An operon encoding three glycolytic enzymes in *Lactobacillus delbrueckii* subsp. *bulgaricus*: glyceraldehyde-3-phosphate dehydrogenase, phosphoglycerate kinase and triosephosphate isomerase

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The structural genes *gap*, *pgk* and *tpi* encoding three glycolytic enzymes, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 3-phosphoglycerate kinase (PGK) and triosephosphate isomerase (TPI), respectively, have been cloned and sequenced from *Lactobacillus delbrueckii* subsp. *bulgaricus* (*L. bulgaricus*). The genes were isolated after screening genomic sublibraries with specific *gap* and *pgk* probes obtained by PCR amplification of chromosomal DNA with degenerate primers corresponding to amino acid sequences highly conserved in GAPDHs and PGKs. Nucleotide sequencing revealed that the three genes were organized in the order *gap*–*pgk*–*tpi*. The translation start codons of the three genes were identified by alignment of the N-terminal sequences. These genes predicted polypeptide chains of 338, 403 and 252 amino acids for GAPDH, PGK and TPI, respectively, and they were separated by 96 bp between *gap* and *pgk*, and by only 18 bp between *pgk* and *tpi*. The codon usage in *gap*, *pgk*, *tpi* and three other glycolytic genes from *L. bulgaricus* differed noticeably from that in other chromosomal genes. The site of transcriptional initiation was located by primer extension, and a probable promoter was identified for the *gap*–*pgk*–*tpi* operon. Northern hybridization of total RNA with specific probes showed two transcripts, an mRNA of 1.4 kb corresponding to the *gap* gene, and a less abundant mRNA of 3.4 kb corresponding to the *gap*–*pgk*–*tpi* cluster. The absence of a visible terminator in the 3'-end of the shorter transcript and the location of this 3'-end inside the *pgk* gene indicated that this shorter transcript was produced by degradation of the longer one, rather than by an early termination of transcription after the *gap* gene.

Keywords: *Lactobacillus delbrueckii* subsp. *bulgaricus*, glycolytic enzymes, differential gene expression, transcript stability, codon usage

INTRODUCTION

Lactobacillus delbrueckii subsp. *bulgaricus* (called *L. bulgaricus* from here on) is one of the bacteria used by

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Abbreviations: GAPDH, glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12); PGK, 3-phosphoglycerate kinase (EC 2.7.2.3); RLM-RACE, RNA-ligase-mediated rapid amplification of cDNA ends; TPI, triosephosphate isomerase (EC 5.3.1.1).

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the food industry for large-scale fermentation of milk into cheese and yoghurt, but despite its economic importance, little is known of its biochemistry and genetics. *L. bulgaricus* produces most (if not all) of the energy required for its growth solely by the glycolytic breakdown of sugars into lactic acid (Kandler & Weiss, 1986), hence the interest in the structure, organization and expression of the genes involved in the central catabolism of lactose into lactic acid. Several glycolytic genes have been already cloned and characterized in *L. bulgaricus*. The genes encoding lactose permease and β -galactosidase constitute an operon (Leong-Morgen-thaler *et al.*, 1991). A monocistronic operon contains

only the *ldhD* gene encoding D-lactate dehydrogenase (EC 1.1.1.28) (Branny *et al.*, 1996). The genes encoding phosphofructokinase (EC 2.7.1.11) and pyruvate kinase (EC 2.7.1.40) are clustered into a bicistronic operon and are transcribed into a single mRNA, even though these enzymes catalyse reactions that are seven steps apart along glycolysis (Branny *et al.*, 1993, 1996).

This study reports the characterization of three other glycolytic genes, the *gap* gene, encoding glyceraldehyde-3-phosphate dehydrogenase (GAPDH; EC 1.2.1.12), the *pgk* gene, encoding 3-phosphoglycerate kinase (PGK; EC 2.7.2.3) and the *tpi* gene, encoding triose-phosphate isomerase (TPI; EC 5.3.1.1). The three enzymes GAPDH, PGK and TPI catalyse the consecutive reactions that convert dihydroxyacetone phosphate into 3-phosphoglycerate in the central part of the Embden-Meyerhof-Parnas pathway. These steps involve successively the keto-aldose isomerization between dihydroxyacetone phosphate and glyceraldehyde 3-phosphate catalysed by TPI, the oxidative phosphorylation of glyceraldehyde 3-phosphate into 1,3-bisphosphoglycerate with NAD⁺ as a coenzyme catalysed by GAPDH, and the phosphoryl transfer from 1,3-bisphosphoglycerate to ADP leading to the formation of ATP catalysed by PGK. These enzymes are thus involved in the coupling between carbon oxidation, NAD⁺ reduction and ATP production that is crucial for the generation of energy by anaerobic fermentation. The three genes *gap*, *pgk* and *tpi* have been cloned and sequenced. They are clustered into one operon that begins with *gap* at the 5'-end and terminates with *tpi* at the 3'-end. The transcription of this operon has also been studied and suggests that the presence of two transcripts could result in an unequal expression of these three genes. Also, the codon usage in this *gap*-*pgk*-*tpi* operon and in other glycolytic genes appeared to be biased compared with that in other genes from *L. bulgaricus*.

