Structural and functional analysis of pCI65st, a 6.5 kb plasmid from *Streptococcus thermophilus* NDI-6

Tadhg O'Sullivan, Douwe van Sinderen and Gerald Fitzgerald

Author for correspondence: Gerald Fitzgerald. Tel: +353 21 902730. Fax: +353 21 903101.
e-mail: g.fitzgerald@bureau.ucc.ie

Department of
Microbiology, University
College, Cork, Ireland

The 6.5 kb cryptic plasmid pCI65st from *Streptococcus thermophilus* NDI-6, a strain isolated from the Indian fermented milk dahi, was subcloned and sequenced. Five putative ORFs were identified. ORF1 could encode a 315 aa polypeptide almost identical to the RepA protein of previously sequenced *S. thermophilus* plasmids, indicating that pCI65st is one of the pC194 group of small Gram-positive rolling-circle plasmids. ORFs 2 and 4 were virtually identical and could specify proteins of approximately 150 aa with significant similarity to the small heat-shock proteins described from a variety of Gram-positive bacteria. ORF3 could encode a 415 aa protein similar to enolase, an enzyme involved in glycolysis and gluconeogenesis. ORF5 could encode a 412 aa protein which had high similarity to the HsdS (specificity) proteins of type I restriction-modification systems. Variants of strain NDI-6 which lacked pCI65st were readily isolated after subculture of the parent strain at 32 °C. The plasmid-bearing parent culture was significantly more resistant to a temperature shift from 42 °C to 62 °C than its plasmid-free variant and expressed proteins which corresponded with the predicted translation products from ORF2 and ORF4. In addition, plasmid-free mutants were lysed in broth by bacteriophages to which the parent culture was resistant.

Keywords: *Streptococcus thermophilus*, plasmid, heat-shock genes, enolase, type I HsdS

INTRODUCTION

The participation of extrachromosomal DNA in key industrial traits of lactic acid bacteria (LAB) is well documented (for reviews see McKay, 1983; Fitzgerald & Hill, 1996). Plasmid DNA is known to mediate traits such as carbohydrate and nitrogen metabolism, exopolysaccharide production, bacteriophage resistance mechanisms and bacteriocin production. While plasmid DNA species have been described in virtually all groups of LAB and are particularly abundant in lactococci, they are relatively rare in thermophilic LAB.

Streptococcus thermophilus is most frequently used in the manufacture of thermophilic fermented milks including yoghurt and yoghurt-like products such as the Middle Eastern laban and the Indian fermented milk,

dahi (Dellaglio, 1988). Strains of this species are also utilized in the manufacture of Swiss and Italian-style cheeses and for short-method Cheddar cheese (Hutkins *et al.*, 1986). Studies on strains from a wide variety of commercial sources indicate that up to 80% of isolates of this species currently in use as starter cultures are plasmid-free (Herman & McKay, 1985; Girard *et al.*, 1987; Somkuti & Steinberg, 1986; Janzen *et al.*, 1992). Where plasmids are present, they rarely exceed 7.6 kb in size and cultures generally have only a single such species. The role of plasmid DNA in *S. thermophilus* is unclear, since no obvious phenotypic trait has been associated with the presence of plasmids, although Mercenier (1990) has indicated that the 6.9 kb element, pA33, can confer altered phenotypic characteristics. Coconcelli *et al.* (1995) have also implicated a 9.6 kb plasmid, pCRB96, in a bacteriophage resistance mechanism. Several *S. thermophilus* plasmids have been sequenced (Mercenier, 1990; Janzen *et al.*, 1992; Somkuti *et al.* 1998) but few genes other than those required for plasmid maintenance have been described.

Abbreviations: LAB, lactic acid bacteria; R/M, restriction-modification; smHsp, small heat-shock protein.

The GenBank accession number for the sequence reported in this paper is AF027167.

Plasmids have played an important role in the development and application of techniques for the genetic manipulation of key industrial traits in food-grade LAB. For this reason it is of interest to examine the sequence of native *S. thermophilus* plasmids with a view to exploring their application as food-grade vectors. We report the cloning and sequence analysis of a 6.5 kb plasmid from *S. thermophilus* dahi isolate NDI-6. Preliminary studies aimed at determining the biological role of this plasmid are described.

METHODS

Bacterial strains, plasmids and growth media. Bacterial strains and plasmids utilized in this study are described in Table 1. *S. thermophilus* cultures were routinely cultivated at 42 °C in Elliker broth (Elliker *et al.*, 1956) supplemented with 10 g beef extract l⁻¹ and referred to as Belliker broth. *Escherichia coli* cultures were cultivated at 37 °C in LB broth (Luria *et al.*, 1960). Streptomycin (Sm) was used to select for resistant *S. thermophilus* NDI-6 mutants at 200 µg ml⁻¹. Ampicillin (Ap) and tetracycline (Tc) were utilized to select for *E. coli* transformants at 100 µg ml⁻¹ and 12.5 µg ml⁻¹, respectively. IPTG and X-Gal were utilized at concentrations of 0.5 mM and 40 µg ml⁻¹, respectively. Fast-slow differential agar supplemented with 1.0 g tryptone l⁻¹ (FSDAT) was prepared as described by Huggins & Sandine (1984).

