The *Lactococcus lactis* triosephosphate isomerase gene, *tpi*, is monocistronic

Michael R. Cancilla,¹ Barrie E. Davidson,¹ Alan J. Hillier,² Nga Y. Nguyen³ and John Thompson⁴

Author for correspondence: Barrie E. Davidson. Tel: +61 3 344 5912. Fax: +61 3 347 7730.
e-mail: davidson@biochemistry.unimelb.edu.au

¹ Russell Grimwade School of Biochemistry, University of Melbourne, Parkville, Victoria 3052, Australia

² Commonwealth Scientific and Industrial Research Organization, Division of Food Science and Technology, Dairy Research Laboratory, Highett, Victoria 3190, Australia

³ Division of Cytokine Biology, Center for Biologics Evaluation and Research, Food and Drug Administration, Bethesda, MD 20892, USA

⁴ Laboratory of Microbial Ecology, National Institute of Dental Research, Bethesda, MD 20892, USA

Triosephosphate isomerase (EC 5.3.1.1) from *Lactococcus lactis* was purified to electrophoretic homogeneity. Approximately 3 mg purified enzyme (specific activity 3300 U mg⁻¹) was obtained from 70 g (wet wt) cells. In solution, triosephosphate isomerase (pI 4.0–4.4) was observed to exist as a homodimer (*M*_r 57 000) of noncovalently linked subunits. The sequence of the first 37 amino acid residues from the NH₂-terminus were determined by step-wise Edman degradation. This sequence, and that of a region conserved in all known bacterial triosephosphate isomerases, was used to design oligonucleotide primers for the synthesis of a lactococcal *tpi* probe by PCR. The probe was used to isolate a molecular clone of *tpi* from a λ GEM11 library of *L. lactis* LM0230 DNA. The nucleotide sequence of *tpi* predicted a protein of 252 amino acids with the same NH₂-terminal sequence as that determined for the purified enzyme and a subunit *M*_r of 26 802 after removal of the NH₂-terminal methionine. *Escherichia coli* cells harbouring a plasmid containing *tpi* had 15-fold higher triosephosphate isomerase activity than isogenic plasmid-free cells, confirming the identity of the cloned gene. Northern analysis of *L. lactis* LM0230 RNA showed that a 900 base transcript hybridized with *tpi*. The 5' end of the transcript was determined by primer extension analysis to be a G located 64 bp upstream from the *tpi* start codon. These transcript analyses indicated that in *L. lactis*, *tpi* is expressed on a monocistronic transcript. Nucleotide sequencing indicated that the DNA adjacent to *tpi* did not encode another Embden–Meyerhoff–Parnas pathway enzyme. The location of *tpi* on the *L. lactis* DL11 chromosome map was determined to be between map coordinates 1.818 and 1.978.

Keywords: *Lactococcus lactis*, triosephosphate isomerase, transcript analysis, biased codon usage, enzyme purification

INTRODUCTION

The Gram-positive bacterium *Lactococcus lactis* plays an essential role in the manufacture of fermented milk products, such as cheese. The fermentation process requires the uptake of lactose as lactose 6-phosphate by the phosphoenolpyruvate:lactose phosphotransferase system, and the subsequent hydrolysis of lactose 6-phosphate into glucose and galactose 6-phosphate by 6-phospho-

D-galactosidase (EC 3.2.1.85). Both products are converted into lactic acid via the Embden–Meyerhoff–Parnas (EMP) pathway: glucose directly and galactose 6-phosphate via dihydroxyacetone phosphate and glyceraldehyde 3-phosphate, products of the tagatose 6-phosphate pathway. Lactose catabolism to lactic acid provides most of the energy needs of this bacterium. The reaction catalysed by triosephosphate isomerase (EC 5.3.1.1), namely the interconversion of dihydroxyacetone phosphate and glyceraldehyde 3-phosphate, is the first common step for the catabolism of the monosaccharide moieties of lactose.

The plasmid-borne genes encoding enzymes responsible for the uptake, phosphorylation and hydrolysis of lactose,

Abbreviations: EMP, Embden–Meyerhoff–Parnas; PTH, phenylthiohydantoin.

The GenBank accession number for the nucleotide sequence reported in this paper is U07640.

and conversion of galactose 6-phosphate into triose-phosphates have been cloned and characterized (Crow *et al.*, 1983; de Vos *et al.*, 1990; van Rooijen & de Vos, 1990; van Rooijen *et al.*, 1991, 1992). The genes required for the uptake and catabolism of glucose into lactic acid are chromosomally encoded, since plasmid-cured strains of *L. lactis* can utilize glucose as the sole carbon source (Efsthathiou & McKay, 1977). We recently discovered that the *L. lactis* genes encoding phosphofructokinase (EC 2.7.1.11), pyruvate kinase (EC 2.7.1.40), and lactate dehydrogenase (EC 1.1.1.27) are organized in a single tricistronic transcriptional unit, the *las* operon (Llanos *et al.*, 1993). This observation is of particular interest since these enzymes are closely regulated at the metabolic level and their relative activities have a major effect on the rates of energy production and lactic acid synthesis in lactic acid bacteria. An operon of this composition has not been reported for any other bacterium and its discovery raises questions about the organization of genes encoding other glycolytic enzymes in *L. lactis*.

Triosephosphate isomerase is one of the most abundant enzymes in bacterial cells (Noltmann, 1972) and has been described as the near-perfect catalyst (Knowles, 1991). In the eubacteria *Escherichia coli*, *Corynebacterium glutamicum* and *Bacillus megaterium* the gene (*tpi*) encoding the enzyme is located in a cluster of two or more genes encoding glycolytic enzymes (Pichersky *et al.*, 1984; Eikmanns, 1992; Schlöpfer & Zuber, 1992). Northern analyses have revealed that this cluster constitutes a polycistronic operon in *C. glutamicum* (Schwinde *et al.*, 1993).