METHODS

Bacterial strains and growth conditions. *L. bulgaricus* was grown in MRS medium (Difco) supplemented with 2% glucose at 42 °C without aeration. *Escherichia coli* strains used for cloning and sequencing procedures were grown in Luria broth (LB) supplemented with 100 µg ampicillin ml⁻¹ when appropriate, at 37 °C with vigorous aeration.

DNA manipulations. Chromosomal DNA of *L. bulgaricus* was isolated as described previously (Branny *et al.*, 1993). Plasmid DNA from *E. coli* was isolated by the alkaline-lysis procedure (Birnboim & Doly, 1979) and further purified on Qiagen columns when necessary. Custom oligonucleotides were purchased from Eurogentec.

Restriction and modifying enzymes were obtained from Boehringer Mannheim or from Promega and used as specified by the manufacturer.

For DNA hybridization, 2 µg chromosomal DNA from *L. bulgaricus* was first cleaved with one or two restriction enzymes, and then fractionated according to the size of

fragments by electrophoresis on 0.8% agarose gels. The DNA bands were transferred onto a nylon membrane Hybond-N (Amersham) by vacuum-supported diffusion. The specific probes (see below) were labelled with digoxigenin-dUTP. Blotting, labelling, hybridization, washing and detection were performed using the non-radioactive DNA Labelling and Detection kit from Boehringer Mannheim, following the manufacturer's instructions.

For sequencing, the overlapping fragments containing the genes of interest were cloned into Bluescript II SK(+)/KS(+) vectors and appropriate subclones were sequenced on both DNA strands using a Pharmacia Automated Laser Fluorescence (ALF) DNA Sequencer. Some of the subclones were sequenced with the Thermosequencing kit from Amersham. All DNA sequences derived from PCR-amplified products were verified by sequencing of three different isolates for correcting any error introduced by *Taq* polymerase. The sequence data were compiled and analysed using the sequence analysis program DNA-Strider 1.2.

Construction of molecular probes. The oligonucleotides designed according to sequences conserved in various GAPDHs and used as PCR primers to amplify the 3'-end of the *gap* gene were GAPF(oward), 5'-TIATGACIACIGTICAC-3' (L/MMTTVH), and GAPR(everse), 5'-CATTCGTTGTC-(A/G)TACCA-3' (WYDNEW). The two oligonucleotides PGKF, 5'-TGGAA(C/T)GGICCIATGGG-3' (WN-GPMG), and PGKR, 5'-GGCA(A/G)TT(C/G)(C/T)TTICCTC-3' [EGK(E/Q/V)LPG], were designed and used similarly to amplify a part of the *pgk* gene. The PCR mix (50 µl final volume) consisted of template DNA (100 ng), 50 pmol amplifiers, dNTP (200 µM), 1 × *Taq* DNA polymerase buffer and 1 U *Taq* DNA polymerase. The reaction was carried out as follows: 94 °C for 5 min and then 30 cycles of 37 °C for 2 min, 72 °C for 1 min, and 94 °C for 1 min. The amplified products of predicted size were isolated and cloned into the pMOSBlue T vector (Amersham). After sequencing, the *gap* and *pgk* fragments were subcloned in the proper orientation into pGEM vectors. The antisense RNA probes specific for the *gap* and *pgk* genes used for Northern (RNA) and Southern (DNA) hybridizations were generated with SP6 RNA polymerase in the presence of digoxigenin-dUTP [with the RNA Labelling kit (SP6/T7) from Boehringer Mannheim].

RNA analysis. *L. bulgaricus* total RNA from mid-exponential growth phase was isolated by the classical hot phenol method, with the only modification that the cell lysate treatment with proteinase K (100 µg ml⁻¹) was for 1 h at 50 °C.

