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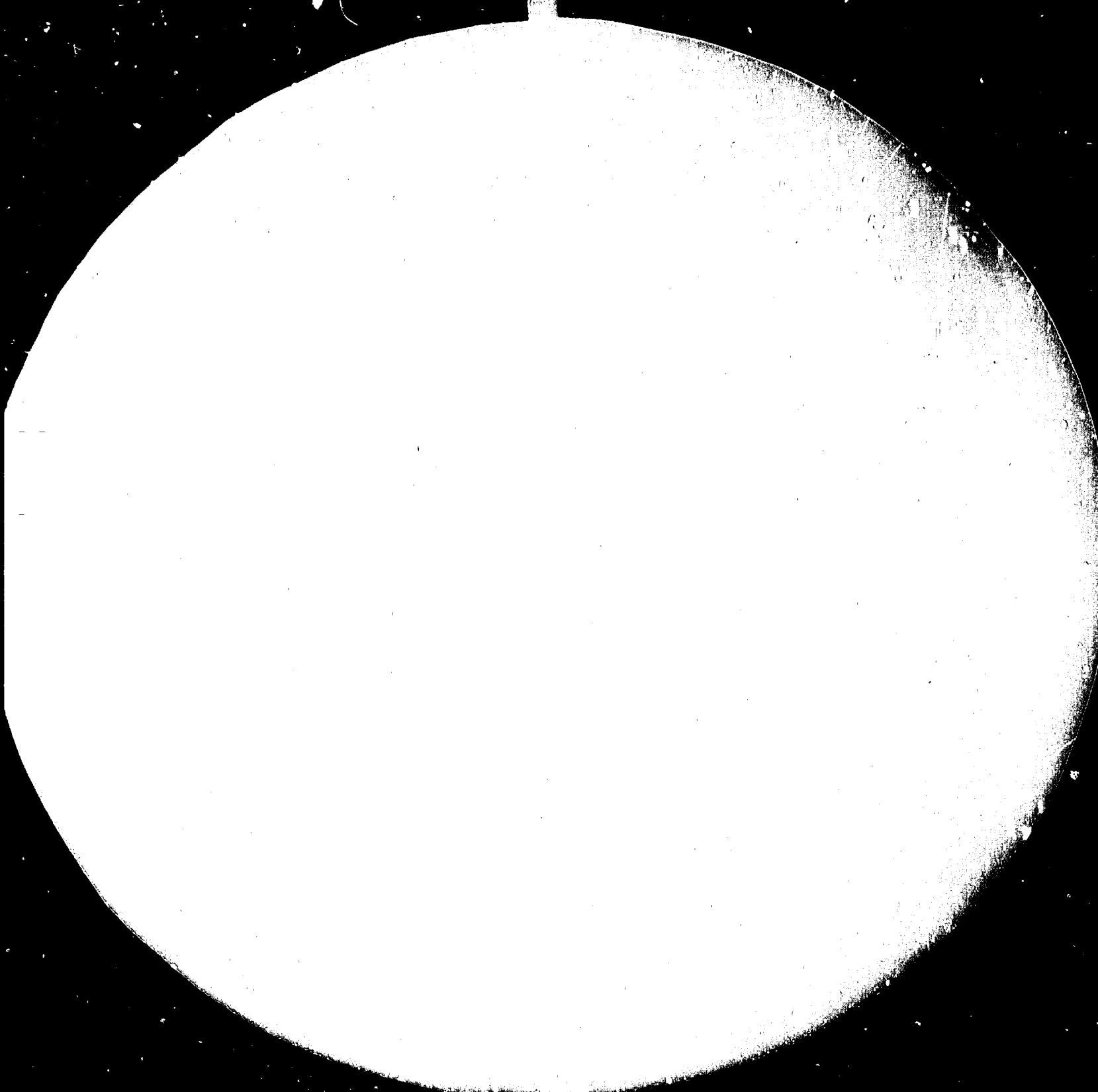