Isolation of plasmid-free derivatives of *S. thermophilus* NDI-6. To reduce the risk of contamination by other plasmid-free *S. thermophilus* strains in the course of this study, a streptomycin-resistant (Sm^R) mutant of *S. thermophilus* was first isolated. Plasmid-free derivatives of this Sm^R mutant were obtained following subculture at 32 °C. After two subcultures at this temperature, cells were harvested from 1.0 ml Belliker broth, washed once and resuspended in 1.0 ml sterile Ringer's solution. Appropriate dilutions were spread-plated on FSDAT to achieve between 100 and 500 c.f.u. per plate. Plates were incubated at 37 °C under anaerobic conditions for 24 h. Representative morphotypes were then selected, inoculated into 10 ml Belliker broth and incubated at 42 °C until an OD₅₈₀ of approximately 1.0 was attained. Cleared lysates of each were then prepared and examined electrophoretically for the presence of plasmid DNA. The presence of the antibiotic-

resistance marker was confirmed by streaking putative plasmid-free derivatives onto Belliker agar containing 200 µg Sm ml⁻¹.

Growth studies. Growth characteristics of parent and plasmid-cured cultures in Belliker broth containing either glucose, lactose or sucrose as sole energy source (1%) were examined. Cultures were subcultured twice in Belliker broth containing the relevant sugar and were then inoculated (2%) into 100 ml broth. Growth was monitored turbidimetrically at 580 nm.

Bacteriophage sensitivity. The bacteriophage sensitivity of cultures was examined against *S. thermophilus* phages in the University College Cork (UCC) collection (43 separate bacteriophages isolated from cheese and yoghurt factories throughout Europe). Each strain was inoculated into 10 ml Belliker broth supplemented with 1 mM CaCl₂ and containing 100 µl of a bacteriophage preparation which had been previously titrated (10⁹ p.f.u. ml⁻¹) on its homologous host. These preparations were incubated at 42 °C and were subcultured a further three times, each in the presence of the phage preparation. Bacteriophage sensitivity was indicated by lysis of the culture.

Heat-shock response. The response of *S. thermophilus* NDI-6 and its plasmid-free mutant to heat shock was assessed using the method described by Auffray *et al.* (1995). Overnight cultures in Belliker broth were inoculated into 10 ml volumes of fresh medium and incubated at 42 °C until an OD₅₈₀ of approximately 1.0 was achieved (3–3.5 h). Duplicate 5.0 ml aliquots of cells were then removed and transferred to 42 or 52 °C. Cells were incubated for a further 30 min at the indicated temperatures and were then harvested by low-speed centrifugation. The cells were washed twice in 5 ml sterile Ringer's solution and resuspended in 5.0 ml fresh Belliker broth pre-incubated at 58, 60 and 62 °C. The resuspended cells were held at the new temperature for a further 2 h. The percentage survival of cells was monitored by determining the viable count at 30 min intervals using the spread-plate method on Belliker agar.

Cell extract preparation. Soluble cell proteins of *S. thermophilus* cultures were prepared by harvesting 50 ml Belliker-broth-grown cells, washing once in Ringer's solution and resuspending in 1.0 ml sonication buffer (1 mM 2-mercaptoethanol, 0.1 M EDTA, 50 mM Tris/HCl, pH 7.0). Cells were

Table 1. Bacterial strains and plasmids

Bacterium or plasmid	Characteristics	Source
<i>S. thermophilus</i> NDI-6	pCI65st host; dahi isolate	Dr S. S. Sannabhadri, University of Anand, Gujarat, India
TOS1	Plasmid-free Sm ^R NDI-6	This study
<i>E. coli</i> XL1-Blue	<i>recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac</i> [F' <i>proAB lacI</i> ^q Z ΔM15 Tn10(Tet ^r)]	Stratagene
Plasmid pBluescript II SK ⁻ (pBS)	Amp ^r , lacZα complementation	Stratagene

disrupted by sonication for 3 min in a Soniprep 150 MSE sonicator operating at half maximum amplitude. Cell debris was removed by centrifugation at 7000 r.p.m. for 1 min in an Eppendorf centrifuge. Protein content was determined by the method of Bradford (1976). Soluble cell proteins of *E. coli* cultures were prepared by the same method from cells grown in 200 ml LB broth.

Assay of enolase (phosphopyruvate hydrolase; EC 4.2.1.11). Enolase activity of cell-free extracts from *S. thermophilus* and *E. coli* cultures was performed as described by Wold (1971).

SDS-PAGE. Soluble cell proteins (15 µg) were separated by the method of Laemmli (1970), using 12.5% and 15% acrylamide gels.

N-terminal sequencing. Proteins were transferred from PAGE gels to PVDF protein transfer membranes (Applied Biosystems) using a Bio-Rad Mini Trans-Blot electrophoretic subculture cell with CAPS transfer buffer at 50 V for 30 min at room temperature. The blot was stained for 5 min with 0.1% Coomassie brilliant blue R-250 in 40% methanol/1% acetic acid and destained in 50% methanol. Relevant bands were excised and their N-terminal sequences were determined using an Applied Biosystems Procise protein sequencer.

DNA preparation and plasmid analysis. *S. thermophilus* DNA was prepared by the method of Anderson & McKay (1983). *E. coli* DNA was prepared by the method of Birnboim & Doly (1979). *E. coli* plasmid DNA for sequencing reactions was prepared using the DNAPrep Spin Plasmid Miniprep kit (Qiagen). *S. thermophilus* plasmid DNA for sequencing reactions was obtained using either the JETstar Plasmid Purification System (Genomed) or caesium chloride density-gradient centrifugation.

Restriction endonuclease digestion and molecular cloning. Restriction endonucleases were purchased from Boehringer or from New England Biolabs and were utilized according to the manufacturers' instructions. The *E. coli* vector pBluescript II SK⁻ (Stratagene) was used for cloning of *SspI*, *DraI* and *EcoRV* restriction-generated fragments of pCI65st. Ligation mixtures were used to electroporate competent cells of *E. coli* XL-1 Blue (Stratagene). Positive selection of recombinant plasmids was effected using blue/white screening on LB agar supplemented with X-Gal and IPTG.

