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Development of High-Level Streptomycin Resistance Affected by a Plasmid in Lactic Streptococci†

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Some lactose-negative (Lac$^-$) mutants of *Streptococcus lactis* C2 and ML3 exhibited development of very high level streptomycin resistance after incubation with subinhibitory concentrations of the drug for 18 to 22 h. These drug-resistant mutants showed no loss of resistance even after 6 months of subculturing in broth without any drug. The parental Lac$^+$ strains did not show mutation to high-level streptomycin resistance. The Lac$^+$ characteristic of the parental strain was conjugally transferred to Lac$^-$ derivatives of C2 and ML3, showing the ability to mutate to high-level resistance. When transconjugants were analyzed for this characteristic, they showed both mutable and nonmutable Lac$^+$ types. The results suggested that genetic information for mutation to high-level streptomycin resistance in lactic streptococci resides on the chromosome, and its expression is affected by a plasmid. The plasmid profiles of strains C2, ML3, C2 Lac$^-$, ML3 Lac$^-$, and two kinds of transconjugants confirmed the presence of a plasmid of approximately 5.5 megadaltons in strains showing no mutation to high-level streptomycin resistance, while strains missing such a plasmid exhibited high-level streptomycin resistance after incubation with subinhibitory concentrations of the drug.

A number of genetic characteristics have been found to be associated with plasmid DNA in lactic streptococci, including genetic determinants for lactose metabolism, a vital characteristic for successful fermentation (6, 8, 11, 12, 16, 17, 19, 20, 24, 25, 28). Lactic streptococci are nutritionally fastidious organisms, which makes it imperative to use antibiotic resistance as a selective marker in the transfer of genetic information from one bacterial culture to another. Although the nature of drug resistance in lactic streptococci is obscure, information on the susceptibility of various cultures to several antibiotics has been noted (3, 23). Recently, it has also been shown that buffering complex growth media with phosphates increases the resistance of lactic streptococci to aminoglycoside antibiotics (26).

During the isolation of streptomycin-resistant mutants from lactose-negative (Lac$^-$) derivatives of *Streptococcus lactis* C2, it was noticed that when some Lac$^-$ strains were incubated with certain concentrations of streptomycin sulfate well below the MIC for 18 to 22 h at 32°C, the culture showed resistance 20- to 1,000-fold higher than the treatment concentrations of the drug. Under similar experimental conditions the parental strain C2 did not show such increase in resistance to streptomycin. The present study was therefore undertaken to examine by genetic analysis the nature of such high-level streptomycin resistance in Lac$^-$ variants and the role of a plasmid in the development of such resistance in lactic streptococci.

MATERIALS AND METHODS

Bacterial strains. The origin and phenotype of bacterial strains used in this investigation are described in Table 1.

Media and growth of culture. The bacterial strains and their Lac$^-$ mutants and transconjugants were maintained by biweekly transfer in sterile (121°C, 10 min) 10% reconstituted nonfat milk after overnight incubation at 32°C. In the case of Lac$^-$ mutants, the milk was supplemented with 1% glucose. The cultures were held at 4°C between transfers. M17 (29) and M17G broth (in which glucose was substituted for lactose) were used to propagate the Lac$^+$ and Lac$^-$ cultures, respectively. Since the presence of phosphate in the media has been shown to increase the resistance levels of lactic streptococci (26), lactic broth (LB; Difco Laboratories) (7), which does not contain phosphate, was used for testing the development of high-level streptomycin resistance in the cultures. Agar plates were made by adding 1.5% agar (Difco) to liquid broth.

The growth of the cultures was tested in LB. The turbidity of an overnight broth culture (16 to 18 h, 32°C) diluted (2%) in fresh broth was followed with a Klett-Summerson colorimeter equipped with a no. 54 filter. In one experiment, the cultures were inoculated into 30 ml of fresh broth in Klett flasks. After 2 h of growth at 32°C, the cultures were split into three flasks, and 20 and 50 mg of streptomycin per ml were added to the first and second flasks, respectively, while the third, without drug, was kept as a control. The growth was then followed by hourly Klett readings.

Detection of Lac$^-$ mutants. Lactic agar plates containing 1% lactose as the primary carbon source and 0.004% bromocresol purple as indicator (18) were used for differentiating Lac$^-$ (white) from Lac$^+$ (yellow) colonies. The bacterial cultures were treated with 6 μg of acriflavine (AF) per ml for 18 to 22 h at 32°C in M17G broth, and 0.1 ml of appropriate dilutions was surface spread. The plates were incubated at 32°C for 24 to 48 h. Lac$^-$ colonies were picked up and purified after two transfers on the indicator plate.