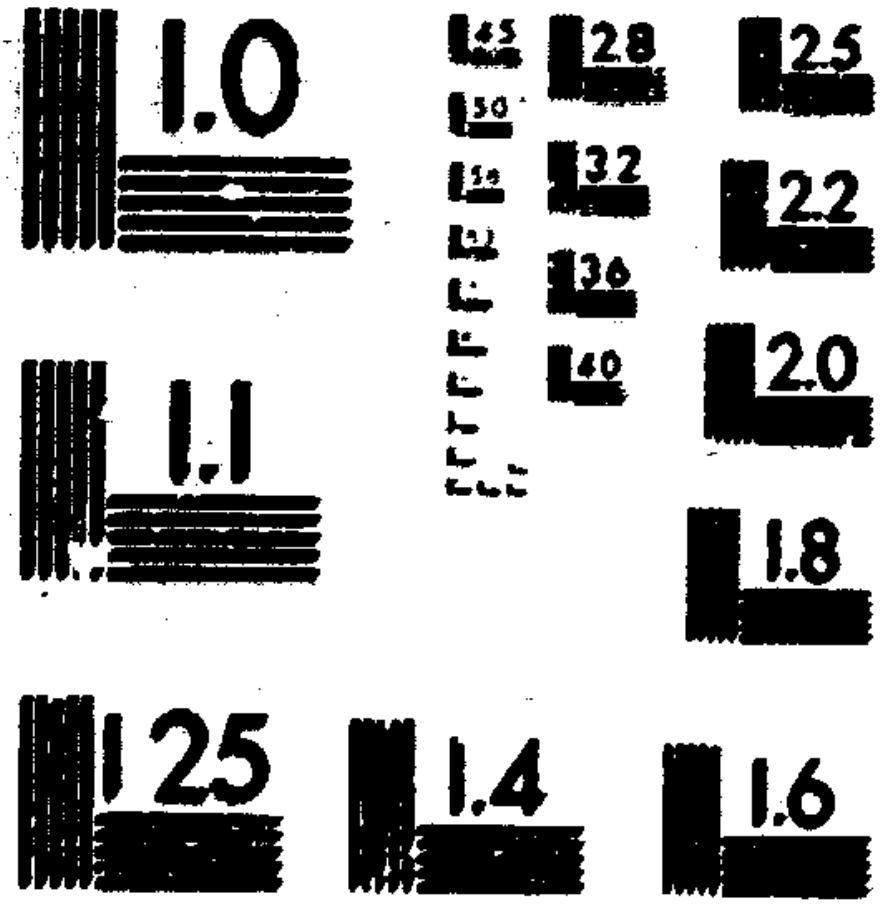

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GENETIC MODIFICATIONS OF LACTIC ACID BACTERIA: PLASMID DIRECTED FUNCTIONS AND DEVELOPMENT OF GENE TRANSFER SYSTEMS*

Prepared by

Larry L. McKay**

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** Professor, University of Minnesota, Department of Food Science and Nutrition, 1134 Eckles Avenue, St. Paul, Minnesota 55108, USA.

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CONTENTS

BACKGROUND

1. Plasmid biology has become an important area of investigation in dairy starter cultures. It is now established that group N streptococci used as starter cultures harbor plasmids of diverse sizes and that some of these plasmids code for properties vital for successful milk fermentation processes. Examples of plasmid-mediated traits in this group of bacteria include lactose utilization, proteolytic activity, citrate utilization, production of antagonistic proteins, nisin resistance, and resistance to bacteriophage. Developments in the above area may lead to amplification of plasmid genes through the isolation of copy number mutants, thus increasing the efficiency of the fermentation process as well as the quality of the final product. Stabilization of these plasmid-mediated traits by integrating the essential genes into the chromosome may also prove beneficial. Thus, the study of plasmid biology in dairy streptococci, as well as other lactic acid bacteria, has become a prerequisite for future strain improvement programs. This knowledge, coupled with transduction, conjugation, protoplast fusion, and transformation systems applicable to dairy streptococci, is essential for gene cloning work within this group of bacteria. The world-wide interest in the genetics of lactic acid bacteria brings us closer to the time when genetically improved strains are actually used in commercial fermentation processes.