In view of the role of triosephosphate isomerase in the homofermentative process in *L. lactis*, we have carried out an analysis of this enzyme and its structural gene. In this report we describe the purification of triosephosphate isomerase from *L. lactis* to electrophoretic homogeneity, its NH₂-terminal amino acid sequence and some properties of the purified enzyme. The availability of the sequence enabled us to clone the gene encoding triosephosphate isomerase, which we have designated *tpi*, and to analyse its structure and transcriptional organization.

METHODS

Bacterial strains, plasmids, bacteriophage, and culture conditions.

The bacterial strains, plasmids, and bacteriophage used in this study are listed in Table 1. *L. lactis* strains LM0230 and DL11 were grown at 30 °C in M17G [M17 medium (Terzaghi & Sandine, 1975), without lactose but supplemented with 0.5% (w/v) glucose]. *L. lactis* K1-42 was grown for purification of triosephosphate isomerase in a previously described medium (Thompson & Saier, 1981). D-Ribose (0.5%, w/v) was used as the carbon source so that the cell extract could also be used to purify fructokinase II, which is derepressed by ribose (Thompson *et al.*, 1991b); triosephosphate isomerase levels are not altered by growth in D-ribose. After overnight growth at 32 °C to stationary phase, the cells were harvested by centrifugation, washed by resuspension and centrifugation from KP buffer (KP buffer is 10 mM potassium phosphate, pH 7.0), and stored at -20 °C until required. The cell yield was approxi-

mately 3.5 g (wet wt) of cells per litre. *E. coli* was grown in 2YT medium (Miller, 1972) at 37 °C with aeration. Antibiotics were used at the following concentrations: erythromycin, 200–400 µg ml⁻¹; ampicillin, 100 µg ml⁻¹; kanamycin, 25 µg ml⁻¹; and tetracycline, 12.5 µg ml⁻¹.

Chemicals and reagents. Protein standards for SDS-PAGE and gel filtration chromatography, isoelectric focusing standards, Ampholine polyacrylamide gel plates, DEAE-Sephacel and phenyl-Sepharose CL-4B were obtained from Pharmacia. Ultrogel AcA 44 was purchased from Sepracor and hydroxylapatite (Fast Flow) from Calbiochem. DL-Glyceraldehyde 3-phosphate diethyl acetal (monobarium salt), α-glycerophosphate dehydrogenase (type III, rabbit muscle), and other reagents, were purchased from Sigma.

Purification of triosephosphate isomerase

The SDS-PAGE procedure of Laemmli (1970) was used to monitor enzyme purity throughout the purification. Preparation of SDS-polyacrylamide gels, electrophoresis conditions, and staining procedures have been described previously (Thompson, 1989). Protein concentrations were determined by use of the Bio-Rad protein dye-binding reagent with BSA as the standard. Except where indicated, all purification steps were carried out at 4 °C.

(i) **Cell disruption and preparation of high-speed supernatant.** Frozen cells of *L. lactis* K1-42 (70 g wet wt) were thawed and resuspended in KP buffer to a volume of 150 ml. The cells were disrupted at 0 °C by three 5 min periods of sonic oscillation with a Branson model 350 sonifier operating at 75% maximum power. Intact cells and debris were removed by centrifugation (25 000 g, 30 min), and the supernatant was dialysed overnight against 4 l KP buffer. The dialysate was concentrated twofold by ultrafiltration through a Diaflo PM-10 membrane (Amicon) and centrifuged (180 000 g, 2 h).

(ii) **Phosphocellulose chromatography.** The high-speed supernatant (54 ml) was transferred to a 2.6 × 24 cm column of phosphocellulose (Whatman P-11) previously equilibrated with KP buffer. The non-adsorbed proteins, which included triosephosphate isomerase, were eluted with KP buffer. The active fractions were pooled and concentrated to 20 ml by ultrafiltration.

(iii) **DEAE-Sephacel chromatography.** The concentrate from step (ii) was transferred to a 2.6 × 24 cm column of DEAE-Sephacel previously equilibrated with 25 mM HEPES, pH 7.5. After washing the column with 150 ml of this buffer, a 600 ml linear NaCl gradient (0.2–0.5 M) in 25 mM HEPES, pH 7.5, was passed through the column and 5 ml fractions were collected. The active fractions (usually fractions 45–51) were pooled and concentrated to 10 ml by ultrafiltration.

(iv) **Phenyl-Sepharose chromatography.** Solid NaCl was added to the concentrate from step (iii) to a final concentration of 1 M. The sample was then applied to a 1 × 28 cm column of phenyl-Sepharose CL-4B equilibrated with KP buffer containing 1 M NaCl. Unadsorbed proteins were eluted with this buffer, then a 300 ml linear decreasing gradient of NaCl (0.6–0.1 M) in KP buffer was applied to the column and 2 ml fractions were collected. The active fractions (fractions 10–24) were pooled and concentrated to 3 ml by ultrafiltration.

(v) **Hydroxylapatite chromatography.** The concentrate from step (iv) was desalted and exchanged for KP buffer by passage through a PD-10 gel filtration column previously equilibrated with KP buffer. The desalted preparation was transferred to a 1 × 5 cm column of hydroxylapatite equilibrated with KP buffer. In contrast to the contaminating proteins, the triosephosphate