DNA sequence analysis. DNA sequencing reactions were performed using an ABI Prism Dye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq DNA polymerase (Perkin Elmer). Sequence determination was performed using an Applied Biosystems 373 automated DNA sequencer employing synthetic oligonucleotides (Oligo 1000M, Beckman Instruments) as primers. Assembly of sequences was performed using the Seqman program of the DNASTAR software package. Database searches were performed using the FASTA (Pearson & Lipman, 1988), BLASTN and TBLASTN (Altschul *et al.*, 1990) programs with sequences from the following databases: SWISS-PROT (release 30); NBRF-PIR (release 42); GenBank translated (release 86) and EMBL (release 38). Sequence alignments were performed using the CLUSTAL method of the Megalign program of the DNASTAR software package.

RESULTS

DNA sequence analysis

pCI65st was sequenced bidirectionally, revealing a circular genome of 6499 bp with a total G + C content of 34.5 mol%, which is typical of small plasmids from *S.*

Fig. 1. Circular map of *S. thermophilus* plasmid pCI65st showing restriction sites and ORFs. The duplicated regions are indicated by double-headed arrows.

thermophilus (Janzen *et al.*, 1992; Hashiba *et al.*, 1993; Somkuti *et al.*, 1998). A map of pCI65st showing ORFs and restriction sites is presented in Fig. 1. Analysis of the plasmid sequence revealed the presence of five putative ORFs, all of which were orientated in the same direction although in different frames. ORF1 could encode a 315 aa polypeptide which was closely related (92% similarity and 85% identity) to the RepA proteins described from other *S. thermophilus* plasmids (Janzen *et al.*, 1992; Hashiba *et al.*, 1993). The presence of this gene suggests that pCI65st belongs to the pC194 group of rolling-circle plasmids (Seery *et al.*, 1993). ORF2 and ORF4 could encode 140–150 aa proteins which had up to 64% similarity to the small heat-shock proteins (smHsp) described from a number of Gram-positive bacteria, including *Clostridium acetobutylicum* (Sauer & Durre, 1993; 40% identity, 64% similarity) and the LAB *Leuconostoc oenos* (Jobin *et al.*, 1997; 42% identity, 63% similarity) and *Lactobacillus delbrueckii* (accession no. Z71782; 41% identity, 63% similarity). ORF2 and ORF4 of pCI65st were essentially identical, apart from a 24 base insertion in ORF2, and comprised part of a duplication which covered 11.5% of the entire plasmid length. Virtually identical ORFs have also been described on other small *S. thermophilus* plasmids (Hashiba *et al.*, 1993; Somkuti *et al.*, 1998). Alignments of the putative heat-shock genes from *S. thermophilus* plasmids with those from *Lactobacillus helveticus* and *Leuc. oenos* are presented in Fig. 2. ORF3 could encode a 415 aa polypeptide with 89% similarity and 74% identity to enolase of *Haemophilus influenzae* (Fleischmann *et al.*, 1995). This enzyme is involved in glycolysis and gluconeogenesis and generally exists in its dimeric form. Like most of the enzymes of glycolysis it is

1	MLNKIQHRNLNTYSVTF	L	D	FYSVTP	LN	FFE	hsp1 (pCIst65)
1	MLNKIQHRNLNTYSVTF	D				FFE	hsp2 (pCIst65)
1	MLNKIQHRNLNTYSVTF	N				FFE	hsp (pER341)
1	MLNKIQHRNLNTYSVTF	D				FFE	Str term acid shock
1	MSHGRLAR	Q	MD	S			hsp Ib
1	MANELMDLR	ND	GLMD	V	S		hsp Leuc
31	DFSR	S	LFNDFKPH				hsp1 (pCIst65)
23	EF	SR	NLFNDFKPH				hsp2 (pCIst65)
23	DF	R	NLFNDFKPH				hsp (pER341)
23	EF	SR	NLFNDFKPH				Str term acid shock
13							hsp Ib
29	P		RDG				hsp Leuc
59	EAE	LP	GIPKEDIQVTYENG	V	L	T	hsp1 (pCIst65)
51	EAE	LP	GIPKEDIQVTYENG	V	L	T	hsp2 (pCIst65)
51	EAE	LP	GIPKEDIQVTYENG	V	L	T	hsp (pER341)
51	EAE	LP	GIPKEDIQVTYENG	V	L	T	Str term acid shock
33	K	I	D	V	P	G	hsp Ib
58	K	I	E	L	P	G	hsp Leuc
89	AV	N	E	D	K	K	hsp1 (pCIst65)
81	AV	N	E	D	K	K	hsp2 (pCIst65)
81	A	A	I	E	D	K	hsp (pER341)
81	AV	N	E	D	K	K	Str term acid shock
63	K	D	S	D	K	K	hsp Ib
88	A	E	E	K	D	K	hsp Leuc
119	E	D	E	I	K	A	hsp1 (pCIst65)
111	E	D	E	I	K	A	hsp2 (pCIst65)
111	E	D	E	I	K	A	hsp (pER341)
111	E	D	E	I	K	A	Str term acid shock
93	K	K	G	I	S	A	hsp Ib
118	E	N	K	I	S	A	hsp Leuc
149	I	E					hsp1 (pCIst65)
141	I	E					hsp2 (pCIst65)
141	I	E					hsp (pER341)
141	I	E					Str term acid shock
123	I	E					hsp Ib
147	I	Q					hsp Leuc

