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The Genes for Phosphofructokinase and Pyruvate Kinase of *Lactobacillus delbrueckii* subsp. *bulgaricus* Constitute an Operon

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In *Lactobacillus delbrueckii* subsp. *bulgaricus*, the *pyk* gene coding for pyruvate kinase and the *pfk* gene coding for phosphofructokinase formed a bicistronic operon transcribed into a 2.9-kb RNA. The nucleotide sequence of the *pyk* gene indicated that the encoded protein possessed an extra C-terminal domain with a potential phosphoenolpyruvate-dependent autophosphorylation site.

We have previously reported the cloning and molecular analysis of the region of the *pfk* gene that codes for phosphofructokinase (Pfk) in *Lactobacillus delbrueckii* subsp. *bulgaricus*. The presence of an open reading frame (ORF) at the 3′ end of the *pfk* gene and the fact that the ORF corresponded to the N-terminal part of pyruvate kinase (Pyk) suggested that the *pfk* and *pyk* genes could form an operon (3). In addition to the *Eco*RI-*Hin*dIII fragment (fragment I in Fig. 1) of 1.2 kb carrying the coding region of the *pfk* gene and the 5′ end of the *pyk* gene (3), three other DNA fragments were cloned in order to characterize this *pfk-pyk* operon (Fig. 1). A total digest of chromosomal DNA from *L. bulgaricus* by *Eco*RI and *Sac*I was screened by hybridization with a digoxigenin-labelled probe specific for the *pfk* gene, and fragment II of 2.8 kb was isolated and sequenced (see Fig. 2). A digoxigenin-labelled probe corresponding to the small *Hin*dIII-*Sac*I fragment of 0.22 kb at the 3′ end of fragment II (Fig. 1 and 2) was used to screen *L. bulgaricus* chromosomal DNA after total digestion by *Bgl*II, which led to the isolation of fragment III of 1.55 kb (Fig. 1).

Attempts to clone the 5′ flanking region of the *pfk* gene were unsuccessful, and fragment IV of 1.5 kb (Fig. 1) was amplified directly from genomic DNA by inverted PCR (29) with the following steps: complete cleavage of chromosomal DNA by *Hin*dIII, self-circularization and ligation of these fragments, linearization by cleavage with *Pst*I, PCR amplification with two primers on either side of the *Pst*I site, and Southern hybridization with a probe specific for the 5′ end of the *pfk* gene.

Nucleotide sequence of the *pfk-pyk* operon. The complete sequence of the 3,479 nucleotides of the *pfk-pyk* operon obtained from fragments I to IV is shown in Fig. 2. There was no significant ORF in the 277 bp upstream of the *pfk* gene, indicating that the *pfk* gene was not preceded by another gene. A putative ribosome-binding site GAGG was found at position 267 upstream of the *pfk* translation initiation codon. No sequence corresponding to a transcription termination signal could be identified in the 100 to 200 nucleotides downstream of the *pfk* gene.

The ORF coding for the *pyk* gene contained 590 codons beginning with an ATG initiation codon at position 1277 and ending with a TAA stop codon at position 3046 and was preceded by a potential ribosome-binding site GGAG at position 1262. A potential rho-independent transcription terminator similar to those described for *Escherichia coli* (23) was identified downstream of the stop codon of the *pyk* gene. Indeed, the segments from positions 3078 to 3090 and 3095 to 3107 were complementary but for one mismatch, suggesting that a stable stem-loop structure could form with a ΔG around 58 kJ/mol at 25°C (28), and several T residues followed this potential mRNA hairpin (Fig. 2).

No significant ORF was found in the 250 nucleotides downstream of the *pyk* gene, indicating that the tandem *pfk-pyk* arrangement was not associated with another gene.