Table 1. Bacterial strains, plasmids and bacteriophage used

Characteristics		Source/reference
Strains		
<i>L. lactis</i>		
K1-42	<i>L. lactis</i> subsp. <i>lactis</i> K1 after spontaneous loss of <i>lac</i> plasmid and Tn5306	Donkersloot & Thompson (1990); Thompson <i>et al.</i> (1991a)
LM0230	Plasmid-cured derivative of <i>L. lactis</i> subsp. <i>cremoris</i> C2 (formerly <i>L. lactis</i> subsp. <i>lactis</i> C2)	Efstathiou & McKay (1977); Salama <i>et al.</i> (1991)
DL11	Prt ⁻ derivative of <i>L. lactis</i> subsp. <i>lactis</i> ATCC 11454	Tulloch <i>et al.</i> (1991)
<i>E. coli</i>		
JM107	<i>endA1 gyrA96 thi-1 hsdR17 supE44 relA1 Δ(lac-proAB)</i> (F' <i>traD36 proAB lacI^qZ ΔM15</i>)	Yanisch-Perron <i>et al.</i> (1985)
SURE	<i>ε14⁻(mcrA) Δ(mcrCB-hsdSMR-mrr)171 sbcC recB recJ umuC::Tn5(Km^r) wvrC supE44 lac gyrA96 relA1 thi-1 endA1[F' proAB lacI^qZ ΔM15]</i>	Stratagene
Plasmids		
pGEM-T	Ap ^r <i>ΔlacZ</i> , 3005 bp vector for cloning PCR products	Promega
pJDC9	Em ^r <i>ΔlacZ</i> , 6.85 kbp cloning vector	Chen & Morrison (1988)
pMU5501	Ap ^r , pGEM-T with 503 bp of <i>tpi</i> prepared by PCR amplification of <i>L. lactis</i> LM0230 DNA	This study
pMU5502	Em ^r , pJDC9 with a 6.0 kbp <i>EcoRI</i> fragment containing <i>tpi</i> from <i>L. lactis</i> LM0230	This study
pMU5503	Em ^r , pJDC9 with a 2.0 kbp <i>DraI</i> fragment containing <i>tpi</i> from <i>L. lactis</i> LM0230	This study
Bacteriophage		
λ268	λGEM11 with a 21.0 kbp partial <i>Sau3AI</i> fragment from <i>L. lactis</i> LM0230 in the <i>XbaII</i> site	This study

isomerase was not adsorbed by this matrix, and the purified enzyme (about 3 mg) was eluted in the wash fractions and concentrated by ultrafiltration.

***M_r* determinations.** The *M_r* of native triosephosphate isomerase in solution was determined by the gel filtration method of Andrews (1964) using a 1.6 × 94 cm column of Ultrogel AcA44 equilibrated with 50 mM potassium phosphate buffer, pH 7, containing 0.1 M NaCl. Standard proteins used to calibrate the column were: BSA (*M_r* 66000), ovalbumin (45000), carbonic anhydrase (31000) and chymotrypsinogen A (25000). SDS-PAGE was used to estimate the subunit *M_r* of the denatured triosephosphate isomerase. Standard proteins used to calibrate the gel were: phosphorylase *b* (97000); BSA, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor (21500) and lysozyme (14400).

Isoelectric focusing and pI estimation. The isoelectric point (pI) of purified triosephosphate isomerase was determined by analytical isoelectric focusing in a Multiphor model 2117 flat-bed electrophoresis unit (Pharmacia). Electrophoresis conditions, and procedures for fixing, staining and destaining of 1 mm thick isoelectric focusing gels were done according to the manufacturer's instructions. The pI calibration standards (pI range 3–10) and precast Ampholine polyacrylamide gel plates (pH range 3.5–9.5) were purchased from Pharmacia.

Triosephosphate isomerase assay. Triosephosphate isomerase catalyses the reversible conversion of D-glyceraldehyde 3-phosphate to dihydroxyacetone phosphate. Enzyme activity was determined spectrophotometrically at 340 nm by moni-

toring the rate of NADH-dependent, α-glycerophosphate-dehydrogenase-mediated conversion of dihydroxyacetone phosphate (produced from glyceraldehyde 3-phosphate) to α-glycerophosphate. This assay would be perturbed by glyceraldehyde-3-phosphate dehydrogenase and NADH oxidase activities in the sample; however, the relative levels of these enzymes in the high-speed supernatant compared with that of triosephosphate isomerase were negligible. One unit of triosephosphate isomerase was defined as the amount of enzyme that catalysed the oxidation of 1 μmol NADH (i.e. equivalent to the conversion of 1 μmol glyceraldehyde 3-phosphate to dihydroxyacetone phosphate) per min at 22 °C. The standard 1 ml assay mixture contained 0.1 M imidazole/HCl buffer (pH 7.0), 0.1 mM NADH, 10 mM MgCl₂, 0.25 mM D-glyceraldehyde 3-phosphate, 5 U α-glycerophosphate dehydrogenase (EC 1.1.1.8) and triosephosphate isomerase.

NH₂-terminal amino acid sequence. Approximately 100 pmol dry, salt-free protein was dissolved in 30 μl sequencing-grade trifluoroacetic acid (0.1 %, v/v), and dried on a glass fibre filter membrane which had been coated with polybrene. The protein was subjected to three filter cycles with an Applied Biosystems model 470A gas phase microsequencer, and PTH-amino acid derivatives were identified on an Applied Biosystems model 120A analyser.

DNA techniques and sequence analysis. *E. coli* cells were transformed by the method of Hanahan (1983). Plasmid DNA from *E. coli* was isolated by alkaline-lysis procedure (Birnbom & Doly, 1979) and was purified by the addition of PEG (to

6.5%, v/v) and NaCl (to a final concentration of 0.4 M) at 4 °C, followed by centrifugation (12000 g, 15 min, 4 °C). Genomic DNA used as template in the PCR was isolated from *L. lactis* LM0230 as described previously (Cancilla *et al.*, 1992). Oligonucleotides were synthesized in an Applied Biosystems model 381A DNA synthesizer. Bacteriophage λ was isolated from plate lysates and λ DNA was purified on Qiagen minicolumns according to the manufacturer's instructions. Digestions, electrophoresis, transfers, hybridizations, and other DNA manipulations were performed as described elsewhere (Sambrook *et al.*, 1989; Tanskanen *et al.*, 1990; Tulloch *et al.*, 1991). DNA sequence determinations were carried out on both DNA strands using an Applied Biosystems model 373A automated DNA sequencer (Llanos *et al.*, 1992). DNA and amino acid sequences were analysed using the ANGIS suite of programs on the University of Sydney SUN computer system. The suite includes the Staden (Staden, 1978) and University of Wisconsin Genetics Computer Group programs.