Induction of high-level streptomycin-resistant mutations. Strains C2, ML3, and their Lac$^-$ variants were grown overnight in lactic broth at 32°C. The fresh broths were then inoculated with overnight cultures (2% inoculum) with and without different concentrations of streptomycin, and culture tubes were incubated for 18 to 22 h at 32°C. In experiments on the time course of induction, streptomycin was added to a final concentration of 50 μg/ml, and the tubes with and without the drug were incubated at 32°C. At regular intervals samples were withdrawn and 0.1 ml of appropriate dilutions was plated on streptomycin-free lactic agar plates

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TABLE 1. *S. lactis* strains used

Strain no.	Relevant phenotype[a]	Source or derivation
C2	Lac$^+$ Mut$^-$ Str^s	This laboratory
RSO13	Lac$^-$ Mut$^+$ Str^s	Spontaneous Lac$^-$ mutant of C2
RSO14	Lac$^-$ Tet^r Ery^r Mut$^+$ Str^s	Tetracycline (5 μg/ml) and erythromycin (5 μg/ml) resistant variant of strain RSO13
RSO15	Lac$^-$ Str^s Mut$^-$	Lac$^-$ mutant of C2
RSO16	Lac$^-$ Str^r Mut$^+$	Streptomycin-resistant variant of RSO13
RSO17	Lac$^+$ Tet^r Ery^r Mut$^+$	Transconjugant of cross C2 × RSO14
RSO18	Lac$^+$ Tet^r Ery^r Mut$^-$	Transconjugant of cross C2 × RSO14
ML3	Lac$^+$ Mut$^-$ Str^s	This laboratory
RSO31	Lac$^-$ Mut$^-$ Str^s	Spontaneous Lac$^-$ mutant of ML3
RSO32	Lac$^-$ Mut$^+$ Str^s	AF-induced Lac$^-$ mutant
RSO33	Lac$^-$ Mut$^+$ Str^s	AF-induced Lac$^-$ mutant
RSO34	Lac$^-$ Mut$^+$ Str^s	Spontaneous Lac$^-$ mutant
RSO35	Lac$^-$ Mut$^+$ Str^r	From RSO34
RSO36	Lac$^-$ Mut$^+$ Tet^r Ery^r	From RSO34
RSO37	Lac$^+$ Mut$^+$ Tet^r Ery^r	Transconjugant of cross ML3 × RSO36
RSO38	Lac$^+$ Mut$^-$ Tet^r Ery^r	Transconjugant of cross ML3 × RSO36

[a] Phenotypic symbols: Lac$^+$, lactose-fermenting ability; Str^s, sensitive to 100 μg of streptomycin per ml; Str^r, resistant to 2×10^4 μg of streptomycin per ml; Mut$^-$, nonmutable to high-level streptomycin-resistance; Mut$^+$, mutable to high-level streptomycin-resistance after incubation with concentrations of streptomycin below the MIC, (presumptive phenotype; the presence of a suppressor mutation in the strain is not excluded).

for total viable cells and on plates containing 10^3 μg of streptomycin per ml to obtain the number of resistant cells.

Isolation of tetracycline- and erythromycin-resistant mutants. Overnight growth of strains RS013 and RS034 in LB was inoculated into fresh broth with and without 0.5 μg of tetracycline per ml, and the tubes were incubated at 32°C for 24 h. The overnight tetracycline-treated culture was further inoculated into fresh broth containing 1 or 5 μg of tetracycline per ml and incubated for 24 to 48 h at 32°C. Single-colony isolates were purified from the resistant broth culture containing 5 μg of tetracycline per ml. Similarly, a culture resistant to 5 μg of erythromycin per ml was then isolated from the tetracycline-resistant strain. These isolates were designated RS014 and RS036, respectively. The strains were maintained in LB containing 5 μg of tetracycline and 5 μg of erythromycin per ml.

Conjugation. Solid-surface matings were carried out as described by McKay et al. (20). In the conjugation, strains RS014 and RS036 were used as recipients and C2 and ML3, sensitive to tetracycline and erythromycin, respectively, served as donors. Cell-free filtrate of the donor strain was mixed with recipient cells and tested for Lac$^+$ transfer. This was done to determine whether the transfer of Lac$^+$ was due to transduction.

Chemicals. The chemicals were from the following sources: acriflavine hydrochloride (AF), Allied Chemicals, Morristown, N.J.; streptomycin sulfate, kanamycin sulfate, neomycin sulfate, tetracycline hydrochloride, and erythromycin, Sigma Chemical Co., St. Louis, Mo.; bromocresol purple, BDH Chemical, Toronto, Ontario.

Plasmid analysis. Plasmid DNA was extracted from the parental strain and its Lac$^-$ mutants and transconjugants by the procedure of Anderson and McKay (1) with the following modifications. The cells were grown in a 60-ml volume of M17G containing 20 mM DL-threonine (13). The reagents were scaled down 10-fold from the recommended concentrations for the preparative protocol. The centrifugation after phenol and chloroform-isoamyl alcohol extraction was performed at 4,000 rpm in a tabletop GLC-1 (Sorvall, general laboratory) centrifuge at room temperature, and DNA was collected after isopropanol precipitation by centrifugation at 12,000 × *g* for 20 min at 4°C in a Sorvall centrifuge. *Escherichia coli* V517 containing multiple plasmids was employed as a single source of covalently closed circular DNA molecules of different sizes (14). Plasmid DNA was extracted as described by Birnboim and Doly (2). The chromosomal DNA was extracted by the method of Marmur (15).

