Analysis of the *lacZ* sequences from two *Streptococcus thermophilus* strains: comparison with the *Escherichia coli* and *Lactobacillus bulgaricus* β -galactosidase sequences

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The *lacZ* gene from *Streptococcus thermophilus* A054, a commercial yogurt strain, was cloned on a 7.2 kb *Pst*I fragment in *Escherichia coli* and compared with the previously cloned *lacZ* gene from *S. thermophilus* ATCC 19258. Using the dideoxy chain termination method, the DNA sequences of both *lacZ* structural genes were determined and found to be 3071 bp in length. When the two sequences were more closely analysed, 21 nucleotide differences were detected, of which only nine resulted in amino acid changes in the proteins, the remainder occurring in wobble positions of the respective codons. Only three bases separated the termination codon for the *lacS* gene from the initiation codon for *lacZ*, suggesting that the lactose utilization genes are organized as an operon. The amino acid sequence of the β -galactosidase, derived from the DNA sequence, corresponds to a protein with a molecular mass of 116860 Da. Comparison of the *S. thermophilus* amino acid sequences with those from *Lactobacillus bulgaricus*, *E. coli* and *Klebsiella pneumoniae* showed 48, 35 and 32.5% identity respectively. Although little sequence homology was observed at the DNA level, many regions conserved in the amino acid sequence were identified when the β -galactosidase proteins from *S. thermophilus*, *E. coli* and *L. bulgaricus* were compared.

Introduction

The bioconversion of lactose to lactic acid is an essential process in dairy fermentations. In lactococci (previously the mesophilic, group N streptococci) and certain lactobacilli, lactose is phosphorylated during its uptake and converted to lactose 6-phosphate, which is then hydrolysed by a phospho- β -galactosidase (Pbg) to yield D-glucose and galactose 6-phosphate. However, in *Streptococcus salivarius* subsp. *thermophilus* (designated

as *S. thermophilus* throughout this paper) lactose transport is not mediated through a phosphoenolpyruvate phosphotransferase system (PEP-PTS; Poolman *et al.*, 1989), but rather the intracellular sugar is hydrolysed by a β -galactosidase (Bgal; Somkuti & Steinberg, 1979).

There has been growing interest in the molecular organization of genes for lactose utilization from dairy lactic acid bacteria (Alpert & Chassy, 1988; Boizet *et al.*, 1988; DeVos & Simons, 1988; DeVos *et al.*, 1989; Porter & Chassy, 1988; Schmidt *et al.*, 1989; Poolman *et al.*, 1989), including those genes from species widely used in the preparation of yogurt and cheeses that require high incubation or cooking temperatures (Herman & McKay, 1986; Poolman *et al.*, 1989; Schmidt *et al.*, 1989). Furthermore, homology studies of lactose genes from dairy starter organisms, which are mainly adapted to the efficient metabolism of lactose, with equivalent genes from *Escherichia coli* and similar organisms, for which this source of energy is minor in the natural environment, could possibly provide information about cellular efficiency and protein structure and function.

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Abbreviations: Ap, ampicillin; Bgal, β -galactosidase; Cm, chloramphenicol; Em, erythromycin; ExoIII, exonuclease III; Km, kanamycin; ORF, open reading frame; PEP-PTS, phosphoenolpyruvate phosphotransferase system; Pbg, phospho- β -galactosidase; RBS, ribosome-binding site; Tc, tetracycline; X-Gal, 5-bromo-4-chloro-3-indolyl β -D-galactopyranoside.

The nucleotide sequence data reported in this paper have been submitted to GenBank and have been assigned accession numbers M36729 (*lacZ* A054) and M37204 (*lacZ* ATCC 19258).

The plasmid cloning vectors that are currently available for the dairy streptococci depend upon selection markers which encode antibiotic resistance. Since such markers are considered unsuitable for use in food fermentations, other markers, such as genes for carbohydrate utilization, should be isolated and characterized. One such example would be the Bgal gene linked to the lactose transport gene from *S. thermophilus*. Herman & McKay (1986) reported the cloning and characterization of the *lacZ* gene (for nomenclature, see Methods) from *S. thermophilus*. The protein encoded by this gene was analysed in *E. coli* maxicells and was determined to have a molecular mass of approximately 105000 Da (Herman *et al.*, 1987). The *lacZ* gene was localized on a 3.85 kb region of a 7.2 kb *Pst*I-generated chromosomal fragment.

In this study, the *lacZ* genes from two *S. thermophilus* strains, A054 (an industrial yogurt strain whose genetics has been well studied; Mercenier & Lemoine, 1989) and ATCC 19258 (the type strain), were subjected to DNA sequence analysis. These strains differ markedly in their acid production/milk coagulation rates and in their sensitivities to various lytic *S. thermophilus* bacterio-

phages. Although strain ATCC 19258 is a 'type' culture, it exhibits relatively few of the desirable characteristics of a starter culture (i.e. fast acid production, milk clotting activity, etc.). The DNA and deduced protein sequences of the *lacZ* genes of these two strains were compared to determine whether their rate of acid production reflects functional differences in their lactose utilization genes. Sequence homologies were also examined among the Bgal genes from *S. thermophilus*, *Lactobacillus bulgaricus* and *E. coli*.

