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Distribution and analysis of plasmids in *Streptococcus thermophilus*

G.A. Somkuti* and D.H. Steinberg

Eastern Regional Research Center, Agricultural Research Service, U.S. Department of Agriculture, 600 E. Mermaid Lane, Philadelphia, PA 19118, U.S.A.

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SUMMARY

In a survey of 35 strains of *Streptococcus thermophilus*, 13 strains were found to harbor plasmid DNA. Most of these strains contained plasmid species varying in size from 2.2 to 7.15 kilobases. Only three strains had more than one plasmid species. Each of the nine distinct types of plasmid DNAs identified had two or more unique recognition sites for restriction endonucleases. The characteristics of the indigenous cryptic plasmids of *S. thermophilus* may allow their development as cloning vectors useful in the genetic engineering of this species and other streptococci that are important in food production

INTRODUCTION

Streptococcus thermophilus is an economically important microorganism. As a starter culture, it plays an instrumental role in the production of fermented dairy foods that require elevated (>40°C) processing temperatures. In these dairy fermentations, the thermophilic streptococci are co-cultured with *Lactobacillus bulgaricus* (yogurt and Italian cheeses) or *L. helveticus*, *L. lactis*, and *Propionibacterium* sp. (Swiss-style cheeses). In the initial phases of fermentation, the primary function of *S. thermophilus* is the synthesis of lactic acid. During the product ripening phase, through various metabolic activities, the thermophilic streptococci contribute to the development of flavor and body properties that characterize the individual products [1].

The genetic improvement of thermophilic streptococci requires a thorough knowledge of the properties of their extrachromosomal elements that may be suitable as cloning vectors for introducing new phenotypic traits into these microorganisms. In sharp contrast to the sizeable body of knowledge already accumulated on the several plasmid-controlled metabolic activities in the mesophilic group of dairy streptococci including *S. lactis*, *S. cremoris* and *S. diacetylactis* [11,14], only limited information has been available about the plasmid biology of thermophilic streptococci [6,9]. The occurrence of plasmids in *S. thermophilus* was first reported in

* To whom correspondence should be addressed.

1981 (G.A. Somkuti and D.H. Steinberg, Absrt. Am. Dairy Sci. Assoc., 1981, DR79, p. 66). In this report we present data on the detailed restriction endonuclease enzyme analysis and the mapping of individual plasmids found in thermophilic dairy streptococci. We also discuss the potential value of these plasmids in the development of recombinant DNA techniques for this group of microorganisms.

MATERIALS AND METHODS

Bacterial strains and culture conditions. All 35 *S. thermophilus* strains used in this study were obtained from the stock culture collection of lactic acid bacteria maintained in our laboratory. Cultures were grown at 37°C in Hogg-Jago-lactose (HJL) medium adjusted to pH 6.5 [18].

DNA isolation and electrophoresis. Plasmid DNAs were isolated from 16-h old *S. thermophilus* cultures grown in 200 ml of DL-threonine (20 mM)-supplemented HJL medium [4], according to a procedure developed in our laboratory [19]. Plasmid DNAs were electrophoresed through 0.7% agarose gels in a vertical slab chamber, at a setting of 100 V (3 V/cm) for 4 h at 18°C, in Tris-borate-EDTA buffer (89 mM Tris/89 mM boric acid/2.5 mM EDTA (pH 8.2), and visualized by UV light (302 nm). *Escherichia coli* V517 plasmids were used as molecular markers to estimate the size of plasmids in megadaltons (Mdal) or kilobases (kb) [12]. Kilobase values were calculated assuming 650 daltons per nucleotide base pair. The unambiguous assignment of covalently closed circular and open circular forms of plasmids in gels was based on two-dimensional horizontal agarose gel electrophoresis and UV light-induced extensive nicking of the supercoiled species, according to the method of Oppenheim [16], with Gel Bond film as the agarose gel support medium (FMC Corporation, Rockland, ME).

The copy number of each plasmid was estimated by the method of Anderson and McKay [2].

Restriction endonuclease analysis and molecular map construction. For restriction endonuclease analysis, individual plasmid DNAs were recovered from preparatory-scale agarose gels by electroelution onto dialysis membranes [22], and further purified by the Elutip-d column procedure (Schleicher & Schuell, Inc., Keene, NH) according to the manufacturer's protocol, and by rapid cesium chloride equilibrium density gradient centrifugation [21]. DNA solutions were concentrated with 2-butanol [20] and precipitated with 2 vol. of ice-cold ethanol. The final pellets were dissolved in Tris-EDTA buffer (10 mM Tris/0.1 mM EDTA (pH 8.0).