Fig. 2. Sequence alignments of the putative small heat-shock proteins (smHsp) encoded by ORF2 and ORF4 of the *S. thermophilus* plasmid pCI65st with those from *S. thermophilus* plasmid pER341 (Somkuti *et al.*, 1998), the acid-shock protein from *S. thermophilus* (P80458), and with smHsps from *Lactobacillus helveticus* (Z71782) and *Leuconostoc oenos* (Jobin *et al.*, 1997). Amino acids are boxed if three or more residues are identical. Numbers in the left hand margin refer to the amino acid positions in the proteins indicated in the right hand margin.

extremely highly conserved across the animal and plant kingdoms. ORF5 could encode a 412 aa protein with significant similarity to the HsdS or specificity proteins from type 1 restriction-modification (R/M) systems, including those of *EcoRI*24II from *E. coli* (Price *et al.*, 1989; 27.5% identity, 52% similarity), *StySBL1* from *Salmonella enterica* (Titheradge *et al.*, 1996; 32.5% identity, 52% similarity) and to the S proteins of R/M systems of *Methanococcus jannaschii* (Cfr1; accession no. L77158; 33% identity, 53% similarity), *Mycoplasma pulmonis* (Dybvig & Yu, 1994; 35% identity, 52% similarity) and *Spiroplasma citri* (Laigret *et al.*, 1996; 36% identity, 56% similarity) (Fig. 3). Significant relatedness (33.6–64.8% similarity) was also noted to various plasmid-borne *hsdS* genes from *Lactococcus lactis* (Schouler *et al.*, 1998a, b; accession numbers U90222, AF034786, AF036486). The ORF5 translation product has a conserved core region typical of HsdS proteins. This region is flanked by two variable domains which specify the target sequence and has high homology to the conserved C and N domains (Kneale, 1994). Interestingly, one of the variable regions from this peptide has a high degree of similarity to the first variable region from the *L. lactis* HsdS subunit encoded by pIL7 (Schouler *et al.*, 1998b).

Other features of pCI65st

In addition to possessing a putative *repA* gene which is

capable of encoding a product virtually identical to that reported for other *S. thermophilus* plasmids, pCI65st showed a remarkable conservation of DNA sequence upstream of the putative *repA*. A potential ribosome-binding site (RBS) exists 50 bases upstream (5' GAA-AGGAG 3') and is identical to that described 47 bases upstream of pST1 (Janzen *et al.*, 1992). A short inverted repeat (IR) exists between the putative RBS and the *repA* start codon (5' CAAATTAATTTG 3') and may partly explain the unusually long distance to the start codon. This would appear to be characteristic of this group of plasmids, as a similar situation has been reported in the *S. thermophilus* plasmids pST1 (Janzen *et al.*, 1992) and pER3-1 (Somkuti *et al.*, 1998). A region which is identical to the proposed *dso* of pST1 (CTTTCTTC-TTATCTTGATACTA) is also located 200 bp upstream of *repA*. Consensus regions for transcription initiation similar to those described from Gram-positive bacteria (Graves & Rabinowitz, 1986) were also identified at -35 (5' TACTTC 3') and -10 (TATAAT). Putative RBSs and promoters were also identified for ORF2 and ORF4 but not for ORF3 or ORF5. A large IR sequence present immediately downstream of ORF4 (and thus following the large repeat which includes ORF4) could form a hairpin loop which may be involved in transcription termination or other regulatory functions. A similar feature has been noted in another recently described smHsp-encoding plasmid from this species (Somkuti *et al.*, 1998).