Methods

Bacterial strains and vectors. The genotypes of the bacterial strains and plasmids used in this study, with their sources, are given in Table 1. *E. coli* strains were grown at 37 °C in YT broth medium (8 g tryptone l⁻¹, 5 g yeast extract l⁻¹, 5 g NaCl l⁻¹) or LB medium (10 g tryptone l⁻¹, 10 g yeast extract l⁻¹, 5 g NaCl l⁻¹) (Miller, 1972). Agar plating media were prepared by the addition of 1.5% (w/v) agar to broth media. Antibiotics were added in the following concentrations when needed: chloramphenicol (Cm), 25 µg ml⁻¹; tetracycline (Tc), 15 µg ml⁻¹; ampicillin (Ap), 50 or 100 µg ml⁻¹. *S. thermophilus* strains were grown at 37 °C or 42 °C in M17 medium (Terzaghi & Sandine, 1975) containing 5% (w/v) glucose or in Elliker medium (Elliker *et al.*, 1956)

Table 1. *Bacterial strains and plasmid vectors*

Strain	Genotype	Reference
<i>E. coli</i>		
C600	F ⁻ <i>thi-1 thr-1 leuB6 lacY1 tonA21 supE44 λ⁻</i>	Appleyard (1954)
XL-1 Blue	<i>recA endA1 gyrA96 thi hsdR17 (r_K m_K) supE44 relA1 λ⁻ lac [F' <i>proAB lacZ</i> M15 Tn10]</i>	Bullock <i>et al.</i> (1987)
JM107	<i>endA1 gyrA96 thi hsdR17 supE44 relA1 λ⁻ Δ(lac-proAB), [F' <i>traD36 proAB lacZ</i> M15]</i>	Yanisch-Perron <i>et al.</i> (1985)
<i>S. thermophilus</i>		
A023	Wild-type (yogurt starter)	This study
A032	Wild-type (yogurt starter)	This study
A054	Wild-type (yogurt starter)	This study
A147	Wild-type (yogurt starter)	Poolman <i>et al.</i> (1989)
ATCC 19258	Wild-type (type culture)	Herman & McKay (1986)
<i>L. lactis</i> subsp. <i>lactis</i>		
LM0230	Lac ⁻	McKay <i>et al.</i> (1980)
LM2301	Lac ⁻ Str ^r	McKay <i>et al.</i> (1980)
7962	Wild-type	Crow & Thomas (1984)
Plasmid	Selective marker(s)	Reference
pBR322	Ap ^r , Tc ^r	Bolivar <i>et al.</i> (1977)
pUC19	Ap ^r	Yanisch-Perron <i>et al.</i> (1985)
Bluescript KS	Ap ^r	Stratagene Cloning Systems
pMG4536	Km ^r Cm ^r	Gasson (1987)
pSA3	Em ^r Cm ^r Tc ^r	Dao & Ferretti (1985)
pRH116	Tc ^r LacZ ⁺	Herman & McKay (1986)
pTG234	Cm ^r LacZ ⁺ (Km ^r)*	This study
pTG236	Cm ^r LacZ ⁺ (Km ^r)*	This study
pCS119	Ap ^r LacZ ⁺	This study
pCS200	Ap ^r LacZ ⁺	This study
pUM101	Em ^r Cm ^r LacZ ⁺	This study

* Km^r was proven an unsuitable marker for the selection of lactococcal or streptococcal transformants.

supplemented with 1% (w/v) beef extract. *Lactococcus lactis* strains were grown in M17/glucose broth at 32 °C. When necessary, Cm or Em was added at a concentration of 5 μ g ml⁻¹ for streptococci and lactococci.

Nomenclature. The β -galactosidase gene, previously designated β -gal (Herman & McKay, 1986; Herman *et al.*, 1987), has been re-designated *lacZ* in accordance with the rules for uniform nomenclature as proposed by Demerec *et al.* (1966). This gene designation is based on structural (sequence homology) and functional properties of the *S. thermophilus* gene, and brings this gene into alignment with the nomenclature used for *E. coli* and *Salmonella typhimurium*. However, because the lactose transport protein of *S. thermophilus* has a unique structure, *lacS* is an appropriate designation for that gene (described by Poolman *et al.*, 1989). It is our hope that researchers in related areas will incorporate the use of this nomenclature in future publications.

Reagents and chemicals. For detection of Bgal-positive subclones, 5-bromo-4-chloro-3-indolyl β -D-galactopyranoside (X-Gal) was used in agar media at a concentration of 40 μ g ml⁻¹. Restriction enzymes and T4 DNA ligase were obtained from BRL and were used as directed by the supplier. Exonuclease III (ExoIII) and mung bean nuclease, used for deletion analysis, were purchased in kit form from Stratagene. For DNA sequencing of the *lacZ* gene of ATCC 19258, dideoxy sequencing reagents and Sequenase were purchased in kit form from US Biochemical Corp., and ³²P-labelled dATP was obtained from New England Nuclear. The *lacZ* gene of strain A054 was sequenced following the procedure of Sanger *et al.* (1977), using the large Klenow fragment of DNA polymerase I purchased from BRL. ³²P-radiolabelling of DNA probes was achieved as described by Maniatis *et al.* (1982).