Agarose gel electrophoresis. Agarose gel electrophoresis was performed in a Tris-acetate buffer as described by Anderson and McKay (1). Gels contained 0.8% agarose, and electrophoresis was performed in a submerged horizontal gel apparatus (Bio-Rad Laboratories Canada, Ltd.) at 100 V for 3.5 to 4 h. Gels were stained with 0.5 μg of ethidium bromide per ml for 30 min and viewed with UV light with a transilluminator (model TM-20; UVP, Inc., San Gabriel, Calif.). Photographs were taken with CPP contrast process pan film or Polaroid type 57 film and a K2 filter (Eastman Kodak Co., Rochester, N.Y.).

RESULTS

Isolation of Lac$^-$ derivatives. AF treatment of strains C2 and ML3 yielded 22 to 25% and 23 to 25% Lac$^-$ variants, respectively. A similar frequency of appearance of Lac$^-$ derivatives in C2 by AF treatment has been reported by McKay et al. (18). Twenty-five individual colonies of AF-induced and spontaneously arising Lac$^-$ types were selected at random from each strain. All the Lac$^-$ variants were then tested for the induction of high-level streptomycin resistance after incubation with low concentrations of streptomycin. Among the spontaneously arising and AF-induced Lac$^-$ variants of C2, 20 and 60%, respectively, showed development of high-level streptomycin resistance. In the case of ML3, 15 and 30% of spontaneously arising and AF-induced derivatives, respectively, showed development of high-level resistance. One such C2 Lac$^-$ variant, referred to as RS013, was chosen for further use in the present study.

Development of streptomycin resistance. Induction of mutation to high-level streptomycin resistance was tested by incubating cells of strain C2 and RS013 with 10, 20, 40, and 50 μg of streptomycin per ml. As shown in Fig. 1, 50 μg of streptomycin per ml treatment gave maximum resistance. There was an exponential increase in the development of high-level streptomycin resistance. It is important that strain C2 did not show any increase in resistance to streptomycin over the untreated control, although the MIC for C2 was similar to that for RS013 (Table 2). When induction of mutation to high-level streptomycin resistance was tested by incubating cells of strains C2 and RS013 with and without streptomycin (50 μg/ml), neither strain showed any cells resistant to 1 mg of streptomycin per ml for at least 2 h. However, after 3 h of incubation, 0.0083% of the cells of strain RS013 incubated with 50 μg of streptomycin per ml showed resistance to 1 mg of streptomycin per ml, and this proportion increased to 0.29% after 6 h without any detectable increase in the total number of viable cells. Under similar experimental conditions, neither C2 with and without streptomycin (50 μg/ml) nor RS013 without streptomycin showed any resistant cells.

One of the main characteristics of inducible resistance is that bacterial strains adapt rapidly to growth in media containing high concentrations of drug after only brief previous exposure of cells to subinhibitory concentrations of the drug (4, 10). This was tested by adding 20 and 50 μg of streptomycin per ml to the cultures at an early exponential stage. As shown in Fig. 2, growth of both strains C2 and RS013 was inhibited by these concentrations of streptomycin. Strain C2 with either 20 or 50 μg of streptomycin per ml showed logarithmic growth, but at a lower rate than the control culture. On the other hand, the growth of RS013 in the presence of 50 μg of streptomycin per ml was completely inhibited. Addition of 20 μg of streptomycin per ml was not as drastic as 50 μg of streptomycin per ml. However, under the experimental conditions, when 0.1 ml of culture with and without drug at each stage was tested on plates containing 10^3 μg of streptomycin per ml, no resistant colonies were found from either strain. In similar experiments with even lower concentrations of streptomycin, such as 5, 10, and 15 μg/ml, the cells did not show any development of high-level streptomycin resistance (data not shown). This indicated that the development of high-level streptomycin resistance may not be inducible. Since the growth of C2 in the presence of 50 μg of streptomycin per ml was relatively unaffected compared with that of RS013 (Fig. 2), a pertinent question arose whether this was why no high-level resistance developed. Therefore, C2 was incubated for 20 to 24 h with 60, 70, 80, 90, and 100 μg of streptomycin per ml and then tested for resistance to 1 mg of streptomycin per ml. None of the treatments resulted in any resistant colonies (data not shown). Thus, it became clear that although the inhibitory concentrations of streptomycin caused development of high-level resistance in RS013, they were ineffective in C2.

Next, the effect of the stage of cell growth on the induction of mutation was determined. From an exponentially growing culture of RS013 in LB, samples were withdrawn at regular intervals for 8 h and used as inocula for fresh broth containing 50 μg of streptomycin per ml. When the cultures were incubated for 20 to 24 h and tested for resistance to 10^3 μg of streptomycin per ml, none of them showed resistance. However, when an inoculum from an overnight culture was used, practically all of the cells showed resistance to 10^3 μg. Thus, it became apparent that the physiological state of cells is very important for such induction (Table 3).

