Note

Plasmid Distribution in Lactic Streptococci

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Received December 23, 1983

Lactic streptococci are industrially important microorganisms used in dairy fermentation and harbor plasmids of a variety of sizes.^{1~3)} It has been shown that plasmid DNA acts as a genetic determinant for practically important functions like proteolytic activity, lactose metabolism and citrate utilization, which are rather unstable genetic elements in lactic streptococci.^{1~7)} Resistance to some inorganic ions⁴⁾ and production of a bacteriocin-like substance, nicin,⁸⁾ are evidently associated with some plasmids.

To find out whether harboring of plasmids is a common feature of lactic streptococci we surveyed some representative strains.

Plasmid screening was performed on exponential cultures grown in 50 ml of lysis broth⁹⁾ containing 10 mM DL-threonine by the method of LeBlanc and Lee¹⁰⁾ with some modifications. Cells were harvested by centrifugation and washed with TH buffer (50 mM Tris-(hydroxymethyl)aminomethane-HCl buffer, pH 8.0), and then resuspended in 0.4 ml of the same buffer followed by the addition of 0.2 ml lysozyme solution (20 mg/ml in TH buffer). After 7 min incubation at 37°C, 0.2 ml of 0.25 M EDTA (pH 8.0) was added. Further lysis was achieved by the addition of 0.2 ml of SLS solution (20% sodium lauryl sulfate in 50 mM Tris(hydroxymethyl)-aminomethane-HCl buffer containing 10 mM EDTA, pH 8.0). Cell lysis was enhanced by 15 sec incubation at 55°C followed by gentle inversion for 15 sec to mix, and this procedure was repeated eight times. Chromosomal DNA in the lysate was denatured by the addition of 0.1 ml of 3 N NaOH, and gentle inversion for 3 min. After alkali denaturation, the lysate was rapidly neutralized by the addition of 0.1 ml of water and 0.2 ml of 2 M TH buffer (2 M Tris(hydroxymethyl)aminomethane-HCl buffer, pH 7.0) and mixed by gentle inversion for 30 sec. An additional 0.2 ml of 2 M TH buffer was added, and the lysate was mixed again as described above. Denatured DNA was removed by SLS-NaCl precipitation by adding 0.25 ml of SLS solution and 0.5 ml of 5 M NaCl solution and immediate mixing by inversion for 1 min. The sample was stored in an ice bath for 1 hr, and the supernatant fraction was collected after centrifugation at 12,000 g for

30 min at 5°C. The fraction was diluted by the addition of 1 ml of water, followed by the addition of 0.2 ml RNase solution (2 mg/ml in water, preheated in boiling water bath for 15 min) to remove RNA. After an hour incubation at 37°C, the sample was extracted with an equal volume of 3% NaCl saturated phenol. The aqueous phase was then extracted with an equal volume of chloroform-isoamyl alcohol (24:1, v/v). 0.2 ml of 3 M sodium acetate solution and 2 volumes of cold ethanol were added to the aqueous phase and, after mixing, the sample was stored at -20°C for 4 hr or overnight. Precipitated plasmid DNA was recovered by centrifugation at 12,000 g for 30 min at 5°C. The plasmid DNA was suspended in 0.1 ml of TES buffer (10 mM Tris(hydroxymethyl)aminomethane-HCl, 10 mM EDTA, 50 mM NaCl, pH 8.0), and a 15 μ l sample was mixed with an equal volume of loading buffer,¹¹⁾ which contained 0.07% bromophenol blue, 7% SLS and 33% glycerol, and then examined by agarose gel electrophoresis.

Agarose gel electrophoresis of the plasmid DNA samples were performed on a horizontal slab gel (120 $\times$ 150 mm) made of 0.5% or 0.7% agarose 1600 (Wako Pure Chem. Ind.) in TBE buffer¹¹⁾ (89 mM Tris (hydroxymethyl)aminomethane-borate, 2.5 mM EDTA, pH 8.3) for 5 hr at 60 V. The developed gel was stained with ethidium bromide and then observed and photographed under long-wave UV light. The plasmid DNA of *Streptococcus faecalis* strain ATCC 14508¹²⁾ prepared by this procedure, which contains pAM α 1 (6 Mdal), pAM β 1 (17 Mdal) and pAM γ 1 (35 Mdal), served as molecular weight markers (Fig. 1).

