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Structural and Functional Properties of the *hsp16.4*-Bearing Plasmid pER341 in *Streptococcus thermophilus*¹

The plasmid pER341 (2798 bp) of *Streptococcus thermophilus* ST134 was sequenced and its open reading frame (ORF) regions were characterized. Analysis of nucleotide sequences showed the putative translation product of ORF1 (*rep*) sharing a high level of homology with replication proteins of several small plasmids present in lactic acid bacteria and staphylococci. This and homology of regions of plus-strand (ORI) and minus-strand (*ssrA*) origin of replication with pC194-class plasmids indicated that pER341 replicates by the rolling-circle mechanism. ORF2 corresponded to a putative *hsp* gene that apparently encodes Hsp16.4, a 142-amino-acid heat stress protein. Hsp16.4 shared significant identity with other small, 18-kDa-class heat stress proteins from prokaryotic and eukaryotic sources. Hsp16.4 is apparently the first plasmidborne low-molecular-weight heat stress protein reported in dairy fermentation bacteria with a potential role in temperature-regulated functions in *S. thermophilus*.

Key Words: *Streptococcus thermophilus* plasmid; plasmidborne *hsp16.4* gene; pER341.

Streptococcus thermophilus (ST)³ is an important species in the diverse group of lactic acid bacteria (LAB) with essential catalytic functions in dairy food fermentations. Unlike its mesophilic LAB counterparts, research on the genetic systems of *S. thermophilus* has intensified only in the past several years.

In the interest of characterizing the biochemical functionality of plasmids and developing gene transfer systems of *S. thermophilus*, plasmid distribution patterns had been studied earlier. Most ST strains were found to carry a limited number of plasmids (Herman and McKay, 1985; Somkuti and Steinberg, 1986) that were classified into homology groups (Somkuti and Steinberg, 1991). Although a few small plasmids have been com-

pletely sequenced and analyzed (Janzen, et al., 1992; Hashiba et al., 1993), no linkage was established between biochemical functions and the presence of plasmids in ST strains.

In this report, we describe the nucleotide sequence and structural features of pER341 (2798 bp), the smaller of the two plasmids present in *S. thermophilus* ST134. The replication system (ORF1) of pER341 was compared with that of a family of gram-positive bacterial plasmids replicating via the rolling-circle mechanism (Gruss and Ehrlich, 1989), while the 142-amino-acid polypeptide (Hsp16.4) product of ORF2(*hsp*) was evaluated for homology with several 18-kDa-class heat stress proteins (Hsps) from prokaryotic and eukaryotic organisms. The data presented here offer the first conclusive evidence for an extrachromosomally encoded *hsp* gene in LAB and a plasmid-linked biochemical function (Hsp production) in *S. thermophilus*.

MATERIALS AND METHODS

Bacterial strains, media, and plasmids. *Streptococcus thermophilus* ST134, the carrier strain of pER341 (ca. 2.8 kb) and pER342 (ca. 9.5 kb), and

¹ Sequence data from this article have been deposited with the GenBank Data Library under Accession No. AF019139.

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³ Abbreviations used: ST, *Streptococcus thermophilus*; LAB, lactic acid bacteria; ORF, open reading frame; Hsp, heat shock protein; TYL, tryptone-yeast-lactose; AGE, agarose gel electrophoresis; TBE buffer, 0.089 M Tris base-0.089 M boric acid-0.002 M Na-EDTA (pH 8); Em, erythromycin; LB, Luria-Bertani; nt, nucleotide(s); R.ENases, restriction endonucleases.

the plasmid-free ST128 were from an in-house culture collection. Cultures were grown in tryptone–yeast extract–lactose (TYL) medium at 37°C as described (Somkuti and Steinberg, 1986). Plasmids were isolated from 16-h-old cultures by a standard procedure (Somkuti and Steinberg, 1986). pER341 and pER342 were resolved by agarose (0.75%) gel electrophoresis (AGE) in TBE buffer (0.089 M Tris base, 0.089 M boric acid, 0.002 M Na-EDTA, pH 8). pER341 was eluted by centrifugation according to the GenElute Agarose Spin Column protocol (Supelco, Inc., Bellefonte, PA). High-purity plasmids were prepared by CsCl-gradient ultracentrifugation (Stougaard and Molin, 1981) or by the Elutip-d column protocol (Schleicher & Schuell, Inc., Keene, NH). Coelectrotransformation of ST128 with pER341 and pVA736 (Em^R) (Macrina et al., 1980) was done under standard conditions (Somkuti and Steinberg, 1988) and cotransformants were scored in TYL–agar (1.5%) plates with 15 µg/ml erythromycin.

