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Isolation and Partial Characterization of Plasmid DNA from *Streptococcus thermophilus*†

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Of 23 strains of *Streptococcus thermophilus* examined, 5 were found to contain a single small cryptic plasmid, designated pHM1 through pHM5. Through analysis by restriction endonuclease mapping and DNA-DNA hybridization, the five plasmids were found to be closely related. They were present in 4 to 18 copies per cell and ranged in size from 1.4 to 2.2 megadaltons. Plasmids pHM1 and pHM5, as well as pHM2 and pHM4, were found to be identical. Single restriction endonuclease sites were observed on each plasmid for *Pvu*II and *Mbo*I. Plasmids pHM2 and pHM3 each had an additional single site for *Hha*I, and pHM1 had an additional single site for *Hin*dIII. The characteristics of these plasmids may make them useful for the development of cloning vectors for use in *S. thermophilus*.

Streptococcus thermophilus is used in the manufacture of yogurt and in cheese varieties in which bacterial survival at elevated temperatures is required. Little is known, however, about the plasmid biology of this organism. Because of the role of plasmids in strain improvement programs that would use gene transfer or recombinant DNA techniques, we attempted to establish the incidence of plasmid DNA in *S. thermophilus* and to characterize any resident plasmids with respect to size, copy number, and single restriction enzyme sites.

The *S. thermophilus* strains used in this study are listed in Table 1. They were maintained by biweekly transfer at 37°C in M17 broth (8) containing 0.5% lactose or sucrose. Sucrose was used preferentially except with the sucrose-negative strain 3641.

Cells for plasmid extraction were harvested by centrifugation from *S. thermophilus* cultures grown in M17 broth for 4 to 5 h from a 1% inoculum of an overnight culture. Plasmid DNA was isolated as described previously (1), except that incubations after the addition of lysozyme and EDTA were lengthened 10 and 5 min, respectively. Preparative amounts of plasmid DNA were obtained from 1 liter of culture and purified by cesium chloride-ethidium bromide density gradient centrifugation (4). Horizontal agarose gel electrophoresis was performed in TAE (40 mM Tris-acetate, 2 mM EDTA [pH 8.0]) buffer (6). Gells (15 by 15 cm) contained 1 to 2.5% agarose and were electrophoresed at 4 V/cm for 4 h. Restriction enzymes were purchased from Bethesda Research Laboratories, Inc., and New England BioLabs, Inc. Restriction digests were performed as described by Maniatis et al. (6). Restriction fragments were separated by agarose gel electrophoresis as described above. Lambda DNA digested with *Hin*dIII and pBR322 digested with *Taq*I or *Hin*fI were used as size standards. Cleavage sites were mapped by single, double, and triple digestions. The copy number for each plasmid in the *S. thermophilus* strains was estimated as previously described (2).

Plasmid hybridization to restriction fragments was conducted with plasmid probes prepared by nick translation of pHM4 and pHM5, for which a ^{35}S-dATP nick translation kit manufactured by New England Nuclear Corp. was used. Restriction fragments were separated by electrophoresis in 2.5% agarose, stained, and photographed. The DNA was denatured, and the gel was prepared for transfer by the instructions provided for the Bio-Rad Trans-Blot cell. The fragments were transferred to a Gene Screen Plus hybridization membrane (New England Nuclear) by electrophoresis in a Trans-Blot Cell with 0.25× TAE buffer at 4°C by applying 10 V for 60 min and then 40 V for 120 min. Hybridization was conducted by method IV in the Gene Screen Plus instruction sheet. The membranes were dried at room temperature, dipped in toluene containing 20% 2,5-diphenyloxazole, and allowed to dry at room temperature. The membranes were exposed to Kodak XAR-2 X-ray film at −80°C for 20 h to 5 days.

The carbon sources utilized by plasmid-containing *S. thermophilus* strains were examined by using Rapid CH test strips (DMS Laboratories, Inc.). The inoculated test strips were incubated at 42°C and observed periodically for 48 h.