One transcript for the *pfk* and *pyk* genes. The sizes of the mRNAs formed upon transcription of the *pfk* and *pyk* genes were determined by hybridizing total *L. bulgaricus* RNA with digoxigenin-labelled antisense RNA probes specific for either the 0.2-kb *Eco*RI-*Kpn*I segment at the 5′ end of the *pfk* gene or the 0.47-kb *Hin*dIII-*Bgl*II fragment in the middle of the *pyk* gene (Fig. 1 and 2). Only one transcript was revealed by the two probes (Fig. 3) with the size of 2.9 kb, which was in close agreement with the added lengths of the *pfk* and *pyk* genes.

The transcription start site was the nucleotide T_{233}, as determined both by cDNA primer extension (4) using primers in the 5′ region of the *pfk* gene (Fig. 4) and by mapping with S1

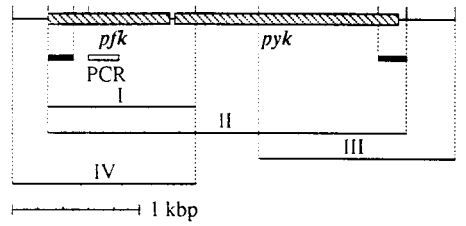

FIG. 1. Physical map of the DNA region around the *pfk-pyk* operon in *L. bulgaricus* constructed from four different DNA fragments, with the positions of the three specific probes used. The DNA fragments are as follows: fragment I, an *Eco*RI-*Hin*dIII fragment cloned and sequenced previously (3); fragment II, an *Eco*RI-*Sac*I fragment identified with the PCR-amplified *pfk* probe labelled PCR; fragment III, a *Bgl*II-*Bgl*II fragment identified with the *Hin*dIII-*Sac*I probe indicated in black; and fragment IV, a *Hin*dIII-*Hin*dIII fragment identified with the *Eco*RI-*Kpn*I probe indicated in black. Also shown are the coding regions corresponding to the *pfk* and *pyk* genes and the restriction sites mentioned in the text. Abbreviations: B, *Bgl*II; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; P, *Pst*I; S, *Sac*I.