Construction of a molecular probe for *tpi*. The following oligonucleotides were used as polymerase chain reaction (PCR) primers: N-TPI, 5'-GG(ACGT)AA(CT)TGGAA(AG)ATG-AA-3', and C-TPI, 5'-CC(ACGT)GT(ACGT)CC(AGT)AT-(ACGT)GCCCCA-3'. N-TPI encoded amino acid residues 8–13 (GNWKMN) of *L. lactis* K1-42 triosephosphate isomerase, and the complementary strand of C-TPI encoded WAIGTG, a conserved sequence in all known bacterial triosephosphate isomerases near residues 171–176. The PCR mix (50 μ l final volume) consisted of template DNA (10–20 ng), N-TPI and C-TPI (250 ng each), deoxynucleoside triphosphates (200 μ M each), 1 $\times$ *Taq* DNA polymerase buffer (Boehringer Mannheim), and 2 U *Taq* DNA polymerase (Boehringer Mannheim). The reaction was carried out in a thermal sequencer (model FTS-1C, Corbett Research) as follows: 95 °C for 3 min, and then 30 cycles of 45 °C for 1 min, 72 °C for 1.5 min, and 95 °C for 0.5 min. The amplified product was isolated and cloned into pGEM-T.

Northern analysis. Total RNA was purified from *L. lactis* LM0230, electrophoresed, transferred to a nylon membrane and hybridized as described previously (Chandry *et al.*, 1994). The size of the *tpi* transcript was determined by comparison of its electrophoretic mobility with those of a set of RNA markers of 0.24–9.5 kb (BRL).

Primer extension analysis. Total RNA (5 μ g) was annealed with 750 ng of the oligonucleotide *tpi*2 (5'-GCACCGATAAC-TGATTC-3') in 5 μ l water, by incubation at 90 °C for 1 min then 42 °C for 5 min. Radiolabelled cDNA was synthesized by using [³²S]dATP α S and AMV reverse transcriptase (Boehringer Mannheim), according to the method of Chandry *et al.* (1994). The products of the four different dideoxy sequencing reactions obtained with the *fmo*l DNA sequencing system (Promega) using pMU5503 as the template and *tpi*2 as the primer, served as the size standards on the 6% (w/v) polyacrylamide/7 M urea gel.

RESULTS

Purification and properties of triosephosphate isomerase

Cell-free extracts of *L. lactis* K1-42 grown with ribose exhibited high levels of triosephosphate isomerase activity (60 U mg⁻¹), and were a suitable starting material for purification of the enzyme (Table 2). The five-stage purification procedure usually provided 3–4 mg of

electrophoretically pure enzyme from 70 g (wet wt) *L. lactis* cells. The purified enzyme remained stable for several months when stored at –20 °C in 50 mM potassium phosphate buffer (pH 7.0), exhibiting little loss of activity upon repeated (at least four times) freezing and thawing. The M_r of native triosephosphate isomerase was estimated to be 57 000 by Ultrogel AcA44 filtration chromatography (data not shown), while SDS-PAGE of the fully reduced enzyme revealed a single polypeptide of M_r 27 000 (Fig. 1, lane 5). These data indicated that triosephosphate isomerase from *L. lactis* exists in the native state as a homodimer of noncovalently linked subunits. Although SDS-PAGE of purified triosephosphate isomerase revealed a single protein band, the same preparation was resolved into two species by analytical electrofocusing (data not shown). The pIs were estimated to be 4.0 for the more sharply focused polypeptide, and 4.4 for the more diffuse species. Whether this microheterogeneity reflected post-translational modification(s), deamidation or proteolysis during purification, or whether it was an artefact of the electrofocusing procedure, is unclear. Microsequencing of the pure triosephosphate isomerase provided a reasonably unambiguous sequence for the first 37 NH₂-terminal residues: Ser-Arg-Lys-Pro-Ile-Ile-Ala-Gly-Asn-(Trp/Cys)-Lys-Met-Asn-Lys-Thr-Leu-(Ala/Ser)-Glu-Ala-Gln-Ala-Phe-Val-Glu-Ala-Val-Lys-Asn-Asn-Leu-Pro-(Pro/Ser)-Ser-Asp-Asn-Val-Glu.

Molecular cloning and sequence analysis of *tpi*

The availability of the NH₂-terminal sequence of *L. lactis* triosephosphate isomerase enabled the use of PCR to generate a specific probe for the *L. lactis* gene (which we designated *tpi*) encoding the enzyme. One primer, N-TPI, was designed from the NH₂-terminal sequence data and the other, C-TPI, was designed from the sequence WAIGTG, which is conserved in all known bacterial triosephosphate isomerase sequences. PCR amplification of *L. lactis* DNA with these primers yielded a fragment of 503 bp, which is the approximate size expected from known triosephosphate isomerase amino acid sequences. The fragment was cloned (pMU5501) (Table 1) and its nucleotide sequence was determined. The predicted amino acid sequence indicated that the fragment contained the expected region of *tpi*. The 503 bp fragment from pMU5501 was then used as a specific probe to screen a λ GEM11 genomic library of *L. lactis* LM0230 (Llanos *et al.*, 1992). One clone (λ 268) hybridized strongly with the probe. DNA was isolated from this clone and Southern analyses were used to construct a restriction map of a 6.0 kbp *Eco*RI fragment located within the λ 268 *L. lactis* DNA insert (Fig. 2). The 6.0 kbp *Eco*RI fragment that hybridized with the *tpi* probe was subcloned from λ 268 into pJDC9, yielding a recombinant plasmid containing *tpi* and its flanking DNA (pMU5502).