The plasmid content of the *S. thermophilus* strains examined and the the plasmid designations used in this study are listed in Table 1. Each plasmid-containing strain possessed a single small plasmid. The size of each plasmid, in megadaltons, was estimated by its electrophoretic mobility relative to the plasmid standards contained in *Escherichia coli* V517 (Table 1). Plasmids pHM1 and pHM5 appeared to have the same mobilities as plasmids pHM2 and pHM4 (data not shown). To determine whether their mobilities were identical, plasmids pHM1 and pHM5 were mixed and electrophoresed in 1.2% agarose, as was a mixture of pHM2 and pHM4. In both cases, only a single band was detectable, suggesting that the mixtures contained plasmids of the same size (data not shown). Because the number of plasmid copies per cell can have important effects on gene dosage and yield of plasmid DNA in isolation procedures, the copy number of each *S. thermophilus* plasmid was estimated (Table 1). All of the plasmids were found to have multiple copies per cell.

Restriction endonucleases were used to determine the relatedness and to develop physical maps of the *S. thermophilus* plasmids. The size of each plasmid, in

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TABLE 1. Distribution, size, and copy number of *S. thermophilus* plasmids

Strain	Source[a]	Plasmid	Plasmid size[b]		Minimum copy no.[c]
			MDa	kb	
3641	NRRL	pHM1	1.43 ± 0.02	2.2	3.9
ST2	M. S. Reddy	pHM2	2.06 ± 0.02	3.2	9.5
R_2ST	M. S. Reddy	pHM3	2.27 ± 0.06	3.5	12.2
CR_2ST	M. S. Reddy	pHM4	2.13 ± 0.08	3.2	17.7
STA	M. S. Reddy	pHM5	1.5 ± 0.04	2.2	4.5
ST401, ST403, ST407, ST7, ST8, CH_1ST, CH_2ST, CH_3ST, K1ST, Y5ST	M. S. Reddy	—[d]			
19258, 19987	ATCC	—			
21, 302, 307	INRA	—			
573	NCDO	—			
6097	CHL	—			
C3	Departmental culture collection	—			

[a] NRRL, Northern Regional Research Laboratories, Peoria, Ill. The strains obtained from M. S. Reddy were isolates from commercial starter cultures or cheese plants (M. S. Reddy, Ph.D. thesis, Iowa State University, Ames, 1974). ATCC, American Type Culture Collection, Rockville, Md. INRA, National Agricultural Research Institute, Jouy-En-Josas, France. NCDO, National Collection of Dairy Organisms, Reading, England. CHL, Chr. Hansen's Laboratory, Inc., Milwaukee, Wis.

[b] The size in megadaltons (MDa) was determined by plasmid mobility, and the size in kilobases (kb) was determined from the sum of restriction fragments. Values are means ± standard deviation.

[c] The copy numbers are the result of averaging duplicate determinations.

[d] —, No plasmids were detectable.

kilobases, was derived from the summation of restriction fragments and was in close agreement to the size, in megadaltons, determined by electrophoretic mobility (Table 1). The number of cleavage sites and the size of the resulting restriction fragments were identical for plasmids pHM1 and pHM5 for every enzyme tested (see Fig. 2). Likewise, identical restriction patterns were observed for pHM2 and pHM4. This result, together with their indistinguishable sizes, indicated that the collection of five plasmids consisted of three unique plasmids, two of which were present in duplicate. Accordingly, the restriction maps of pHM1 and pHM5 and of pHM2 and pHM4 were combined (Fig. 1). For simplicity, only the cleavage sites of enzymes that cleaved the plasmids one or two times are indicated on the maps. Upon examination, it was apparent that the restriction maps of pHM2 and pHM4 were very similar to that of pHM3. The only difference between these maps was that the 500-base-pair *Eco*RI-*Pvu*II fragment of pHM2 or pHM4 corresponded to an 800-base-pair *Eco*RI-*Pvu*II fragment in pHM3. This 300-base-pair difference accounted for the entire size difference between the plasmids. We have not found an enzyme that cleaves pHM3 and does not cleave pHM2 or pHM4.