DNA isolation. DNA fragments were isolated from agarose gels using a modification of the method of Winberg & Hammerskjola (1980). DEAE membranes (BA45; Schleicher & Schuell) replaced DEAE paper. Plasmid DNA was isolated from *E. coli* by either the rapid boiling method of Holmes & Quigley (1981) or the method of Macrina *et al.* (1983).

Deletion analysis. Deletions in the *lacZ* gene of ATCC 19258 were generated by the procedure of Henikoff (1984) using the Stratagene ExoIII deletion kit. For deletions from the *SalI* end of the *lacZ* (ATCC 19258) fragment, pKS200 was completely digested with *SalI* and *KpnI* prior to ExoIII digestion. Bidirectional deletions from the *PstI* end were generated after first digesting pKS200 with *BamHI*.

Transformation. *E. coli* strains were transformed using the procedure of Hanahan (1983). For *L. lactis*, the protoplast transformation developed by Kondo & McKay (1982) was used. Plasmid DNA was introduced into *S. thermophilus* using the spheroplast transformation procedure of Mercenier *et al.* (1988). Alternatively, electroporation was used to introduce DNA into *L. lactis* and *S. thermophilus* (A. Mercenier and others, unpublished).

Nucleotide sequencing procedure. A 5.6 kb chromosomal insert carrying the *lacZ* gene from strain A054 was sequenced after generating multiple overlapping clones by a shotgun approach (Bankier & Barrell, 1983) using the sequencing vectors M13tg130 and M13tg131 (Kieny *et al.*, 1983). Alternatively, deletions were generated from both ends of the 3.0 kb *lacZ* (ATCC 19258) fragment in pCS200 using the method described above. After restriction digestion, and ExoIII and mung bean nuclease treatments, the deletion fragments were released from the original plasmid vector DNA and subsequently subcloned into the M13 cloning/sequencing vectors M13mp18 and M13mp19 (Yanisch-Perron *et al.*, 1985). In addition, *EcoRI* and *BglII* restriction fragments were subcloned directly into M13 vectors to cover regions not accessible for sequencing using the deletion products. Dideoxy sequencing of the *lacZ* (ATCC 19258) gene was achieved using a modified T7 DNA polymerase (Sequenase; Sanger *et al.*, 1977;

Tabor & Richardson, 1987). Synthetic primers were employed to complete the sequence of both strands of DNA. The IntelliGenetics DNA and protein analysis package on a SUN 2/120 computer, programs purchased from DNA-Star (Madison, WI, USA), and the Larson & Messing (1982) DNA sequence analysis programs facilitated sequence analysis of both *lacZ* genes.

β -Galactosidase assays. Cell-free extracts of *S. thermophilus* ATCC 19258 were prepared in the following manner. Cultures were grown overnight at 42 °C in 200 ml M17 broth supplemented with 1% (w/v) maltose or lactose as carbon source. After harvesting, the cells were washed twice with 0.1 M-sodium phosphate buffer (pH 7.0) and resuspended in 5 ml fresh buffer. Mechanical breakage of the cells was achieved using an Eaton pressure cell, and cell debris was removed by centrifugation. Bgal activity was then determined by the method of Miller (1972). For *S. thermophilus* A054, enzyme activity was determined following the procedure described by Somkuti & Steinberg (1979) after lysis of a spheroplast suspension prepared as described by Mercenier *et al.* (1988).

Results

Subcloning of the 5.7 kb fragments carrying the lacZ gene from strains A054 and ATCC 19258

The *lacZ* gene from the yogurt starter strain A054 was cloned on a 7.2 kb *PstI* fragment into pBR322, as reported previously for the *lacZ* gene from *S. thermophilus* ATCC 19258 (Herman & McKay, 1986). This plasmid was designated pTG234 and is equivalent to pRH116 described by Herman & McKay (see Fig. 1). Comparative restriction and deletion analyses of pRH116 and pTG234 showed physical similarity between the respective chromosomal inserts. The 5.7 kb *PstI*-*SalI* *lacZ* (A054) fragment was subcloned from pTG234 into pMG4536 (Gasson, 1987) downstream from the promoter A (P_A) of this plasmid. The resulting construct, pTG236, was introduced into *E. coli* JM107 (a Lac⁻ recipient), *L. lactis* LM2301 and *S. thermophilus* A054. Bgal activity was detected in the two former strains by streaking the recombinant strains on X-Gal medium. In A054, the intracellular enzyme activity was measured and found to be increased only by a factor of two over that observed without pTG236 (data not shown).

The 5.7 kb *PstI*-*SalI* *lacZ* (ATCC 19258) fragment from pRH116 was subcloned into the corresponding sites of pUC19 (Yanisch-Perron *et al.*, 1985), and the new Bgal-positive construct was designated pCS119 (Fig. 1). Using the *BamHI*-*SphI* sites provided by the pUC19 portion of pCS119, the *lacZ* fragment was further subcloned into the *BamHI*-*SphI* sites of pSA3 (Dao & Ferretti, 1985) and transformed into *E. coli*. Plasmid DNA of the new construction, pUM101 (Fig. 1), was used to transform *L. lactis* LM2301. Transformants were Bgal positive on X-Gal plates, but only after 36–48 h, suggesting that enzyme activity or production in LM2301(pUM101) was lower than for LM2301

Fig. 1. Subcloning of the *Pst*I-*Sal*I *lacZ* fragment from *S. thermophilus* ATCC 19258 and construction of pCS119 and pUM101, the latter being an *E. coli*-streptococcal shuttle vector containing *lacZ* (ATCC 19258). A linear map of the *Pst*I-*Pst*I fragment from *S. thermophilus* A054 is also included.