DNA manipulations and analysis. Fragments of pER341 generated by digestion with several restriction endonucleases (R.ENases, BRL Life Technologies, Inc., Gaithersburg, MD) were subcloned in pUC19 at corresponding polylinker sites either directly (*Hind*III and *Eco*RI fragments) or after blunt ending with Klenow fragment or T₄ DNA polymerase, at the *Hinc*II site (*Hha*I, *Hinf*I, and *Ase*I fragments), and transformed into competent cells of *Escherichia coli* DH5α (BRL Technologies). Recombinants were scored on Luria–Bertani (LB) agar plates (1% tryptone, 0.5% each of yeast extract and NaCl, and 1.5% agar), with 100 µg/ml ampicillin and 50 µg/ml X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside).

Recombinant plasmids were isolated by an alkaline lysis protocol (Ausubel et al., 1987), purified, and sequenced in triplicate by the dideoxynucleotide chain termination method (Sanger et al., 1977) with a T7 Autoread Sequencing Kit using the 24-base M13 universal (forward) and the 17-base M13 reverse primer in an ALF DNA Sequencer unit (Pharmacia, New Brunswick, NJ). Sequences were analyzed with the Clone Manager (Version 4) program (Scientific and Educational Software, Inc.,

Durham, NC). Sequence comparisons with other plasmid DNAs were made with BLASTP and BLASTX programs (Altschul et al., 1990). Multiple sequence alignments were carried out with DNASIS WINDOWS 2.1 (Hitachi Software Engineering America, San Bruno, CA) or CLUSTAL-W (Thompson et al., 1994) program.

Isolation of total RNA and Northern blot analysis. Total RNA was isolated from control and heat-stressed cells of plasmid-free ST128 and ST128 transformed with pER341 (ST128.Hsp). Cultures grown in TYL (25 ml) at 37°C to an OD₆₆₀ of 0.4 (ca. 4 h) were incubated further at 37°C for 30 min (control cells) or transferred to a 50°C water bath for 30 min (heat stressed cells). Total RNA was recovered according to the method of Chomczynski and Sacchi (1987) by using TRIzol reagent (BRL Life Technologies). Cells collected by centrifugation were resuspended in 2.5 ml of a mutanolysin solution (1.6 U/µl, Sigma Chemical Co., St. Louis, MO) supplemented with 10 mg/ml lysozyme (Sigma) and incubated for 15 min at 37°C. After resuspension of the protoplast pellets in 1.5 ml of TRIzol, isolation of total RNA followed the protocol recommended by the manufacturer. Final RNA pellets were resuspended in 50–100 µl of distilled water. Electrophoresis of RNA was performed in 1.2% (w/v) denaturing formaldehyde agarose gels according to a published procedure (Selden, 1992), and resolved RNAs were transferred overnight to Nytran membranes (Schleicher & Schuell) in 20× SSC buffer as described (Selden, 1992). Hybridization and chemiluminescent detection were done with the Rad-Free Probe Labeling and Detection System (Schleicher & Schuell). A 258-bp *Hinf*I fragment of ORF2 in pER341 (nt 2014–1756) was biotinylated according to the method of Leary et al. (1983) and used as the hybridization probe. Size determination was performed with an RNA ladder (0.24, 1.4, 2.4, 4.4, 7.5, and 9.5 kb; BRL Life Technologies) as a standard.

RESULTS

Restriction Map and Nucleotide Sequence

The restriction map of pER341 was constructed by single and coupled digestions with

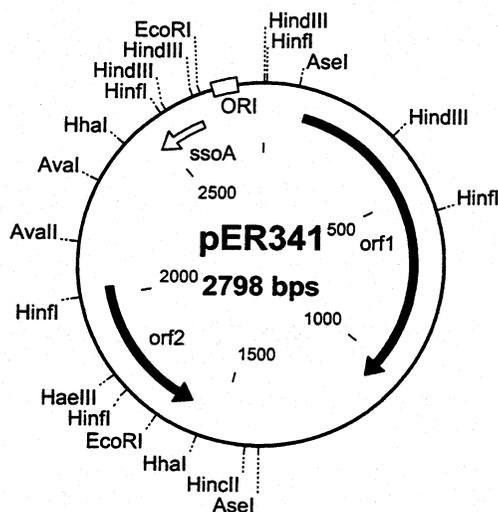


FIG. 1. Restriction endonuclease map of pER341. Arrows designate ORF1 (*rep*) and ORF2 (*hsp*). ORI, plus-strand origin of replication; *ssoA* (formerly *palA*), minus-strand origin of replication.

an array of R.ENases, using a *HindIII* site as the starting point (Fig. 1). The molecular mass of pER341 was accurately determined to be 2798 bp by replicate (3X) nucleotide sequence analyses (Fig. 2). The plasmid had two major ORF regions, ORF1 (nt 119–1063) and ORF2 (nt 2029–1601), with putative promoter regions positioned on opposing strands. The translation products of the two ORFs corresponded to putative polypeptides with 332 and 142 amino acid residues, respectively. The calculated G+C content (33.7%) of pER341 was lower than the range of reported G+C contents (37–40%) of the *S. thermophilus* chromosome (Hardie and Whiley, 1992).

