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Alves, Alexandra M.C.R.; Meijer, Wim G.; Vrijbloed, Jan W.; Dijkhuizen, Lubbert

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Characterization and Phylogeny of the *pfp* Gene of *Amycolatopsis methanolica* Encoding PP_i-Dependent Phosphofructokinase

ALEXANDRA M. C. R. ALVES, WIM G. MEIJER, JAN W. VRIJBLOED, AND LUBBERT DIJKHUIZEN*

Department of Microbiology, Groningen Biomolecular Sciences and Biotechnology Institute (GBB), University of Groningen, 9751 NN Haren, The Netherlands

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The actinomycete *Amycolatopsis methanolica* employs a PP_i-dependent phosphofructokinase (PP_i-PFK) (EC 2.7.1.90) with biochemical characteristics similar to those of both ATP- and PP_i-dependent enzymes during growth on glucose. A 2.3-kb *Pvu*II fragment hybridizing to two oligonucleotides based on the amino-terminal amino acid sequence of PP_i-PFK was isolated from a genomic library of *A. methanolica*. Nucleotide sequence analysis of this fragment revealed the presence of an open reading frame encoding a protein of 340 amino acids with a high degree of similarity to PFK proteins. Heterologous expression of this open reading frame in *Escherichia coli* gave rise to a unique 45-kDa protein displaying a high level of PP_i-PFK activity. The open reading frame was therefore designated *pfp*, encoding the PP_i-PFK of *A. methanolica*. Upstream and transcribed divergently from *pfp*, a partial open reading frame (*aroA*) similar to 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase-encoding genes was identified. The partial open reading frame (*chiA*) downstream from *pfp* was similar to chitinase genes from *Streptomyces* species. A phylogenetic analysis of the ATP- and PP_i-dependent proteins showed that PP_i-PFK enzymes are monophyletic, suggesting that the two types of PFK evolved from a common ancestor.

The actinomycete *Amycolatopsis methanolica* is one of the few known gram-positive bacteria which can use methanol as a growth substrate (14). Until recently, research on *A. methanolica* focused on methanol metabolism (2) and on regulation of aromatic-amino-acid biosynthesis (16, 17). It was shown previously that *A. methanolica* metabolizes glucose via the Embden-Meyerhof-Parnas pathway (2).

In most organisms, the glycolytic flux is controlled at the level of phosphofructokinase (PFK) (44), which catalyzes the irreversible ATP-dependent phosphorylation of fructose-6-phosphate (EC 2.7.1.11). The ATP-dependent PFKs (ATP-PFKs) are allosterically regulated, have a high K_m for their substrate fructose-6-phosphate, and a neutral pH optimum. Three classes can be recognized. The bacterial enzyme has a tetrameric structure of identical subunits each with a molecular mass of 35 kDa and is allosterically regulated by phosphoenolpyruvate and ADP (44). Like the bacterial enzyme, the mammalian enzyme is composed of four identical subunits, although the subunits are substantially larger (85 kDa). The yeast enzyme is an octamer composed of two nonidentical subunits with molecular masses of 112 and 118 kDa. Sequence comparisons among the three classes of ATP-PFK proteins indicate that the yeast and mammalian enzymes have arisen as a result of gene duplication and fusion events. The activities of the yeast and mammalian enzymes are generally regulated by citrate, ATP, and fructose-2,6-bisphosphate (20, 44).

In some bacteria, plants, and protozoa, a PP_i-dependent PFK (PP_i-PFK) (EC 2.7.1.90) is encountered (33). In contrast to the ATP-PFK, the PP_i-dependent enzyme catalyzes the reversible phosphorylation of fructose-6-phosphate (36). A correlation between anaerobic metabolism and the presence of PP_i-PFK has led to the suggestion that this enzyme is better adapted to anaerobiosis than its ATP-dependent counterpart is. In *Propionibacterium freudenreichii* and a number of protists, a homodimeric PP_i-PFK that is not allosterically regulated is present (33, 35). This class of PP_i-PFK is characterized by a low K_m for fructose-6-phosphate and an acidic pH optimum for the phosphorylation of fructose-6-phosphate. In plants, PP_i-PFK is present as a heterotetrameric enzyme composed of two nonidentical subunits with molecular masses of 60 and 65 kDa (3). As with the yeast and mammalian enzymes, the activity of the plant PP_i-PFK is regulated by fructose-2,6-bisphosphate, the K_m for fructose-6-phosphate is high, and the enzyme has a neutral pH optimum for the glycolytic reaction.

We have previously purified and characterized the PP_i-PFK of *A. methanolica* (2). As is typical for PP_i-PFK enzymes, the enzyme activity was not allosterically regulated and catalyzed the reversible phosphorylation of fructose-6-phosphate. However, the *A. methanolica* PFK has a number of biochemical characteristics in common with the bacterial ATP-dependent enzymes: it is a tetramer of identical subunits, the K_m for fructose-6-phosphate is high, and it has a neutral pH optimum. Furthermore, the amino-terminal amino acid sequence of this enzyme is more similar to ATP-dependent than to PP_i-dependent enzymes.