For Northern hybridizations, 10 µg total RNA from *L. bulgaricus* was electrophoresed on an agarose denaturing gel, transferred to a nylon membrane and hybridized to each of the antisense RNA probes at 50 °C, in the presence of 50% (v/v) formamide. The washing and detection were performed using the Nucleic Acid Detection kit from Boehringer Mannheim. The size of RNAs was determined by comparison to the 0.24–9.5 kb RNA markers set from Gibco-BRL.

For the primer extension experiments, 50 µg vacuum-dried total RNA was dissolved in 100 µl 40 mM PIPES buffer, at pH 6.4, containing 1 mM EDTA, 0.4 M NaCl and 80% (v/v) formamide. After the addition of 20 pmol of a ³²P-end-labelled primer GMP1L (5'-CGGCCAAAACCGTTAATACCAATC-TTAAC-3'), a denaturation at 95 °C for 5 min and a hybridization at 50 °C for 8 h, the mixture was precipitated by ethanol, washed with 70% (v/v) ethanol and lyophilized. The pellet was dissolved in reverse transcription buffer containing 1 mM MnCl₂, 200 µM of each dNTP and 40 U RNasin

(Promega). cDNA was synthesized with *Thermus thermophilus* DNA polymerase for 1 h at 70 °C. In the presence of Mn^{2+} ions, this enzyme gave much clearer results than avian myeloblastosis virus (AMV) reverse transcriptase. All the RNA present was degraded by a treatment of 30 min at 37 °C with RNase A at a final concentration of 50 $\mu\text{g ml}^{-1}$. The cDNA synthesized upon primer extension was precipitated by ethanol, dried under vacuum, dissolved in 3 μl TE buffer and 3 μl loading dye, and loaded on a 6% polyacrylamide sequencing gel. The size markers obtained by using the oligonucleotide GMP1L as primer for the sequencing reaction were loaded on the same gel and used to characterize the product of primer extension.

The 3'-end of the processed *gap* transcript was determined using RNA-ligase-mediated rapid amplification of cDNA ends (RLM-RACE) (Liu & Gorovsky, 1993). Eighty picomoles of the 16-mer oligonucleotide DFG (5'-TCATCGACTTCGG-CAT-3') was phosphorylated with polynucleotide kinase and ligated to 10 μg total RNA using T4 RNA ligase. Free oligonucleotides were then eliminated using the RNaid kit from Bio101. cDNA synthesis was primed using the 16-mer oligonucleotide ANTIDFG (5'-ATGCCGAAGTCGATGA-3'), the sequence of which is complementary to that of DFG. The first strand reverse transcription reaction was carried out with AMV reverse transcriptase (Promega) according to the manufacturer's instructions. Excess primer was removed as described above. Half of the cDNA synthesized was used as a template for PCR amplification with one primer, GAP3END (5'-GGTTCGTA CTTTGTTCGACTT-3'), designed from a sequence located near the 3'-end of the *gap* gene, the other primer being the ANTIDFG oligonucleotide. The amplification product was gel-purified, cloned into the pMOSBlue T vector, and sequenced.

Amplification of the 5'-end and promoter region of the *ygaP* gene by inverted PCR. Chromosomal DNA was digested to completion with *SmaI*, and the mixture of fragments was allowed to ligate under conditions favouring self-circularization. The product of this ligation was used as a template for PCR amplification with the primers ORFU (5'-TTGCCCC-TGGCAGACTGCATT-3') and ORFD (5'-GAATTCAGCT-CTTCGCCAGCC-3').

RESULTS AND DISCUSSION

Molecular cloning of DNA fragments containing the *gap*, *pgk* and *tpi* genes

Alignment of the amino acid sequences known for the GAPDHs and PGKs from different organisms revealed a number of highly conserved regions (Fothergill-Gilmore & Michels, 1993). For each protein, the sequences of two conserved segments were used to design a pair of degenerate oligonucleotide primers for the production of a specific probe by direct PCR amplification of chromosomal DNA. Each PCR yielded a single amplification product of the size expected from the alignment of amino acid sequences: 430 bp for the *gap* DNA fragment obtained with the two primers GAPF(oward) and GAPR(everse), and 220 bp for the *pgk* DNA fragment obtained with the primers PGKF(oward) and PGKR(everse). Each of these two amplification products was cloned and its nucleotide sequence determined. Each DNA fragment contained an ORF giving an amino acid sequence in which all the residues important for the