Identification of the *rep* Gene of pER341

Putative transcription and translation initiation signals were identified in the 5' proximal region of ORF1 (Fig. 2). A Shine–Dalgarno (AGAAAG) region was located 58-bp upstream from the start codon of ORF1. Although this location is unusually distant in comparison to the commonly found spacings of 7–8 bp, similar findings were reported for certain *S. thermophilus* gene systems (Janzen et al., 1992;

Constable and Mollet, 1994). A promoter region with the –10 (TATGAT) and –35 (TTGATT) consensus hexanucleotide sequences was identified 21 bp upstream from the ribosome binding site which implied that ORF1 is likely to be expressed *in vivo*.

The nucleic acid and deduced amino acid sequences of ORF1 (nt 119–1063) were aligned with sequences available from GenBank databases using BLASTN and BLASTP programs. The comparison indicated a high degree (85% or more) of homology of the 314-residue ORF1 gene product with replication proteins (*Rep*) of small gram-positive bacterial plasmids that replicate by the rolling-circle mechanism (Gruss and Ehrlich, 1989). These included the *Reps* of the nearly identical *S. thermophilus* plasmids pST1 (315 residues) (Janzen et al., 1992) and pER8 (315 residues) (Somkuti and Solaiman, unpublished data), and pST1-No.29 (315 residues) (Hashiba et al., 1993). Other *rep* genes with products showing less extensive homology are present in plasmids pWC1 (54%, 316 residues, *Lactococcus lactis* subsp. *lactis*) (Pillidge et al., 1996); pC30i1 (47%, 317 residues, *Lactobacillus plantarum*) (Skaugen, 1989); pKH8 (50%, 334 residues, *Staphylococcus aureus*) (Moon et al., 1996); and pSK108 (50% 334 residues) (*S. epidermidis*) (Leelaporn et al., 1995). Multiple sequence alignment of ORF1 (*rep*) in pER341 with *rep* gene products of these plasmids is shown in Fig. 3.

The consensus sequence of pC194-class plus-strand origins of replication (ORI) (Novick, 1989) was located in a region about 185 bp upstream from the ORF *rep*. Alignment analysis with the aid of the CLUSTAL-W program (Thompson et al., 1994) showed that the putative pER341 origin shares ca. 70% homology with the pC194-class sequence (Fig. 4).

Secondary structure analysis with the DNASIS program indicated the potential occurrence of a stem-loop (hairpin) structure by the palindromic sequence *ssoA* (formerly known as *palA*, see Khan, 1997) located between nt 2477 and 2619 (Fig. 2). The free energy of formation (ΔG) for this structure was calculated to be -57.7 kcal/mol. The nucleotide sequence of this palindromic structure is highly homologous to that of the *ssoA*-

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          -35                -10                SD
1  AAGCTTGATT CTCAGTCAAA ACTTTGATAT GATTTAGTCA CCACAACAAA ACATAGAAA
                                     orf1
61  TGAGACGAGT ACAAACTTTT TTTGCGAATT AATTCAATTT TTGTATTCAA ATTATAGCCT

121  GGTAGATGAC GTAAATCAAG AGGAAGTCCT TTTAGATAAA AATAAAAATG GAAAAGATAG
      M V D D   V N Q   E E V   L L D K   N K N   G K D

181  AGACTGGCGA GGACGAAAAA TATTAAGTTT GAAATTAGCT GATATATTTA AAGAATTGCA
      R D W R   G R K   I L S   L K L A   D I F   K E L

241  GTACAAGAAG ACTTTTGTTG AAAGAGTAAT ATCATGTGGT GATACTTTGC GATTTATTCA
      Q Y K K   T F V   E R V   I S C G   D T L   R F I

301  AAAGCAAGAT GGTAGCCTAA AGCTCTATCA AGCATACTTT TGTAAAAACA AGCTTTGCCC
      Q K Q D   G S L   K L Y   Q A Y F   C K N   K L C

361  AATATGTAAC TGGAGACGTT CAATGAAATA CTCTTATCAG ACATCAAAAA TCGTTGATGA
      P I C N   W R R   S M K   Y S Y Q   T S K   I V D

421  GGCTATAAAA CAAGAACCTA AAGGACGCTT TCTCTTTCTG ACATTGACTG TTAAGAATAT
      E A I K   Q E P   K G R   F L F L   T L T   V K N

481  TGAGGGAAAA GCCTTGAATA GCACGATTAG TCAGCTCACA AAATCATTG ACCGTTTATT
      I E G K   A L N   S T I   S Q L T   K S F   D R L

541  CAAGCGTGCT AAAGTTCAA GGAATCTGTT GGGATATTG CGTTCAGTTG AAGTGACCCA
      F K R A   K V Q   R N L   L G Y L   R S V   E V T