I. INTRODUCTION

2. Plasmid biology is fast becoming an important area of research in lactic acid bacteria used for milk, meat, and plant fermentation processes, as well as probiotics. Our laboratory has been examining mesophilic dairy streptococci for the presence of plasmid DNA and attempting to find or develop DNA transfer systems in order to allow genetic studies to proceed with this group of bacteria. The present discussion will concentrate on these investigations but is not intended to be a comprehensive review. For recent reviews the reader is directed to Antonie van Leeuwenhoek 49:209-352 (1983) which is a series of papers from the Symposium on Lactic Acid Bacteria in Foods: Genetics, Metabolism, and Applications.

3. Three metabolic properties of these organisms, vital for sucessful dairy fermentations, include the ability to ferment lactose to lactic acid, to release amino acids and peptides from casein (proteolytic activity), and the ability of Streptococcus diacetylactis to utilize citrate and produce diacetyl (butter aroma). It is now known that from parental strains one can isolate variants which have lost their ability to ferment lactose (Lac^-), to produce proteinase (Prt^-) and/or to utilize citrate (Cit^-). Phenotypic data suggested that these metabolic traits were plasmid mediated.

II. PLASMID BIOLOGY

4. Plasmids can be defined as unstable genetic entities within the bacterial cell, separate from the bacterial chromosome. Loss of plasmids cause the cell to lose the ability to perform functions dictated by the plasmid DNA. Plasmids can now be extracted from the dairy streptococci and separated from chromosomal

DNA through the use of cesium chloride-ethidium bromide density gradient centrifugation and the various plasmids are then separated according to size using electron microscopy or agarose gel electrophoresis. It is well established that the mesophilic streptococci used in dairy fermentation processes harbor plasmids of diverse sizes (Chopin and Langella, 1982; Davies and Gasson, 1981; LeBlanc, et al, 1980; McKay and Baldwin, 1982; Pechmann and Teuber, 1980). Further, it has been demonstrated that these organisms characteristically contain many plasmid species. The number observed ranges from 2 to 13, but most strains appear to contain 4 to 7 distinct plasmid sizes. Most of the plasmids observed in these organisms are cryptic, but some carry identifiable metabolic properties (McKay, 1983). In the group N streptococci, phenotypic, physical, and genetic evidence has been accumulating for plasmid encoded lactose (McKay, 1982; St. Martin, et al, 1982), galactose (Crow, et al, 1983; Park and McKay, 1982) and sucrose utilization (Gasson, 1984), as well as for production of nisin and bacteriocins (Gasson, 1984; Dobrzanski, et al, 1982; Fuchs, et al, 1975; Kozak, et al, 1974; Scherwitz, et al, 1983) proteinases (McKay, 1983; Otto, et al, 1982), nisin resistance (Gasson, 1984; McKay and Baldwin, 1984), production of diplococcin by certain Streptococcus cremoris strains (Davey, 1984; Davey and Pearce, 1982), and for resistance to certain phages (McKay and Baldwin, 1984). Phenotypic and physical evidence for plasmid linkage of metabolic traits in mesophilic dairy streptococci has also been obtained for citrate (Kempler and McKay, 1981), glucose, mannose, and xylose utilization (LeBlanc, et al, 1980), modification-restriction systems (Chopin, et al, 1984; Sanders and Klaenhammer, 1981), and phage adsorption (Sanders and Klaenhammer, 1983; Vos, et al, 1984). Due to the importance of plasmids in food fermentation processes, attempts to elucidate the functional properties of cryptic plasmids in lactic acid bacteria must continue.

III. GENETIC STUDIES

5. Dairy streptococci have been poorly characterized genetically, probably due to their fastidious nature, the absence of selective markers and because gene transfer systems in this group have only recently been developed. Due to the importance of genetics in strain improvement programs, the results presented now will concentrate on the development of gene transfer systems in this group of bacteria.