structure and function of the corresponding enzyme, GAPDH or PGK, were conserved. These *gap* and *pgk* DNA fragments were subcloned in the proper orientation into pGEM vectors and specific antisense RNA probes were generated with SP6 RNA polymerase. Chromosomal DNA from *L. bulgaricus* was partially digested with several restriction endonucleases, and each mixture of fragments was analysed by Southern blotting using hybridization with the specific *gap* and *pgk* RNA probes to identify the fragments carrying the corresponding genes (data not shown). Three fragments were thus characterized: fragment I was obtained by *PstI* cleavage and hybridization with the *gap* probe only, fragment II by *EcoRI* cleavage and hybridization with the *gap* and *pgk* probes, and fragment III by *EcoRI*-*PstI* double cleavage and hybridization with the *pgk* probe only (Fig. 1). These overlapping fragments (Fig. 1) were cloned in the pBluescript II vector. The positive clones were identified by colony hybridization and the recombinant plasmids were isolated. A nested set of unidirectional deletions of these plasmids was subsequently constructed with exonuclease III and mung bean nuclease, essentially as described by Henikoff (1984). Dideoxy sequencing reactions were carried out with 5'-end-labelled primers and determined with the ALF DNA sequencer from both strands. DNA sequencing of fragment I showed the presence of a *SmaI* site that could be used in inverted PCR to obtain fragment IV (Fig. 1). The successive steps of *SmaI* cleavage of chromosomal DNA, self-circularization of the fragments, linearization by *PstI* cleavage, PCR amplification of chromosomal DNA with primers diverging from the *PstI* site, and cloning and sequencing of the amplified fragment were used to determine the sequence of fragment IV and of the 5'-half of the first ORF (Fig. 1).

Nucleotide sequence of the *gap*, *pgk* and *tpi* genes and their flanking DNA segments

The essential features and the distances between them of the nucleotide sequences of the *gap-pgk-tpi* operon and flanking regions obtained from the four DNA fragments described in Fig. 1 are shown in Fig. 2. The complete sequence contained four ORFs in the same orientation, and the 3'-end also contained an incomplete ORF oriented in the reverse direction. The ORFs are indicated as boxes in Fig. 2. By a search comparison with the BLAST program, the first ORF was similar to the *ygaP* gene from *Bacillus megaterium*, which encodes a hypothetical protein of 37.7 kDa (Schlpfer & Zuber, 1992). The ATG codon at position 70 was preceded by a weak potential RBS, and was assumed to correspond to the start codon of this ORF. This ORF would end at the stop codon at position 1216, and could encode a protein of 382 residues. This protein would have a size similar to that of the product of the *ygaP* gene, with about 35% sequence identity. A region of dyad symmetry followed by several T residues was present at positions 1189-1216, and could correspond to a rho-independent terminator. Transcripts corresponding to this region would form a stem-loop secondary structure

Fig. 1. Physical map of the DNA region around the *gap-pgk-tpi* operon in *L. bulgaricus* constructed from four different DNA fragments, with the positions of the two specific probes used. The DNA fragments were identified as follows: fragment I was a *Pst*I fragment recognized by the *gap* probe; fragment II was an *Eco*RI fragment recognized by the *gap* probe only; fragment III was an *Eco*RI fragment recognized by the *pgk* probe only; and fragment IV was a *Sma*I fragment obtained by inverted PCR. The regions corresponding to *gap* and *pgk* probes are indicated in black. Also shown are the coding regions corresponding to the genes *ygaP*, *gap*, *pgk*, *tpi* and part of *ycsE* and the relevant restriction sites.

Fig. 2. Essential features and the distances between them of the nucleotide sequences of the *gap-pgk-tpi* operon and flanking regions obtained from the four DNA fragments described in Fig. 1. The ORFs are indicated as boxes with only the ATG start and the TAA or TAG stop codons. The transcription starts at T₁₁₂₉ and A₁₁₃₀ are in bold, and the -35 and -10 sequences of the corresponding potential promoter are given explicitly, together with the putative RBSs preceding the *ygaP*, *gap*, *pgk* and *tpi* genes. The actual sequences of inverted repeats (indicated by opposing arrows below the sequence) followed by several Ts that could be either rho-independent terminators or mRNA stabilizing stem-loop structures are also shown explicitly. The arrow and interruption in the coding region of *pgk* indicate the 3'-end determined for the shorter 1.4 kb mRNA corresponding to the *gap* gene only.

with a calculated free energy of -59 kJ mol^{-1} (Tinoco *et al.*, 1973). Primer extension experiments failed to indicate the presence of a promoter, suggesting that transcription of this putative *ygaP* gene started at a distant site upstream of the ORF and therefore that this gene was the last gene in an operon.

