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High-Frequency Conjugation Associated with *Streptococcus lactis* Donor Cell Aggregation

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Conjugal transfer of the *Streptococcus lactis* 712 lactose plasmid was found to occur at a low frequency. Variants of this plasmid were selected which had much greater donor abilities and which also exhibited an unusual cell aggregation phenotype.

To grow rapidly in milk, the dairy starter organism *Streptococcus lactis* must use lactose as a source of energy and also digest milk protein to satisfy its essential amino acid requirements. The uptake of lactose by the phosphoenolpyruvate transferase system and the production of proteinase enzymes are both plasmid-controlled properties (3, 6, 8).

Transfer of plasmids by conjugation-like processes has been reported previously in the enterococci (2, 5, 7). A similar mechanism for transfer of lactose genes by *S. lactis* has been briefly reported previously (Gasson and Davies, Soc. Gen. Microbiol. Quart. 6:87, 1979), and further details of the process are presented here.

MATERIALS AND METHODS

Bacterial strains. All strains used in this work were derived from *S. lactis* NCDO 712. This strain was found to be lysogenic, but prophage-cured derivatives were readily isolated (Gasson and Davies, submitted for publication). One such strain (SH4109) was used here to preclude possible confusion with transduction of lactose genes (10). Table 1 lists the strains constructed for use in the mating experiments, and the derivation of progeny strains is shown in the following tables. Antibiotic-resistant mutants were made by UV mutagenesis.

Media and reagents. The M17 medium of Terzaghi and Sandine (12) was the routinely used growth medium. For growth or selection of lactose-utilizing strains, the original M17 medium containing lactose was used, but whenever lactose-defective strains were involved, glucose was substituted as the energy source. The bromocresol purple lactose indicator agar was described previously by McKay et al. (9), and the clear, "citrated" milk agar was described previously by Stadhouders (11). Where appropriate, these media were supplemented with 0.5% (wt/vol) lactose or glucose and 0.01% (wt/vol) Casamino Acids (Difco Laboratories). Dilutions were made in Ringer solution (Oxoid Ltd.). Streptomycin (Sigma Chemical Co.) and spectinomycin (The Upjohn Co.) were used at a final concentration of 200 μg/ml. Bovine pancreatic DNase I and lysozyme were obtained from Sigma Chemical Co., and diethyl pyrocarbonate and ethidium bromide were obtained from British Drug House.

Solutions used for the physical isolation of plasmid DNA were a lysis mixture (0.1 M Tris-hydrochloride, 0.05 M EDTA, 0.8 M NaCl, 1% sodium dodecyl sulfate [pH 8.0]) and E buffer (0.04 M Tris-hydrochloride, 0.02 M sodium acetate, 0.001 M EDTA [pH adjusted to 8.2 with acetic acid]).

Detection of lactose and proteinase phenotypes. Lactose-utilizing strains were differentiated from lactose-defective strains by the pH change during growth on lactose indicator agar. Both the lactose and the proteinase phenotypes were detected by use of citrated milk agar. Efficient growth of a test isolate on citrated milk agar led to acid production and a cloudy precipitation zone around a stab. When an isolate was defective for either lactose utilization or proteinase production, growth on citrated milk agar was very slow, and no acid precipitation zone was found. The specific nature of such a defect was defined by performing stab tests on three supplemented variants of citrated milk agar. Glucose or Casamino Acids was added to remove the dependence on lactose utilization and proteinase production for rapid growth.

Plasmid-cured derivatives. Curing of the lactose and proteinase genes was achieved by overnight growth of a 0.1% inoculum in glucose-M17 broth, either at 30°C in the presence of 5 μg of ethidium bromide per ml, or at the near-inhibitory temperature of 40°C. After the curing treatment, cells were plated on lactose indicator agar, and any white, lactose-defective colonies were scored for lactose and proteinase phenotypes by using the citrated milk agar tests described above.

Mating experiments. Mating mixtures were prepared by using equal quantities of overnight donor and recipient cultures. For broth mating, these mixtures were diluted in fresh glucose-M17 broth (10-fold for 18-h mating experiments and 2-fold for 2-h mating experiments) and incubated at 30°C. For plate mating, 0.2 ml of undiluted mating mixture was spread onto a glucose-M17 agar plate and incubated for 18 h at 30°C. The bacterial lawn was suspended by cutting three agar plugs with an 18-mm-diameter cork borer and gently shaking them in 5 ml of Ringer solution. Filter-mating experiments involved collecting 2.5 ml of mating mixture on a 0.45-μm (pore diameter) filter (Millipore Corp.) and incubating this on the surface of a glucose-M17 agar plate for 2 h at 30°C. Cells were suspended in 5 ml of Ringer solution.