1	MAKIDDSVKK	E	VPELRFKGF	T	D	DWEERKL	-	GELANIVG	--	hds	pCI65st																																	
1	MAKIDDSVKK	K	VPELRFKGF	T	N	DWEERKL	-	GELSNIVG	--	hds	pIL7																																	
1	MAKIDDSVKK	R	VPELRF	F	G	F	T	N	D	WEEERK	F	F	E	S	I	A	S	T	I	D	F	R		hds	pIL103																			
38	G	G	T	P	S	T	S	N	P	E	Y	W	D	G	D	I	D	W	Y	A	P	A	E	I	G	E	Q	S	Y	V	--	-	S	K	S	K	K	T	I		hds	pCI65st		
38	G	G	T	P	S	T	S	N	P	E	Y	W	D	G	D	I	D	W	Y	A	P	A	E	I	G	E	Q	S	Y	V	--	-	S	K	S	K	K	T	I		hds	pIL7		
41	G	R	T	E	K	K	L	G	M	D	-	W	S	D	S	G	Y	L	A	L	S	A	L	N	V	K	N	G	L	I	D	P	L	A	D	A	H	Y	G	D		hds	pIL103	
75	T	E	L	G	L	K	N	S	S	A	R	I	L	P	V	G	T	V	L	F	T	S	R	A	G	I	G	N	T	A	I	L	A	K	E	A	T	T	N	Q		hds	pCI65st	
75	T	E	L	G	L	K	K	S	S	A	R	I	L	P	V	G	T	V	L	F	T	S	R	A	G	I	G	N	T	A	I	L	A	K	E	A	T	T	N	Q		hds	pIL7	
80	E	K	L	Y	R	K	W	M	S	G	R	E	L	K	R	G	Q	V	L	F	T	T	E	L	P	M	G	N	V	L	Q	V	P	D	D	N	G	Y	I	L		hds	pIL103	
115	G	F	Q	S	I	V	-	-	P	D	Q	N	K	L	D	S	Y	F	I	F	S	R	T	N	E	L	K	R	Y	G	E	V	T	G	-	-	A	G	S	T		hds	pCI65st	
115	G	F	Q	S	I	V	-	-	P	D	Q	N	K	L	D	S	Y	F	I	F	S	R	T	N	E	L	K	R	Y	G	E	V	T	G	-	-	A	G	S	T		hds	pIL7	
120	S	Q	R	T	V	A	F	E	T	K	E	D	M	M	T	N	D	E	L	A	V	L	L	K	S	P	L	V	F	N	N	L	S	A	L	S	S	G	L	T		hds	pIL103	
151	F	V	E	V	S	G	K	Q	M	S	K	M	S	I	M	V	P	-	E	L	S	E	Q	Q	K	I	G	S	F	F	K	Q	L	D	E	T	I	T	L	H		hds	pCI65st	
151	F	V	E	V	S	G	K	Q	M	S	K	M	S	I	M	V	P	-	E	L	S	E	Q	Q	K	I	G	S	F	F	K	E	L	D	N	T	I	A	L	H		hds	pIL7	
160	A	K	G	V	S	Q	K	S	L	K	G	L	S	I	T	V	P	L	D	I	D	E	Q	Q	K	I	G	S	F	F	K	H	L	D	D	T	I	A	L	H		hds	pIL103	
190	Q	R	K	L	D	L	L	K	E	Q	K	K	G	L	F	L	Q	K	M	F	P	K	N	G	A	K	V	P	E	L	R	F	A	G	F	A	D	D	W	E		hds	pCI65st	
190	Q	R	K	L	D	L	L	K	E	Q	K	K	G	L	F	L	Q	K	M	F	P	K	N	G	A	K	V	P	E	L	R	F	A	G	F	A	D	D	W	E		hds	pIL7	
200	Q	R	K	L	D	L	L	K	E	Q	K	K	G	L	F	L	Q	K	M	F	P	K	N	G	A	K	V	P	E	L	R	F	A	G	F	A	D	D	W	E		hds	pIL103	
230	R	K	L	G	E	L	A	N	I	V	G	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	hds	pCI65st	
230	R	K	L	G	D	I	T	K	I	S	T	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	hds	pIL7		
240	R	K	L	G	D	I	A	P	L	R	G	G	Y	A	F	K	S	S	K	F	R	K	G	V	E	I	V	R	I	S	N	I	L	S	S	G	E	V	G		hds	pIL103		
254	G	D	I	D	W	Y	A	P	A	E	I	G	E	Q	S	Y	V	S	K	S	K	K	T	I	T	E	L	G	L	K	N	S	S	A	R	I	L	P	V	G		hds	pCI65st	
252	G	K	Y	D	F	Y	T	S	G	I	K	Y	R	I	D	V	A	A	F	E	G	P	S	I	T	I	A	G	N	G	A	T	V	G	Y	M	H	L	A		hds	pIL7		
280	G	D	F	A	Y	I	D	E	Q	D	K	D	K	Y	I	L	P	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	hds	pIL103		
294	T	V	L	F	T	S	R	A	G	I	G	N	T	A	I	L	A	K	E	A	T	T	N	Q	G	Y	L	S	I	V	P	D	Q	N	K	L	D	S	Y	F		hds	pCI65st	
292	D	N	K	L	E	N	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	hds	pIL7			
312	K	V	S	I	L	S	Q	T	D	V	D	K	V	L	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	hds	pIL103			
334	I	F	S	R	T	-	N	E	L	-	K	R	Y	G	E	V	T	G	A	G	S	T	F	V	E	V	S	G	R	Q	M	S	K	M	S	I	M	V	P	E		hds	pCI65st	
314	I	F	S	E	I	G	N	K	L	-	P	K	K	I	K	Q	E	A	R	T	G	N	I	P	Y	I	V	M	D	M	L	T	E	L	K	L	S	I	P	Q		hds	pIL7	
344	I	S	T	I	V	R	S	E	L	-	F	M	M	Q	L	E	S	V	L	V	S	G	A	Q	P	N	V	S	S	K	E	I	D	S	F	N	F	M	I	P	I		hds	pIL103
372	L	-	S	E	Q	Q	K	I	G	L	F	F	R	H	L	D	D	T	I	T	F	H	Q	R	K	L	D	L	-	-	-	-	-	-	-	-	-	-	-	-	hds	pCI65st		
353	N	N	S	E	Q	Q	K	I	G	S	F	F	K	Q	L	D	D	T	I	A	L	H	Q	R	K	L	A	F	V	E	R	T	E	N	T	I	G	F	L	Q	K		hds	pIL7
384	L	V	Q	E	Q	Q	K	I	G	S	F	F	K	L	D	D	T	I	A	L	H	Q	R	K	L	D	L	-	-	-	-	-	-	-	-	-	-	-	-	-	hds	pIL103		
410	M	F	I																																					hds	pCI65st			
393	M	F	V																																					hds	pIL7			
423	M	F	V																																					hds	pIL103			

Fig. 3. Sequence alignments of the putative HsdS protein from a type I R/M system encoded by ORF5 of the *S. thermophilus* plasmid pCI65st with HsdS proteins from *Lactococcus lactis* plasmids pIL7 and pIL103 (Schouler et al., 1998b). Amino acids are boxed if three or more residues are identical. Numbers in the left hand margin refer to the amino acid positions in the proteins indicated in the right hand margin. Note the high degree of homology in the core, N and C domains and the similarity of the N-terminal variable region to that of the HsdS from pIL7.

Plasmid-free variants of *S. thermophilus* NDI-6

Plasmid-free derivatives were readily isolated after subculture of strain NDI-6 at 32 °C. After plating on FSDAT, variants with altered colony morphology were observed. When cleared lysates of these colonial variants were compared with the parent culture by gel electrophoresis, the plasmid band corresponding to pCI65st was absent. The presence of the Sm^R marker confirmed that this culture was a derivative of the NDI-6 parent. Plasmid-free variants (a representative of which was designated TOS1) grew at the same rate as the parent culture in broth ($k = 1.38 \text{ h}^{-1}$) regardless of the sugar source; however, during growth on glucose, final turbidity was invariably higher in the case of the parent culture. In addition, the parent culture gave rise to slightly larger colonies on glucose agar.