TABLE 2. Drug resistance of *S. lactis* strains

Strain	MIC[a] (μg/ml)			
	Kanamycin	Neomycin	Streptomycin	Tetracycline
C2	200	400	100	2.0
RSO13	200	400	100	2.0
RSO15	200	400	100	ND[b]
RSO16	200	400	$>10^4$	2.0
ML3	100	400	100	1.0
RSO17	200	400	100	ND
RSO18	200	400	100	ND

[a] The MIC was defined as the lowest antibiotic level at which no colonies formed after plating 1×10^8 to 5×10^8 cells. RSO17 and RSO18 represent Mut^+ and Mut^- types, respectively.
[b] ND, Not determined.

Kinetics of development of resistance. Since the maximum number of resistant cells emerged from treatment with 50 μg of streptomycin (Fig. 1), this concentration was used for kinetic studies. There was drastic killing of RS013 cells when incubated with 50 μg of streptomycin per ml for about 10 h (Fig. 3). After 10 h, the survivors started growing, and when these cells were plated on lactic agar plates with and without 10^3 μg of streptomycin per ml the total number of viable cells

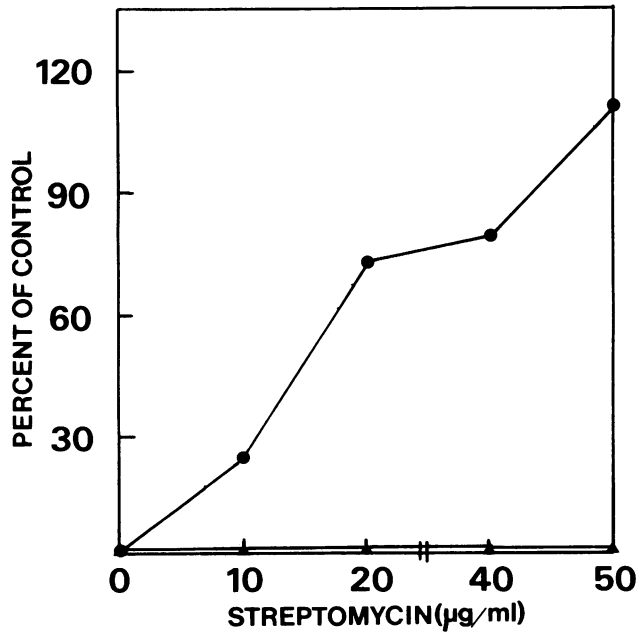

FIG. 1. Development of high-level resistance (10^3 μg of streptomycin per ml) as a function of streptomycin concentration in strains C2 (▲) and RS013 (●). Percent of control = [(colonies on plates with drug)/(colonies on drug-free plates)] × 100.

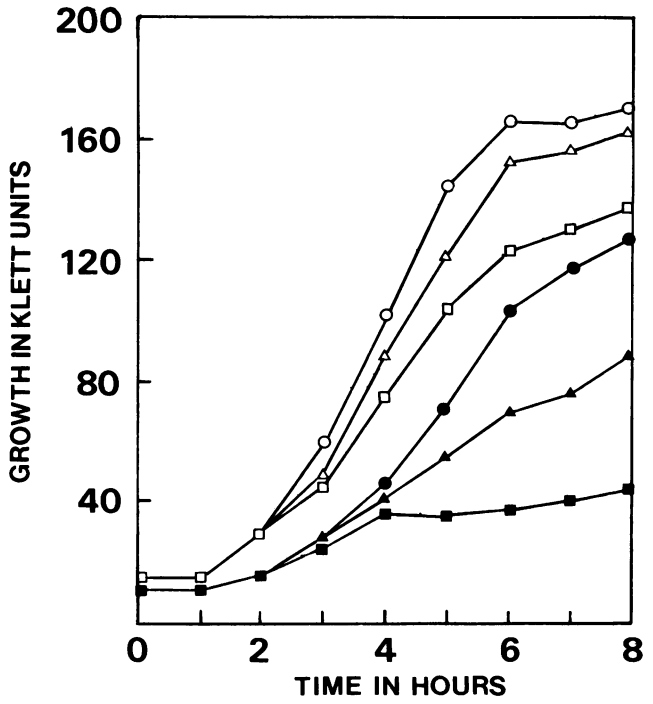

FIG. 2. Growth of strains C2 and RS013 with and without streptomycin. C2 (open symbols) and RS013 (closed symbols): control (○, ●) and 20 (△, ▲) and 50 (□, ■) μg of streptomycin per ml.

TABLE 3. Effect of stage of growth on development of high-level streptomycin resistance in strain RSO13

Culture time (h)	Growth (CFU/ml)	
	Drug-free plates[a]	Plates containing streptomycin (10^3 μg/ml)[b]
0	3.54×10^7	<2.5
2	1.39×10^8	<2.5
4	1.07×10^9	<2.5
6	1.82×10^9	<2.5
8	1.80×10^9	<2.5
16–18	1.19×10^9	1.10×10^9

[a] Fresh broth containing 50 μg of streptomycin per ml was inoculated with 2% inoculum from each stage of growth.
[b] Development of resistance to 10^3 μg of streptomycin per ml after 20 to 24 h of incubation of cells with 50 μg of streptomycin per ml.

on both kinds of plates was equal. This clearly showed that cells which developed resistance to 50 μg of streptomycin per ml also exhibited resistance to 10^3 μg of streptomycin per ml. These cells were later tested for resistance to 2×10^4 to 5×10^4 μg of streptomycin per ml and were found to be resistant. One such streptomycin-resistant mutant of RS013, termed RS016, was selected for testing cross-resistance to kanamycin, neomycin, and tetracycline. There was no difference between RS013 and RS016, showing that the development of streptomycin resistance was quite specific. The stability of the streptomycin resistance of strain RS016 was tested for 6 months by subculturing in LB without streptomycin. The strain was still resistant to 10^3 μg of streptomycin per ml.