The three other ORFs oriented in the same direction were located between nucleotides 1256 and 2272, between nucleotides 2369 and 3580, and between nucleotides 3599 and 4357. These ORFs could be identified unambiguously by comparison of the corresponding amino acid sequences with those in the SWISS-PROT database. The first (in the 5' to 3' direction) ORF revealed significant identity to several GAPDHs, the

second ORF to several PGKs and the third ORF to several TPis. The extent of identity was sufficient to conclude that ORF1 corresponded to the *gap* gene encoding GAPDH, ORF2 corresponded to the *pgk* gene encoding PGK and ORF3 corresponded to the *tpi* gene encoding TPI. The level of identity was even high enough to identify the sites of initiation of translation of each gene by aligning their N-terminal amino acid sequences with those of other GAPDHs, PGKs and TPis. The probable starts of translation of the *gap*, *pgk* and *tpi* genes are the ATG codons located at positions 1256, 2369 and 3599, respectively. In addition, these ATG start codons were preceded by a potential RBS (Fig. 2). The coding sequences were thus separated by 96 bp between *gap* and *pgk*, and by 18 bp between *pgk*

and *tpi*. The nucleotide sequences of these three ORFs predicted a polypeptide chain of 338 amino acids (with a molecular mass of 36 541 Da) for GAPDH, a chain of 403 amino acids (with a molecular mass of 42 685 Da) for PGK and a chain of 252 amino acids (with a molecular mass of 27 365 Da) for TPI. The amino acid sequences respectively deduced for the products of the *gap*, *pgk* and *tpi* genes clearly showed homologies with the sequences of GAPDHs, PGKs and TPis from other bacteria. For GAPDH, the degree of identity ranged between 64% with the *E. coli* enzyme and 46% with the *Zymomonas mobilis* enzyme; for PGK, the identity ranged from 60% with the *Thermotoga maritima* enzyme to 43% with the *Z. mobilis* enzyme; and for TPI, the identity ranged from 66% with the *Lactococcus lactis* enzyme to 40% with the *Mycoplasma genitalium* enzyme. When conservative amino acid replacements were taken into account, the similarities between the sequences from *L. bulgaricus* and the others rose to values between 78 and 61% for GAPDHs, 74 and 61% for PGKs, and 78 and 58% for TPis. These very high degrees of similarity confirmed that the *gap*, *pgk* and *tpi* genes of *L. bulgaricus* indeed encode the enzymes GAPDH, PGK and TPI. Southern hybridizations of restricted chromosomal DNA with the specific *gap* and *pgk* probes clearly indicated the presence of a single copy of each gene (data not shown).

Stem-loop structures formed by mRNAs sometimes serve as rho-independent transcription terminators (d'Aubenton Carafa *et al.*, 1990), so the nucleotide sequence was searched for potential stem-loop sites. Downstream of the *gap* gene, a region of dyad symmetry followed by several T residues was identified between positions 2311 and 2337 (Fig. 2). This sequence predicts that a rather stable stem-loop structure could be formed by the mRNA with a free energy of -91 kJ mol^{-1} . A second inverted-repeat sequence followed by TTT was found at positions 4384–4411 downstream of the *tpi* gene. The mRNA transcribed from this region could also form a stem-loop structure, albeit less stable, with a free energy of $-54.5 \text{ kJ mol}^{-1}$. The presence of these stem-loop structures resembling rho-independent terminators indicates a possible termination of transcription after the *gap* and/or the *tpi* genes (see below).

The DNA segment on the 3'-side of the *tpi* gene did not reveal any coding sequence of significant length for 1.4 kb, but an incomplete ORF oriented in the reverse direction was identified further downstream (Fig. 1). The amino acid sequence translated from this ORF showed about 30% identity to the putative product of the *ycaE* gene from *Bacillus subtilis*, which would encode a protein with a chain of molecular mass 29.7 kDa and of unknown function (Yamane *et al.*, 1996).

Determination of the sizes and ends of transcripts

The total RNA extracted from *L. bulgaricus* was analysed by Northern hybridization with antisense RNA probes specific for the 3'-end of the *gap* gene, the 3'-end

Fig. 3. Determination of the size of the mRNA of the *gap*, *pgk* and *tpi* genes. Total RNA from *L. bulgaricus* was size-fractionated by electrophoresis on a formamide-agarose gel and hybridized with a digoxigenin-labelled *gap* (lane A) or *pgk* (lane B) probe. The numbers and arrows on the right correspond to the sizes and migration positions of RNA standards.