Bacteriophage sensitivity

In broth cultures, *S. thermophilus* NDI-6 was insensitive to all 43 of the *S. thermophilus* bacteriophages in the UCC phage bank while its plasmid-free mutant, TOS1, was consistently lysed in broth by bacteriophage ϕ bas19. Because this phage does not readily form plaques on this culture in sloppy agar assays (unlike the situation when it is titrated on its homologous host) it is not known whether the apparent resistance of the parent culture is due to the activity of a functional R/M system.

Enolase activity

Enolase activity was determined in cell-free extracts of the plasmid-bearing *S. thermophilus* culture NDI-6 and its plasmid-free mutant, and in *E. coli* XL-1 Blue which had been transformed with either pBS or the same plasmid carrying a 2.44 kb *SspI* fragment of pCI65st (from 3728 to 6274 kb) which included ORF5. No difference in enolase activity was observed between the parent and plasmid-free cultures at any of the stages of growth examined. However, 75 % more enolase activity [0.68 versus 0.39 U (mg protein)⁻¹] was detected in stationary-phase cells of *E. coli* XL-1 Blue culture harbouring the recombinant plasmid than in the same culture carrying only the cloning vector, pBS. This suggests that while the product of ORF3 may not possess enolase activity in its *S. thermophilus* host, it is capable of such activity in *E. coli*.

Heat-shock response

No significant difference was observed in the response of strain NDI-6 and its plasmid-free derivative TOS1 to an increase in temperature from 42 °C up to 58–62 °C with a prior sublethal shock at 52 °C. The death rate of both cultures at the various challenge temperatures assessed was broadly similar. However, in the absence of a prior heat shock, the death rate of the plasmid-free mutant increased significantly in comparison to that of the

Fig. 4. Heat-survival curves of *S. thermophilus* NDI-6 (■, ●) and its plasmid-free derivative TOS1 (◆, ▲) in Belliker broth at 62 °C. Prior to exposure to 62 °C, cells were preincubated at 42 °C (■, ◆) or 52 °C (●, ▲). The experiment was repeated at least three times; typical results are shown.

parent culture. This difference in response was particularly marked at 62 °C when, over the 2 h test interval, both the parent and plasmid-free cultures exhibited 10% survival when subjected to a prior sublethal heat shock. In the absence of a heat shock, the survival of the parent culture decreased to 1%; however, the survival of the plasmid-free culture decreased to 0.0001%. Typical heat survival data for strain NDI-6 and its plasmid-free variant are presented in Fig. 4.

SDS-PAGE analysis

The electrophoretic examination of the soluble cell proteins revealed the presence of two small proteins, of 16.4 and 17.3 kDa, in the *S. thermophilus* NDI-6 parent culture which were not observed in its plasmid-free mutant. During growth in Belliker broth containing 2% lactose as carbohydrate source, the intensity of these bands increased, corresponding with decreasing pH of the growth medium. The data are summarized in Fig. 5. The N-terminal sequences (20 aa) of the 16.4 and 17.3 kDa proteins which were overexpressed at low pH match precisely the deduced sequences of the proteins encoded by ORF2 and ORF4 of pCI65st. A similar expression of a 16 kDa family of smHsps was observed during growth of a *S. thermophilus* culture by Gonzalez-Marquez *et al.* (1997). No other differences in protein profile between the parent culture and its plasmid-free mutant were noted. The SDS-PAGE profile of *E. coli* XL-1 Blue transformants carrying pBS with the 2.44 kb insert which includes ORF3 was compared with that of the same culture carrying only pBS. Several differences were observed, notably the presence of a heavier band of approximately 92 kDa, which would be consistent with the dimeric form of enolase, in the culture harbouring the insert. However, other differences were also noted

Fig. 5. SDS-PAGE of the soluble proteins of *S. thermophilus* NDI-6 (lanes 2, 3 and 4) and its plasmid-free mutant TOS1 (lanes 5, 6 and 7) during growth in Belliker broth containing 20 g lactose l⁻¹. The pH and OD₅₈₀ of the cultures at the time of harvesting are indicated below lanes 2–7. Lane 1 contains protein size markers.

and no difference in band intensities was observed at 46 kDa (results not presented).

DISCUSSION

Data from this study indicate that the smHsp-encoding genes described on pCI65st and on other small cryptic plasmids from *S. thermophilus* may have a role in the heat-shock response other than that which is induced in non-plasmid-bearing strains. The increased resistance of the plasmid-bearing parent culture to elevated temperature challenge in the absence of a prior sublethal heat shock indicates that the presence of pCI65st provides a level of uninduced resistance to stress. The fact that the optimum growth temperature for this species is 42 °C, and that pCI65st is easily lost during subculture at reduced growth temperatures, suggests that the presence of genes encoding smHsps may confer some advantage or may influence plasmid maintenance during growth at elevated temperatures. These genes could also have a role in the response to other stresses such as acid, salt or ethanol. Similar proteins have been described relatively recently from other LAB. The 18 kDa smHsp (Lo18) of *Leuconostoc oenos* is induced by heat, acid and alcohol shock (Jobin *et al.*, 1997). A similar smHsp has been induced by heat shock in *Lactobacillus helveticus* (Timpone *et al.*, 1996). Somkuti *et al.* (1998) recently described increased synthesis of smHsp RNA in *S. thermophilus* during heat stress but did not demonstrate the presence of the relevant protein.