Transfer frequencies were expressed as the number

TABLE 1. *Properties of bacterial strains used in gene transfer experiments*

Strain[a]	Lysogeny[b]	Plasmid presence		Counterselective markers[c]		Derivation
		Lactose	Proteinase	Streptomycin	Spectinomycin	
NCDO 712	+	+	+	S	S	
SH4109	−	+	+	S	S	NCDO 712
SH4045	−	−	−	R	S	SH4109
SH4065	−	−	−	S	R	SH4109

[a] NCDO, National Collection of Dairy Organisms. All strains prefixed SH (Shinfield) were derived at the National Institute for Research in Dairying.
[b] +, Present; −, absent.
[c] S, Sensitive; R, resistant.

of progeny colonies per recipient colony formed at the end of the mating period. This transfer frequency expression was chosen because the long-term mating experiments allowed considerable growth of the mating mixture between initiation of an experiment and selection of progeny.

DNase treatment. To inactivate any free DNA present in a donor culture, 20 mM $MgSO_4$ and 40 μg of DNase I per ml were added, and the mixture was incubated at 37°C for 15 min.

Physical isolation of DNA. A 50-ml amount of late-exponential-phase culture, grown in M17 broth, was harvested; the cells were then washed twice in chilled distilled water and suspended in 36 ml of 25% (wt/vol) sucrose solution. A 4-ml amount of a solution of 10 mg of lysozyme per ml of water was added, and the tube was gently swirled for 5 min at 5°C. Tubes were cooled and centrifuged at 1,500 × *g* for 30 min at 5°C. The pellet was warmed to room temperature and gently suspended in 1 ml of 25% sucrose in distilled water. Diethyl pyrocarbonate (50 μl) was added, followed by 1 ml of the lysis mixture. After each addition, tubes were gently rolled to mix the contents. Lysates produced during a 10-min incubation at room temperature were cooled on ice, and 0.5 ml of 5 M NaCl was added with gentle mixing. After 30 min on ice, cleared lysates were prepared by centrifuging at 32,000 × *g* for 30 min at 5°C. To remove protein, the supernatant was gently poured into a fresh tube, and an equal quantity of chloroform-isoamyl alcohol (24:1) was added. Tubes were gently rolled for 5 min, cooled on ice for 10 min, and centrifuged at 14,000 × *g* for 20 min at 5°C. The upper phase was retained, and 0.3 M sodium acetate and, after cooling to below −20°C, 2 volumes of ethanol were added. The mixture was rolled very gently and left for 3 h or overnight at −20°C to allow precipitation of nucleic acid. Tubes were centrifuged at 11,000 × *g* for 20 min at −20°C, and the precipitate was suspended in a 0.1 volume of *N*-tris(hydroxymethyl)methyl-2-aminomethane-sulfonic acid (TES).

Plasmid DNA was subjected to vertical agarose gel electrophoresis by using 0.7 and 1.0% agarose in E buffer. Electrophoresis was continued for 2 h at 100 V or 5 h at 50 V, and gels were stained for 20 min in E buffer containing 0.5 μg of ethidium bromide per ml. Plasmid bands were visualized with a transilluminator (model C61; Ultra-violet Products) and photographed with FP4 film (Ilford) and a no. 25 Wratten filter (Kodak).

RESULTS

Detection of lactose gene transfer. The abilities of the phage-free strain SH4109 to utilize lactose and to produce proteinase were readily lost after treatment with curing agents or growth at the near-inhibitory temperature of 40°C. A lactose- and proteinase-defective, streptomycin-resistant strain (SH4045) was used as a recipient in mating experiments with strain SH4109 as the donor. Mating experiments were carried out in broth or on the surface of agar plates. A low frequency of lactose gene transfer (10^{-7} progeny per recipient) occurred during plate mating, but no transfer ($<6.3 \times 10^{-9}$ progeny per recipient) was detected in broth. No colonies arose on control plates after equivalent periods of incubation.