*Hae*III, *Dra*I, *Hind*III, *Ssp*I and *Pvu*II fragments from the 6.0 kbp insertion in pMU5502 were cloned in pJDC9, and the nucleotide sequences of these fragments were determined. The 1138 bp nucleotide sequence shown in Fig.

Table 2. Purification of triosephosphate isomerase from *L. lactis* K1-42

Purification step*	Enzyme activity (U)	Amount of protein (mg)	Sp. act. (U mg ⁻¹)	Purification (-fold)	Yield (%)
High-speed supernatant	127 000	2160	60	1	100
Phosphocellulose P-11 chromatography	84 500	1460	60	1	67
DEAE-Sephacel chromatography	31 100	220	140	2.3	25
Phenyl-Sepharose chromatography	28 700	12.3	2300	38	23
Hydroxylapatite chromatography	11 500	3.5	3300	55	9

* See Methods for details.

Fig. 1. Purification and M_r determination of triosephosphate isomerase from *L. lactis* K1-42. SDS-PAGE (12.5%, w/v, acrylamide) of samples from each stage of triosephosphate isomerase purification: high-speed supernatant (lane 1); phosphocellulose (lane 2); DEAE-Sephacel (lane 3); phenyl-Sepharose (lane 4); hydroxylapatite (lane 5). The numbers on the right are the molecular sizes of standard proteins (see Methods) electrophoresed in the right and left hand lanes. The arrow indicates the subunit of purified triosephosphate isomerase in lane 5 (M_r 27 000).

3 includes the region that hybridized with the *tpi* probe and contains an ORF of 253 codons. Using the first ATG in the ORF as the translation-initiation codon, the deduced amino acid sequence at the NH₂-terminus of the ORF was identical to the NH₂-terminal sequence we had determined for triosephosphate isomerase from *L. lactis* K1-42. This observation provided strong evidence that the ORF is *tpi*. The nucleotide sequence predicted a chain length of 252 amino acids and a subunit M_r of 26 802 for *L. lactis* triosephosphate isomerase, assuming the post-translational removal of the NH₂-terminal methionine, as observed in our protein sequence data. The predicted subunit M_r agreed with the observed subunit M_r of 27 000

Fig. 2. Restriction map of the 6.0 kbp *EcoRI* insert in pMU5502 containing *L. lactis tpi*. The digestion sites shown are for *EcoRI* (E), *HindIII* (H), *PvuII* (P), *SspI* (Ss), *DraI* (D), *Sau3AI* (Sa), and *HaeIII* (Ha). The positions of *tpi*, ORFs a, b and c are indicated by rectangles, with arrows showing their orientations. Solid portions of the rectangles indicate sequenced regions. The locations of the *tpi* probe generated by PCR and the oligonucleotide *tpi2* used in primer extension analysis are indicated.

for triosephosphate isomerase (Fig. 1). The pI of the protein predicted from the *tpi* sequence was 4.4, which is also in agreement with the values that were determined experimentally. A putative ribosome-binding site was identified 8 bp upstream from the *tpi* initiation codon (Fig. 3). The 3' flanking DNA of *tpi* contained an inverted-repeat sequence followed by TTT (Fig. 3). Transcripts of this region could form a stem-loop structure with a calculated free energy value (Tinoco *et al.*, 1973) of $-15.6 \text{ kcal mol}^{-1}$ ($-65.3 \text{ kJ mol}^{-1}$). A stem-loop structure followed by UUU is likely to be a rho-independent transcription terminator (d'Aubenton Carafa *et al.*, 1990), causing RNA polymerase to stop transcribing at bp 988 (Fig. 3).

Expression of *L. lactis tpi* in *E. coli*

The predicted amino acid sequence (Fig. 3) provided evidence that the lactococcal DNA in pMU5502 contained *tpi*. To establish this point, we looked for the expression of the *L. lactis tpi* gene in *E. coli* cells harbouring pMU5502. Cell extracts from *E. coli* SURE(pMU5502)

TTAGAAAGACACGAGATGCAATTAAATTATTGGAAGAAACATGATAGACCCGAAATTTACTGATGATGAGTTGATTTCTGCAATTTTTTTCACAATGTGATAAAATAGACCATGTAAAA	120
GATGGATTAAAGTCCAAAATTTAAGAGGCTTAAAGGCCCTATAAGGAAATTAGAATGTCACGTAAACCAATTATCGCTGGTAACGGAAATGAATAAACTTTGTGAGAAGCACAA	21 240
A F V E A V K N N L P S S D N V E S V I G A P A L F L A P M A Y L R O G S E L K	61
GCTTTTGTGAAGCTGTTAAAAATAACTTGCCTTCATCTGATAATGTTGAATCAGTTATCGGTGCACCTGCTCTTTCTCGCTCCTATGGCTTACCTTCGTCAAGGTTCAGAATTGAAA	360
L A A E N S Y F E N A G A F T G E N S P A A I V D L G I E Y I I I G H S E R R E	101
CTTGCTGTGAAAATAGCTACTTCGAAAATGCTGGAGCAATTCACCTGGTGAACAGCCAGCTGCAATCGTTGACCTTGGTATTGAATACATCATCATCGGTACAGCGAAGCTCGTGAA	480
Y F H E T D E D I N K K A K A I F A A G A T P I L C C G E T L E T F E A G K T A	141
TATTTCCAGAACTGACGAAGACATCAACAAAAAGCAAAAGCAATCTTCGCTGCTGGAGCAACTCCAATCCTTTGTTGGTGAACTTTGGAACTTTTGAAGCTGGTAAACAGCT	600
E W V S G Q I E A G L A G L T A E Q V S N L V I A Y E P I W A I G T G K T A T N	181
GAATGGGTTTCAGGTCAAATCGAAGCTGGTCTTCAGGATTGACTGCTGAACAAGTTTCAAACTTAGTTATCGCTTACGAACCAATCTGGGCAATCGGAACCTGGTAAAACTCGCAGCAAAAC	720
E I A D E T C G V V R S T V E K L Y G K E V S E A V R I Q Y G G S V K P E T I E	221
GAAATTGCTGACGAACTTGTGGTGTGTACGTTCAACAGTTGAAAACTTTACGGAAGAAGTTTCAGAAGCTGTACGTATCCAATACGGTGGATCAGTAAACCTGAAACAATCGAA	840
G L M A K E N I D G A L V G G A S L E A D S F L A L L E M Y K *	252
GGATGTAGGCTAAAGAAAACATCGACGGTGCCCTCGTTGGTGAGCTTCACTTGAAGCTGATAGCTTCCTTCATTGCTTGAATGTACAAATAATTTTTAAATATGGGTGAATGAAA	960
GTACTGACAGCTCTGTCAGTACTTTTTTCGAGCTTTTTTGAAGCTTTTGTATAGGATAGCATTTTACAGATATGCTAGTATCTCTATTAGTAAATTTTTTGATAAAATAAATAGATGACA	1080
TCTATTTAAGGGGAGAAAAATGTGTACTAGTTTCAACTAAAATCAAGTGATGGTGG	1138