601  CAATGAAAAT GATAATAGCT ATCACCTCA TATTCATGTT TTGATGATGA TGAGACCGAG
      H N E N   D N S   Y H P   H I H V   L M M   M R P

661  TTATTTCAA GGTGGAAT ACATTTACA AAAAGAGTGG GGCAATCTTT GGTCTCAATC
      S Y F K   G G N   Y I S   Q K E W   G N L   W S Q

721  ATTGAAAGTA TCTTACAAGC CTATGATTGA CATTAGAAGC GTCAAAGAGA CTGGCAAAGG
      S L K V   S Y K   P M I   D I R T   V K E   T G K

781  ACTAAGGGGA GCGGTCTTGG AAAGTCAAAA ATATCCAAC TAAACCAATA AATTAGACAT
      G L R G   A V L   E T A   K Y P T   K P I   K L D

841  TGAAAATAAG CAAGTTGTTG ATGATCTATA CAACGGTTTA TATCGAAAAC GCCAACTTGG
      I E N K   Q V V   D D L   Y N G L   Y R K   R Q L

901  TTATGGTGGC TTATTTAAAA TAATCAAAAA ACAACTTGCC CTAGATGATG TTGAAAGTGG
      G Y G G   L F K   I I K   K Q L A   L D D   V E S

961  CGATTTAATT CATACGTCTG ACGATAAAGA AACCTATCA AAGGTACAG AAATTGTAGC
      G D L I   H T S   D D K   E T L S   K G T   E I V

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FIG. 2. Complete nucleotide sequence of pER341 numbered from the *Hind*III site shown as the zero coordinate in Fig. 1. Only the sense strand is shown. ORFs and deduced amino acid sequences of Rep and Hsp16.4 are indicated. Potential Shine Dalgarno (SD) sequences, putative promoter hexamers -10 and -35, and *ssrA* are underlined. Convergent arrows indicate axes of dyad symmetry (D1, putative terminator sequence for *hsp16.4*; D2).

type minus-strand origin of replication (M-O) (Gruss and Ehrlich, 1989) of pC194-class small plasmids (Fig. 5). The conserved recombination site RS_B (Novick, 1989) was also located at the end of this structure (see Fig.5, italics).

Characterization of ORF2(*hsp*) in pER341

ORF2(*hsp*) in pER341 was positioned counter-clockwise with respect to ORF1(*rep*), spanning coordinates 2029 and 1601 (Figs. 1 and 2). A

1021 GATTTGGAAC GCTACAAAAC AAAATTATTA TTTAAAAAAA TAAAAGCAAG CTCCTCGAAA
A I W N A T K Q N Y Y L K K -
1081 GAGGGGCTTT TTCTAAACTC ATTTAAATG GTATAGACAT CTTATGAAAA ACTGGAAAAA
1141 TTGTGCGTGA CGTAAGGAAA TACCTTGTCG TGGCTCTCCT AGGCTCATAA TAGGAGCGGG
1201 GGTTCGTTTTA ATAGCGTCTA GGATAATTTA TATCACCTG CTGTAAAAATC GTGCTAAGGG
D2
1261 CGTTTTAGAG GGGTTTTAGTG ATGTCCTAGA AAAATCAATA TTTTAACATT CTCAATTGA
1321 GCTGGTTCCT ACATTCCAAA ATTTTCGTTAG GTTAATGTTT TGATGCTAAT TATCAAGATA
1381 GATTTAGATG TGAGAGAAAA ATTAATAGAG AGACAGGGGA AATTATCAGT GGTGTTGACG
1441 AATTATAAAG ATTTTTTTGA TAAATAATTT TAGACAAGAG CGAAGCGAAT TTTAACAGAC
1501 ACGCTTGACG GAAAAAAGT ACAGTTTCAC GTGAGTAATC TGTACTTTAA AAGGCGCCTA
D1
1561 TAAAATTTAT AGACGCCTTT TAAAGTAATT TTTTAAATTA CTAICTCAATT GGAATAGATG
- E I P I S T
1621 TTTTATTTTC TTTGTTACTA TCTTTTGGTA AGGTTACTTT AAGAATTCCTA TCTGAATAAG
K I E K N S D K P L T V K L I G D S Y S
1681 AAGCCTTTAT TTCGTCTTCT TTAACATTTT CTAATAAATA TTGACGTCGG ACACCTTGTTA
A K I E D E K V N E L L Y Q R R V S T L
1741 AACTACGTTTC GCTATGAATC AATTTTCCTT TTTCATCTTC GATTGCTGCA TCAATTTGGC
S R E S H I L K G K E D E I A A D I Q R
1801 GTTGGCCACT AATTGTTAAT ACTCCGTTTT CATAATTAAC TTGAATGTCT TCTTTAGGAA
Q G S I T L V G N E Y N V Q I D E K P I
1861 TACCAGGAAG TTCAGCTTCT ACAACATATT CATTATCAGT TTCATGAATA TCTGTTTTGA
G P L E A E V V Y E N D T E H I D T K I
1921 TTAAATTGGA CTTTAAATCG TTGAATAAAT TACGTCTAAA GTCTTCAAAA AAATTAAG
L N S K L D N F L N R R F D E F F N F P
orf2
1981 GTGTCATACT ATATATGTCT GAGTTACGTT GTTGAATCTT ATTTAACATA ATAATCACCC
T M S Y I D S N R Q Q I K N L M
SD -10 -35
2041 CTTTCTATAT TCTCTTTCAA TTTTATTAT AGTCAAAAAA GGTCAGTTTT CAAGTATTAT