All three plasmid maps have single cleavage sites for the enzymes *Mbo*I and *Pvu*II. The *Pvu*II site of each plasmid was arbitrarily chosen as the 0 reference point, and the other enzyme cleavage sites were mapped relative thereto. Plasmid pHM1 and pHM5 also have single cleavage sites for *Hin*dIII, and plasmids pHM2, pHM3, and pHM4 have single cleavage sites for *Hha*I. Plasmids pHM2, pHM3, and pHM4 have two *Eco*RI cleavage sites, whereas pHM1 and pHM5 have no detectable *Eco*RI sites. *Hin*fI cleaved pHM1 and pHM5 twice, but cleavage of pHM2, pHM3, and pHM4 by *Hin*fI resulted in at least five fragments. The enzyme *Taq*I cleaved pHM1 and pHM5 at four sites, whereas it cleaved pHM2, pHM3, and pHM4 at six to seven sites. No cleavage sites were detected in any of the plasmids for the enzymes *Bam*HI or *Hpa*II.

Because of the similarities in the restriction maps, the homology between the five *S. thermophilus* plasmids was examined by DNA-DNA hybridization. Restriction frag-

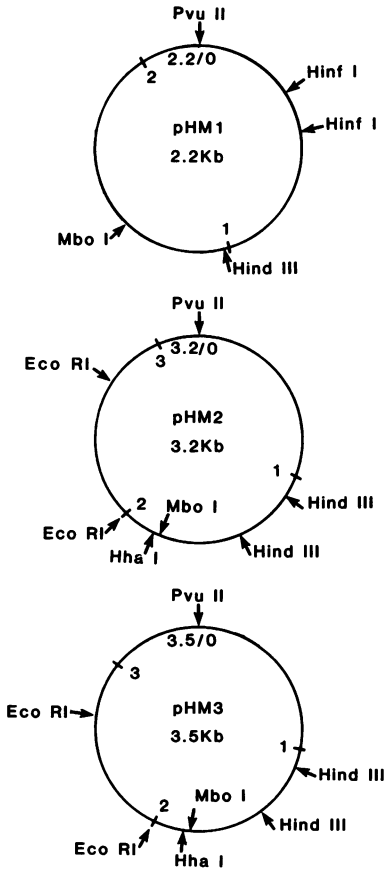

FIG. 1. Restriction maps of *S. thermophilus* plasmids. The maps of pHM1 an pHM5 are identical, as are the maps of pHM2 and pHM4. Restriction sites of the enzymes which cleaved the plasmids one or two times are shown. The *Pvu*II site on each plasmid was arbitrarily chosen as the 0 reference point, and distances from it are indicated in kilobases.