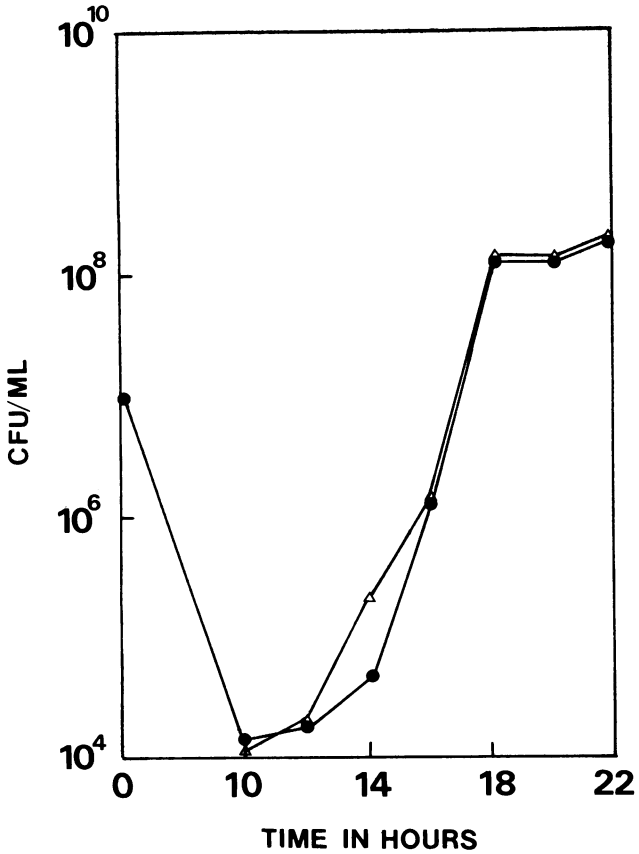

FIG. 3. Kinetics of development of mutation. Total viable cells (●) and streptomycin-resistant cells (△).

Effect of transfer of Lac$^+$ characteristics on the development of streptomycin resistance. Since Lac$^-$ cells occurring spontaneously or isolated after treatment with plasmid-curing agents showed development of high-level streptomycin resistance, it was of interest to test the effect of the transfer of Lac$^+$ on the development of such resistance. To obtain evidence for conjugal transfer of genetic information controlling the development of high-level streptomycin resistance, transfer of Lac$^+$ was used as a phenotypic marker for the selection of transconjugants. In conjugation, recipients with two antibiotic resistance markers were used to ensure that spontaneously arising antibiotic-resistant donors would not be confused with genuine transconjugants. Cells of donor strains C2 and ML3 were mixed with recipient strains RS014 and RS036, respectively, and Lac$^+$ recombinants were scored as shown in Table 4. Twenty-five Lac$^+$ transconjugants were isolated at random and purified. Single-colony isolates of 25 transconjugants from each cross together with the recipient and donor were then tested for induction to high-level streptomycin resistance by incubation with 50 μg of streptomycin per ml for 18 to 20 h at 32°C. Sixteen and 20 transconjugants of C2 and ML3 crosses, respectively, were found to develop resistance to 10^3 to 10^4 μg of streptomycin per ml. The absence of Lac$^+$ colonies from the mixture of donor cell filtrates and recipient cells showed that transduction was not involved in transferring *lac* genes from C2 and ML3 to RS014 and RS036, respectively.

Plasmid analysis of *S. lactis* C2, ML3, Lac$^-$ variants, and Lac$^+$ transconjugants. To determine whether the absence of induction to high-level streptomycin resistance in these *S. lactis* strains was due to the presence of a plasmid, clear lysates of parental strains, two types of Lac$^-$ variants, and transconjugants showing mutability and nonmutability were prepared. These preparations were then analyzed by agarose gel electrophoresis for the presence of different plasmid species (Fig. 4 and 5). Strain C2 in the present investigation did not show the six identical plasmid bands reported earlier (13). However, it became clear from the plasmid profiles (Fig. 4, lanes 2, 3, and 7) that strains C2, RS015, and RS018 carried a plasmid band (shown by arrow) which was missing in strains RS013, RS016, and RS017 (Fig. 4, lanes 4, 5, and 6). These strains which were missing the plasmid band were also mutable to high-level resistance, while those with that

TABLE 4. Transfer of genetic information controlling induction to high-level streptomycin resistance with the Lac$^+$ characteristic from *S. lactis* C2 to RSO14 and from ML3 to RSO36