Fig. 3. Nucleotide sequence of *L. lactis tpi* and its flanking DNA and the deduced amino acid sequence of *L. lactis* triosephosphate isomerase. The translated region of 253 codons starts with an ATG at nucleotide positions 178–180. The underlined G indicates the start point of transcription determined by primer extension analysis. The likely –35 and –10 hexanucleotides of the promoter (van de Guchte *et al.*, 1992) upstream of the transcription start point are overlined. Double underlining indicates a sequence which exhibits complementarity to the sequence 3'-UCUUUCCUCCA-5' at the 3' end of *L. lactis* 16S rRNA (Ludwig *et al.*, 1985) and is the likely ribosome-binding site. The arrows above the sequence indicate an inverted repeat that is a likely rho-independent terminator.

had a 15-fold higher level of triosephosphate isomerase activity (81 U mg^{-1}) than those from plasmid-free *E. coli* SURE (5.3 U mg^{-1}). These data confirmed that pMU5502 contained *tpi* and indicated that the gene was expressed in *E. coli*. By using the determined specific activity of pure *L. lactis* triosephosphate isomerase (3300 U mg^{-1} ; Table 2) the amount of *L. lactis* triosephosphate isomerase expressed in *E. coli* SURE(pMU5502) was calculated to be approximately 2% of the soluble cellular protein. SDS-PAGE of the two extracts (data not shown) revealed an increased amount of a polypeptide of the expected size (M_r 27000) in *E. coli* SURE(pMU5502).

Sequence analysis of the DNA adjacent to *tpi*

Subcloning experiments from pMU5502 provided constructs which allowed the determination of partial nucleotide sequences of the DNA adjacent to *tpi* (Fig. 2). Computer analysis of these sequences was used to identify potential ORFs. The predicted amino acid sequences of these ORFs (designated a, b and c, Fig. 2) were compared with the sequences in the GenPeptide data base using the FASTA program (Lipman & Pearson, 1985). ORFs a and b shared 30% identity over a combined total of 145 amino acids with enzyme II encoded by *ptsG* from *Brevibacterium lactofermentum* (*C. glutamicum*) (GenBank accession no. L18875), and ORF c shared 25% identity over 147 amino acids with the conjugated bile acid hydrolase encoded by *cbh* from *Lactobacillus plantarum* (GenBank accession no. M96175). Neither ORF exhibited any significant homology with known EMP pathway enzymes. Although additional nucleotide sequence and biochemical analyses need to be undertaken to establish the identity of ORFs a,

b and c, the present data indicate that other genes encoding EMP pathway enzymes are not adjacent to *tpi*.

Transcript analyses

Northern analysis of *L. lactis* LM0230 RNA demonstrated that a *tpi* probe hybridized strongly with a single 900 base transcript (Fig. 4a). Primer extension from oligonucleotide *tpi*2 using total RNA as template yielded one cDNA product (Fig. 4b). The mobility of the product was identical to that of the product in the C track, corresponding to bp 115 in Fig. 3. We concluded from this observation that the G at bp 115 was the 5' end of the *tpi* transcript; a transcript extending from this 5' end to its 3' end equivalent at bp 988 would be 873 bases long. This value agrees with the experimentally determined size of 900 bases for the *tpi* transcript, and indicates that *L. lactis tpi* is expressed as a monocistronic mRNA.

Codon usage in *L. lactis tpi*

Codon usage in *L. lactis tpi* exhibits a marked bias towards particular codons and away from others. Of the 61 possible amino acid codons, 24 are not used in this gene. While the low G+C content (37 mol%) of *L. lactis* DNA may contribute to the non-usage of codons with a G or C in the third position, it cannot be the reason for the preference for T compared with A in the third position of Thr, Leu, Ile, Ala, Arg, and Gly codons. These data, when combined with compilations of codon usage for other genes encoding glycolytic enzymes (*las* operon) (Llanos *et al.*, 1993) suggest that a strong bias exists in the codon usage of glycolytic genes in *L. lactis*.

Fig. 4. Transcript analyses of *L. lactis tpi*. (a) Northern blot analysis of *L. lactis* LM0230 RNA. Total RNA from *L. lactis* LM0230 was electrophoresed, transferred to a nylon membrane, and hybridized with 32 P-labelled 503 bp *tpi* probe. The numbers on the left indicate the sizes of RNA standards that migrated to the positions indicated. The arrow indicates the 900 base *tpi* transcript. (b) Primer extension analysis of the 5' end of *tpi*. The autoradiograph shows electrophoretic separation of: lane 1, 10% of the primer extension reaction; and lane 2, 20% of the primer extension reaction of cDNA products generated as described in Methods using oligonucleotide *tpi2* as primer. Lanes A, C, G, and T, electrophoretic separation of the products of dideoxynucleotide sequencing using oligonucleotide *tpi2* as primer and plasmid pMU5503 as a template. The number on the right corresponds to the nucleotide sequence numbering in Fig. 3.