The restriction endonucleases *Acc*I, *Ava*I, *Ava*II, *Bam*HI, *Bst*EII, *Eco*RI, *Hha*I, *Hin*dIII, *Hin*fI, *Hpa*II, *Kpn*I, *Pst*I, *Sal*I, *Xba*I and *Xho*I that are known to yield DNA fragments with cohesive ends, and the endonucleases *Hae*III, *Hpa*I, *Pvu*II and *Tha*I that yield DNA fragments with blunt ends, were used to digest approximately 1 μg DNA according to the manufacturer's instructions (Bethesda Research Laboratories, Rockville, MD). Buffer conditions for each enzyme were adjusted by adding 10-fold concentrated digestion buffers in appropriate volumes to samples of plasmid DNA. Reactions were allowed to proceed for 2.5 h and then stopped by the addition of 0.1 vol. of stop solution (20% glycerol/200 mM EDTA/0.01% bromophenol blue). Restriction endonuclease fragments were electrophoresed through 1.2% agarose gels in Tris-borate-EDTA (pH 8.2). Molecular masses of DNA fragments were estimated with a standard curve based on the *Hin*dIII and *Tha*I digests of λDNA and ϕ174RF DNA, respectively (Bethesda Research Laboratories, Inc.).

Molecular maps of plasmid DNAs were constructed by comparing double and triple digests of each plasmid with different combinations of endonucleases and digestion patterns produced by each enzyme alone. Double and triple digestions were carried out either simultaneously or sequentially, depending on the buffer conditions recommended for each restriction endonuclease. Alternatively, multiple enzyme digestions were also carried out in 'universal buffer' [15].

Metabolic activities of plasmid-carrying and plasmid-free strains Plasmid-free and plasmid-carrying *S. thermophilus* strains that belong to either the gal^+ or gal^- group of this species [19] were tested

for ability to ferment 20 different carbohydrates including polyols. Fermentation tests were carried out in HJ base medium supplemented with 0.1% of each carbohydrate. Tubes were incubated for 48 h at 37°C and examined turbidimetrically. The antibiotic susceptibility of *S. thermophilus* strains was tested with 'Dispens-o-Disc' (Difco, Detroit, MI) antibiotic filter paper disks. Antibiotics tested included three compounds of the penicillin group, two compounds of the cephalosporin group, four compounds of the aminoglycoside group, erythromycin, chloramphenicol, rifampin, novobiocin, polymixin B, and two sulfa drugs. Plates were scored after 24 h incubation at 37°C. The urease activity of strains was tested in Urea R Broth (Difco).

RESULTS

Occurrence and size of plasmid DNA in S. thermophilus strains

A total of 13 or 37% of the 35 *S. thermophilus* strains surveyed were found to contain plasmid DNA. The agarose gel electrophoretic profiles of the different types of plasmid DNAs and the calculated size of each plasmid are shown in Fig. 1 and Table 1, respectively. There were nine distinct size classes of plasmids, ranging in size from 2.2 kb to 14.75 kb, that could be isolated from *S. thermophilus* cultures. Only three strains, ST134, ST137, and ST138 harbored more than a single plasmid. The plasmids designated pER8 (2.2 kb) and pER16 (4.46 kb) were found in several strains, while pER371 and pER372 were detected in two strains. All other plasmids were present only in single strains. Some plasmids from different strains had sizes that were nearly identical such as pER341(2.77 kb) and pER371(2.70 kb), or pER13(4.23 kb) and pER16(4.46).

Characterization of plasmids with restriction endonucleases and construction of physical maps

The nine distinct types of plasmid DNAs found in *S. thermophilus* strains were analysed with 19 restriction endonucleases.

The construction of physical maps for *S. thermophilus* plasmids was achieved by double and triple digestions of plasmid DNAs and their isolated fragments. This approach allowed the unambigu-