IV. TRANSDUCTION

6. A temperate bacteriophage was demonstrated in S. lactis C2 by exposing the cells to UV irradiation, inoculating the irradiated cells into broth, and following the change in turbidity (McKay and Baldwin, 1973). The turbidity increased for about 2 h at which time the suspension lysed, as indicated by rapid clearing of the culture. The existence of phage was confirmed by obtaining electron micrographs of the phage particles. Further results indicated that a transducing phage had been isolated since UV induced lysates prepared from S. lactis C2 converted lactose-, maltose-, or mannose-negative recipient cells to the respective carbohydrate-positive phenotype (McKay, et al, 1973). Thus, transduction, a genetic exchange system involving the use of a bacteriophage, was found and it provided a genetic means for examining the spontaneous loss of lactose metabolism and other traits in this organism.

7. Plasmid analysis has revealed that the Lac^+ Prt^+ parental culture S. lactis C2 harbored a 30 Mdal plasmid which was lost when the cells became Lac^- Prt^-. Upon transduction, the original phenotype was restored when a plasmid of approximately 21 Mdal was acquired by the Lac^- Prt^- recipient. The plasmid is smaller than the 30 Mdal lactose plasmid because the phage head can only accommodate or package a piece of DNA of 20-22 Mdal. When the Lac^+ transductant lost the 21 Mdal plasmid, the cells became Lac^-; thus transduction analysis confirmed

linkage of the lac genes to plasmid DNA (McKay et al, 1976). This temperate phage from C2 was also shown to transduce streptomycin resistance (McKay et al, 1980). Other transducing phages have now also been found. Davies and Gasson have found that S. lactis 712 transferred the plasmid gene for lac, prt, and erythromycin resistance (Davies and Gasson, 1981). In our laboratory a transducing phage in S. lactis $C_2$0 (McKay, et al, 1980) and S. cremoris C3 were also noted (Snook, et al, 1981).

V. STABILIZATION OF LACTOSE-FERMENTING ABILITY

8. Since stabilization of lactose-fermenting ability is a desirable trait in dairy starter cultures, we used the temperate phage from S. lactis C2 to isolate Lac^+ transductants in which the Lac plasmid, or a portion of it, had become integrated into the chromosome. Hence, stabilization of the lactose genes was accomplished (McKay and Baldwin, 1979). This conclusion was based on the following observations.

9. First was the effect of successive transfers in the presence of acriflavine (AF) on the Lac^+ transductants. The parental culture S. lactis C2 possessed 88% Lac^- cells after 6 consecutive transfers. Lac^+ transductants normally contained from 12 to 22% Lac^- cells after 10 consecutive transfers. However, no Lac^- cells were detected in 4 Lac^+ transductants even after 10 consecutive transfers. This suggested that AF was ineffective in causing the conversion from Lac^+ to Lac^- in these transductants and that lactose metabolism had been stabilized. Indeed, when the Lac^+ transductants were examined for plasmid DNA, those which were curable by AF possessed a 22 Mdal plasmid, while in those unaffected by AF no detectable plasmid could be observed.

10. This stabiiization was also demonstrated by growing the culture under conditions non-conducive for maintenance of the lactose plasmid. When S. lactis C2, the parental strain, was grown continuously in a chemostat with glucose as

the fermentable sugar, over 90% of the cells in the growth chamber were Lac$^-$ after 120 h, whereas no Lac$^-$ variants could be detected when a stabilized transductant was grown in the chemostat for over 150 h.

11. The final proof that lactose metabolism had become integrated came from examining the kinetics of UV inactivation of transducing ability. According to Arber (1960), the transducing frequency of chromosomal genes may be stimulated by exposure of the transducing lysate to low UV doses, whereas transduction of plasmid linked determinants would show an exponential decrease. Therefore, an Arber experiment was performed to confirm whether lactose-fermenting ability was plasmid or chromosomal linked. The results were consistent for plasmid encoded lactose utilization in the parental culture, but for a chromosomal location in the stabilized strains.

12. Stabilization of the lac genes by causing the lactose plasmid or a portion of it to be incorporated into the chromosome now allows a study of the genes regulating lactose metabolism and may provide a means for stabilizing other metabolic properties, in lactic acid bacteria, which are vital for use in food or dairy fermentation processes.