For all of the 38 investigated strains of lactic streptococci the presence of plasmid DNA was demonstrated by agarose gel electrophoresis, as shown in Table I. The number of electrophoretically distinct plasmids in a strain varied from 1 to up to 12, ranging in molecular weight between 1 and 80 Mdal. But under these conditions, accurate estimation of the molecular size of plasmid DNA from the migration distance through the agarose gel was difficult when the plasmid was larger than 40 Mdal. Some strains of the species *S. cremoris* were very rich in plasmids, however, it did not appear to provide a basis for species differentiation. The plasmid pattern of lactic streptococci is inherent for each strain.

Further investigation was carried on the spontaneous or acridine orange induced lactose negative (Lac⁻) derivatives of ten strains to determine the involvement of plasmid DNA in coding for the lactose utilization.

S. lactis 527 was found to harbor 4 distinct plasmid species of 47, 41, 11 and 4.9 Mdal. The plasmid profiles of several Lac⁻ mutants isolated after growth in broth containing acridine orange are shown in Fig. 1. The only plasmid missing from these Lac⁻ variants was the 47 Mdal plasmid, compared to the lactose fermenting (Lac⁺) parental culture. This result provided strong correlative evidence that the ability of 527 to utilize lactose was linked to the 47 Mdal plasmid.

TABLE I. PLASMID CONTENT OF LACTIC STREPTOCOCCI

Strain	Origin ^a	Plasmid content (mol. wt. $\times 10^6$) ^b		
<i>S. lactis</i> 527	NIAI	47.2 $\pm$ 4.92 4.92 $\pm$ 0.21	40.8 $\pm$ 3.78	10.6 $\pm$ 1.18
<i>S. lactis</i> 565	ISC	59.0 $\pm$ 3.00 31.5 $\pm$ 0.50 1.47 $\pm$ 0.02	44.0 $\pm$ 2.00 9.95 $\pm$ 0.05	39.5 $\pm$ 0.50 4.95 $\pm$ 0.15
<i>S. lactis</i> 712	ISC	36.6 $\pm$ 2.87 5.34 $\pm$ 0.16 1.95 $\pm$ 0.17	32.7 $\pm$ 4.88 4.24 $\pm$ 0.41	12.8 $\pm$ 1.35 2.87 $\pm$ 0.31
<i>S. lactis</i> 7963	ATCC	52.8 $\pm$ 8.35 31.5 $\pm$ 0.82	44.8 $\pm$ 6.50	37.1 $\pm$ 5.52
<i>S. lactis</i> 19435	ATCC	21.5 $\pm$ 2.96	7.96 $\pm$ 0.64	
<i>S. lactis</i> H-7	NIAI	38.0 $\pm$ 6.50 5.54 $\pm$ 0.24	30.7 $\pm$ 4.72 4.97 $\pm$ 0.31	13.9 $\pm$ 1.66