The recombinant colonies were of two morphological types. Approximately one-half of the colonies were normal and soft, but the others were unusually hard and compact, retaining their integrity when moved with a loop. Broth cultures from the hard, variant colonies contained skeins of adhering cells, and, unlike normal cultures, they failed to suspend after mild centrifugation. Centrifuged cells remained as a solid pellet which disintegrated into smaller aggregates only after violent mixing in a Vortex blender. We postulate that this variant phenotype is controlled by a gene denoted *lax* (lax meaning loose or not compact). Hence, strains producing colonies of normal morphology were Lax^+, whereas strains exhibiting cell aggregation were Lax^-. The donor properties of both types of progeny colonies were further investigated.

Donor properties of Lax^+ progeny. The donor properties of 15 Lax^+ progeny strains (normal morphology), isolated from five separate mating experiments, were investigated. The frequencies of lactose gene transfer by these prog-

eny strains were determined in mating experiments with the spectinomycin-resistant recipient strain SH4065 (Table 2). Seven progeny strains reproduced the properties of the original donor strain SH4109. Lactose gene transfer was low, and the progeny colonies produced were again of two morphologies: 50% Lax^+ and 50% Lax^-. All seven strains were able to digest milk protein, showing that in the original mating, lactose gene transfer had been accompanied by transfer of the proteinase determinant. The remaining eight progeny strains transferred their lactose genes at a higher frequency (Table 2) and produced only normal Lax^+ progeny colonies. Seven of these strains had not received the proteinase determinant in the original mating experiment, whereas the eighth, SH4220, had done so (Table 2).

Donor properties of Lax^- progeny. Nine Lax^- variant progeny strains were isolated in independent mating experiments. Their lactose gene donor abilities were determined in broth and on plates by using the spectinomycin-resistant recipient strain SH4065 (Table 3). All Lax^- progeny strains were more efficient donors of the lactose genes than the parent strain SH4109. However, six strains (e.g., SH4054) achieved significantly higher frequencies of lactose gene transfer than the other three (e.g., SH4055). Mating in broth was always poor in comparison with mating on the surface of an agar plate. Selected transfer of the lactose genes was sometimes accompanied by cotransfer of the proteinase determinant, but, contrary to the observations for Lax^+ progeny strains (Table 2), no correlation between the presence of the proteinase activity and a low lactose gene transfer frequency was found.

A separate determination of transfer of the Lax^- cell aggregation phenotype was made. After mating, up to 1,000 recipient-strain colonies were screened by touching them with sterile toothpicks to detect the presence of any compact Lax^- colonies (Table 3). The more efficient Lax^- donor strains transferred lactose genes and the Lax^- phenotype at similar frequencies. The less efficient Lax^- donor strains failed to transfer the Lax^- characteristics at a frequency above the 10^{-3} limit of resolution imposed by the screening procedure. Colonies selected for lactose gene transfer and detected as having received the Lax^- characteristics were tested to determine whether the nonselected gene had been cotransferred. In all of the mating experiments, the lactose genes and the *lax* gene were transferred together. For example, in the mating with SH4054 as the donor (Table 3), 450 lactose-utilizing progeny colonies that were selected all proved to be Lax^- colonies, and 100 Lax^- progeny colonies, identified by screening recipient

TABLE 3. *Donor properties of progeny strains with Lax^- cell aggregation morphology*[a]

Donor strain	Proteinase activity[b]	Transfer frequency		Lac^+ transfer frequency in broth mating
		Lac^+	Lax^-	
SH4109[c]	+	1.0×10^{-7}		$<6.3 \times 10^{-9}$
SH4054	−	2.7×10^{-2}	1.5×10^{-2}	3.7×10^{-6}
SH4055	−	3.7×10^{-6}	$<1.0 \times 10^{-3}$	$<2.0 \times 10^{-9}$
SH4056	+	5.8×10^{-2}	3.0×10^{-2}	3.4×10^{-6}
SH4057	+	1.2×10^{-2}	2.6×10^{-2}	2.0×10^{-6}
SH4058	+	7.7×10^{-2}	1.3×10^{-2}	1.8×10^{-6}
SH4059	+	3.1×10^{-5}	$<1.0 \times 10^{-3}$	2.2×10^{-8}
SH4060	+	1.8×10^{-6}	$<1.0 \times 10^{-3}$	$<2.5 \times 10^{-9}$
SH4061	+	2.6×10^{-2}	1.5×10^{-2}	1.1×10^{-5}
SH4062	+	1.2×10^{-2}	6.9×10^{-2}	5.9×10^{-6}

[a] Mating experiments were carried out for 18 h either on the surface of an agar plate or in broth. Frequencies for the Lax^- morphology were determined by screening recipient-strain colonies selected after mating. The recipient strain used was SH4065, except for the original mating with SH4109, when the recipient strain was SH4045.