Gonzalez-Marquez *et al.* (1997) reported that a family of 16 kDa proteins is overexpressed during acid stress in another plasmid-bearing culture of *S. thermophilus*. N-terminal sequencing of one of the proteins involved indicates that it is similar or identical to the putative heat-shock genes described from pCI65st and other small *S. thermophilus* plasmids. The two putative smHsps encoded by pCI65st have also been demonstrated to be overexpressed late in the growth cycle and may therefore also be acid-inducible, suggesting that the resistance of the parent culture to heat shock may be induced by growth in acid conditions.

The plasmid location of ORF3, whose putative product has high similarity to enolase, is difficult to explain but the presence of additional plasmid copies could be linked to some of the phenotypic characteristics associated with the plasmid, such as larger colony size and higher final turbidities during growth on glucose. However, increased levels of enolase activity were not observed in the plasmid-bearing parent culture during this study. Enolase homologues have been reported to be induced by heat shock in a variety of species, including *Bacillus subtilis* (Miller *et al.*, 1991) and the presence of an enolase gene in association with smHsp-encoding genes would thus be consistent with a role for pCI65st in the stress response. In this respect, it would be of interest to compare the plasmid and chromosomal copies of this gene in *S. thermophilus* and to investigate the level and role (if any) of the protein in stress situations.

The significance of ORF5, which is capable of encoding an HsdS protein from a type I R/M system, on pCI65st is not clear. Type I R/M systems consist of three subunits. In addition to HsdS, restriction (HsdR) and modification (HsdM) proteins are required for functional expression of the system (Bickle & Kruger, 1993). Plasmid-free mutants of strain NDI-6 acquire sensitivity to a bacteriophage to which the parent culture is resistant, but the mechanism of the resistance of the parent culture has yet to be elucidated. A similar ORF has been described in another *S. thermophilus* plasmid, pCRB33, which has been reported to confer bacteriophage resistance upon some *S. thermophilus* cultures (Coconcelli *et al.*, 1995), possibly by activation of a type I R/M system. Schouler *et al.* (1998b) recently described the existence of plasmid-borne *hsdS* genes in lactococci and demonstrated the interaction of such plasmid-borne specificity subunits with chromosomally encoded HsdR and M proteins. In this manner, combinatorial variation of R/M specificities could result from the possession of more than one such *hsdS* gene. It would be of interest to assess the impact of this plasmid in known phage-sensitive hosts of *S. thermophilus*, particularly considering the likely involvement of another small *S. thermophilus* plasmid, pCRB33, which harbours a similar ORF, in bacteriophage resistance.

The presence of genes capable of encoding putative heat-shock proteins, an HsdS and enolase on an *S. thermophilus* plasmid sheds further light on the relevance of these heretofore cryptic replicons in thermophilic species of LAB and may have implications in the

manipulation of commercial cultures of this species in the food industry. Work is currently in progress to further examine the functionality of ORFs described on pCI65st and to evaluate the influence of this plasmid in other backgrounds.

ACKNOWLEDGEMENTS

We acknowledge the expert technical assistance of Aine Healy and Sinead Geary. This work was financially supported by the Centre International de Recherche, Daniel Carasso of the Danone group.

REFERENCES

- Altschul, S. F., Gish, W., Millar, W., Meyers, W. E. & Lipman, D. J. (1990). Basic local alignment search tool. *J Mol Biol* **215**, 403–410.
- Anderson, D. G. & McKay, L. L. (1983). A simple and rapid method for isolating large plasmid DNA from lactic streptococci. *Appl Environ Microbiol* **46**, 549–552.
- Auffray, Y., Lecesne, E., Hartke, A. & Boutibonnes, P. (1995). Basic features of the *Streptococcus thermophilus* heat shock response. *Curr Microbiol* **30**, 87–91.
- Bickle, T. A. & Kruger, D. H. (1993). Biology of DNA restriction. *Microbiol Rev* **57**, 434–450.
- Birnboim, H. C. & Doly, J. (1979). Rapid alkaline extraction procedure for screening recombinant DNA. *Nucleic Acids Res* **7**, 1513–1523.
- Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilising the principle of protein dye binding. *Anal Biochem* **72**, 248–254.
- Cocconcelli, P. S., Callegari, M. L., Porro, D. & Morelli, L. (1995). Plasmid recombination mediated by IS-like structures in *S. thermophilus* T003. In *Abstracts of Posters, European Commission Biotechnology and FAIR Programmes Conference on Lactic Acid Bacteria: From Fundamental Research to Innovative Applications*, Abstract F-8. Cork, Ireland, 22–26 October, 1995.
- Dellaglio, F. (1988). Starters for fermented milks. Section 3, Thermophilic starters, Chapter 11. *Bull Int Dairy Fed* **227**, 27–33.
- Dybvig, K. & Yu, H. (1994). Regulation of a restriction and modification system via DNA inversion in *Mycoplasma pulmonis*. *Mol Microbiol* **12**, 547–560.
- Elliker, P. R., Anderson, A. W. & Hannesson, G. (1956). An agar culture medium for lactic acid streptococci and lactobacilli. *J Dairy Sci* **89**, 1611–1612.
- Fitzgerald, G. F. & Hill, C. (1996). Genetics of starter cultures. In *Dairy Starter Cultures*, pp. 25–46. Edited by T. M. Cogan & J.-P. Accolas. New York: VCH Publishers.
- Fleischmann, R. D., Adams, M. D., White, O. & 37 other authors (1995). Whole-genome random sequencing and assembly of *Haemophilus influenzae* Rd. *Science* **269**, 496–512.
- Girard, F., Lautier, M. & Novel, G. (1987). DNA:DNA homology between plasmids from *Streptococcus thermophilus*. *Lait* **67**, 537–544.
- Gonzalez-Marquez, H., Perrin, C., Bracquart, P., Guimont, C. & Linden, G. (1997). A 16 kDa protein family overexpressed by *Streptococcus thermophilus* in acid environments. *Microbiology* **143**, 1587–1594.
- Graves, M. C. & Rabinowitz, J. C. (1986). In vivo and in vitro transcription of the *Clostridium pasteurianum* ferredoxin gene. Evidence for 'extended' promoter elements in gram positive organisms. *J Biol Chem* **261**, 11409–11415.