of the *pgk* gene, or the 5'-end of the *tpi* gene. Hybridization with the *gap* probe showed two transcripts, a shorter and abundant mRNA of 1.4 kb and a longer and less abundant one of 3.4 kb, whereas hybridization with either the *pgk* (Fig. 3) or the *tpi* (data not shown) probes showed only one transcript of 3.4 kb. The length of 3.4 kb of the larger RNA was sufficient to correspond to the transcription of the three genes *gap*, *pgk* and *tpi*. The shorter abundant RNA of 1.4 kb could be the result of either a frequent termination of transcription after the *gap* gene, or an efficient degradation of the full-length transcript of 3.4 kb. The size of 1.4 kb found for the shorter dominant RNA was slightly larger than that of 1.2 kb expected from the distance between the transcription start (see below) and the putative termination site at positions 2311–2337 immediately after the *gap* gene. Therefore, this shorter mRNA could be longer than the *gap* gene, and contain also the *gap-pgk* intercistronic region of 96 bp and even a segment of the 5'-part of the *pgk* gene. The actual mapping of the 3'-end of the shorter mRNA by the RLM-RACE method indeed showed that this transcript

Fig. 4. Mapping of the 3'-end of the 1.4 kb *gap* message by the RLM-RACE method. The sequence shown corresponds to the 3'-end of mRNA, ligated DFG oligonucleotide, and sequencing vector. The sequence of the coding strand is shown on the right.

extended for 139 bp within the *pgk* gene with its 3'-end located 235 bp downstream of the potential terminator (Fig. 4). Therefore the shorter mRNA encoding the *gap* gene did not appear to be formed by early termination but by degradation of a longer transcript. This degradation yielded products of different stabilities: a segment of 1.4 kb at the 5'-end of the long transcript remained stable, while the segment of 2 kb at the 3'-end was completely degraded. This processing might thus involve an initial specific endonucleolytic cleavage and the marked segmental stabilization of the fragment on the 5'-side of the cleavage site.

The transcription start site was determined by primer extension of the cDNA. Primers complementary to the coding region of the *gap* gene showed that transcription was initiated at the nucleotide T₁₁₂₉ and/or A₁₁₃₀ (Fig. 5). The promoter was identified by its similarity to the consensus sequences found in Gram-positive bacteria

Fig. 5. Determination of the transcription initiation site of the *gap-pgk-tpi* operon by cDNA primer extension, showing that the major products correspond to initiation at residues T₁₁₂₉ and A₁₁₃₀. Lane 1, products of primer extension; lanes A, C, G and T, sequence ladder generated with the same primer and the non-coding DNA strand as a template. The relevant DNA sequence is shown on the right. Asterisks indicate the positions of the deduced start sites. The putative -10 hexameric segment of the promoter is boxed.

and *E. coli*, and its probable sequence was TTGGCA-16 bp-CATCAA. This promoter and the site of transcription initiation were therefore located in the coding region of the putative *ygaP* gene. Primer extension experiments were also performed to identify a possible promoter upstream of the *pgk* gene, but no transcription start was found within the *gap-pgk* intercistronic region using a primer complementary to the 5'-end of the *pgk* gene.

Comparison of the organization and the transcription of the *gap*, *pgk* or *tpi* genes between different bacteria

The above results clearly showed that the three glycolytic genes *gap*, *pgk* and *tpi* of *L. bulgaricus* are clustered into an operon. These genes are probably transcribed into a single mRNA starting at a given initiation site, which would be largely degraded later into a smaller stable product. It can be noted that the

order of the genes along the DNA, *gap-pgk-tpi*, is different from the order of the corresponding enzymes along glycolysis, TPI-GAPDH-PGK.

In several bacteria, the *gap* gene is clustered with other genes encoding enzymes of the glycolytic pathway. *Corynebacterium glutamicum* and *B. megaterium* possess the same gene cluster of *gap*, *pgk* and *tpi* as that observed in *L. bulgaricus* (Eikmanns, 1992; Schläpfer & Zuber, 1992). The *gap* gene of *Z. mobilis* is associated with the *pgk* gene in a bicistronic operon (Eddy *et al.*, 1991). In contrast, the *gapA* gene encoding the active GAPDH in *E. coli* is not clustered with any other genes (Branlant & Branlant, 1985), and the *pgk* gene is associated with the *fda* gene encoding another glycolytic enzyme, fructose-1,6-bisphosphate aldolase (EC 4.1.2.13), and the cryptic *gapB* gene (Alefounder & Perham, 1989). Also, in *Lactoc. lactis* the *gap* and *tpi* genes were both found to be monocistronic (Cancilla *et al.*, 1995a, b). Therefore, the genes encoding the same glycolytic enzymes do not have the same organization in different prokaryotes, but there is no obvious explanation for this variability.