Mating pair		No. of Lac$^+$ Tet^r Ery^r clones/ml		
Donor (Lac$^+$ Tet^s Ery^s)	Recipient (Lac$^-$ Tet^r Ery^r)	Donor alone	Recipient alone	Mixture of donor and recipient[a]
C2	RSO14	<1.5	<1.5	6.70×10^3
Filtrate of C2	RSO14	<1.5	<1.5	<1.5
ML3	RSO36	<1.0	<1.5	1.11×10^3
Filtrate of ML3	RSO36	<1.5	<1.5	<1.5

[a] 25 Lac$^+$ transconjugants were tested for development of high-level streptomycin resistance; 16 were found to develop resistance (mutable) and 9 showed no development of high-level resistance (nonmutable) in the C2 cross; in ML3, 20 were mutable and 5 were nonmutable.

plasmid band were nonmutable. The plasmid profiles of 25 transconjugants were also examined by agarose gel electrophoresis. All 16 transconjugants which were mutable appeared to lack that plasmid, while in all nine nonmutable transconjugants, a 5.5-megadalton (MDa) plasmid band was present. Next, the plasmid profiles of strain ML3 together with its six different Lac$^-$ variants and transconjugants were analyzed (Fig. 5). ML3 (Fig. 5, lane 1) showed five plasmid bands identical to those found by Anderson and McKay (1). The molecular sizes estimated by comparison with *E. coli* V517 plasmid markers were found to be 1, 2, 5.5, 33, and 60 MDa. The most important plasmid for this investigation is one below the chromosomal DNA band (Fig. 5, lanes 1, 2, and 9), which is about 5.5 MDa. Again, the strains carrying it were nonmutable and all other Lac$^-$ variants or transconjugants missing this plasmid were mutable. There was a slight trace of some kind of molecule below the chromosomal band (lanes 4, 6, 7, and 8). To see whether the intensity of such bands could be increased by increasing the concentration of DNA, plasmid profiles were examined by doubling the concentration. There was no increase in the intensity of this band, while others did increase. These strains were also mutable. It can thus be safely concluded that the 5.5-MDa plasmid was involved in this phenomenon.

DISCUSSION

In the present study it was noticed that some Lac$^-$ variants of strains C2 and ML3 developed very high-level streptomycin resistance (>10^4 μg/ml) after incubation with streptomycin sulfate at concentrations below the MIC. This was true with Lac$^-$ variants isolated either spontaneously or after treatment with conventional plasmid-curing agents. There were higher numbers of Lac$^-$ variants showing induction of high-level resistance to the drug among the AF-induced Lac$^-$ population than in spontaneously occurring Lac$^-$ derivatives. It is interesting that AF has been shown to be mutagenic to bacteria and bacteriophages (21, 31). However, under similar experimental conditions the parental Lac$^+$ cells did not show such high-level resistance, although

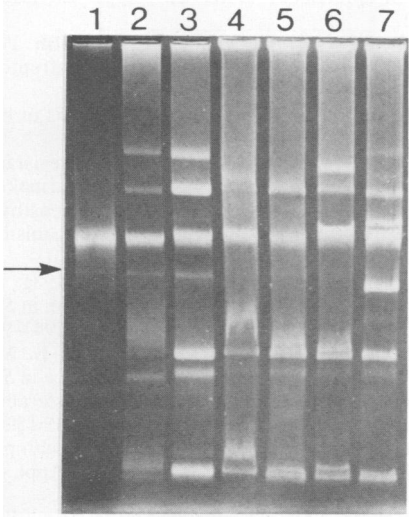

FIG. 4. Plasmid profiles of *S. lactis* strains. Lanes: 1, strain C2, chromsomal DNA band; 2, C2, Lac$^+$ Mut$^-$; 3, RS015, Lac$^-$ Mut$^-$; 4, RS013, Lac$^-$ Mut$^+$; 5, RS016, Lac$^-$ Mut$^+$ Str^r; 6, RS017, Lac$^+$ Mut$^+$ transconjugants; 7, RS018, Lac$^+$ Mut$^-$ transconjugant. Arrow shows the 5.5-MDa plasmid.