(pTG236). Additional Bgal assays showed approximately 100-fold lower enzyme activity in *L. lactis* when compared with the same gene product in *E. coli* (data not shown).

Analysis of the 5.6 kb insert of A054

For sequencing purposes, overlapping fragments of the *lacZ* (A054) insert were generated using a shotgun approach (Bankier & Barrell, 1983). This required prior *in vitro* mutagenesis of the unique *Mlu*I site present in pTG234 and resulted in the generation of a 5.6 kb *Pst*I–*Pst*I fragment. This fragment was isolated, religated to itself, and further sonicated to produce a population of random blunt-end fragments. These fragments were then subcloned into the *Sma*I sites of M13tg130 and M13tg131. As a result, 32 overlapping clones were generated for the sequencing of one strand, while 38 were isolated for sequencing of the other strand (data not shown).

To determine whether the *lacZ* gene resides on the same DNA fragment in different strains of *S. thermophilus*, chromosomal DNA from four industrial yogurt starter cultures (A023, A032, A054 and A147) was isolated and digested with *Pst*I. The fragments were separated by agarose gel electrophoresis and screened by Southern blot hybridization (Southern, 1975) using high-stringency conditions and 32 P-labelled *lacZ* (A054) gene as a probe. All *S. thermophilus* strains tested carried *lacZ* on a 7.2 kb *Pst*I–*Pst*I fragment (data not shown). When DNA from *E. coli* C600 (Appleyard, 1954) or *L. lactis* 7962 (Crow & Thomas, 1984) was screened in the same manner, no hybridization signal was detected (data not shown) even though *L. lactis* 7962 produces both a Bgal and a Pbg (Crow & Thomas, 1984).

Deletion analysis of the *lacZ* (ATCC 19258) fragment

In order to localize the *lacZ* (ATCC 19258) gene on the 5.7 kb *Pst*I–*Sa*I fragment of pCS119, deletions were generated using ExoIII (Henikoff, 1984; see Fig. 2). DNA was first deleted from the *Sa*I end. Restriction analysis of the deletion fragments showed that the 590 bp *Hind*III fragment had previously been incorrectly mapped to the right of the large 3.0 kb *Hind*III fragment. Deletion analysis also aided in determining the location of the right end of the *lacZ* (ATCC 19258) gene. Isolate 137 (Fig. 2) was Bgal positive but isolate 145 was Bgal negative, indicating that one boundary of the *lacZ* (ATCC 19258) gene was located at or near the corresponding *Hind*III site. The left end of the *lacZ* gene was localized by deleting sequences bidirectionally from the *Bam*HI site. Isolate 68, which retained Bgal activity, had lost approximately 600 bp of DNA, suggesting that

Fig. 2. Map of *lacZ* (ATCC 19258) fragment deletions generated using ExoIII/mung bean nuclease. Deletions 28–137 and 68 were Bgal positive while deletions 145–559 did not display enzyme activity.

the *lacZ* structural gene resides on a 3.2 kb region of DNA.

Based on previous work by Herman & McKay (1986) suggesting that the *lacZ* (ATCC 19258) gene might be contained on the largest *Hind*III fragment, the 3.0 kb *Hind*III fragment from pCS119 was subcloned into Bluescript KS (see Table 1). This fragment produced constitutive Bgal activity in the new construct, pCS200. Because enzyme activity was constitutive in Bluescript KS, which contains an inducible *E. coli*-derived *lacZ* promoter upstream from the polylinker, it was thought that the *S. thermophilus lacZ* (ATCC 19258) gene carried its own promoter.

DNA sequencing and comparison of the *lacZ* genes from ATCC 19258 and A054

By nucleotide sequencing, it was determined that the *lacZ* genes from strains A054 and ATCC 19258 were actually contained on two *Hind*III fragments, one of 3098 bp, and one of 76 bp (Fig. 1). The eight carboxy-terminal residues of the protein plus the termination codon are encoded by the 76 bp fragment. The size of the *S. thermophilus lacZ* structural gene was determined to be 3071 bp and most probably encodes a protein of 1026 amino acid residues. With a calculated average pI value of 5.90, the Bgal protein has a calculated molecular mass of 116860. Analysis of nucleotide percentages gave

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      -35          -10
TCACCAATTATTGAGAAAAATGGTCTTGATGACACAGTACTTGCTCACTGTAACATAATTCAGAAAAATTCAGTGCTTCCATCTTGAACAAAAAGTTGGAG
-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+
AAAAGGTAGAAGCTTTGTCTGAAGTTATTACCTTCAAAAAAGGAGAATAATCTATGAACATGACTGAAAAATTCAAACTATTTAAACGATCCAAAGAT
-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+
                                     m n m t e k i q t y l n d p k i
      -35          -10          RBS          ***
TGTTAGCGTTAATACTGTTGATGCTCACTCAGATCATAAGTATTTGAATCTCTTGAAGAATTTCTGAAGGGGAGATGAAGTTAAGACAATCTCTTAAT
-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+
v s v n t v d a h s d h k y f e s l e e f s e g e m k l r q s l n