FIG. 2—Continued

putative ribosomal binding site (AAAGGGG) located 8 bp upstream from the start codon of ORF2(*hsp*) is preceded by a 5'-TTGAAA(16 bp) TATAAT-3' promoter region (Fig. 2) that bears similarity to consensus promoter sequences re-

ported for other LAB strains (Koivula et al., 1991; Slos et al., 1991; Constable and Mollet, 1994).

The putative gene product of ORF2(*hsp*) was a 142-amino-acid polypeptide with a calculated molecular mass of 16,433 Da and a predicted *pI*

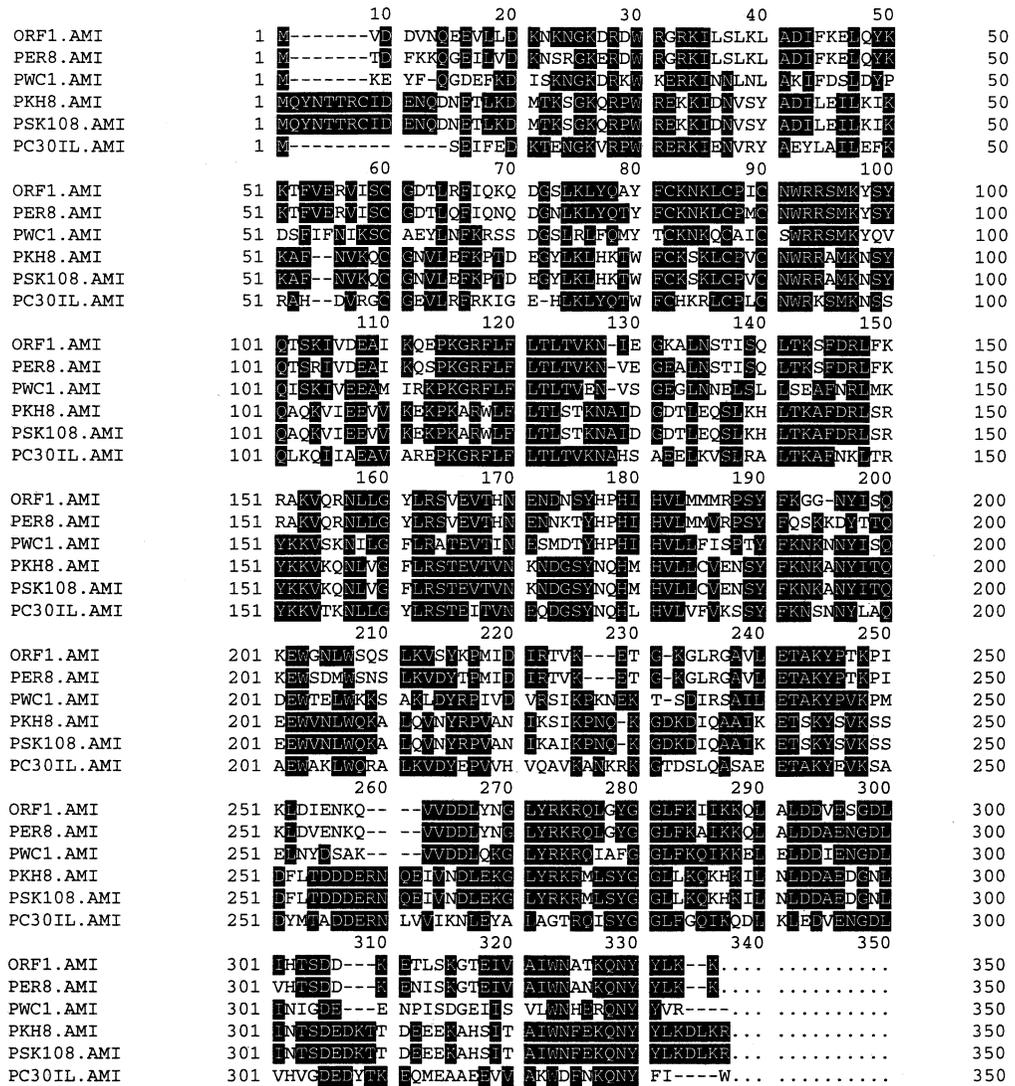


FIG. 3. Multiple sequence alignment of replication proteins of small rolling-circle plasmids from gram-positive bacteria. Shaded areas indicate 50% match.