<i>S. lactis</i> H28	NIAI	49.1 $\pm$ 11.9 10.0 $\pm$ 1.09	39.5 $\pm$ 9.13 4.80 $\pm$ 0.24	38.5 $\pm$ 9.25
<i>S. lactis</i> HL-1	NIAI	49.5 $\pm$ 2.50 31.0 $\pm$ 0.00 6.40 $\pm$ 0.00	40.5 $\pm$ 2.50 19.5 $\pm$ 0.50 5.40 $\pm$ 0.00	37.5 $\pm$ 1.50 11.0 $\pm$ 0.00
<i>S. lactis</i> SC10	NIRD	53.4 $\pm$ 8.42 31.7 $\pm$ 5.50 4.86 $\pm$ 0.16	41.9 $\pm$ 5.66 18.9 $\pm$ 2.89	38.3 $\pm$ 5.62 9.88 $\pm$ 1.67
<i>S. lactis</i> SIN	NIAI	42.0 $\pm$ 6.74 5.07 $\pm$ 1.11	34.4 $\pm$ 5.75 4.36 $\pm$ 0.47	12.6 $\pm$ 1.39 2.73 $\pm$ 0.57
<i>S. lactis</i> W2	ISC	8.89 $\pm$ 0.85	7.69 $\pm$ 0.76	4.41 $\pm$ 0.13
<i>S. lactis</i> W8	ISC	8.30 $\pm$ 0.30 4.15 $\pm$ 0.05	7.30 $\pm$ 0.30	5.60 $\pm$ 0.00
<i>S. cremoris</i> 122-1	ISC	9.60 $\pm$ 1.80 3.90 $\pm$ 0.10	8.40 $\pm$ 1.60	4.15 $\pm$ 0.15
<i>S. cremoris</i> 924	NIRD	37.7 $\pm$ 7.25 14.6 $\pm$ 0.96 5.83 $\pm$ 0.16 3.42 $\pm$ 0.36	27.5 $\pm$ 4.07 12.2 $\pm$ 0.97 5.24 $\pm$ 0.30 2.34 $\pm$ 0.22	22.7 $\pm$ 3.91 8.50 $\pm$ 0.99 4.35 $\pm$ 0.35 1.90 $\pm$ 0.12
<i>S. cremoris</i> 19257	ATCC	38.3 $\pm$ 4.67 6.38 $\pm$ 0.68 3.18 $\pm$ 0.24	23.5 $\pm$ 2.90 5.33 $\pm$ 0.66 2.88 $\pm$ 0.18	8.46 $\pm$ 0.65 3.58 $\pm$ 0.17 2.19 $\pm$ 0.11
<i>S. cremoris</i> E8-1	ISC	42.5 $\pm$ 4.50	36.0 $\pm$ 5.00	
<i>S. cremoris</i> F-16	NIAI	8.00 $\pm$ 0.10 3.90 $\pm$ 0.10	7.05 $\pm$ 0.05 2.30 $\pm$ 0.00	4.25 $\pm$ 0.05
<i>S. cremoris</i> FI-8	NIAI	76.0 $\pm$ 0.00		
<i>S. cremoris</i> H-17	NIAI	29.0 $\pm$ 2.00	10.1 $\pm$ 0.70	
<i>S. cremoris</i> H41-7	NIAI	32.5 $\pm$ 7.27 13.4 $\pm$ 1.55 5.50 $\pm$ 0.29	29.0 $\pm$ 5.87 11.0 $\pm$ 1.20	25.8 $\pm$ 5.74 5.50 $\pm$ 0.73
<i>S. cremoris</i> H41-21	NIAI	41.5 $\pm$ 3.50 12.7 $\pm$ 1.25 4.80 $\pm$ 0.20	34.7 $\pm$ 0.25 10.4 $\pm$ 0.85	15.1 $\pm$ 1.15 5.50 $\pm$ 0.30
<i>S. cremoris</i> H-61	NIAI	62.5 $\pm$ 8.99 14.7 $\pm$ 2.12 2.55 $\pm$ 0.25	31.2 $\pm$ 3.68 11.4 $\pm$ 1.19 2.10 $\pm$ 0.35	20.5 $\pm$ 0.56 7.68 $\pm$ 0.91 1.79 $\pm$ 0.48
<i>S. cremoris</i> HC-1	NIAI	84.0 $\pm$ 0.00 17.5 $\pm$ 0.50 4.95 $\pm$ 0.05 1.78 $\pm$ 0.03	75.0 $\pm$ 1.00 8.55 $\pm$ 0.15 2.75 $\pm$ 0.15	27.2 $\pm$ 1.25 5.45 $\pm$ 0.05 2.15 $\pm$ 0.05
<i>S. cremoris</i> HI-5	ISC	52.5 $\pm$ 2.50 18.0 $\pm$ 1.00	20.5 $\pm$ 1.50 7.05 $\pm$ 0.05	19.2 $\pm$ 1.25 1.96 $\pm$ 0.14