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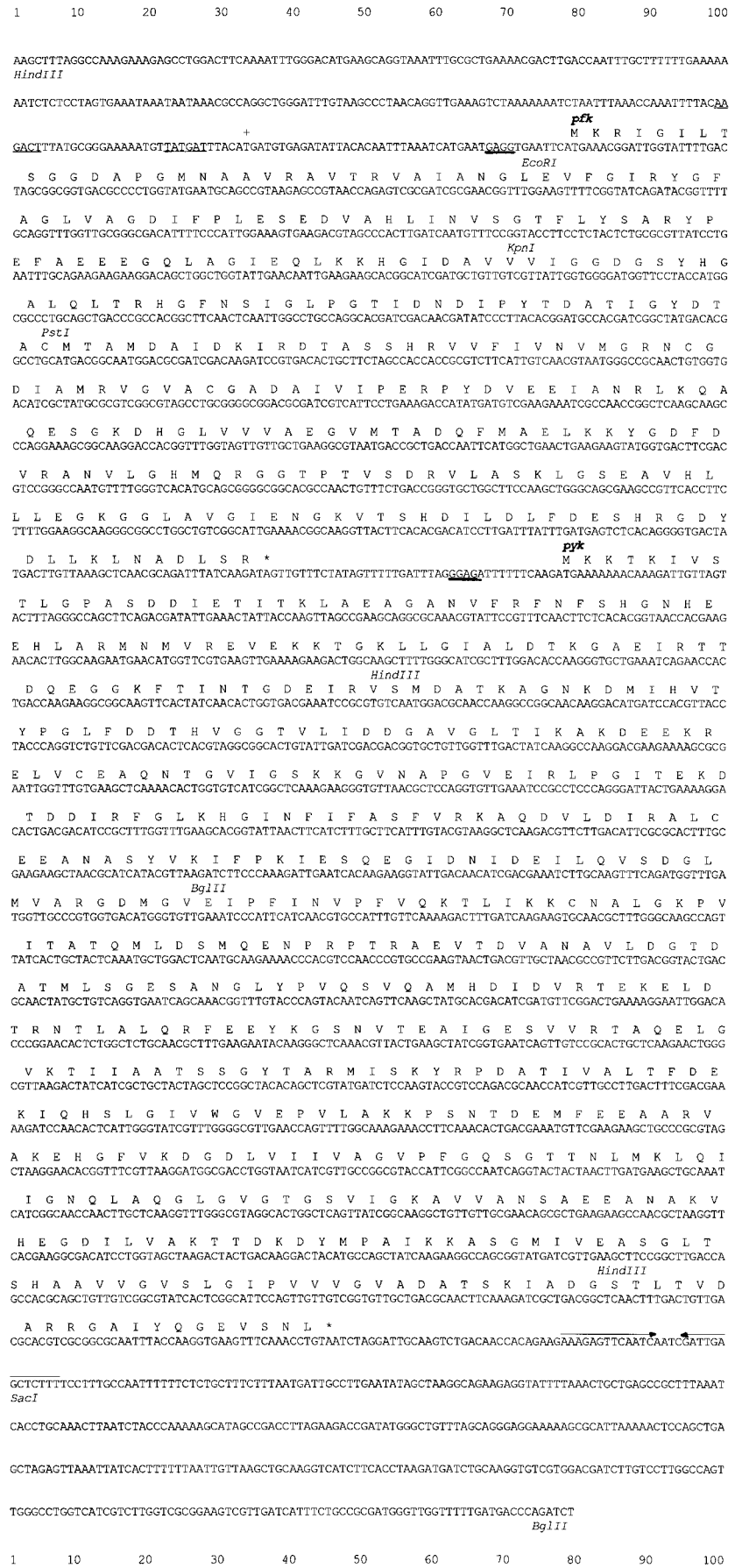
1 10 20 30 40 50 60 70 80 90 100
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HindIII
AATCTCTCCTAGTGAAATAAATAATAAACGCCAGGCTGGGATTTGTAAGCCCTAACAGGTTGAAAGTCTAAAAAAATCTAATTTAAACCAAATTTTACAA
pfk
M K R I G I L T
GACTTTATGCGGGAAAAATGTTATGATTTACATGATGTGAGATATTACACAATTTAAATCATGAATGAGGTGAATTCATGAAACGGATTGGTATTTTGAC
EcoRI
S G G D A P G M N A A V R A V T R V A I A N G L E V F G I R Y G F
TAGCGGCGGTGACGCCCCTGGTATGAATGCAGCCGTAAGAGCCGTAACCAGAGTCGCGATCGCGAACGGTTTGGAAGTTTTCGGTATCAGATACGGTTTT
A G L V A G D I F P L E S E D V A H L I N V S G T F L Y S A R Y P
GCAGGTTGGTTGCGGGCGACATTTTCCCATTGGAAAGTGAAGACGTAGCCCACTTGATCAATGTTTCCGGTACCTTCCTCTACTCTGCGCGTTATCCTG
KpnI
E F A E E E G Q L A G I E Q L K K H G I D A V V V I G G D G S Y H G
AATTTGCAGAAGAAGAAGGACAGCTGGCTGGTATTGAACAATTGAAGAAGCACGGCATCGATGCTGTTGTCGTTATTGGTGGGGATGGTTCCTACCATGG
A L Q L T R H G F N S I G L P G T I D N D I P Y T D A T I G Y D T
CGCCCTGCAGCTGACCCGCCACGGCTTCAACTCAATTGGCCTGCCAGGCACGATCGACAACGATATCCCTTACACGGATGCCACGATCGGCTATGACACG
PstI
A C M T A M D A I D K I R D T A S S H R V V F I V N V M G R N C G
GCCTGCATGACGGCAATGGACGCGATCGACAAGATCCGTGACACTGCTTCTAGCCACCACCGCGTCTTCATTGTCAACGTAATGGGCCGCAACTGTGGTG
D I A M R V G V A C G A D A I V I P E R P Y D V E E I A N R L K Q A
ACATCGCTATGCGCGTCGGCGTAGCCTGCGGGGCGGACGCGATCGTCATTCCTGAAAGACCATATGATGTCGAAGAAATCGCCAACCGGCTCAAGCAAGC
Q E S G K D H G L V V V A E G V M T A D Q F M A E L K K Y G D F D
CCAGGAAAGCGGCAAGGACCACGGTTTGGTAGTTGTTGCTGAAGGCGTAATGACCGCTGACCAATTCATGGCTGAACTGAAGAAGTATGGTGACTTCGAC
V R A N V L G H M Q R G G T P T V S D R V L A S K L G S E A V H L
GTCCGGGCCAATGTTTTGGGTCACATGCAGCGGGGCGGCACGCCAACTGTTTCTGACCGGGTGCTGGCTTCCAAGCTGGGCAGCGAAGCCGTTCACCTTC
L L E G K G G L A V G I E N G K V T S H D I L D L F D E S H R G D Y