tion with pER341 (*hsp16.4*) and pVA736(*erm*), growing at 37°C, and also from cells that were heat stressed by exposure to 50°C for 30 min. Each RNA extract was used for Northern blot analysis with the biotinylated 258-bp fragment which is internal to *hsp16.4* as the probe. Hybridization with this probe revealed a signal corresponding to a transcript ca. 600 nt long (Fig. 7), which was detectable only in cells that carried pER341. The signal detected with RNA from heat-stressed cells was markedly stronger

than the signal obtained with nonstressed control cells.

Cells of ST128 and ST128 transformed with pER341 were also tested for increased heat tolerance by a procedure outlined for *S. thermophilus* by Auffrey et al. (1995). Both types of culture showed the same basic response to heat stress; i.e., following exposure to lethal temperatures the number of survivors increased in heat-stressed cultures (data not shown). However, the presence of pER341 apparently did not

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pER341 2656   -AAAAGAGGGCTGAAAGCCCTAAAAAGCAGTCGAAAGACTGCTTTCTTCTTATCTTGAT
pC194  1053 (C) TTTTGTGTTTTTTAAAGGATTTGAGCGTAGCGAAAAATCCTTTTCTTCTTATCTTGAT
                *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *
                *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *

pER341      ACTAGTAGAAATAATTACGGGGAATTTCAAGCCAGTATGGTATTAACCCCTTGATAT
pC194      AATAAGGGTAACTATTGCCGG-----CGAGGCTAGT--TACCCTTAAGTTATTGGTAT
                *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *
                *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *

pER341      TACTGGTTTTTTAGCACAA 2793
pC194      GACTGGTTTTA-AGCGCAA 925 (C)
                *****  ***  ***

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FIG. 4. CLUSTAL-W alignment of plus-strand origin of replication. The underlined sequences represent the highly conserved double-strand origin nick site (indicated by the arrow). Complete matches are marked by asterisks. Sequence of staphylococcal pC194 was retrieved from GenBank (Accession No. J01754).

result in increased survival benefit for heat-stressed ST128/pER341.

DISCUSSION

We have completed the sequencing of the 2798-bp plasmid pER341 in *S. thermophilus* ST134 and characterized its two ORF regions.

Analysis of the nucleotide sequence showed pER341 sharing a high identity with other small cryptic plasmids reported in *S. thermophilus* (Janzen et al., 1992; Hashiba et al., 1993). In addition to these two *S. thermophilus* strains, the 314-amino-acid polypeptide product of ORF1(*rep*) in pER341 also shared high homology with *rep* gene products described in lactococci (Pillidge et al., 1996), lactobacilli (Skau-gen, 1989), and staphylococci (Leelaporn et al.,

1995; Moon et al., 1996). Further, multiple sequence alignment analyses indicated that both putative regions of plus-strand and minus-strand origins of replication in pER341 shared high identity with pC194-class plasmids, which allowed its classification as a pC194-type rolling-circle replicon (Gruss and Ehrlich, 1989).

The data provided convincing evidence that ORF2(*hsp*) in pER341 was responsible for the heat-induced synthesis of a Hsp with an apparent molecular mass of 16,433 Da. Although chromosomally located synthesis of high-molecular-weight Hsps in response to heat stress was previously demonstrated in both lactococci (Auffray et al., 1992) and *S. thermophilus* (Auffray et al., 1995), neither the production of small Hsps nor plasmid involvement in the pro-

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pER341 2625 (C)  AAGCTTTTTATTCCAATTTCTCTT---GTCAGCAAGTA-----ACCTTTCGCTG
pT181  3932 (C)  TATTGCTTTATTCCAATTTCTTATTG--GTCGGAACCTACAGGGCTTTATGTGTTGTTG
pC194  2045      CGCTTCTTTATTCCAATTTGCTTTATTGACGTTGAGCCTCGGAACCCCTTAACAATCCCAAA
                *****  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *

pER341      ATTTCTCGACTTCGTGTCCTAATCGCAAGCTTTTCAGTCTTGCGACTCAGACACATTCAAGT
pT181      AGTTGGTACTTTCTTGGGATTAATCCAATTCAAGTCCAACCAACTCGCTAACAAGTTAG
pC194      ACTTGTGCAATGGTCGGCTTAATAGCTCACGCTATGCCGAC--ATTGCTGCAAGTTTA
                *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *

pER341      CTAAATCGCTCAGCTTACTGA-CAAG-AGTTTTCTCGGCATAAATCGCCTGG 2469 (C)
pT181      CTAACACATAGCCCATTTCCAACCAATAAGTTTTCTCGGCATAAAT-GCATGT 3764 (C)
pC194      GTTAAGGGTTCTTCTCAACGCACAATAAATTTTCTCGGCATAAAT-GCGTGG 2213
                *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *

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FIG. 5. CLUSTAL-W alignment of *ssaA* elements of pER341 and staphylococcal plasmids pC194 (GenBank Accession No. J01754) and pT181 (GenBank Accession No. J01765). The italicized sequences denote the conserved regions of recombination site RS_B. Conserved nucleotides are marked with asterisks.