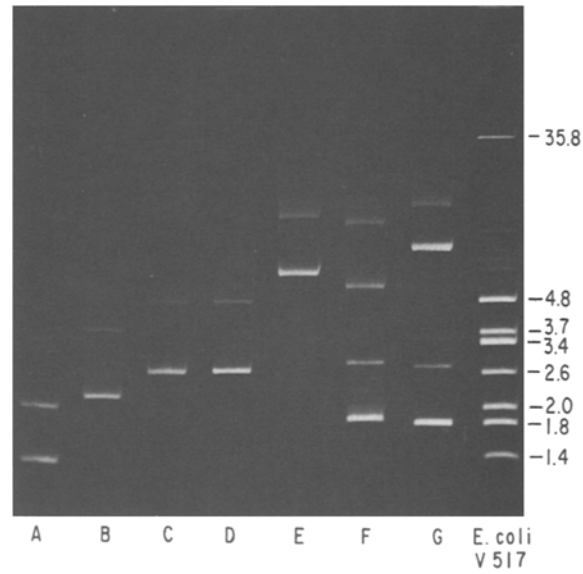

Fig. 1. Agarose gel electrophoresis profiles of plasmid DNAs isolated from *S. thermophilus*. Lane A, ST108 (pER); lane B, ST136 (pER36); lane C, ST113 (pER13); lane D, ST116 (pER16); lane E, ST135 (pER35); lane F, ST134 (pER341 and pER342); lane G, ST137 (pER371 and pER372); less intense bands in each lane are open circular forms of plasmid DNAs; sizes of standard *E. coli* V517 plasmids are in Mdal.

Table 1

Plasmids of *Streptococcus thermophilus*

Plasmid designation	Molecular mass*		Host strains
	Mdal	kb	
pER8	1.43	2.20	ST101, ST108, ST119, ST120, ST121
pER371	1.75	2.70	ST137, ST138
pER341	1.80	2.77	ST134
pER36	2.40	3.70	ST136
pER13	2.75	4.23	ST113
pER16	2.90	4.46	ST107, ST116, ST126
pER342	6,20	9.54	ST134
pER35	7.15	11.0	ST135
pER372	9.58	14.75	ST137, ST138

* Plasmid sizes were determined from a standard curve of the log of mass (Mdal or kb) of known plasmids of *E. coli* V517 versus their respective R_F values in 0.7% agarose gels.

ous alignment of all restriction fragments in seven plasmids (Figs. 2 and 3), with the exception of the *Hha*I and *Hin*fI fragments of pER371 that were too numerous and too small in size. For each map, one of the single restriction sites was chosen arbitrarily as the reference point for the alignment of fragments.

The most commonly found plasmid was pER8(2.2 kb) which occurred in five different strains of *S. thermophilus* (Table 1). pER8 was cleaved by only 5 of 19 restriction endonucleases tested, and had single restriciton sites for *Hin*dIII, *Hpa*I and *Pvu*II (Fig. 2). Two linear fragments were generated from this plasmid following digestion with *Bst*EII (1.43 and 0.77 kb) and *Hin*fI (2.0 and 0.2 kb). The copy number of pER8 was approximately 4–6 per cell.

Plasmid pER371(2.7 kb) was the smaller of the two plasmids detected in strains ST137 and ST138 (Table 1). This plasmid possessed single recognition sites for *Acc*I *Bst*EII and *Xba*I (Fig. 2). Two fragments were recovered from pER371 after digestion with *Hae*III (1.37 and 1.32 kb), *Hpa*II (1.54 and 1.15 kb), *Pvu*II (2.-48 and 0.21 kb), and *Tha*I (2.16 and 0.53 kb). In addition, pER371 was cleaved by both *Hha*I and *Hin*fI into six or more fragments. Restriction sites for these two enzymes could not be assigned on the map. The copy number of pER371 was estimated as 8–10 per cell.

Similarly, plasmid pER341 (2.77 kb) was the smaller of the two plasmids found in strain ST134 (Table 1). It was nearly identical in size to pER371 but its digestion pattern was unique (Fig. 2). Plasmid pER341 was cleaved by seven endonucleases and had unique sites for *Ava*I, *Ava*II and *Hae*III. Two fragments were recovered following digestions with *Eco*RI (1.76 and 1.01 kb), *Hha*I (1.9 and 0.87 kb) and *Hin*dIII (2.16 and 0.61 kb). Digestion with *Hin*fI cleaved pER341 into four fragments (1.29, 0.56, 0.54 and 0.38 kb). Plasmid pER341 had a copy number of about 10–12 per cell.