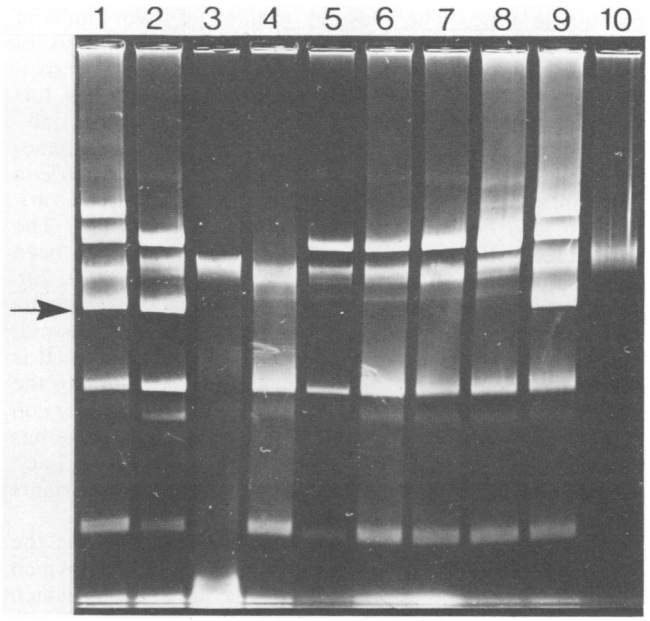

FIG. 5. Plasmid profiles of *S. lactis* strains. Lanes: 1, ML3, Lac$^+$ Mut$^-$; 2, RS031, Lac$^-$ Mut$^-$; 3, RS032, Lac$^-$ Mut$^+$; 4, RS033, Lac$^-$ Mut$^+$; 5, RS034, Lac$^-$ Mut$^+$; 6, RS035, Lac$^-$ Mut$^+$ Str^r; 7, RS036, Lac$^-$ Mut$^+$ Tet^r Ery^r; 8, RS037, Lac$^+$ Mut$^+$ transconjugant; 9, RS038, Lac$^+$ Mut$^-$ transconjugant; 10, chromsomal band. Arrow shows the 5.5-MDa plasmid involved in affecting development of high-level streptomycin resistance.

the MIC of streptomycin for Lac$^+$ and Lac$^-$ colonies was similar (Table 2). The development of such high-level streptomycin resistance observed in the present report is similar in some respects to the classic "inducible" drug resistance found in other organisms, mostly in pathogenic bacteria (4). However, one of the main criteria for inducible resistance is the total reversion of resistance to sensitivity, which was not found with strain RS016 even after 6 months of subculturing in absence of the antibiotic. Since the inducible phenotypic resistance is generally lost after several generations in a medium without antibiotic, as opposed to genotypic resistance, which normally persists in the absence of the drug (22, 27), the resistance in strain RS016 appears to be of the latter type. Furthermore, when the Lac$^-$ variants mutable to high-level streptomycin resistance, were crossed with the parental strain and Lac$^+$ transconjugants were analyzed, two kinds of transconjugants emerged. One kind showed mutability to high-level streptomycin resistance and were also missing the plasmid, while the other Lac$^+$ transconjugants were nonmutable and carried that plasmid.

A possible explanation is based on the hypothesis that the mutation to high-level streptomycin resistance is under the control of chromosomal loci and is recessive to a nonmutable marker associated with a plasmid. If this is true, one would expect two kinds of Lac$^-$ mutants. Those missing this plasmid would be capable of developing high-level streptomycin resistance, and others with the plasmid would be nonmutable. The data obtained from the plasmid analysis of both Lac$^-$ variants and Lac$^+$ transconjugants support this hypothesis. Examination of plasmid DNA from two kinds of Lac$^-$ variants (mutable and nonmutable) revealed that the mutable type of Lac$^-$ strain was missing a plasmid of approximately 5.5 MDa, while it was present in the

nonmutable type. The plasmid profiles of two kinds of transconjugants recovered from the conjugal matings of strains C2 and ML3 with their mutable-type Lac$^-$ derivatives further revealed that the donors could transfer this plasmid together with the Lac$^+$ phenotype to some Lac$^-$ cells. The cotransfer of this plasmid with the Lac$^+$ phenotype varied from 20 to 25% in ML3 matings to 36 to 40% in C2 matings. This also indicated that the mutable-type variants can also be isolated from normal Lac$^+$ strains. The lactose metabolism in *S. lactis* C2 and ML3 has already been shown to be plasmid encoded and transferable (12, 19, 20). Thus, it is possible that in conjugal matings some recipients received both the Lac$^+$ plasmid and this particular plasmid, while other recipients received only the Lac$^+$ plasmid. It is interesting that plasmid-determined susceptibility to the drugs kasugamycin and atabrine has been shown in *E. coli* (5, 30). A hypothesis similar to the one proposed here has been suggested to explain the increased sensitivity of Lac$^+$ *S. lactis* cells to copper compared with their Lac$^-$ variants (6).

An alternative that also needs to be considered is the presence of a suppressor associated with the plasmid which suppresses the expression of a chromosomal locus which determines streptomycin resistance. The present results do not conclusively discriminate between these possibilities. However, the results presented in this report clearly show the effect of a plasmid in inhibiting the expression of high-level streptomycin resistance in lactic streptococci.

At present it is difficult to assess the importance of streptomycin-resistant lactic streptococci in dairy fermentation, although Wulf and Sandine (32) have pointed out the significance of the use of streptomycin-resistant strains in Cheddar cheese manufacture to overcome antibiotics in the milk supply. The importance of the use of Lac$^-$ cells for accelerating the ripening process in Cheddar cheese has also been emphasized (9).

Streptomycin-resistant Lac$^-$ mutants have generally been isolated for genetic recombination studies through repeated transfer of cells in broth containing increasing concentrations of streptomycin sulfate (20). The results presented not only show the role of a plasmid in the development of drug resistance in lactic streptococci, but also demonstrate a very simple technique for the isolation of high-level, stable streptomycin-resistant mutants.