TTTTGGAAGGCAAGGGCGGCCTGGCTGTCGGCATTGAAAACGGCAAGGTTACTTCACACGACATCCTTGATTTATTTGATGAGTCTCACAGGGGTGACTA
pyk
D L L K L N A D L S R *
M K K T K I V S
TGACTTGTTAAAGCTCAACGCAGATTTATCAAGATAGTTGTTTCTATAGTTTTTGATTTAGGGAGATTTTTTCAAGATGAAAAAAACAAAGATTGTTAGT
T L G P A S D D I E T I T K L A E A G A N V F R F N F S H G N H E
ACTTTAGGGCCAGCTTCAGACGATATTGAAACTATTACCAAGTTAGCCGAAGCAGGCGCAAACGTATTCCGTTTCAACTTCTCACACGGTAACCACGAAG
E H L A R M N M V R E V E K K T G K L L G I A L D T K G A E I R T T
AACACTTGGCAAGAATGAACATGGTTCGTGAAGTTGAAAAGAAGACTGGCAAGCTTTTGGGCATCGCTTTGGACACCAAGGGTGCTGAAATCAGAACCAC
HindIII
D Q E G G K F T I N T G D E I R V S M D A T K A G N K D M I H V T
TGACCAAGAAGGCGGCAAGTTCACTATCAACACTGGTGACGAAATCCGCGTGTCAATGGACGCAACCAAGGCGGCAACAAGGACATGATCCACGTTACC
Y P G L F D D T H V G G T V L I D D G A V G L T I K A K D E E K R
TACCCAGGTCTGTTCGACGACACTCACGTAGGCGGCACTGTATTGATCGACGACGGTGCTGTTGGTTTGACTATCAAGGCCAAGGACGAAGAAAAGCGCG
E L V C E A Q N T G V I G S K K G V N A P G V E I R L P G I T E K D
AATTGGTTTGTGAAGCTCAAAACACTGGTGTCATCGGCTCAAAGAAGGGTGTTAACGCTCCAGGTGTTGAAATCCGCCTCCCAGGGATTACTGAAAAGGA
T D D I R F G L K H G I N F I F A S F V R K A Q D V L D I R A L C
CACTGACGACATCCGCTTTGGTTTGAAGCACGGTATTAACTTCATCTTTGCTTCATTTGTACGTAAGGCTCAAGACGTTCTTGACATTCGCGCACTTTGC
E E A N A S Y V K I F P K I E S Q E G I D N I D E I L Q V S D G L
GAAGAAGCTAACGCATCATACGTTAAGATCTTCCCAAAGATTGAATCACAAGAAGGTATTGACAACATCGACGAAATCTTGCAAGTTTCAGATGGTTTGA
BglII
M V A R G D M G V E I P F I N V P F V Q K T L I K K C N A L G K P V
TGGTTGCCCGTGGTGACATGGGTGTTGAAATCCCATTCATCAACGTGCCATTTGTTCAAAAGACTTTGATCAAGAAGTGCAACGCTTTGGGCAAGCCAGT
I T A T Q M L D S M Q E N P R P T R A E V T D V A N A V L D G T D
TATCACTGCTACTCAAATGCTGGACTCAATGCAAGAAAACCCACGTCCAACCCGTGCCGAAGTAACTGACGTTGCTAACGCCGTTCTTGACGGTACTGAC
A T M L S G E S A N G L Y P V Q S V Q A M H D I D V R T E K E L D