TABLE I. (continued).

Strain	Origin ^a	Plasmid content (mol. wt. $\times 10^6$) ^b		
<i>S. cremoris</i> HI-5S	NIAI	46.5 $\pm$ 2.00	34.0 $\pm$ 1.00	12.2 $\pm$ 0.25
<i>S. cremoris</i> Ho-6	NIAI	3.15 $\pm$ 0.05	2.27 $\pm$ 0.02	
		36.5 $\pm$ 1.50	30.2 $\pm$ 0.75	25.0 $\pm$ 2.00
		18.2 $\pm$ 1.75	11.1 $\pm$ 0.85	7.80 $\pm$ 0.30
		1.77 $\pm$ 0.02	1.50 $\pm$ 0.00	
<i>S. cremoris</i> HP	ISC	83.0 $\pm$ 1.00	8.15 $\pm$ 0.25	7.15 $\pm$ 0.15
		3.70 $\pm$ 0.00	3.35 $\pm$ 0.05	2.35 $\pm$ 0.05
		1.90 $\pm$ 0.00		
<i>S. cremoris</i> ML	ISC	55.0 $\pm$ 7.03	38.4 $\pm$ 4.40	29.7 $\pm$ 3.76
		23.8 $\pm$ 3.33	15.5 $\pm$ 3.16	12.1 $\pm$ 0.88
		10.3 $\pm$ 1.23	8.87 $\pm$ 0.92	5.70 $\pm$ 0.55
		5.10 $\pm$ 0.55		
<i>S. diacetilactis</i> 2316	NIAI	41.1 $\pm$ 1.00	36.5 $\pm$ 0.59	
<i>S. diacetilactis</i> 13675	ATCC	33.0 $\pm$ 0.00	11.9 $\pm$ 0.90	5.40 $\pm$ 0.00
		5.10 $\pm$ 0.00		
<i>S. diacetilactis</i> D4	NIAI	41.5 $\pm$ 1.50	36.0 $\pm$ 3.00	15.0 $\pm$ 0.00
		3.00 $\pm$ 0.10		
<i>S. diacetilactis</i> D16	NIAI	38.1 $\pm$ 5.59	34.8 $\pm$ 5.80	30.8 $\pm$ 4.32
		29.5 $\pm$ 4.23	13.6 $\pm$ 1.79	10.3 $\pm$ 1.25
		5.57 $\pm$ 0.33	5.01 $\pm$ 0.28	
<i>S. diacetilactis</i> DRC-1	NIRD	53.3 $\pm$ 6.79	43.0 $\pm$ 5.19	29.5 $\pm$ 3.83
		12.5 $\pm$ 1.23	11.3 $\pm$ 1.04	9.70 $\pm$ 1.24
		5.52 $\pm$ 0.26	5.21 $\pm$ 0.36	4.73 $\pm$ 0.21
<i>S. diacetilactis</i> DRC-2	NIRD	31.1 $\pm$ 2.96	26.0 $\pm$ 2.49	22.0 $\pm$ 2.42
		10.1 $\pm$ 1.38	7.92 $\pm$ 1.16	4.81 $\pm$ 0.35
		4.21 $\pm$ 0.29	4.16 $\pm$ 0.05	
<i>S. diacetilactis</i> N-7	NIAI	34.2 $\pm$ 6.22	23.3 $\pm$ 3.27	
<i>S. species</i> CVT7	NIAI	31.8 $\pm$ 2.79	26.5 $\pm$ 2.45	20.1 $\pm$ 0.84
		16.0 $\pm$ 0.83	12.0 $\pm$ 0.99	8.74 $\pm$ 0.83
		5.37 $\pm$ 0.26		
<i>S. species</i> CVT21	NIAI	32.4 $\pm$ 5.09	23.4 $\pm$ 2.56	14.8 $\pm$ 1.98
		12.7 $\pm$ 1.03	8.48 $\pm$ 0.73	5.50 $\pm$ 0.36

^a NIAI; National Institute of Animal Industry. ATCC; American Type Culture Collection. ISC; Iowa State College. NIRD; National Institute for Research in Dairying.

^b Each value indicates the mean $\pm$ standard deviation.

FIG. 1. Agarose Gel Electrophoresis of Plasmid DNA Isolated from *S. lactis* 527 and Its Derivatives.

A, *S. faecalis* 14508 used as molecular weight markers; B, *S. lactis* 5273 and 5271, Lac^- mutants (missing a 47 Mdal plasmid) of *S. lactis* 527; D, *S. lactis* 527 (wild type).

By the same criterion, lactose utilization has been associated with a 32Mdal plasmid in *S. cremoris* H41-7; a 38 Mdal plasmid in *S. diacetilactis* D16; a 43 Mdal plasmid in *S. diacetilactis* DRC-1; a 34 Mdal plasmid in *S. diacetilactis* N-7 and a 32 Mdal plasmid in *S. species* strains CVT7 and CVT21, which are the intermediate types of *S. cremoris* and *S. diacetilactis*, respectively. However, the other three strains tended to lack relatively larger molecular weight plasmids simultaneously, so we could not designate particular plasmids related to the lactose utilization. In *S. lactis* 712, Lac^- mutants lacked 37 and 33 Mdal plasmids. Lac^- mutants of *S. lactis* SC10 lacked 53, 42 and 38 Mdal plasmids and the Lac^- mutant of *S. cremoris* H-61 lacked 63 and 31 Mdal plasmids (data not shown).

These data agreed with the findings of McKay and his co-workers who indicated that lactose metabolism was determined by a 30~45 Mdal plasmid in lactic strepto-

FIG. 2. Agarose Gel Electrophoresis of Plasmid DNA Isolated from *S. Species* CVT21, *S. diacetilactis* DRC-1 and Their Derivatives.