[b] +, Active; −, not active.

[c] Original mating from which the remaining donor strains were selected as Lax^- progeny colonies.

TABLE 2. *Donor properties of progeny strains with normal Lax^+ morphology*[a]

Donor strain	Proteinase activity[b]	Lactose gene transfer frequency	Morphology of progeny
SH4109[c]	+	1.0×10^{-7}	Lax^+ and Lax^-
SH4088, SH4128, SH4210, SH4211, SH4212, SH4213, and SH4214	+	0.8×10^{-7} to 3.9×10^{-7}	Lax^+ and Lax^-
SH4196, SH4138, SH4215, SH4216, SH4217, SH4218, and SH4219	−	1.0×10^{-5} to 9.0×10^{-5}	Lax^+ only
SH4220	+	2.1×10^{-5}	Lax^+ only

[a] Mating experiments were carried out for 18 h on the surface of an agar plate, and transfer frequencies are expressed as the number of progeny colonies per recipient at the end of the mating period. Recipient strains were SH4045 for the original mating and SH4065 for subsequent experiments with the progeny strains.

[b] +, Active; −, not active.

[c] Original mating from which the remaining donor strains were selected as progeny colonies.

colonies after mating, were all able to use lactose as a carbon source. This apparent linkage between *lax* and lactose genes was confirmed in curing experiments. Representative lactose-utilizing, Lax^- progeny strains (SH4054, SH4055, SH4056, and SH4057) were grown either in the presence of ethidium bromide or at the near-inhibitory temperature of 40°C and plated on lactose indicator agar to detect lactose-defective cured cells. Loss of lactose genes was always accompanied by a change from the Lax^- cell aggregation morphology to the normal Lax^+ morphology. Curing of the lactose genes always caused the Lax^- phenotype to be lost.

Evidence for transfer of lactose genes by a conjugation-like process. The Lax^- donor strain SH4054 was used to investigate the nature of the process by which transfer of the lactose genes occurred. An overnight culture of this strain was divided into three aliquots; one was kept as a control, another was treated with DNase, and the third was used to prepare a sterile, cell-free filtrate. These donor culture preparations were used in 2-h mating experiments, either in broth or on Millipore filters. Transfer of the *lax* and lactose genes by filter mating occurred at the highest frequency observed during this work (0.56 per recipient). Treatment with DNase had no effect on this high transfer frequency (0.6 per recipient) or on the frequency of transfer in broth (8.4×10^{-4} without DNase and 1.5×10^{-3} with DNase). Conversely, a cell-free donor culture filtrate had no detectable lactose gene transfer activity. These results, together with the precautions taken to eliminate bacteriophages from the system, appear to exclude both transformation and transduction as mechanisms for the lactose gene transfer that was observed. Taking into account the importance of cell aggregation for high-frequency transfer, the involvement of a conjugation-like process is strongly suggested.

Plasmids. The plasmid complement of strain 712 was studied (Fig. 1). Plasmid profiles for the donor strain SH4109, the recipient strain SH4045, and three Lax^- progeny strains in which lactose gene transfer had been selected were compared. Although the lactose- and proteinase-cured recipient strain lost two bands that were present in the parent strain, no change in the plasmid profile of this parent strain occurred after transfer of the lactose genes by conjugation. Similar results were obtained for Lax^+ progeny strains.

Although further lactose- and proteinase-negative derivatives of SH4109 were found with these same two bands missing, one lactose- and proteinase-negative strain was also isolated in which these bands remain. Conversely, a lactose- and proteinase-positive strain was found in which these bands were absent. It is likely that the bands lost from SH4045 represent readily cured cryptic plasmids, frequently eliminated during the isolation of lactose- and proteinase-defective strains. Therefore, despite the presence of physically demonstrable plasmids, the lactose and proteinase plasmids of strain SH4109 have yet to be physically identified.