- Hashiba, H., Takigushi, R., Joho, K., Aoyama, K. & Hirota, T. (1993). Identification of the replication region of *Streptococcus thermophilus* no. 29 plasmid pST1. *Biosci Biotechnol Biochem* **57**, 1646–1649.
- Herman, R. E. & McKay, L. L. (1985). Isolation and partial characterisation of plasmid DNA from *Streptococcus thermophilus*. *Appl Environ Microbiol* **50**, 1103–1106.
- Huggins, A. R. & Sandine, W. E. (1984). Differentiation of fast and slow milk-coagulating isolates in strains of lactic streptococci. *J Dairy Sci* **67**, 1674–1679.
- Hutkins, R., Halambeck, S. M. & Morris, H. A. (1986). Use of galactose-fermenting *Streptococcus thermophilus* in the manufacture of Swiss, Mozzarella and short-method Cheddar cheese. *J Dairy Sci* **69**, 1–8.
- Janzen, T., Kleinschmidt, J., Neve, H. & Geis, A. (1992). Sequencing and characterization of pST1, a cryptic plasmid from *Streptococcus thermophilus*. *FEMS Microbiol Lett* **95**, 175–180.
- Jobin, M.-P., Delmas, F., Garmyn, D., Divies, C. & Guzzo, J. (1997). Molecular characterization of an 18-kilodalton small heat shock protein associated with the membrane of *Leuconostoc oenos*. *Applied Environ Microbiol* **63**, 609–614.
- Kneale, G. G. (1994). A symmetrical model for the domain structure of type I DNA methyltransferases. *J Mol Biol* **243**, 1–5.
- Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.
- Laigret, F., Gaurivaud, P. & Bove, J. M. (1996). The unique organisation of the *rpoB* region of *Spiroplasma citri*: a restriction and modification system gene is adjacent to *rpoB*. *Gene* **171**, 95–98.
- Luria, S. E., Adams, J. N. & Ting, R. C. (1960). Transduction of lactose-utilising ability among strains of *Escherichia coli* and *Shigella dysenteriae* and the properties of the transducing phage particles. *Virology* **12**, 348–390.
- McKay, L. L. (1983). Functional properties of plasmids in lactic streptococci. *Antonie Leeuwenhoek* **49**, 259–274.
- Mercenier, A. (1990). Molecular genetics of *Streptococcus thermophilus*. *FEMS Microbiol Rev* **87**, 61–78.
- Miller, B. S., Kennedy, T. E. & Streips, U. N. (1991). Molecular characterization of specific heat shock proteins in *Bacillus subtilis*. *Curr Microbiol* **22**, 231–236.
- Pearson, W. R. & Lipman, D. J. (1988). Improved tools for biological sequence comparison. *Proc Natl Acad Sci USA* **85**, 2444–2448.
- Price, C., Lingner, J., Bickle, J., Firman, T. A. & Glover, S. W. (1989). Basis for changes in DNA recognition by the *EcoR*124 and *EcoR*124/3 type I DNA restriction and modification enzymes. *J Mol Biol* **205**, 115–125.
- Sauer, U. & Durre, P. (1993). Sequence and molecular characterization of a DNA region encoding a small heat shock protein of *Clostridium acetobutylicum*. *J Bacteriol* **175**, 3394–3400.
- Schouler, C., Clier, F., Lerayer, A. L., Ehrlich, D. S. & Chopin, M. C. (1998a). A type I restriction–modification system in *Lactococcus lactis*. *J Bacteriol* **180**, 407–411.
- Schouler, C., Gautier, M., Ehrlich, D. & Chopin, M. C. (1988b). Combinational variation of restriction–modification in *Lactococcus lactis*. *Mol Microbiol* **28**, 169–178.
- Seery, L. T., Nolan, N. C., Sharp, P. M. & Devine, K. M. (1993). Comparative analysis of the pC194 group of rolling circular plasmids. *Plasmid* **30**, 185–196.
- Somkuti, G. A. & Steinberg, D. H. (1986). Distribution and analysis of plasmids in *Streptococcus thermophilus*. *J Ind Microbiol* **1**, 157–163.
- Somkuti, G. A., Solaiman, D. K. Y. & Steinberg, D. H. (1998). Structural and functional analysis of the *hsp*16.4-bearing plasmid pER341 plasmids in *Streptococcus thermophilus*. *Plasmid* **40**, 61–72.
- Timpone, D. E., Furster, K. M., Wei, L., Broadbent, J. R. & Steele, J. L. (1996). A small heat shock protein from *Lactobacillus helveticus*. In *Fifth Symposium on Lactic Acid Bacteria – Genetics, Metabolism and Applications*, abstract G60. Veldhoven, The Netherlands, 8–12 September 1996.
- Titheradge, A. J. B., Ternent, D. & Murray, N. E. (1996). A third family of allelic *hsd* genes in *Salmonella enterica*: sequence comparisons with related proteins identify conserved regions implicated in restriction of DNA. *Mol Microbiol* **22**, 437–447.
- Wold, F. (1971). Enolase. In *The Enzymes*, vol. 5, pp. 499–538. Edited by P. D. Boyer. New York: Academic Press.

Received 1 July 1998; revised 5 October 1998; accepted 13 October 1998.