GCAACTATGCTGTCAGGTGAATCAGCAAACGGTTTGTACCCAGTACAATCAGTTCAAGCTATGCACGACATCGATGTTCGGACTGAAAAGGAATTGGACA
T R N T L A L Q R F E E Y K G S N V T E A I G E S V V R T A Q E L G
CCCGGAACACTCTGGCTCTGCAACGCTTTGAAGAATACAAGGGCTCAAACGTTACTGAAGCTATCGGTGAATCAGTTGTCCGCACTGCTCAAGAACTGGG
V K T I I A A T S S G Y T A R M I S K Y R P D A T I V A L T F D E
CGTTAAGACTATCATCGCTGCTACTAGCTCCGGCTACACAGCTCGTATGATCTCCAAGTACCGTCCAGACGCAACCATCGTTGCCTTGACTTTCGACGAA
K I Q H S L G I V W G V E P V L A K K P S N T D E M F E E A A R V
AAGATCCAACACTCATTGGGTATCGTTTGGGGCGTTGAACCAGTTTGGCAAAGAAACCTTCAAACACTGACGAAATGTTCGAAGAAGCTGCCCGCGTAG
A K E H G F V K D G D L V I I V A G V P F G Q S G T T N L M K L Q I
CTAAGGAACACGGTTTCGTTAAGGATGGCGACCTGGTAATCATCGTTGCCGGCGTACCATTCGGCCAATCAGGTACTACTAACTTGATGAAGCTGCAAAT
I G N Q L A Q G L G V G T G S V I G K A V V A N S A E E A N A K V
CATCGGCAACCAACTTGCTCAAGGTTTGGGCGTAGGCACTGGCTCAGTTATCGGCAAGGCTGTTGTTGCGAACAGCGCTGAAGAAGCCAACGCTAAGGTT
H E G D I L V A K T T D K D Y M P A I K K A S G M I V E A S G L T
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HindIII
S H A A V V G V S L G I P V V V G V A D A T S K I A D G S T L T V D
GCCACGCAGCTGTTGTCGGCGTATCACTCGGCATTCCAGTTGTTGTCGGTGTTGCTGACGCAACTTCAAAGATCGCTGACGGCTCAACTTTGACTGTTGA
A R R G A I Y Q G E V S N L *
CGCACGTCGCGGCGCAATTTACCAAGGTGAAGTTTCAAACCTGTAATCTAGGATTGCAAGTCTGACAACCACAGAAGAAAGAGTTCAATCAATCGATTGA
GCTCTTTTCCTTTGCCAATTTTTTCTCTGCTTTCTTTAATGATTGCCTTGAATATAGCTAAGGCAGAAGAGGTATTTTAAACTGCTGAGCCGCTTTAAAT
SacI
CACCTGCAAACTTAATCTACCCAAAAAGCATAGCCGACCTTAGAAGACCGATATGGGCTGTTTAGCAGGGAGGAAAAAGCGCATTAAAAACTCCAGCTGA
GCTAGAGTTAAATTATCACTTTTTTAATTGTTAAGCTGCAAGGTCATCTTCACCTAAGATGATCTGCAAGGTGTCGTGGACGATCTTGTCCTTGGCCAGT
TGGGCCTGGTCATCGTCTTGGTCGCGGAAGTCGTTGATCATTTCTGCCGCGATGGGTTGGTTTTTGATGACCCAGATCT
BglII
1 10 20 30 40 50 60 70 80 90 100