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LITERATURE CITED

1. **Anderson, D. G., and L. L. McKay.** 1983. Simple and rapid method for isolating large plasmid DNA from lactic streptococci. Appl. Environ. Microbiol. **46**:549–552.
2. **Birnboim, H. C., and J. Doly.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7**:1513–1523.
3. **Cogan, T. M.** 1972. Susceptibility of cheese and yoghurt starter bacteria to antibiotics. Appl. Environ. Microbiol. **23**:960–965.
4. **Connamacher, R. H.** 1976. Inducible bacterial resistance. Antibiot. Chemother. **20**:8–66.
5. **Danbara, H., and M. Yoshikawa.** 1975. Plasmid-determined epistatic susceptibility to kasugamycin. Antimicrob. Agents Chemother. **8**:243–250.
6. **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.
7. **Elliker, P. R., A. W. Anderson, and G. Hannesson.** 1956. An agar culture medium for lactic acid streptococci and lactobacilli. J. Dairy Sci. **39**:1611–1612.
8. **Fuchs, P. G., J. Zajdel, and W. T. Dobrzanski.** 1975. Possible plasmid nature of the determinant for production of the antibiotic nisin in some strains of *Streptococcus lactis*. J. Gen. Microbiol. **88**:189–192.
9. **Grieve, P. A., B. A. Lockie, and J. R. Dudley.** 1983. Use of *Streptococcus lactis* Lac$^-$ mutants for accelerating Cheddar cheese ripening. I. Isolation, growth and properties of a C2 Lac$^-$ variant. Aust. J. Dairy Technol. **38**:10–13.
10. **Hyder, S. L., and M. M. Streitfeld.** 1973. Inducible and constitutive resistance to macrolide antibiotics and lincomycin in clinically isolated strains of *Streptococcus pyogenes*. Antimicrob. Agents Chemother. **4**:327–331.
11. **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.
12. **Kempler, G. M., and L. L. McKay.** 1981. Biochemistry and genetics of citrate utilization in *Streptococcus lactis* subsp. *diacetylactis*. J. Dairy Sci. **64**:1527–1539.
13. **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.
14. **Macrina, F. L., D. J. Kopecko, K. R. Jones, D. J. Ayers, and S. M. McCowen.** 1978. A multiple plasmid-containing *Escherichia coli* strain: convenient source of size reference plasmid molecules. Plasmid **1**:417–420.
15. **Marmur, J.** 1961. A procedure for the isolation of deoxyribonucleic acid from micro-organisms. J. Mol. Biol. **3**:208–218.
16. **McKay, L. L.** 1982. Regulation of lactose metabolism in dairy streptococci, p. 153–182. *In* R. Davies (ed.), Developments in food microbiology, vol. 1. Applied Science Publishers Ltd., Essex, England.
17. **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.
18. **McKay, L. L., K. A. Baldwin, and E. A. Zottola.** 1972. Loss of lactose metabolism in lactic streptococci. Appl. Microbiol. **23**:1090–1096.
19. **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. **23**:45–52.
20. **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.
21. **Orgel, A., and S. Brenner.** 1961. Mutagenesis of bacteriophage T4 by acridines. J. Mol. Biol. **3**:762–768.
22. **Pitton, J. S.** 1972. Mechanisms of bacterial resistance to antibiotics. Ergeb. Physiol. Biol. Chem. Exp. Pharmakol. **65**:17–93.
23. **Reinbold, G. W., and M. S. Reddy.** 1974. Sensitivity or resistance of dairy starter and associated microorganisms to selected antibiotics. J. Milk Food Technol. **37**:517–521.
24. **Sanders, M. E., and T. R. Klaenhammer.** 1981. Evidence for plasmid linkage of restriction and modification in *Streptococcus cremoris* KH. Appl. Environ. Microbiol. **42**:944–950.
25. **Scherwitz, K. M., K. A. Baldwin, and L. L. McKay.** 1983. Plasmid linkage of a bacteriocin-like substance in *Streptococcus lactis* subsp. *diacetylactis* WM4 and its transferability to *Streptococcus lactis*. Appl. Environ. Microbiol. **45**:1506–1512.
26. **Sinha, R. P.** 1984. Effect of buffering media with phosphates on antibiotic resistance of lactic streptococci. Appl. Environ. Microbiol. **47**:1175–1177.
27. **Sirotnak, F. M.** 1976. Chromsomal mutation to drug resistance in bacteria. Antibiot. Chemother. **20**:67–86.
28. **St. Martin, E. J., L. N. Lee, and D. J. LeBlanc.** 1982. Genetic analysis of carbohydrate metabolism in streptococci, p. 232–233. *In* D. Schlessinger (ed.), Microbiology—1982. American Society for Microbiology, Washington, D.C.

29. **Terzaghi, B. E., and W. E. Sandine.** 1975. Improved medium for lactic streptococci and their bacteriophages. Appl. Microbiol. **29**:807–813.
30. **Yoshikawa, M., and M. G. Sevag.** 1967. Sensitivity of *Escherichia coli* to atabrine conferred by R factor and its potential clinical significance. J. Bacteriol. **93**:245–253.
31. **Witkin, E. M.** 1947. Mutations in *Escherichia coli* induced by chemical agents. Cold Spring Harbor Symp. Quant. Biol. **12**: 256–269.
32. **Wulf, J., and W. E. Sandine.** 1983. Isolation and characterization of fast acid-producing antibiotic-resistant mutants of lactic streptococci. J. Dairy Sci. **66**:1835–1842.