GGAAAAATGAAAAATTCAGTATGCTCAGAAATACAAATCAGGTTTTAAAGACTTTTATAAAACAGAAATTTGATGAACTGATTTGAATTTATCAATGTAC
-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+
g k w k i h y a q n t n q v l k d f y k t e f d e t d l n f i n v p

CAGGTCATTTAGAGCTTCAAGGTTTTGGTTCTCCACAATATGTGAATACCAATATCCTTGGGATGGTAAGAAATTCCTTCGTCACCTCAAGTTCTCTCA
-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+
g h l e l q g f g s p q y v n t q y p w d g k e f l r p p q v p q

AGAATCAATGCTGTTGCATCATACGTTAAACATTTTACCTTGAATGATGCATTAAGATAAAAAAGTATTTATCTCATTCCAAGGGGTGCTACTTCC
-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+
e s n a v a s y v k h f t l n d a l k d k k v f i s f q g v a t s

ATCTTTGTATGGTCAATGGTAAGTCTCGTAGGATACAGTGAAGATTCATTACACCTAGTGAATTTGAAATAGTGATTACCTTGTGAAGTGATAACA
-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+
i f v w v n g n f v g y s e d s f t p s e f e i s d y l v e g d n k

AGTTGGCGTAGCTGTTTATCGTTACTCTACAGCAAGCTGGTTGGAAGACCAAGACTTCTGGAGACTTTACGGTATTTTATGAGATGTTTACTTGATGC
-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+
l a v a v y r y s t a s w l e d q d f w r l y g i f r d v y l y a

TATTCCAAAAGTTCAGTTCAAGATCTCTTTGTTAAGGGAGATTATGATTACCAACAAAAAGCAGGTCAATTGGATATTGATTGAAGACTGTTGGTGAT
-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+
i p k v h v q d l f v k g d y d y q t k a g q l d i d l k t v g d

TATGAAGACAAGAGATTAAATATGTTCTTTAGATTATGAAGGCATCGTTACAGAAGGTGATGCATCTGTTAATGGTGACGGTGAACATCTGTAAGTC
-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+
y e d k k i k y v l s d y e g i v t e g d a s v n g d g e l s v s l

TTGAAATCTTAAATCAAACTTGGAGTCTGAAAGTCTTAACTTTACGATTTGATCCTTCATGTTTGGATGATGACCAAGTTGTTGAAGTCGTTCC
-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+
e n l k i k p w s a e s p k l y d l i l h v l d d d q v v e v v p

AGTTAAAGTTGGATTTAGACGCTTTGAAATTAAGATAAACTTATGCTTTTGAATGGTAAGAGAATTGTCTTTAAAGGGGTAAACAGACACGAATTTAAC
-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+
v k v g f r r f e i k d k l m l l n g k r i v f k g v n r h e f n

GCTAGAACAGGACGTTGTATCACTGAAGAAGATATGCTTTGGGATATCAAAGTGATGAACAACATAACATCAATGCTGTTGCTACTTCACACTATCCTA
-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+
a r t g r c i t e e d m l w d i k v m k q h n i n a v r t s h y p n

ACCAACACGTTGGTATGAATGTGTGATGAATATGGACTTTATGTTATCGATGAAGCCAACCTTGAACACACGGTACATGGCAAAAACCTTGGTCTATG
-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+
q t r w y e l c d e y g l y v i d e a n l e t h g t w q k l g l c

CGAACCTTCATGAATATCCCAGCTAGTGAACAGAATGGTTGCTGCTGTTGTTGGATCGTGCCAATAACATGTTCCAACGCGATAAGAACCAGCTAGT
-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+
e p s w n i p a s e p e w l p a c l d r a n n m f q r d k n h a s

GTTATCATTTGGTCTTGTTGAATGAATCATATGCTGGTAAAGATATTGCTGACATGGCTGATTACTTCGAGTAGTTGACAATACTCGTCCAGTTCACT
-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+
v i i w s c g n e s y a g k d i a d m a d y f r s v d n t r p v h y

ATGAAGGTGTTGCATGGTGCCTGAGTTTGATTACATTACAGACATCGAAAGTCGTATGTATGCGAAACCAGCTGATATCGAAGAATACCTCACAACTGG
-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+
e g v a w c r e f d y i t d i e s r m y a k p a d i e e y l t t g

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TAAACTAGTTGATCTTTCAAGCGTTAGTGATAAACACCTTTGCTTCAGGTAACTAACTAACAAACCTCAAAAACCTTATATTTTCATGTGAATACATGCAC
-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+ 1800
k l v d l s s v s d k h f a s g n l t n k p q k p y i s c e y m h

ACAATGGGTAACCTGGTGGTGGATTGCAACTCTACACTGACTTAGAGAAATATCCAGAATACCAAGGTGGATTATTTGGGACTTCATTGACCAAGCTA
-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+ 1900
t m g n s g g g l q l y t d l e k y p e y q g g f i w d f i d q a i

TTTACAAAACACTTCCAAATGGTAGCGAATTCCTATCATATGGTGGTGACTGGCATGATAGACCTTCTGACTACGAATTTGTGGAAATGGTATCGTCTT
-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+ 2000
y k t l p n g s e f l s y g g d w h d r p s d y e f c g n g i v f