FIG. 2. Nucleotide sequences of the *pfk* and *pyk* genes and flanking regions obtained from four DNA fragments isolated independently (Fig. 1), with the deduced amino acid sequences of Pfk and Pyk from *L. bulgaricus*. Asterisks indicate stop codons. The −35 and −10 hexameric sequences of the potential promoter upstream of the transcription start T_{233} are underlined. The putative ribosome-binding sites preceding the *pyk* and *pyk* genes are underlined with thick lines. The opposing arrows above the sequence downstream of the *pfk* gene indicate the inverted repeat attributed to a rho-independent terminator (23). Also shown are the restriction sites that were used in cloning various DNA fragments, obtaining specific probes, and performing inverse PCR amplification (Fig. 1).

nuclease (32). The promoter sequences identified by their similarity to the consensus sequence found in gram-positive bacteria and *E. coli* (8, 19) were AAGACT-17 bp-TATGAT (underlined in Fig. 2). This promoter even had one TG doublet upstream of the −10 sequence, a feature that was suggested to be characteristic of promoters from gram-positive bacteria (10, 30). Extension of a primer in the 5′ region of the *pyk* gene did not reveal a transcription start site in or immediately upstream of the intergenic region.

Three pieces of evidence lead to the conclusion that the two *pfk* and *pyk* genes were transcribed as one operon into a unique mRNA of 2.9 kb: (i) the same mRNA was recognized by both the *pfk* and *pyk* probes, (ii) a rho-independent terminator was present downstream of the *pyk* gene and a potential promoter upstream of the *pfk* gene, and (iii) no other ORF was found in the vicinity of the *pfk* and *pyk* genes.

In *Bacillus stearothermophilus*, the *pfk* and *pyk* genes also form an operon (25), but in *E. coli*, the *pfkA* gene coding for the major Pfk is not followed by the *pyk* gene (9). In *Lactococcus lactis*, the *pfk* and *pyk* genes are clustered into one operon together with the *ldhL* gene that codes for L-lactate dehydrogenase (LdhL) located downstream of *pyk* (18). *L. bulgaricus* has no LdhL but has a LdhD (13), and hybridization of total RNA with a *ldhD* probe revealed only one transcript with a size of 1 kb (data not shown), which corresponds to a monocistronic mRNA coding for LdhD with a molecular mass of 37,000 Da (16).

The association between the *pfk* and *pyk* genes in the same operon was found in several organisms such as *L. bulgaricus* (3), *L. lactis* (18), *Spiroplasma citri* (5), *Mycoplasma genitalium* (7), *B. stearothermophilus* (25), and *Bacillus psychrophilus* and *Bacillus licheniformis* (27). In the absence of specific polar effects on mRNA translation and/or decay, the expression of these genes into a unique mRNA will produce comparable amounts of Pfk and Pyk chains. However, the activities of these enzymes must be controlled separately to accommodate the differences in flux through the steps that they catalyze: indeed, some of the glycolytic intermediates may be used as precursors for the synthesis of pentose phosphates, phospholipids, or other important compounds. Also, part of the phosphoenolpyruvate (PEP) may be used by the phosphotransferase system instead of being a substrate for Pyk. This need for fine differential control of Pfk and Pyk could explain why these enzymes are allosteric in most organisms (6). In *L. bulgaricus*, the regulation of Pfk and Pyk is coordinated, since the substrate of one enzyme is an allosteric effector of the other (15, 17). A change in the concentration of one metabolite, either fructose-6-phosphate or PEP, will thus affect the activities of both enzymes.

Comparison of the sequence of Pyk from *L. bulgaricus* with the sequences of other Pyk proteins. The amino acid sequence and calculated mass of Pyk agreed with the N-terminal sequence and the mass obtained previously with the pure protein (17). The molecular mass of 62,880 Da was significantly higher than the values of around 50,000 Da reported for many bacterial Pyk proteins (6). This larger size was explained by the presence of a C-terminal extension of about 110 residues, which has been found only in a few Pyk proteins from bacilli, those from *B. stearothermophilus* (25) and *B. psychrophilus* and *B. licheniformis* (27). Except for this C-terminal extension, the sequence of Pyk from *L. bulgaricus* was similar to the sequences of Pyk proteins from other bacteria (6, 12, 18, 21, 25, 27). The similarity ranged from 65% (55% identical residues) with the *B. stearothermophilus* enzyme down to 35% (29% of identical residues) with the *E. coli* enzyme II. Most of the residues involved in catalysis (14, 20, 26, 31) were conserved in the *L. bulgaricus* enzyme, suggesting that the active site was built only by the 480 or so N-terminal residues, and not by the extra C-terminal domain.