Plasmid pER36 (3.7 kb) was present only in ST136 (Table 1), and had an approximate copy number of 6–8 per cell. It was digested by seven restriction endonucleases of which *Acc*I, *Hha*I and *Kpn*I had unique recognition sites (Fig. 2). Three

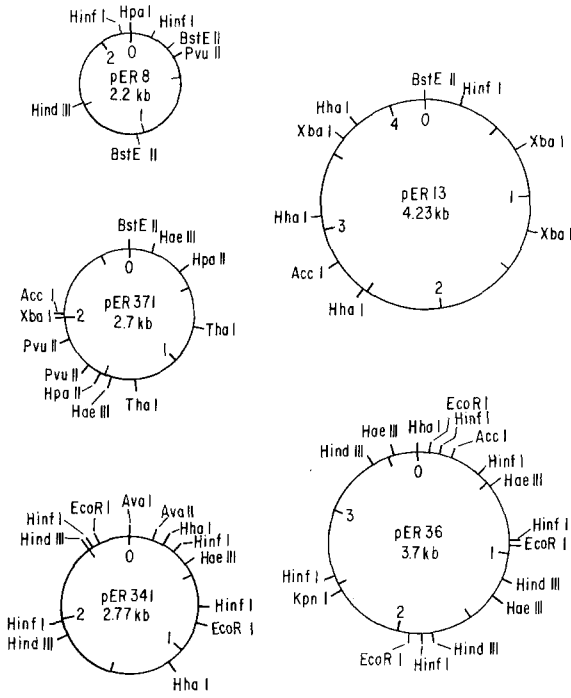

Fig. 2. Restriction enzyme maps of plasmids pER8, pER371, pER341, pER36 and pER13. Circular maps were generated with the aid of a computer program and are drawn to scale with respect to one another. The maps are calibrated in kilobases.

fragments were recovered following the digestion of pER36 with either *Eco*RI 1.87, 0.93 and 0.9 kb), *Hae*III (2.3, 0.78 and 0.62 kb), or *Hin*dIII (1.73, 1.36 and 0.61 kb). Digestion with *Hin*fI produced four fragments (1.30, 0.94, 0.78 and 0.68 kb).

The somewhat larger pER13 (4.23 kb) was also detectable in only one strain, ST113 (Table 1), and had unique recognition sites for *Acc*I, *Bst*EII and *Hin*fI (Fig. 2). This plasmid had a copy number of about 10 per cell. Calculated fragment masses were 3.0, 0.7 and 0.53 kb for *Hha*I, and 2.28, 1.40 and 0.54 kb for *Xba*I.

Nearly identical in size to pER13, the plasmid pER16 (4.46 kb) was found in three strains of *S. thermophilus* (Table 1), and was characterized by unique restriction sites for *Hae*III, *Hha*I, *Hpa*I and *Xba*I (Fig. 3). Two fragments were found after digestion with *Bst*EII (3.3 and 1.15 kb) and *Hin*dIII (3.23 and 1.23 kb), whereas five fragments were detected after *Hin*fI digestion (1.26, 1.20, 0.93, 0.57

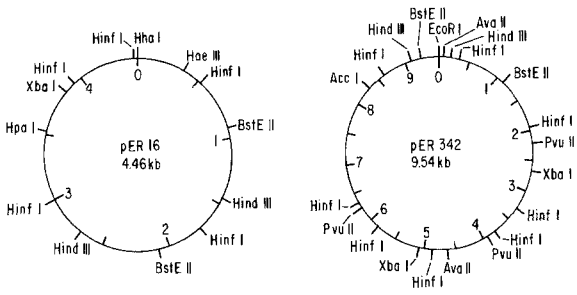

Fig. 3. Restriction enzyme maps of plasmids pER16 and pER342. Map coordinates are given in kilobases; only pER16 is drawn to scale with respect to plasmids shown in Fig. 1.

and 0.5 kb). This plasmid was estimated to have a copy number of 8–10 per cell.

Plasmid pER342 (9.54 kb), the larger of the two plasmid DNAs present in strain ST134 (Fig. 3 and Table 1), had a copy number of about 8–10 per cell. Unique recognition sites on this plasmid were mapped for *Acc*I and *Eco*RI. Two linear fragments were generated from pER342 with *Ava*II (4.98 and 4.55 kb), *Bst*EII (8.13 and 1.4 kb), *Hin*dIII (8.85 and 0.68 kb), and *Xba*I (7.1 and 2.43 kb). Digestion with *Pvu*II yielded three fragments (5.5, 2.31 and 1.72 kb) and eight fragments were recovered following *Hin*fI digestion (2.28, 1.66, 1.3, 1.25, 1.1, 0.96, 0.5 and 0.48 kb).