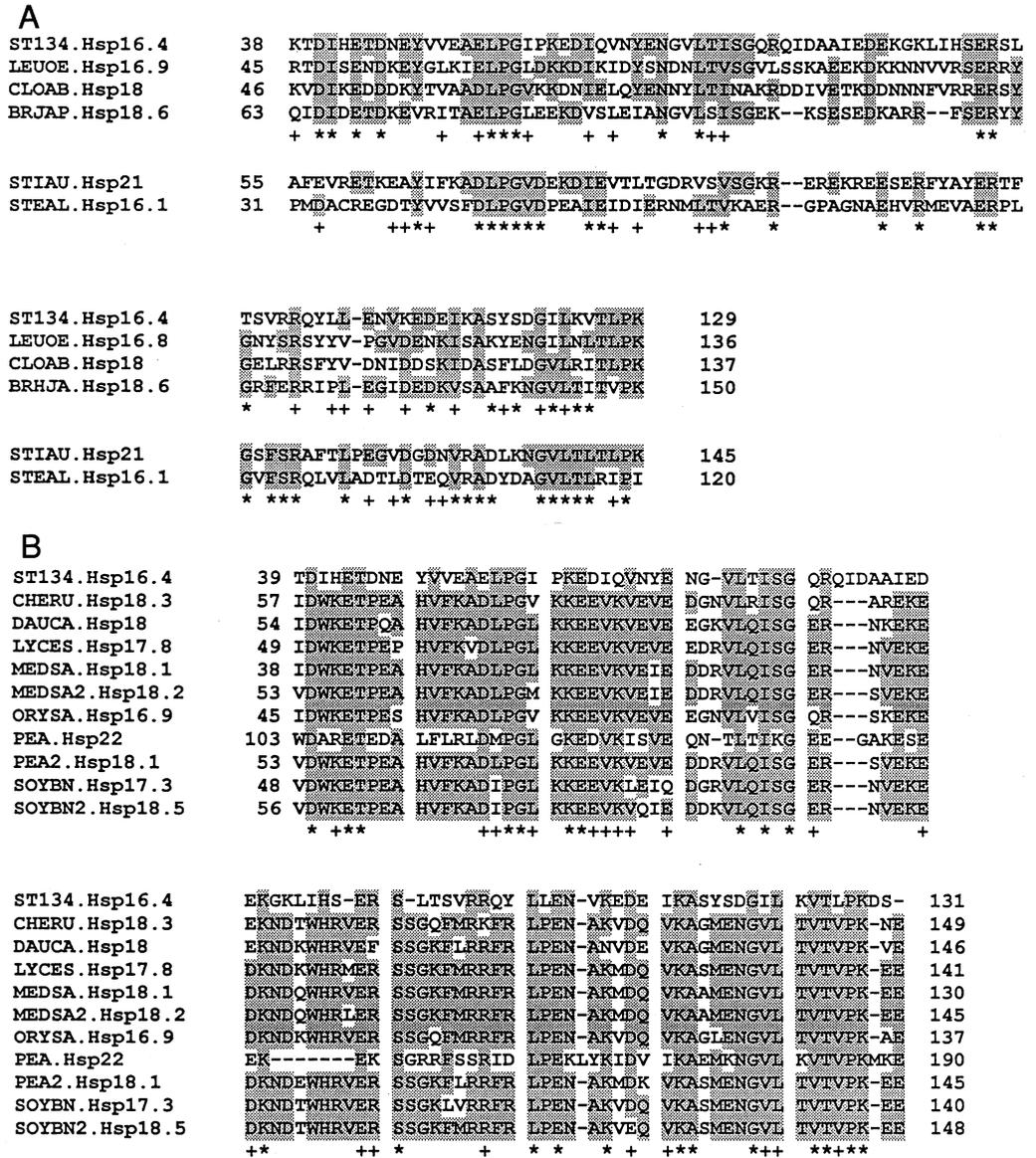


FIG. 6. Sequence alignment of conserved parts of *S. thermophilus* Hsp16.4 and other small 18-kDa-class small heat shock proteins. (A) Comparison with prokaryotic Hsps. ST134, *S. thermophilus* (this work); LEUOE, *Leuconostoc oenos* (Jobin et al., 1997); CLOAB, *Clostridium acetobutylicum* (Sauer and Durre, 1993); BRJAP, *Bradyrhizobium japonicum* (Narberhaus et al., 1996); STIAU, *Stigmatella aurantiaca* (Heidelbach et al., 1993); STEAL, *Streptomyces albus* (Servant and Mazodier, 1995). (B) Comparison with Hsps from eukaryotic sources. ST134, *S. thermophilus* (this work); CHERU, *Chenopodium rubrum* (Knack et al., 1992); DAUCA, *Daucus carota* (Darwish et al., 1991); LYCES, *Solanum lycopersicum* (Fray et al., 1990); MEDSA and MEDSA2, *Medicago sativa* (Gyorgyey et al., 1991); ORYSA, *Oryza sativa* (Tzeng et al., 1992); PEA, *Pisum sativum* (Lenne et al., 1995); PEA2, *Pisum sativum* (Lauzon et al., 1990); SOYBN, *Glycine max* (Nagao et al., 1985); SOYBN2, *Glycine max* (Raschke et al., 1988). Shaded area, 50% match; *, perfect match; +, conservative substitutions. Numbers on the left and right delineate the stretches of Hsps compared.

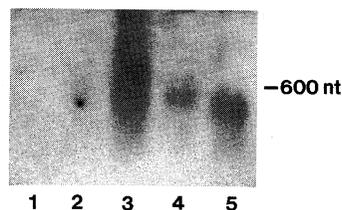


FIG. 7. Northern blot analysis of *S. thermophilus hsp16.4* transcripts. The same amount of total RNA preparation (10 μ g) was separated from cells of plasmid-free ST128 (negative control), ST134, and ST128 transformed with pER341, and used for hybridization. Cultures were grown at 37°C (lanes 2 and 4) or heat stressed at 50°C (lanes 1, 3, and 5). The probe corresponds to the *hsp16.4* structural gene (*HinfI* fragment, nt 2,014–1,756) in pER341. Fragment size (number of nucleotides, nt) indicated to the right, was based on comparison with size markers.

cess has been reported in these bacteria. On the other hand, the Hsp16.4 of *S. thermophilus* ST134 showed an impressive level of identity with other 18-kDa-class Hsps from prokaryotic and eukaryotic sources, particularly with Hsp18 of *C. acetobutylicum* (Sauer and Durre, 1993), and Lo18 of *L. oenos* (Jobin et al., 1997), which are chromosomally encoded proteins. As observed also by others (Heidelbach et al., 1993), higher identity scores were found in the C-terminal region, where the consensus motif characteristic of small Hsps is present.