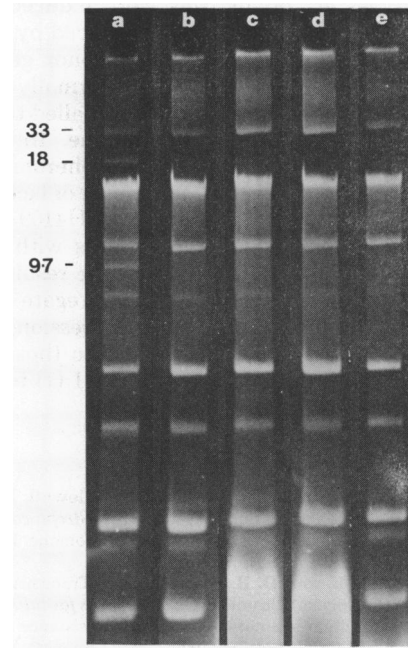

FIG. 1. *Plasmid profiles. Plasmids of the donor strain SH4109 (a), the lactose-cured recipient strain SH4045 (b), and three lactose-utilizing Lax^- progeny strains (c to e) are shown. Plasmid DNA, prepared by the cleared-lysate procedure (see the text), was separated on a 0.7% agarose gel by electrophoresis at 50 V for 5 h. Molecular weights ($\times 10^6$) are shown for representative plasmid bands.*

DISCUSSION

The conjugation-like system of *S. lactis* 712 was capable of transferring lactose genes to 60% of recipients during a mating experiment. This approaches the efficiency with which the classical *Escherichia coli* sex factor F is transferred and offers considerable potential for studying the genetics of group N streptococci.

High-frequency transfer of lactose genes was achieved by donor strains which exhibited an unusual cell aggregation morphology, and the transfer process may be related to that described by Dunny et al. (1) for hemolysin plasmid transfer in *Streptococcus faecalis*. In the experiments

of Dunny et al., donor cells were induced to aggregate by a sex pheromone produced by the recipient cells. Expression of the donor genes responsible for aggregation was normally repressed, and the recipient strains signalled their derepression via the sex pheromone. In the transfer system of *S. lactis*, no sex pheromone has been detected. The low frequency of lactose gene transfer in matings involving SH4109 imposed a strong selection for variants with increased donor ability. The cells of the resulting high-frequency donors tended to aggregate and appeared to have changed gene expression, allowing a transfer system analogous to those of hemolysin plasmids pAMγ1 and pPD1 (1) to be constitutively expressed.

LITERATURE CITED

1. **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. U.S.A. **75**:3479–3483.
2. **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**:785–790.
3. **Efstathiou, J. D., and L. L. McKay.** 1976. Plasmids in *Streptococcus lactis*: evidence that lactose metabolism and proteinase activity are plasmid linked. Appl. Environ. Microbiol. **32**:38–44.
4. **Finnegan, D. J., and N. S. Willetts.** 1972. The nature of the transfer inhibitor of several F-like plasmids. Mol. Gen. Genet. **119**:57–66.
5. **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.
6. **Klaenhammer, T. R., L. L. McKay, and K. A. Baldwin.** 1978. Improved lysis of group N streptococci for isolation and rapid characterization of plasmid deoxyribonucleic acid. Appl. Environ. Microbiol. **35**:592–600.
7. **Marder, H. P., and F. H. Kayser.** 1977. Transferable plasmids mediating multiple-antibiotic resistance in *Streptococcus faecalis* subsp. *liquefaciens* Antimicrob. Agents Chemother. **12**:261–269.
8. **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.
9. **McKay, L. L., K. A. Baldwin, and E. A. Zottola.** 1972. Loss of lactose metabolism in lactic streptococci. Appl. Microbiol. **23**:1090–1096.
10. **McKay, L. L., B. R. Cords, and K. A. Baldwin.** 1973. Transduction of lactose metabolism in *Streptococcus lactis* C2. J. Bacteriol. **115**:810–815.
11. **Stadhouders, T.** 1961. The hydrolysis of protein during the ripening of cheese: some methods to accelerate the ripening. Neth. Milk Dairy J. **15**:161–164.
12. **Terzaghi, B. E., and W. E. Sandine.** 1975. Improved medium for lactic streptococci and their bacteriophages. Appl. Microbiol. **29**:807–813.