Strain ST135 was the only one to harbor pER35 (11 kb) which was digested by seven restriction enzymes, and apparently had single sites for *Bam*HI, *Bst*EII, *Hae*III, *Pst*I, and *Xho*I. The largest plasmid found was pER372 (14.75 kb). This plasmid was present in strains ST137 and ST138, together with the smaller pER371 (Table 1). pER372 was restricted by 17 enzymes and had unique recognition sites for five endonucleases (*Eco*RI, *Pst*I, *Sal*I, *Xba*I, and *Xho*I). Molecular maps for pER35 and pER 372 have not been completed.

Carbohydrate fermentation, antibiotic resistance, urease activity and plasmids

In ten strains of the gal$^-$ group, plasmids were totally absent. On the other hand, two gal$^-$ strains harbored plasmids, ST134 (pER341, pER342) and ST120 (pER8). One of these plasmids, pER8, also occurred in several gal$^+$ strains. Thus, the gal$^+$ phenotype was not associated with the presence of a plasmid in *S. thermophilus*. Antibiotic resistence patterns were essentially identical for plasmid-free and plasmid-containing strains (data not shown). A total of eight strains were urease negative but this group included both plasmid-free and plasmid-harboring strains (ST101, ST108, ST119, and ST126). Therefore, involvement of plasmids in either antbiotic resistance or urease activity in any of the *S. thermophilus* strains was most likely nonexistent.

DISCUSSION

The results of this study provide the most detailed information to date on the distribution and physical characteristics of plasmids in *S. thermophilus*. Plasmids have been reported either to be absent [23] or present in some *S. thermophilus* strains but without providing further detail [6].

Recently, Herman and McKay [9] reported limited restriction endonuclease data on three small cryptic plasmids (pHM1, 2.2 kb; pHM2, 3.2 kb, and pHM3, 3.5 kb) in *S. thermophilus* strains. All three plasmids had a single restriction site for *Pvu*II [9]. In our studies, only pER8 was susceptible to this enzyme. Although pER8 and pHM1 are apparently identical in molecular mass (2.2 kb), restriction data with *Bst*EII and *Hpa*I as well as ten additional endonucleases used in this study, are lacking for pHM1 [9]. The limited scope of restriction endonuclease analysis pf pHM2 and pHM3 [9] does not permit their direct comparison with any of the plasmids described in this study.

Unlike the mesophilic dairy streptococci (*S. lactis, S. cremoris, S. diacetylactis*) that carry a relatively large number of plasmid DNAs [7,10,17], some of which have been reported to encode carbohydrate and citrate metabolism, proteinase activity, bacteriocin production, resistance to inorganic ions, bacteriophages, and antibiotics [14], and even resistance to UV light [5], many *S. thermophilus* strains either lack or at best harbor only one or two plasmid DNAs. In our study, the presence of plasmids in *S. thermophilus* could not be associated with

several obvious phenotypic traits such as carbohydrate-fermenting ability, antibiotic resistance, or urease activity. Thus, at this point, the indigenous plasmids of *S. thermophilus* remain cryptic. Similar conclusions were reached in another, more limited study which attempted to relate the presence of plasmids in *S. thermophilus* to capacity for acid production from carbohydrates [9].

Since *S. thermophilus* is a harmless microbe and has been consumed by humans as a component of fermented food products for hundreds of years, its genetic engineering via recombinant DNA techniques appears to be a reasonable objective. The endowment of this thermo-tolerant microbe with novel genetic traits (e.g., expansion of carbohydrate-fermenting capacity, overproduction of enzymes, synthesis of sweeteners and recombinant DNA products, etc.) would vastly improve its usefulness in food and non-food applications. However, a prerequisite for achieving progress in this area is the development of cloning vectors, preferably from the native plasmids of *S. thermophilus.*

Of particular interest are those characteristics of of *S. thermophilus* plasmids that meet some of the essential requirements for a useful molecular cloning vector. Thus, they are relatively small in size, apparently stably maintained in high copy numbers, and each plasmid has at least two or more unique recognition sites for restriction endonucleases where insertion of passenger DNA is possible. The lack of identifiable selection markers may be overcome by the construction of chimeric plasmids in which replication genes remain functional. For example, chimeras from the *Ava*II fragments of pER341 and pGB351, a plasmid that encodes chloramphenicol resistance [3] may be possible to construct. Other candidates may be the *Xba*I fragments of pER371 or pER16, and pUB110 that codes for kanamycin resistance [8], or the *Kpn*I fragments of pER36 and pVA736, a chimeric plasmid itself that encodes erythromycin resistance [13]. The usefulness of chimeric plasmids in the development of recombinant DNA techniques for *S. thermophilus* is currently under investigation.

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