Northern blot analysis revealed that at the mRNA level, *hsp16.4* was clearly a heat stress-regulated gene, responding to a temperature upshift with enhanced transcription. This adaptive response resulted in a greater abundance of ca. 600-nt mRNA in heat-stressed cells of ST134 (original host of pER341) and transformed ST128/pER341 as shown by hybridization with a *hsp16.4* probe. The absence or very low level of detectability of *hsp16.4* mRNA in these cells under normal (37°C) growth conditions suggests that at best only low constitutive expression occurred. This was unlike many major Hsp genes that are known to be essential for cell growth and are constitutively expressed even in the absence of environmental stresses (Straus et al., 1987). In view of these observations, it was reasonable to assume that under stress conditions, Hsp16.4 would have survival benefit to the host. However, when plasmid-free *S. ther-*

mophilus ST128 was transformed with pER341 resulting in high-level *hsp16.4* transcription following heat stress, the presence of this gene failed to show a positive impact at the organismal level as the number of survivors remained apparently unchanged following exposure to lethal temperatures. Although the function of Hsp16.4 in *S. thermophilus* remains unclear, as is the case with other members of the low-molecular-weight prokaryotic Hsp family (Yuan et al., 1996), it may contribute to maintaining the integrity of the cytoplasmic membrane, a possible role that has been suggested for small Hsps in *S. aurantiaca* (Lunsdorf et al., 1995) and *L. oenos* (Jobin et al., 1997).

Analysis of the 5'-nucleotide sequence flanking *hsp16.4* showed the presence of a putative promoter region homologous to LAB consensus -10 and -35 sequences. However, as in the case of other small bacterial Hsps (*C. acetobutylicum*, *L. oenos*, *S. albus*), there was no indication in the area upstream to *hsp16.4* of a 9-bp inverted repeat with potential for a stem-loop structure that is believed to be involved in regulating the expression of heat shock genes (Zuber and Schumann, 1994).

We have shown that pER341, one of the two plasmids present in *S. thermophilus* ST134, harbors *hsp16.4*, encoding a small 18-kDa-class Hsp. The production of a Hsp is apparently the first biochemical function found to be linked to plasmid DNA, and Hsp16.4 is also the first low-molecular-weight heat stress protein reported in this species. The utility of *S. thermophilus hsp16.4* in temperature-controlled expression of heterologous genes in LAB is currently under investigation.

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