TGCAGATCGTACCCAACTCCAAAACCTCAAAACAGTTAAACATCTTTACTCTAATATTAAGATTGCTGTTGATGAAAAATCAGTAACTATCAAGATGAT
-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+ 2100
ACGCTCTAGCATGGGATTGAGGTTTGAAGTTTGTCAATTTGTAGAAATGAGATTATAATTCTAACGCAACTACTTTTGTAGTCATTGATGTTCTTACTA
a d r t l t p k l q t v k h l y s n i k i a v d e k s v t i k n d

AATCTCTCGAAGATCTTCTGCTTACTTTCTAGCTAGAGTTTACGAAGATGGTAAAAAGTTAGTGAAAGTGAATATCACTTTGATGTTAAACCAG
-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+ 2200
n l f e d l s a y t f l a r v y e d g r k v s e s e y h f d v k p g

GCGAAGAACCAACTTCCAGTTAACTTTGTAGTCGAGGCTTCAAATTCGAACAAATTTACGAAGTTGCTTGTGTTCTGAGGGAAGCAACTGAATGGGC
-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+ 2300
e e a t f p v n f v v e a s n s e q i y e v a c v l r e a t e w a

TCCTAAAGGTCATGAAATGTTCTGGTCAATATGTTGTTGAAAAGATTAGCACTGAAACACCAGTTAAAGCACCTTTGAATGTTGTTGAAGGCGACTTC
-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+ 2400
p k g h e i v r g q y v v e k i s t e t p v k a p l n v v e g d f

AACATCGGTATTCAGGACAAAACCTCTCAATCTTGCTTTCACGTGCACAAAATACTTTAGTATCTGCTAAGTATAATGGTGTGTAATTCATTGAGAAAG
-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+ 2500
n i g i q g q n f s i l l s r a q n t l v s a k y n g v e f i e k g

GTCCTAAACTAGCTTCACTCGTGCTTACACTGACAACGATCGTGGTGGTATCCATTCGAAATGGCAGGCTGGAAGGTTGCTGGAATATAGTAA
-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+ 2600
p k l s f t r a y t d n d r g a g y p f e m a g w k v a g n y s k

AGTTACAGATACTCAAATCAAATCGAAGACGACTCTGTTAAAGTACTTATGTTTCATGAATGCCAGGCTTGTCTGATGTCGAAGTTAAGGTAACCTAT
-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+ 2700
v t d t q i q i e d d s v k v t y v h e l p g l s d v e v k v t y

CAAGTTGATTACAAGGGTCGAATCTTTGTACTGCAAACTATGATGGTAAAGCAGGTTTGCCAACTTCCCTGAATTTGGTCTAGAATTTGCTATCGGTT
-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+ 2800
q v d y k g r i f v t a n y d g k a g l p n f p e f g l e f a i g s

CACAATTTACAAACCTTAGCTATTATGGATACGGTGCAGAGAAAGCTACCGTGATAAACTTCTGGTGCCTATCTTGGTGCATATGAACATCTGTTGA
-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+ 2900
q f t n l s y y g y g a e e s y r d k l p g a y l g r y e t s v e

AAAGACATTTGCTCCATATCTAATGCCACAAGAACTGTTAATCACTATGTTACTCGTGAATTCACAGTATCTGATGATAACCAATATGGTCTTAAATTC
-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+ 3000
k t f a p y l m p q e s g n h y g t r e f t v s d d n h n g l k f

ACCGCACTTAATAAGCATTGCAATTCAGTGCTTTGCGTAACAGTACTGAACAAATGAAAATGCTCGTCACCAATATGAGTTGCAAGAACTCTGATGCTA
-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+ 3100
t a l n k a f e f s a l r n s t e q i e n a r h q y e l q e s d a t

CATGGATTAAAGTTCTTCTGCTGCTCAATGGGTGATGGTGGTGACGACACATGGGGTCTCCAGTTTCATGACGAATTTCTGCTTAGCTCAGCAGATAGCTA
-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+ 3200
w i k v l a a q m g v g g d d t w g a p v h d e f l l s a d s y

TCAATTAAGCTTCATGATTGAACCACTAAATAG
-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+ 3234
q l s f m i e p l n .

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Fig. 3. Nucleotide sequence of the *S. thermophilus lacZ* (A054) gene and the deduced amino acid sequence. Putative ribosome-binding sites (RBS), transcription initiation (−10, −35) sites and start sites (***) are indicated.

63.2% A-T and 36.8% G-C, which corresponds with the approximate values calculated for the thermophilic streptococci (Farrow & Collins, 1984; Kilpper-Balz *et al.*, 1982). Hydrophobicity analysis of the deduced amino acid sequence showed the Bgal protein to be very hydrophilic, as expected for a cytoplasmic protein (Kyte & Doolittle, 1982).

DNA sequences obtained both upstream (842 bp) and downstream (1528 bp) from the *lacZ* gene from A054 indicate the presence of at least two additional incomplete open reading frames (ORFs), designated ORF280 and ORF231 (A. Mercenier and others, unpublished observations). ORF280, the upstream sequence, is nearly identical to the 3' region of the DNA sequence of the *S. thermophilus* lactose transport gene (*lacS*) from the yogurt starter strain A147 (Poolman *et al.*, 1989). Only three bases separate the termination codon of the *lacS* sequence from the first initiation codon of the *lacZ* gene, suggesting that a polycistronic message is produced and that the lactose genes exist as an operon. This was indeed confirmed by Northern analysis (Thomas, 1980; Poolman *et al.*, 1990; P. Slos & A. Mercenier, unpublished observations). At this time, the function of ORF231 is unknown but, because it lies 835 bp downstream from the *lacZ* gene, it seems unlikely that ORF231 is related to the lactose operon.

