antibiotic resistance determinants. FEMS Microbiol. Rev. **46**:p7.

75 **Sanders, M.E., P.J. Leonhard, W.D. Sing, and T.R. Klaenhammer.** 1986. Conjugal strategy for construction

of fast acid-producing, bacteriophage-resistant lactic streptococci for use in dairy fermentations. Appl. Environ. Microbiol. 52:1001-1007.

76 **Sandine, W.E.** 1987. Looking backward and forward at the practical applications of genetic researches on lactic acid bacteria. FEMS Microbiol. Rev. 46:205-220.

77 **Sandine, W.E., P.R. Elliker, L.K. Allen, and W.C. Brown.** 1962. II. Genetic exchange and variability in lactic Streptococcus starter organisms. J. Dairy Sci. 45:1266.

78 **Schaeffer, P., B. Cami, and R.D. Hotchkiss.** 1976. Fusion of bacterial protoplasts. Proc. Natl. Acad. Sci. USA 73:2151-2155.

79 **Scherwitz, K.M., K.A. Baldwin, and L.L. McKay.** 1983. Plasmid linkage of a bacteriocin-like substance in Streptococcus lactis subsp. diacetylactis strains WM4: transferability to Streptococcus lactis. Appl. Environ. Microbiol. 45:1506-1512.

80 **Snook, R.J., and L.L. McKay.** 1981. Conjugal transfer of lactose-fermenting among Streptococcus cremoris and Streptococcus lactis strains. Appl. Environ. Microbiol. 42:904-911.

81 **Snook, R.J., L.L. McKay, and G.G. Ahlstrand.** 1981. Transduction of lactose metabolism by Streptococcus cremoris C3 temperate phage. Appl. Environ. Microbiol. 42:897-903.

82 **Somkuti, G.A., and D.H. Steinberg.** 1988. Genetic transformation of Streptococcus thermophilus by electroporation. Biochimie 70:579-585.

83 **Steele, J.M., and L.L. McKay.** 1986. Partial characterization of the genetic basis for sucrose metabolism and nisin production in Streptococcus lactis. Appl. Environ. Microbiol. 51:57-64.

84 **Steele, J.M., and L.L. McKay.** 1989. Conjugal transfer of genetic material in lactococci: a review. J. Dairy Sci. 72:3388-3397.

85 **Steele, J.M., and L.L. McKay.** 1989. Conjugal transfer of genetic material by Lactococcus lactis subsp. lactis 11007. Plasmid 22:32-43.

86 **Stojanova, L.G., and N.S. Egorov.** 1990. Fusion of protoplasts of Lactococcus lactis for getting superior production of nisin. FEMS Microbiol. Rev. 87:p35.

87 **Tang, M., K. Owens, P. Pietri, X. Zhu, R. McVeigh, and B.K. Ghosh.** 1989. Cloning of the crystalline cell wall protein gene of Bacillus licheniformis NM 105. J. Bacteriol. 171:6637-6648.

88 **Terzaghi, B.E., and W.E. Sandine.** 1975. Improved medium for lactic streptococci and their bacteriophages. Appl. Microbiol. 29: 807-813.

89 **Thomas, T. D.** 1985. Role of lactic acid bacteria and their improvement for production of better fermented animal products. N. Z. J. Dairy Sci. Technol. 20:1-

10.

90 **Thompson, J.K., and M.A. Collins.** 1988. Evidence for the conjugal transfer of the broad host range plasmid pIØ501 into strains of Lactobacillus helveticus. J. Appl. Bacteriol. 65:309-319.

91 **Tortorello, M., J. Adsit, D. Krug, D. Antczak, and D. Dunny.** 1985. Monoclonal antibodies to cell surface antigens involved in sex pheromone induced mating in Streptococcus faecalis. 1986. J. Gen. Microbiol. 132:857-864.

92 **Tortorello, M.L., and G.M. Dunny.** 1985. Identification of multiple cell surface antigens associated with the sex pheromone response of Streptococcus faecalis. J. Bacteriol. 162:131-137.

93 **Van der Lelie, D., F. Chavarri, G. Venema, and M.J. Gasson.** 1991. Identification of a new genetic determinant for cell aggregation associated with lactose plasmid transfer in Lactococcus lactis. Appl. Environ. Microbiol. 57:201-206.

94 **Van der Lelie, D., J.M.B.M. van der Vossen, and G. Venema.** 1987. Interspecific protoplast fusion between Bacillus subtillus and lactic acid bacteria. FEMS Microbiol. Rev. 46:p10.

95 **Vescovo, M., L. Morelli, V. Bottazzi, and M.J. Gasson.** 1983. Conjugal transfer of broad-host-range plasmid pAMβ1 into enteric species of lactic acid bacteria.

Appl. Environ. Microbiol. 46:753-755.

96 **Viljanen, P., and J. Boratynski.** 1991. The susceptibility of conjugation resistance transfer in Gram-negative bacteria to physiochemical and biochemical agents. FEMS Microbiol. Rev. 88:43-54.

97 **Walsh, P.M., and L.L. McKay.** 1981. Recombinant plasmid associated with cell aggregation and high-frequency conjugation of Streptococcus lactis ML3. J. Bacteriol. **146:** 937-944.

98 **Willetts, N., and B. Wilkins.** 1984. Processing of plasmid DNA during bacterial conjugation. Microbiol. Rev. **48:**24-41.

99 **Wycoff, H.A., W.E. Sandine, and J.K. Kondo.** 1991. Transformation of dairy Leuconostoc using plasmid vectors from Bacillus, Escherichia, and Lactococcus hosts. J. Dairy Sci. 74:1454-1460.

100 **Zink, A., J.R. Klein, and R. Plapp.** 1991. Transformation of Lactobacillus delbruekii spp. lactis by electroporation and cloning of origins of replication by use of a positive selection vector. FEMS Microbiol. Lett. **78:**207-212.