There are also differences in the mechanisms by which bacteria control the differential expression of the genes that belong to the same operon as the *gap* gene. In *C. glutamicum*, the transcription of the *gap-pgk-tpi* cluster involved mono-, di- and tricistronic mRNAs. The shorter transcript contained the sequence of the *gap* gene and was produced by termination of the transcription at a site downstream of the *gap* gene. The longer transcript contained the sequences of all three genes, and an intermediate bicistronic mRNA containing the sequences of the *pgk* and *tpi* genes was transcribed from a site of reinitiation located in front of the *pgk* gene (Schwinde *et al.*, 1993). In *Z. mobilis*, the expression of the *gap-pgk* cluster yielded two transcripts: a more abundant mRNA of 1.2 kb containing only *gap*, and a minor mRNA of 2.7 kb containing both genes. Transcript mapping and turnover studies indicated that the shorter mRNA corresponding to the *gap* gene was a stable degradation product of the full-length transcript. Thus differential expression of the *gap* and *pgk* genes was mainly achieved by the increased stability of the 5'-segment of the transcript and the subsequent increased translation of *gap* (Eddy *et al.*, 1991; Burchhardt *et al.*, 1993). Several arguments suggested that this was also the case in *L. bulgaricus*, with a shorter transcript corresponding to the *gap* gene alone (1.4 kb) deriving from a longer transcript corresponding to the entire *gap-pgk-tpi* cluster (3.4 kb) by segmental degradation: (i) the *gap* message showed a defined 3'-end and extended for 139 bp within the *pgk* gene (Fig. 4); (ii) the 3'-segment of the *gap* message did not reveal any stem-loop terminator-like structure; (iii) the sequence of a potential rho-independent terminator was indeed present immediately after the 3'-end of the *gap* gene, but it was probably not exploited since no corresponding transcript was observed.

The factors that are responsible for the selective degradation of the 3'-segment of the tricistronic mRNA

and the stabilization of its 5'-segment are unknown, but it could be that the stem-loop structure located in the intercistronic region between the *gap* and *pgk* genes could serve as a recognition site for an endonucleolytic attack and/or contribute to the stability of the shorter *gap* transcript. The untranslated 5'-segment of the *gap* and *gap-pgk-tpi* transcripts contained another stem-loop structure, that corresponding to the terminator of transcription for the putative *ygaP* gene. The shorter mRNA with the *gap* message had thus potential elements of secondary structures in both its 5'- and 3'-segments, which could explain its stability and its relative abundance (Chen & Belasco, 1990; Liu *et al.*, 1992).

Codon usage in *L. bulgaricus* glycolytic genes

Genes that are strongly expressed in *E. coli* and *Saccharomyces cerevisiae* show a highly biased codon usage (Gouy & Gautier, 1982; Sharp *et al.*, 1986), probably related to the relative abundance of tRNA species (Kurland, 1991). A similar biased codon usage has been reported for the glycolytic genes of *E. coli* (Médigue *et al.*, 1991) and *Lactoc. lactis* (Cancilla *et al.*, 1995a). The codon usage in the *gap*, *pgk* and *tpi* genes was close to that in three other glycolytic genes from *L. bulgaricus*, *pfk*, *pyk* and *ldhD*. However, the codon usage common to these six glycolytic genes showed some differences from that in 10 other chromosomal genes known in *L. bulgaricus* (Table 1). On one hand, several codons were used less frequently in glycolytic genes, with the following being unused or used only once: CUC (Leu), CUA (Leu), AUA (Ile), UCG (Ser), CCC (Pro), CCG (Pro), ACA (Thr), UAU (Tyr), CAU (His), CAG (Gln), AAU (Asn), GAG (Glu), UGU (Cys), CGA (Arg), AGU (Ser), AGG (Arg), GGA (Gly). On the other hand, some codons were used more frequently in glycolytic genes, such as those for Phe (UUC), Val (GUU), Ser (UCA), Pro (CCA), Thr (ACU), Ala (GCU), Arg (CGU) and Gly (GGU). In general, NNC codons were used more than NNU, and NNA codons more than NNG. This bias in the NNC/U and NNG/A pairs of codons did not correspond to the GC drift postulated for the strongly expressed genes in *L. bulgaricus* (Pouwels & Leunissen, 1994). Fermentation provides the energy for the growth of *L. bulgaricus*, and the glycolytic genes are probably strongly expressed, even though they may not all be expressed to the same high level. This knowledge of the codon usage in *L. bulgaricus* will be useful for designing probes for the identification of new genes, and for predicting the level of gene expression (Gribskov *et al.*, 1984).