Copy number and chromosomal location of *tpi*

EcoRI, *DraI*, *HaeIII*, *NruI*, and *SspI* digests of *L. lactis* LM0230 chromosomal DNA were resolved by electrophoresis through agarose, and transferred to a nylon membrane. Hybridization of the membrane with a 32 P-labelled *tpi* probe (data not shown) yielded the following hybridizing fragments: 6.0 kbp (with *EcoRI*), 1.8 kbp (*DraI*), 1.0 kbp (*HaeIII*), > 10.0 kbp (*NruI*), and 1.5 and 1.35 kbp (*SspI*). The measured sizes of these fragments corresponded with the sizes predicted from restriction mapping (Fig. 2) and sequence data (Fig. 3). These observations indicated the presence of a single copy of *tpi* in the *L. lactis* LM0230 chromosome.

A physical map of the *L. lactis* DL11 chromosome has been constructed by pulsed-field gel electrophoresis (Tulloch *et al.*, 1991). To locate *tpi* on this map, samples of high- M_r DNA from *L. lactis* DL11 were digested with *SmaI*, *NotI* plus *SmaI*, and *NotI*, then separated by pulsed-field gel electrophoresis, and transferred to a nylon membrane. The membrane was probed with 32 P-labelled

tpi and fragments of 830, 160, and 160 kbp were observed to hybridize (data not shown). The 830 and 160 kbp bands corresponded to fragments *SmA* and *NtF*, respectively (Tulloch *et al.*, 1991). Since it has previously been shown that *NtF* is located within *SmA*, we concluded that the single copy of *tpi* is located on the *L. lactis* DL11 chromosome within fragment *NtF*, between coordinates 1.818 and 1.978 on the physical map (Tulloch *et al.*, 1991).

DISCUSSION

The data reported above describe the enzymic and genetic characterization of triosephosphate isomerase from *L. lactis*. Electrophoretically homogeneous enzyme was purified from *L. lactis* and its NH_2 -terminal amino acid sequence was determined. PCR was then used to synthesize a specific lactococcal probe that could be used to isolate a molecular clone of *tpi*, the gene encoding triosephosphate isomerase. The NH_2 -terminal sequence of the purified enzyme was utilized to design one low-redundancy PCR primer while the sequence WAIGTG, which is conserved in all known triosephosphate isomerases (Fig. 5; Fothergill-Gilmore & Michels, 1993), was used for the second.

Proof that our clone encoded triosephosphate isomerase was obtained by (i) the identity of its predicted NH_2 -terminal amino acid sequence with that determined for the purified enzyme, and (ii) the observation of elevated levels of triosephosphate isomerase activity in *E. coli* cells that contained the clone. The purified enzyme was shown to exist in solution as a homodimer (M_r 57000) of non-covalently linked subunits, and the sequence analysis of *tpi* indicated that the enzyme subunits are identical and have an M_r of 26802. The codon usage in *L. lactis tpi* was found to exhibit a strong bias. This was in direct agreement with the codon usage for genes of other *L. lactis* chromosomal genes encoding EMP pathway enzymes (Llanos *et al.*, 1993), and noticeably different to data compiled from 67 other lactococcal genes (Chopin, 1993). By analogy with the situation in *E. coli* (Gouy & Gautier, 1982) and yeast (Sharp *et al.*, 1986), this observation suggests that *tpi* is a strongly expressed gene.

The predicted amino acid sequence of *L. lactis* triosephosphate isomerase shares considerable identity with the amino acid sequences of other bacterial triosephosphate isomerases (Fig. 5). The identity ranges from 60% with the *Bacillus megaterium* enzyme down to 33% with the enzyme from *Moraxella* sp. Extensive structural and mutagenic studies with triosephosphate isomerase from various organisms have resulted in the identification of amino acid residues involved in subunit interaction, ligand binding and catalysis (Knowles, 1991; Lolis *et al.*, 1990; Wierenga *et al.*, 1992). Each subunit contains a catalytic centre that consists of a number of highly conserved amino acid residues (Wierenga *et al.*, 1992). These conserved amino acids (Lys-12, His-96 and Glu-168) were readily identified in the predicted sequence of triosephosphate isomerase from *L. lactis* (Fig. 5).

Northern blot analyses with *L. lactis* RNA showed that *tpi* is expressed in a 900 base transcript. Given the length of