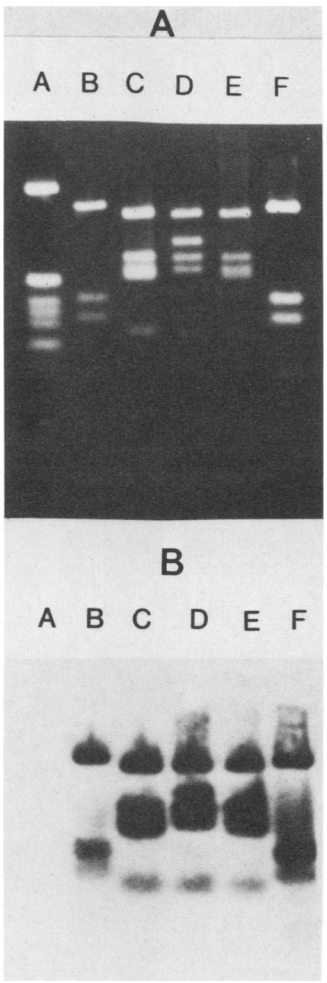

FIG. 2. Restriction enzyme fragments separated in 2.5% agarose (A) and resulting autoradiogram (B) after transfer of fragments to a membrane and hybridization to ^{35}S-labeled pHM4. Plasmid pBR322 was digested with *Hin*fI and generated fragment bands from top to bottom in lane A (panel A) of 1.63, 0.517 and 0.506, 0.396, 0.344, 0.298, 0.221, and 0.220, 0.154, and 0.075 (not visible) kilobases, respectively. Plasmids pHM1 and pHM5 were digested with *Taq*I; pHM2, pHM3, and pHM4 were triply digested with *Eco*RI, *Hin*dIII, and *Pvu*II. Lane A contains pBR322 fragments, and lanes B to F contain fragments of pHM1, pHM2, pHM3, pHM4, and pHM5, respectively.

ments of pBR322 and the five plasmids were hybridized with either pHM4 or pHM5 which had been nick translated in the presence of ^{35}S-labeled ATP. An ethidium bromide-stained agarose gel showing restriction fragments before transfer to a hybridization membrane and the resulting autoradiogram after hybridization with pHM4 are shown in Fig. 2. Plasmid pHM4 hybridized to fragments from all *S. thermophilus* plasmids but not to fragments from pBR322. The same result was observed when pHM5 was used as the hybridization probe (data not shown). Both pHM4 and pHM5 hybridized to all the fragments from each plasmid. This result indicates that the plasmids are extensively homologous.

To determine whether carbohydrate utilization could be correlated with the presence of plasmids, we examined the ability of each plasmid-containing strain to produce acid from 49 carbon sources, including raffinose, xylose, and arabinose. All carbon sources from which acid was produced by plasmid-containing strains were typical of those from which acid was produced by the plasmid-free type strain, 19258 (data not shown).

This study indicates that unlike the group N lactic streptococci, *S. thermophilus* appears to have a low incidence of plasmids (5 of 23 strains). No strain contained more than one plasmid, and all plasmids identified were small. The reason for the paucity of plasmids in *S. thermophilus* as compared with the ubiquity of plasmids in the group N streptococci is unknown. Reports in the literature on the incidence of plasmid DNA in *S. thermophilus* are almost nonexistent. In a review article, Davies and Gasson (3) cited unpublished data that 18 of 53 *S. thermophilus* strains contained plasmids. An abstract submitted by Somkuti (G. A. Somkuti and D. H. Steinberg, Abstr. Am. Dairy Sci. Assoc. 1981, DR79, p. 66) reported that examination of 36 strains of *S. thermophilus* and *Lactobacillus bulgaricus* resulted in the finding of 26 strains that contained up to five plasmids per strain. The latter report did not clarify the proportion of *S. thermophilus* plasmid-containing strains or the genus of the multiple plasmid-containing strains. The group of five plasmids reported here comprised three unique plasmids which shared extensive regions of homology and were subject to cleavage by many of the same restriction enzymes. The similarities in these plasmids suggest that they originated from a common ancestral plasmid. Among the group N streptococci, many phenotypic traits which are important in dairy fermentations are plasmid mediated (5, 7). This does not appear to be the case in *S. thermophilus*, because most strains examined by us, including type strain 19258, were plasmid free. The plasmid-containing strains did not extend the range of carbohydrates utilized by *S. thermophilus*. The *S. thermophilus* plasmids remain cryptic.

To apply recombinant DNA technology to *S. thermophilus*, a suitable cloning vector will be required. An ideal plasmid for use in the development of an *S. thermophilus* cloning vector would (i) be stably maintained in *S. thermophilus*, (ii) be small, (iii) be present in high copy number, and (iv) contain unique restriction enzyme sites for the insertion of selectable markers and for cloning purposes. The plasmids identified in this study satisfy all four of the above criteria and are being further investigated for development into cloning vectors for use with dairy streptococci or other lactic acid bacteria used in food fermentation processes.

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