VI. RECOMBINATION-DEFICIENT MUTANT OF S. lactis

13. A recombination-deficient (Rec$^-$) mutant of S. lactis would be beneficial in characterizing the recombination events occurring in dairy streptococci. Such a mutant was isolated on the basis of its sensitivity to methyl methanesulfonate. The mutant also displayed sensitivity to UV-irradiation. The inability of the mutant to mediate homologous recombination was demonstrated by transduction of plasmid-linked lactose-fermenting ability but not chromosomally-mediated streptomycin resistance (Anderson and McKay, 1983a).

VII. CONJUGATION

14. A second mechanism of genetic exchange between bacteria is conjugation. Conjugation is a sexual process requiring physical contact during which there is

transfer of genetic material from the donor cell to the recipient cell. Conjugal transfer of plasmid DNA among group N streptococci has been demonstrated and the conjugal transfer of plasmids responsible for lactose-fermenting ability has provided additional proof that this trait is plasmid mediated in many strains. Results obtained by Kempler in our laboratory demonstrated conjugation using S. lactis subsp. diacetylactis 18-16 as the donor and a plasmid-cured derivative of S. lactis C2 as the recipient (Kempler and McKay, 1979). Phenotypic data, as well as physical evidence, indicated that the genes responsible for lactose metabolism in 18-16 were linked to the 41 Mdal plasmid. Transfer of this plasmid from 18-16 to the Lac^- plasmid cured devivative converted the strain to the Lac^+ phenotype, providing genetic evidence that the 41 Mdal plasmid in 18-16 is the lactose plasmid.

15. Results obtained by Walsh from our laboratory demonstrated conjugation using S. lactis ML3 as the donor in conjugal matings (Walsh and McKay, 1981). This strain possessed at least four plasmids of 55, 8.5, 3.0, and 1.5 kilobases (kb). Lactose metabolism is linked to the 55 kb plasmid; the other plasmids are cryptic. The recipient was a plasmid-cured derivative of S. lactis C2. All the transconjugants appeared to contain a single plasmid of nearly twice the size of the lactose plasmid in the donor. Although all of the transconjugants from the mating contained a plasmid of approximately 104 kb, we did observe a difference in growth behavior among them. The majority did not grow as a uniform suspension of cells in broth, but rather formed aggregates of cells that did not disperse even after vigorous vortexing. The appearance of cell aggregates was similar to that described by Dunny, et al. (1978) for S. faecalis mating mixtures and by S. lactis 712 (Gasson and Davies, 1980). There were differences between the ML3 system and the S. faecalis system. In the ML3 system, Lac^+ transconjugants alone, but not the original parental mating mixtures formed aggregates when grown in broth and cell-free filtrates of

clumping strains did not induce non-clumpers to aggregate or to mate at higher frequency.

16. The relationship between cell aggregation and conjugation frequency was also examined. When ML3 was the donor, lactose-fermenting ability was transferred at a frequency of 10^{-6}. A non-clumping transconjugant transferred at the lower frequency of 10^{-7}. A clumping transconjugant transferred at a frequency of 1.7 to 10^{-1}. From the ML3 systems, we proposed that some strains of group N streptococci possess a transfer factor that is either chromosomal or plasmid mediated. Translocation of this factor onto the lactose plasmid greatly increases the transfer frequency of the plasmid, and in the ML3 system results in cell aggregation.

17. Further analysis of this system was limited by our inability to isolate required quantities of plasmid DNA. However, we recently developed a methodology which overcomes this limitation (Anderson and McKay, 1983b). As a result, restriction mapping was employed to characterize the 104 kb cointegrate lactose plasmids from 15 independent transconjugants, derived from S. lactis ML3, as well as the 55 kb lactose plasmid (pSK08) and a previously uncharacterized 48.4 kb plasmid (pRS01) from S. lactis ML3 (Anderson and McKay, 1984b). The data revealed that the 104 kb plasmids were cointegrates of pSK08 and pRS01 and were structurally distinct. The replicon fusion event occurred within adjacent 13.8 kb or 7.3 kb PvuII fragments of pSK08, and interrupted apparently random regions of pRS01. Correlation of the transconjugants' clumping and conjugal transfer capabilities with the interrupted region of pRS01 also identified pRS01 regions coding for these properties. In the 104 kb plasmids, the pRS01 region was present in both orientations with respect to the pSK08 region. Replicon fusion occurred in the Rec$^-$ strains, and appeared to introduce a 0.8-1.0 kb segment of