Mor	MQAWVI	Q PAT HDVN LDDLCAAISTTKQMSHDNSARCQIMV	S IHLA VNGR KDT ..I CA QDV GHSASV	Y	C AQQ	A
Vib	M QALVM	L A KG.SVE LINGLVDAAKDNATVAVCP	V.. IPQVEA TADT..AITYG QDC.DVNTS		N AVMLKEF	
Cgl	MA L	LDHQ.Q IGT QKLAF A KEYFEKVD.....VAVTV	FT..DIRSVQT VE DKL VTFG QDV.SQHS	Y	V ASMLAK	
Eco	M H LVM	L GSRH.MVHEL SNLRKE AGVAGCA.....VA AP	EM..YIDMAKREAE ..HIM G Q V.DLNLS		T A MLK I	
Tma	IT L L	H I . KK SLLV E HDVKEFI VC	FT..A SEVGEILS R..NI G Q V.FY DQ		I LMLQEI	
Bst	M	H A . VQ D GHV PA E I.....VC	F .. DRLVQAD T..D IG QTM.H ADQ	Y	V VMLK	
Bme	M	V . TS E GAV PES D.....VC	.. DRLVEATK T..D IG Q M.H EN		V V LA	
Lla	MSRKPIIAGNWKMKTLKLS.EAQAPVEAVKNNLPSSDNVE.....SVIGAPAL...FLAPMAYLRQGS...ELKLAENS.YFENAGAFTEGNSPAIIVDL					87
con	GNWK		P		GA TGE S	
Mor	ATWT L	Q HQ SNDALLQ MIHALSQDLGVVF I	SQ QYDTKQ LTVIDN LVVIKEFITQOPE ID LPTR I	V	VP V	
Vib	CK TLV	VIHG SS VVAD FAVTPEN LV V I	Q E KAV EA L.....Q VVTKSGITSLN A	G	V P	
Cgl	NCSWVVV	HN S LVAA ALSN IS V V	P IR THV Y VE T.....RKS D ELA T	V	V SA	
Eco	AQ	T HK S L A FAVLKEQ L V I	EAEN E VCARD V KTQG AFEGA	V	S P	
Tma	V V V	RI K D F R V VLEK M	V ER K L FCV EK V.....RE FY DK EAKRV	V	RV P	
Bst	VT V L	QM A VQ VL A TR LI I	S ER Q NAV AS V.....KA P KQA		SS P	
Bme	VN V L	M A TV Q TI A KH LT V	N EY QDQ KTV AN V.....QKA D KQT		SS A	
Lla	GIEYIIIGHSERREYFHETDEDINKKAKAIFAAGATPILCCGETLETFEAGKTAENVSGQI.....EAGLAGLTAEQVSNLVIAIEPIWAIGTGKTATN					181
con	GHSERR E K	C GE	Q		I YEP WAIGT	
Mor	AEVSA HKHIQ LAG.FADSL N.MTVL	NADNANSFA DPM	K TIATAFSQASI*	33 %		
Vib	Q IPAHI WLAEQ.DAA ANK Q L	NSAE FGQAD	G D VE SKVISGASA*	40 %		
Cgl	AD Q V KAI GLIVE A D A GL L	A VAEIVGQPDV	G DGEALPK AANAASVA*	43 %		
Eco	AQ QAVHKFI DHIA V.DANIA Q I	NASNAAE F QPD	K A AVIVKAAEAQA*	43 %		
Tma	QQ Q VHAFI KLLSEM DE TAGSI L	I DNFL IVQKD	G K.E IE ARIMRGVIS*	47 %		
Bst	D NSV HI V SR F P AA I	DN RDFL QQQ	P PA Q V AGRHE*	58 %		
Bme	G N V AYI V AEQFSQD AD	AN KEY SQSD	Q AG *	60 %		
Lla	EIADETCCGVVRSTVEKLYGKEVSEAVRIQYGGSVKPTIEGLMAKENIDGALVGGASLEADSFLALLENYK*					252
con	YGGG	DG LVGGASL				

Fig. 5. Homology among the deduced amino acid sequences of triosephosphate isomerases from *L. lactis* LM0230 and other bacteria. The sequences are of triosephosphate isomerase from: *Moraxella* sp. (Mor) (Rentier-Delrue *et al.*, 1993a), *Vibrio* sp. (Vib) (GenBank accession no. L27493), *C. glutamicum* (Cgl) (Eikmanns, 1992), *E. coli* (Eco) (Pichersky *et al.*, 1984), *Thermatoga maritima* (Tma) (GenBank accession no. L27492), *B. stearothermophilus* (Bst) (Rentier-Delrue *et al.*, 1993b), *B. megaterium* (Bme) (Schlöpfer & Zuber, 1992), *L. lactis* (Lla) (this study), and the conserved amino acids from the above (con). The first amino acid for each protein is the NH₂-terminal amino acid. Spaces indicate positions where sequences are identical to that of *L. lactis*; dots indicate gaps inserted to maximize the alignment; double underlining indicates catalytic residues in the triosephosphate isomerase active site (Wierenga *et al.*, 1992); asterisks show the COOH-terminal extremity of each sequence; the percentages show the identity between each sequence and the *L. lactis* sequence.

the triosephosphate isomerase coding region (756 bp), and the distance between the start and termination sites of transcription (873 bp) we conclude that *tpi* is monocistronic. In a number of other bacterial species *tpi* has been found to be adjacent to genes encoding other enzymes of the EMP pathway. For example, in the Gram-positive bacteria *C. glutamicum* and *B. megaterium* a gene cluster of *gap* (encoding glyceraldehyde-3-phosphate dehydrogenase, EC 1.2.1.12), *pgk* (encoding 3-phosphoglycerate kinase, EC 2.7.2.3) and *tpi* has been discovered (Eikmanns, 1992; Schlöpfer & Zuber, 1992). The three enzymes encoded by the cluster catalyse sequential reactions in the pathway. Transcriptional analyses of the *gap-pgk-tpi* cluster from *C. glutamicum* identified *tpi*-containing transcripts corresponding to *gap-pgk-tpi* and *pgk-tpi* (Schwinde *et al.*, 1993). In *E. coli*, *tpi* is near *pfk* (Shimosaka *et al.*, 1982), the gene encoding phosphofructokinase, another enzyme involved in sugar metabolism. However, nucleotide sequence analysis has revealed that they have opposite orientations and must therefore be transcribed convergently from different promoters (Hellinga & Evans, 1985). No experimental data have been reported on the size or composition of the *E. coli* *tpi* transcriptional unit or the identity of the upstream gene. Analysis of the nucleotide sequence of the DNA adjacent to *tpi* in *L. lactis* (Fig. 2) indicates that neither *pgk*, *gap*, *pfk* nor any other gene encoding an EMP pathway enzyme is present. Thus, the clustering (and possible cotranscription) of *tpi* and other genes encoding EMP pathway enzymes found in some Gram-positive bacteria and *E. coli* does not occur in *L. lactis*. Curiously,

L. lactis contains a different grouping of other genes for this pathway: an operon containing *pfk*, *pyk* and *ldh* (Llanos *et al.*, 1993). There seems therefore to be some diversity among prokaryotes in the arrangement of their EMP pathway genes. The possible reasons for this evident diversity are not apparent.

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