Computer analysis of the region upstream from the first initiation codon (believed to be used in *S. thermophilus*) showed the existence of a consensus ribosome-binding site (RBS; positions 139-145) only 8 bp upstream from the translational start site. However, consensus -35 and -10 regions were only found as far as 80 bp upstream from the putative RBS (see Fig. 3), though this does not exclude the possibility of a functional, secondary internal *lacZ* promoter in *S. thermophilus*. Relatively large distances between promoter regions and RBSs have been proposed as in the case of the dihydrofolate reductase gene of *Lactobacillus casei* (Andrews *et al.*, 1985). An inverted repeat of 29 bp was identified at positions 3276-3305, 42 bp downstream from the termination codon (UAG). The hairpin structure that it forms also contains a terminal poly(U) region, suggesting that this structure is a rho-independent terminator.

The cloned fragment carrying the *S. thermophilus lacZ* gene did not contain the 5' region of the lactose transport gene, and is most probably lacking the authentic lactose promoter on the originally cloned *PstI-PstI* fragment. However, Bgal expression was observed in strains XL-1 Blue and JM107 (*E. coli* Lac⁻ recipients) transformed with *E. coli* or *E. coli*-streptococcal vectors containing the 5.7 kb *PstI-SalI* or 3.0 kb *HindIII* fragment in either orientation. Because of the high A-T content of *S. thermophilus* DNA, cloned sequences could be fortuitously

recognized by *E. coli* as transcriptional starts. Also, it is likely that other in-frame initiation codons (ATG) could be used by *E. coli*. In front of the second (*lacZ* ATCC 19258) or third (*lacZ* A054), in-frame ATG, at positions 277-279 (see Fig. 3), a putative RBS at positions 267-273 (TGAGGGG in ATCC 19258, TGAAGGG in A054) was found. This RBS shares strong sequence homology with both the *trpA* (GAGGG) and T7 (TGAGGG) RBSs in *E. coli* (Stormo *et al.*, 1982). In addition, putative -10 and -35 sequences were identified at base positions 241-246 (TATTTT) and 217-222 (GTTGAC in ATCC 19258 and GTTGAT in A054) respectively. The -10 sequence contains the highly conserved and characteristic first, second and last nucleotides required for a Pribnow box, while the putative -35 sequence is homologous with the -35 sequence of the *E. coli trp* operon (Rosenberg & Court, 1979). Similar promoter sequences have been isolated from *Lactococcus lactis* subsp. *cremoris* chromosomal DNA which have strong sequence homology to both *E. coli* and *B. subtilis* promoters (Van der Vossen *et al.*, 1987). The transcriptional unit from this putative promoter region encodes a protein having a molecular mass of 112195 Da. This is similar to the value of 105000 Da determined previously by *E. coli* maxicell analysis (Herman *et al.*, 1987).

Comparison of the DNA sequences of *lacZ* (ATCC 19258) and *lacZ* (A054) showed 21 base differences within the structural gene (Table 2). Of these, only nine resulted in amino acid residue differences between the LacZ proteins, and of these nine, four were conservative. The remaining 12 base differences were in wobble positions having no effect on the protein sequence. Base 270, which is a guanine in *lacZ* (ATCC 19258) and an adenine in *lacZ* (A054), lies within or near the secondary RBS and might alter the translation efficiency of the putative secondary transcriptional unit.

Analysis of the LacZ protein sequence

When the predicted amino acid sequences were compared, the Bgals from *L. bulgaricus*, *E. coli* and *Klebsiella pneumoniae* exhibited 48, 35 and 32.5% identity respectively with the *S. thermophilus* protein (Schmidt *et al.*, 1989; Kalnins *et al.*, 1983; Buvinger & Riley, 1985). The amino acid sequences from *S. thermophilus*, *L. bulgaricus* and *E. coli* were aligned (Fig. 4), and distinct regions of strong conservation were identified. Predictions of secondary structures with the algorithm of Chou & Fasman (1974) showed that most of these regions were contained either within beta sheets or at the ends of beta sheets at alpha turns in the protein structure. From this information, it can be hypothesized that domains having catalytic functions or providing structural integrity were

Table 2. Base and amino acid differences between *lacZ* (A054) and *lacZ* (ATCC 19258)

Base position*	Base change (A054 to 19258)	Codon position†	Amino acid change	Conservative change
161	T→C	2	Met→Thr	No
183	T→C	3	None	—
222	T→C	3	None	—
257	A→C	2	Glu→Ala	No
270	A→G	3	None	—
453	A→G	3	None	—
1051	G→A	1	Asp→Asn	No
1200	C→T	3	None	—
1566	C→T	3	None	—
1722	C→A	3	Ser→Arg	No
1828	C→G	1	Gln→Glu	No
1995	C→T	3	None	—
2104	C→G	1	Leu→Val	Yes
2152	G→A	2	Ser→Glu	Yes
2367	T→C	3	None	—
2394	C→T	3	None	—
2513	G→A	2	Ser→Asn	Yes
2574	C→A	3	None	—
2592	T→C	3	None	—
2709	T→C	3	None	—
3148	A→T	1	Thr→Ser	Yes

* Base position relative to Fig. 3.