This C-terminal extension contained a sequence that is conserved in several enzymes which catalyze a reaction with PEP as a substrate or a product and a phosphoryl-enzyme as an intermediate (1, 22). In these enzymes, the phosphoryl group given by or going into PEP is transiently carried by a histidine residue that belongs to a consensus sequence called the A region (2): -G-G-X-T-X-H-[STA]-[STAV]-[LIVM]-[LIVM]-[STA]-R-. This finding suggested that some interaction could take place between PEP and the C-terminal domain of Pyk, possibly leading to the phosphorylation of His-542. For instance, the C-terminal domain could be a phosphotransferase that catalyzes the transfer of a phosphoryl group from PEP to a yet unknown acceptor, with a transient phosphorylation of its His-542 as an intermediate step. In this case, Pyk from *L. bulgaricus* would be a bifunctional enzyme, with two active sites using both PEP as a substrate to phosphorylate ADP and another target that could be either a small metabolite or a protein. Protein phosphorylation has indeed been observed in gram-positive bacteria in regulatory processes such as catabolic repression (11, 24). Alternatively, the C-terminal domain could still become phosphorylated on His-542 but without transferring the phosphoryl group to a given acceptor, thus being devoid of a genuine phosphotransferase activity. This phosphorylation of His-542 could be due to another protein or to the C-terminal domain itself and would modify the properties of Pyk. Therefore, *L. bulgaricus* could possess two forms of Pyk (a phosphorylated and a nonphosphorylated one) with different properties instead of two different Pyk proteins as in *E. coli* (6, 26). Further work is now in progress to determine the actual function of this C-terminal extension, but its homology with a site of N phosphorylation already suggests an important regulatory role.

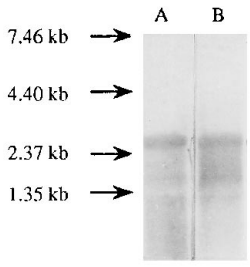

FIG. 3. Determination of the size of the mRNA containing the *pfk* and *pyk* genes. Total RNA from *L. bulgaricus* was size fractionated by electrophoresis on a formamide-agarose gel and hybridized with a digoxigenin-labelled *pfk* (lane A) or *pyk* (lane B) probe. The numbers and arrows on the left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

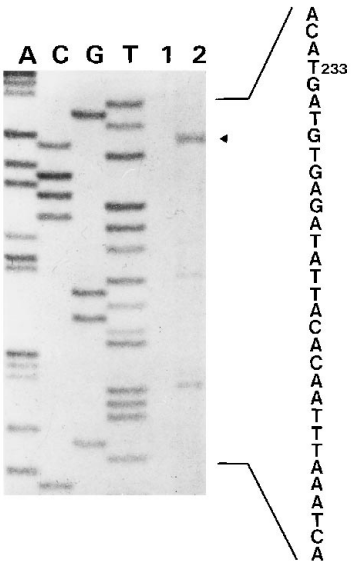

FIG. 4. Determination of the transcription initiation sites of the *pfk-pyk* operon by cDNA primer extension (4), showing that the major product and longest transcript corresponds to initiation at the residue T_{233}. Lane 1, control without primer; lane 2, products of primer extension; lanes A, C, G, and T, sequence ladder generated with the same primer and the noncoding DNA strand as a template. The relevant DNA sequence is shown on the right.

Nucleotide sequence accession number. The nucleotide sequence data reported in this study will appear in the EMBL sequence database under accession number X71403.

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