A, *S. species* CVT211, a $\text{Lac}^- \text{Cit}^+$ mutant (missing a 32 Mdal plasmid) of *S. species* CVT21; B, *S. species* CVT21 (wild type); C, D, *S. species* CVT21b and CVT21a, $\text{Lac}^+ \text{Cit}^-$ mutants (missing a 23 Mdal plasmid) of *S. species* CVT21; E, *S. diacetilactis* DRC-1 (wild type); F, *S. diacetilactis* DRC-1b, a $\text{Lac}^+ \text{Cit}^-$ mutant (missing a 5.2 Mdal plasmid) of *S. diacetilactis* DRC-1; G, *S. diacetilactis* DRC-1a, a $\text{Lac}^+ \text{Cit}^+$ mutant (missing some cryptic plasmids) of *S. diacetilactis* DRC-1.

cocci.²⁾ For example, Lac^- variants of *S. lactis* C2 lacked a 30 Mdal plasmid; a 30, 40 or 45 Mdal plasmid was missing in Lac^- variants of *S. lactis* strains ML3, C10 and M18, respectively; Lac^- *S. cremoris* B1 was missing a 36 Mdal plasmid and *S. diacetilactis* 18-16 Lac^- variants were missing a 41 Mdal plasmid.

As shown in Fig. 2, we found evidence that citrate utilization was also associated with plasmid DNA. *S. species* strain CVT21 was able to produce diacetyl from citrate and found harbor 6 distinct plasmid species. The plasmid profiles of two citrate negative (Cit^-) mutants of *S. species* CVT21 are shown in Fig. 2 (lanes C and D). These mutants were missing a 23 Mdal plasmid, which was presumed to be associated with citrate utilization. In *S. diacetilactis* DRC-1, some plasmids (30, 13, 11, 5.5 and 5.2 Mdal) were lost in a Cit^- variant (Fig. 2, lane F). On the other hand, a $\text{Lac}^+ \text{Cit}^+$ variant was obtained, which lacked some plasmids (30, 13, 11 and 5.5 Mdal) but was

still able to utilize citrate (Fig. 2, lane G). In comparison with these variants, the absence of a 5.2 Mdal plasmid seemed to be related to the loss of the ability to utilize citrate and the other plasmids missing seemed to be cryptic.

It was also reported by Kempler and McKay that variants of *S. diacetilactis* DRC-1 and 18-16 which failed to produce diacetyl from citrate had lost a 5.5 Mdal plasmid and the absence of the plasmid was especially associated with the loss of citrate permease activity.³⁾

Lactic streptococci harbor many species of plasmids, but the plasmid functions were not distinctly understood in detail except for some plasmids which proved to be encoded genes for lactose fermentation. In these cases, the function has been confirmed by the fact that the phenotype was transferred by conjugation, fusion, transduction and transformation.^{2,5,6,13)}

REFERENCES

- 1) D. B. Clewell, *Microbiol. Rev.*, **45**, 409 (1981).
- 2) F. L. Davies and M. J. Gasson, *J. Dairy Res.*, **48**, 363 (1981).
- 3) G. M. Kempler and L. L. McKay, *J. Dairy Sci.*, **64**, 1527 (1981).
- 4) R. Otto, W. M. de Vos and J. Gavrielli, *Appl. Env. Microbiol.*, **43**, 1272 (1982).
- 5) J. K. Kondo and L. L. McKay, *Appl. Env. Microbiol.*, **43**, 1213 (1982).
- 6) M. J. Gasson, *J. Bacteriol.*, **154**, 1 (1983).
- 7) V. L. Crow, G. P. Davey, L. E. Pearce and T. D. Thomas, *J. Bacteriol.*, **153**, 76 (1983).
- 8) K. M. Scherwitz, K. A. Baldwin and L. L. McKay, *Appl. Env. Microbiol.*, **45**, 1506 (1983).
- 9) T. R. Kleanhammer, L. L. McKay and K. A. Baldwin, *Appl. Env. Microbiol.*, **35**, 592 (1978).
- 10) D. J. LeBlanc and L. N. Lee, *J. Bacteriol.*, **140**, 1112 (1979).
- 11) J. A. Meyer, D. Sanchez, L. D. Elwell and S. Falkow, *J. Bacteriol.*, **127**, 1529 (1976).
- 12) D. B. Clewell, Y. Yagi, G. M. Dunny and S. K. Schultz, *J. Bacteriol.*, **117**, 283 (1974).
- 13) M. J. Gasson, *FEMS Microbiol. Lett.*, **9**, 99 (1980).