Concluding remarks

In *L. bulgaricus*, the genes encoding the enzymes GAPDH, PGK and TPI, which catalyse three adjacent steps in glycolysis, are clustered into a single operon. The expression of this operon probably involves a processing of the long primary transcript and an increased synthesis of GAPDH as compared to PGK and

Table 1. Codon usage in *L. bulgaricus*

Amino acid	Codon	Chromosomal genes*		Glycolytic genes†	
		Sum	%	Sum	%
Phe	TTT	122	44	14	21
Phe	TTC	153	56	54	79
Leu	TTA	65	12	7	4
Leu	TTG	154	27	85	50
Leu	CTT	62	12	30	18
Leu	CTC	73	13	6	4
Leu	CTA	22	4	—	0
Leu	CTG	181	32	41	24
Ile	ATT	86	30	36	24
Ile	ATC	194	68	115	75
Ile	ATA	6	2	1	11
Met	ATG	126	100	55	100
Val	GTT	164	37	135	65
Val	GTC	176	40	24	12
Val	GTA	54	12	41	20
Val	GTG	50	11	8	4
Ser	TCT	49	12	9	9
Ser	TCC	83	20	11	11
Ser	TCA	78	19	62	60
Ser	TCG	14	3	—	0
Pro	CCT	40	17	13	17
Pro	CCC	22	9	—	0
Pro	CCA	104	45	63	80
Pro	CCG	67	29	2	2
Thr	ACT	135	38	106	76
Thr	ACC	125	35	24	17
Thr	ACA	40	11	3	2
Thr	ACG	59	16	7	5
Ala	GCT	145	30	125	55
Ala	GCC	200	42	49	22
Ala	GCA	77	16	42	19
Ala	GCG	54	11	10	4
Tyr	TAT	72	27	6	12
Tyr	TAC	194	73	45	88
‡	TAA	8	80	4	67
‡	TAG	2	20	2	33
His	CAT	31	26	1	2
His	CAC	90	74	47	98
Gln	CAA	113	47	55	93
Gln	CAG	126	53	4	7
Asn	AAT	103	36	4	4
Asn	AAC	180	64	98	96
Lys	AAA	230	37	8	5
Lys	AAG	386	63	146	95
Asp	GAT	150	37	17	10
Asp	GAC	254	63	145	90
Glu	GAA	333	82	150	98
Glu	GAG	75	18	3	2
Cys	TGT	14	30	4	29
Cys	TGC	32	70	11	71
‡	TGA	0	0	—	0
Trp	TGG	74	100	12	100

Table 1 (cont.)

Amino acid	Codon	Chromosomal genes*		Glycolytic genes†	
		Sum	%	Sum	%
Arg	CGT	20	9	35	45
Arg	CGC	56	26	22	29
Arg	CGA	9	4	—	0
Arg	CGG	75	35	10	13
Ser	AGT	56	14	3	3
Ser	AGC	133	32	17	17
Arg	AGA	41	19	9	12
Arg	AGG	12	6	1	1
Gly	GGT	78	17	91	45
Gly	GGC	235	52	104	51
Gly	GGA	39	9	1	0.5
Gly	GGG	99	22	7	3.5
Total:					
Codons		6305		2402	
Amino acids		6295		2234	

*Based on the nucleotide sequences of the following *L. bulgaricus* chromosomal genes as given by the GenBank database under the accession numbers given in parentheses: *lacY*, lactose permease (M55068); *lacZ*, β -galactosidase (M55068); ORF306 (D10020); *glnA*, glutamine synthetase (D10020); *hdbA*, D-hydroxyisocaproate dehydrogenase (X65222); ISL3 (X79114); *asnA*, asparagine-tRNA ligase (X89438); *asnS1*, aspartate-ammonia ligase (X89438); *pepLP*, proline iminopeptidase (L10712); *prtB*, proteinase (L48487).

†Based on the nucleotide sequences of the glycolytic genes: *gap*, *pgk* and *tpi* (this study); *pfk*, phosphofructokinase (X71403); *pyk*, pyruvate kinase (X71403); *ldhD*, D-lactate dehydrogenase (X60220).

‡Stop codon.

TPI. In most bacteria, these enzymes do not show any allosteric regulation (Fothergill-Gilmore & Michels, 1993), and the activity of each enzyme mainly reflects its amount in the cell. The differential expression of these genes, probably involving mRNA processing and possibly biased codon usage, would allow the equilibration of the activity levels of 'adjacent' enzymes having different kinetic properties, so as to maintain a regular glycolytic flux with no accumulation or depletion of intermediates.

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