DNA within the junction fragments. The degradation of the cointegrate plasmids was monitored by examining the lactose plasmids from nonclumping derivatives of clumping transconjugants. These plasmids displayed either precise or imprecise excision of pRS01 sequences or had dramatically reduced copy numbers. Both alterations occurred by Rec$^-$ independent mechanisms. Alteration of a transconjugant's clumping phenotype also occurred by rec independent inversion of a 4.3 kb KpnI-PvuII fragment within the pRS01 sequences of the cointegrate plasmid.

18. To construct strains for industrial use, it will be necessary to have an efficient system for moving genes from one strain to another. We have identified clumping and transfer regions in pRS01 which correlate with high frequency conjugation. It may be possible to move this region onto other non-conjugative plasmids in order to get rapid mobilization of the plasmid into a desired strain.

VIII. PRODUCTION OF ANTAGONISTIC PROTEINS

19. The production of inhibitory substances by dairy streptococci has been well documented. Some of these substances have been shown to inhibit spoilage and pathogenic organisms. Additionally they provide the potential for influencing the dominance of one strain over another in mixed or multiple strain starter cultures used for dairy fermentations. During examination of S. diacetylactis WM4 for conjugation of lactose-fermenting ability, we found that this strain was also capable of conjugally transferring the ability to produce the inhibitory substance to S. lactis (Scherwitz, et al, 1983). The ability to produce the inhibitory substance was linked to an 88 Mdal plasmid in WM4. The ability to transfer the genetic factor controlling inhibitory substance production to other lactic acid bacteria may ultimately lead to construction of additional strains capable of inhibiting spoilage or pathogenic organisms during milk, meat, or

plant fermentation processes.

20. Gasson recently demonstrated conjugal transfer of nisin-producing ability into a strain of S. lactis (Gasson, 1984). Nisin is a polypeptide antibiotic produced by some S. lactis strains and it has application as a food preservative. The possible linkage of nisin-producing ability to plasmid DNA may lead to the construction of a "super-nisin"-producing derivative by genetic approaches (Gasson, 1984). Such a strain may have commercial value.

IX. NISIN RESISTANCE

21. In yet another conjugation system we found that S. diacetylactis DRC3 contains a 40 x 10^6 dalton plasmid (pNP40) which is transferred to a plasmid-cured derivative of S. lactis C2 (McKay and Baldwin, 1984). Transconjugants containing pNP40 acquired resistance to nisin. It was subsequently established that pNP40 codes for nisin resistance. This finding may have application in the development of cloning vectors applicable to microorganisms used in dairy and food fermentation processes since traditional antibiotic selection markers may be unacceptable due to possible transmission of the drug resistance plasmid.

X. PHAGE RESISTANCE

22. In addition to the above findings, it was found that when these transconjugants acquired pNP40, they became resistant to phage growth at 21°C and 32°C, but not at 37°C. Further results substantiated that pNP40 was also coding for temperature-dependent phage resistance. The increasing evidence for association of plasmids with phage resistance in lactic streptococci provides a genetic mechanism to explain the rapid development of phage sensitive dairy starter cultures and the manipulation of these plasmids by genetic engineering techniques could be one approach for obtaining phage resistant mutants for commercial purposes. It may also be possible to develop phage resistant strains by

conjugally transferring the appropriate plasmid to selected phage sensitive strains of dairy streptococci as well as to other lactic acid bacteria. In the case of pNP40, nisin could possibly serve as the selective marker. It may also be of value to combine the different genetic loci for phage resistance in a single strain and to stabilize the phage resistant phenotype by integrating the phage resistance genes into the chromosome or into a high copy number plasmid.