† Position in the 3-base codon where position 3 is the wobble position.

maintained. Two examples are very apparent. The region containing the putative active-site residues for Bgal, determined in the *E. coli* protein to be at amino acid positions Glu-461 (Herrchen & Legler, 1984) and Tyr-503 (Naider *et al.*, 1972; Fowler *et al.*, 1978), and the region involved in the formation of the tetrameric structure of Bgal at the carboxyterminal region of the protein (Mandecki *et al.*, 1981), are all highly conserved and are included within three of the conserved regions in Fig. 4. This suggests that all four Bgals may have evolved from a common ancestral gene.

Discussion

In this study, the *lacZ* gene from *S. thermophilus* strain A054, a yogurt starter strain, was cloned and compared by DNA sequence analysis to the homologous gene from the type strain *S. thermophilus* ATCC 19258. A054 and ATCC 19258 differed markedly in their abilities to coagulate milk (4–5 h at 44 °C for A054 versus more than 10 h for ATCC 19258; A. Mercenier and others, unpublished observations). At the DNA level, however only 21 base differences were identified between the *lacZ* genes from strains A054 and ATCC 19258, of which only nine resulted in amino acid changes (five non-conservative and four conservative) in the protein sequence

(Table 2). The few DNA base changes observed and the subsequent amino acid substitutions in the protein demonstrate that there is little difference between these genes (less than 1%). The differences in milk clotting rates could be due to a number of reasons, including differences in the efficiency of lactose uptake or proteolytic capacity.

In comparing the DNA sequences of the *lacZ* genes from *E. coli* and two lactic acid bacteria, *S. thermophilus* and *L. bulgaricus*, little or no sequence identity was apparent. When the deduced protein sequences were aligned, however, several observations were made. The sequences of the *S. thermophilus* and *L. bulgaricus* proteins showed 48% similarity. When all three protein sequences were aligned, regions of conserved protein sequence were apparent that most likely represent domains for secondary structure maintenance and enzymatic function. It is interesting to note that no protein similarity could be detected between the Bgals and the Pbgs of other dairy starters.

So far, the molecular organization of the lactose utilization genes appears to be very similar in *S. thermophilus* and *L. bulgaricus*, the two symbiotic organisms used in making yogurt. Lactose is transported into both organisms by a hybrid protein which, in part, resembles certain PEP-PTS components (Poolman *et al.*, 1989). The genes that encode these protonmotive-force-

linked lactose transport systems demonstrate 60% similarity in *S. thermophilus* and *L. bulgaricus* (*lacS* and *lacL*, respectively; Poolman *et al.*, 1989). In both species, they are separated from the respective structural gene of the lactose-hydrolysing enzyme by only three bases. In the case of *L. bulgaricus*, it has been shown by sequencing of the amino-terminal end of the purified Bgal that the first ATG in frame (located 3 bp downstream from *lacL*) was indeed the translational start (Schmidt *et al.*, 1989). By analogy, and as supported by Northern analysis, this is probably the case for *S. thermophilus* as well. We believe that in both species the lactose genes are organized, at least in part, as an operon. However, in the case of *S. thermophilus*, a second initiation codon, preceded by a putative RBS and promoter sequence, could be identified 112 bp downstream from the first ATG start site. All constructions carrying the minimal *lacZ* insert complemented the Lac⁻ defect in *E. coli lacZ* mutant strains (leading to blue colonies on X-Gal plates). This suggests that the putative internal promoter, carried on the 3 kb *HindIII* fragment, could be recognized at least in *E. coli*. Whether these sequences are utilized by *S. thermophilus* as a minor re-initiation site is not known. Secondary transcription and translation may be a fortuitous consequence in *E. coli* due to the A-T richness of the *S. thermophilus* DNA. This hypothesis is also supported by the slightly reduced size of the cloned Bgal from *S. thermophilus* ATCC 19258 observed in transformed *E. coli* maxicells (Herman *et al.*, 1987). It is notable that, while there is a definite similarity between the Bgals of *E. coli* and *S. thermophilus*, these two species differ completely in their respective lactose transport systems. No similarity could be detected between the *lacS* protein of *S. thermophilus* and the lactose permease (*lacY*) from *E. coli*.

In general, the codon usage deduced from the *lacZ* nucleotide sequence fits well with that reported from the *lacS* gene of *S. thermophilus* (Poolman *et al.*, 1989). For example, the codons CCC (proline), CCG (proline) and CGG (arginine) were not found in either gene. Other rare codons in one gene, for example ACG (threonine) in *lacS* and CUG (leucine) in *lacZ*, were absent in the other gene. As expected from the DNA base composition of *S. thermophilus* (63% A-T), there is a strong propensity, as determined from the existing sequences, for A-T richness.

Further work that may be of interest with the *lacZ* gene might include a more detailed study of the secondary *lacZ* promoter. This aspect has potential in the development of new *E. coli*-streptococcal-lactococcal shuttle vectors. Considering that the *lacZ* gene lacks its own promoter, it would make an ideal marker for a promoter probe vector, whereby promoter sequences from different lactic acid bacteria could be selected.

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