XI. TRANSFORMATION

23. As illustrated above, the development of gene transfer systems in dairy streptococci has increased our knowledge of their genetics and plasmid biology. Conjugation and transduction have now been well documented (see review by Gasson, 1983), and transformation (Kondo and McKay, 1982), transfection (Geis, 1982), and protoplast fusion (Gasson, 1980) have been reported. Although these gene transfer mechanisms have aided in studying the genetics and plasmid biology of these organisms, the development of a more efficient plasmid transformation system is vital for further genetic studies and for the use of recombiant DNA technology for strain improvement.

24. Recently we described polyethylene glycol (PEG)-induced transformation of S. lactis protoplasts using plasmid DNA (Kondo and McKay, 1980). The frequency of transformation was low (approx. 8.5 transformants/ug DNA) using pLM2103, a 23 Mdal transductionally-shortened plasmid coding for lactose utilization. To increase the transformation frequency, the parameters affecting the PEG-induced plasmid transformation of S. lactis LM0230 protoplasts were examined (Kondo and McKay, 1984). In contrast to spreading protoplasts over the surface of an agar medium, their incorporation into soft agar overlays enhanced regeneration of protoplasts and eliminated variability in transformation frequencies. PEG with a molecular weight of 3350 at a final concentration of 22.5% yielded optimal

transformation. A 20 min PEG treatment of protoplasts in the presence of DNA was also necessary for maximal transformation. The number of transformants recovered increased as the protoplast and DNA concentration increased over the range of 3.0×10^6 to 3.0×10^8 protoplasts and 0.25 to 4.0 ug DNA per assay, respectively. Using these parameters, transformation was increased to 5×10^3 to 4×10^4 transformants per ug DNA.

XII. CLONING IN S. lactis

25. The feasibility of using this S. lactis protoplast transformation system and pGB301, an S. sanguis cloning vector, for molecular cloning of lactose metabolism was then examined. Experiments were designed to insert BglII and BclI restriction endonuclease fragments of pLM2001, a 33 kb plasmid coding for lactose metabolism, into the single BclI site of pGB301. BglII restriction digestion of pLM2001 yielded at least six fragments ranging from 17.9 to 0.3 kb while BclI restriction digestion generated at least 8 fragments ranging from 19.4 to 0.4 kb. Recombinant DNA molecules formed from insertion of these fragments into pGB301 transformed S. lactis LM0230 at frequencies of 10 to 100-fold lower than the covalently closed circular form of pGB301. Thirty-six Lac^+ transformants containing plasmids with BclI inserts and 16 Lac^+ transformants containing plasmids with BglII inserts were obtained. Fragments cloned which code for lactose metabolism were determined by restriction endonuclease digestion and Southern blot hybridization. The lactose-metabolizing genes appear to reside on the 17.9-kb BglII and the 19.4-kb BclI fragments of pLM2001. Recombinant plasmids isolated from Lac^- transformants showed that a broad spectrum of fragments had been cloned. These results indicate that pGB301 is a suitable cloning vector and the transformation frequency is sufficient for cloning plasmid-coded genes in S. lactis.

26. The development of a transformation system in dairy streptococci was a key

for performing recombinant DNA experiments for strain improvement. New avenues of investigation are now open for the study of gene expression, regulation, and plasmid development in these industrially important bacteria.

XIII. CONCLUSION

27. In conclusion, it is now well established that lactic acid bacteria used in starter cultures harbor plasmids of diverse sizes and that some of these plasmids code for properties vital for successful fermentation processes. Attempts to elucidate the functional properties of cryptic plasmids must continue. Amplification of desired plasmid genes should be possible through the isolation of copy number mutants (Anderson and McKay, 1984) which may increase the efficiency of the fermentation process as well as the quality of the final product. Stabilization of plasmid-mediated traits by integrating the genes into the chromosome may also prove beneficial. Clearly, the study of plasmid biology in dairy and food starter cultures has become a prerequisite for future strain improvement programs. This knowledge, coupled with the developing plasmid transfer systems applicable to lactic acid bacteria, is essential for gene cloning work within this group of organisms.

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