These characteristics of the PP_i-PFK of *A. methanolica*, which are intermediate between those of ATP-PFKs and other PP_i-PFKs, prompted us to characterize this enzyme in greater detail. This paper describes the cloning, primary structure, and phylogeny of this unusual PP_i-PFK from *A. methanolica.*

* Corresponding author. Mailing address: Department of Microbiology, University of Groningen, Kerklaan 30, 9751 NN Haren, The Netherlands. Phone: (31)503632153. Fax: (31)503632154. Electronic mail address: l.dijkhuizen@biol.rug.nl.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant genotype or characteristics[a]	Source or reference
Strains		
E. coli		
DH5α	*supE44Δ lacU169* (ϕ80*lacZ*Δ M15)*hsdR17 recA1 endA1 gyrA96 thi-1–relA1*	Bethesda Research Laboratories
P678.54	F *thr-1 ara-13 leu-6 azi-8 tonA2 lacY1 minA1 glnU44 gal-6 minB2 rpsL135 malA1 xyl-7 mt1-2 thi-1*	1
MC1061	*hsdR mcrB araD139* Δ(*araABC-leu*)*7679* Δ*lacX74 galU galK rspL thi*	31
A. methanolica WVI	pMEA300-free strain	46
Plasmids		
pWV138	Km^r, *E. coli-A. methanolica* shuttle vector	46
pAA601	Ap^r, 6-kb *Apa*I fragment in pBluescriptKS$^+$	This study
pAA101	Ap^r, 2.3-kb *Pvu*II fragment in pBluescriptKS$^+$, with *pfp* in same orientation as the *lac* promoter	This study
pAA108	Ap^r, 2.3-kb *Pvu*II fragment in pBluescriptKS$^+$ with *pfp* in opposite orientation to the *lac* promoter	This study
pAA123	Km^r, 12.3-kb fragment in pWV138	This study
pBluescriptKS$^+$	Ap^r, phagemid derived from pUC18, *lacZ*	Stratagene

[a] Km^r, kanamycin resistant; Ap^r, ampicillin resistant.

MATERIALS AND METHODS

Bacterial strains and plasmids. Bacterial strains and plasmids used in this study are listed in Table 1.

Media and growth conditions. *Escherichia coli* strains were grown on Luria-Bertani (LB) medium at 37°C (40). *A. methanolica* was grown on complete medium (47) at 37°C. When appropriate, the following supplements were added: ampicillin, 100 μg/ml; 5-bromo-4-chloro-3-indolyl-β-D-galactoside, 20 μg/ml; isopropyl-β-D-thiogalactoside (IPTG), 0.1 mM; kanamycin, 25 μg/ml. Agar was added for solid media (1.5% [wt/vol]).

DNA manipulations. Plasmid DNA was isolated by the alkaline lysis method of Birnboim and Doly (4) and then subjected to equilibrium centrifugation in a cesium chloride gradient for preparative DNA isolations (40). Chromosomal DNA was isolated following cell lysis with sodium dodecyl sulfate (SDS) as described by Hintermann et al. (25). DNA-modifying enzymes were obtained from Boehringer (Mannheim, Germany) and were used according to the manufacturer's instructions. *E. coli* DH5α was electrotransformed with the electrocell manipulator model 600 (Biotechnology & Experimental Research Inc., San Diego, Calif.) according to the manufacturer's instructions. Other DNA manipulations were done according to standard protocols (40).

Southern hybridizations. Chromosomal DNA from *A. methanolica* was digested with the appropriate restriction enzymes, separated on a 0.8% (wt/vol) agarose gel, and blotted onto a high-bond nylon membrane supplied by Qiagen (Basel, Switzerland), via the alkaline transfer method (40). Southern hybridizations were done at 68°C with oligonucleotides (100 pmol) labeled with the DIG oligonucleotide tailing kit from Boehringer. The membrane was subsequently washed twice with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% (wt/vol) SDS for 5 min and twice with 0.1× SSC–0.1% (wt/vol) SDS for 5 min.

Construction of a genomic library. Plasmid pWV138 was derived from pMEA300, which is indigenous to *A. methanolica* (46, 47); it was digested with *Bam*HI and subsequently dephosphorylated with calf intestine phosphatase according to the manufacturer's instructions (Boehringer). Chromosomal DNA of *A. methanolica* was partially digested with *Sau*IIIA. DNA fragments ranging in size from 5 to 10 kb were purified from agarose gels and subsequently ligated into the *Bam*HI site of pWV138. *E. coli* MC1061 was transformed with 0.02 μg of ligation mixture. Transformants were scraped off the LB plates and combined into six pools each containing approximately 2,500 colonies.

Isolation and labeling of *E. coli* minicells. *E. coli* P678.54 was transformed with the appropriate plasmids, and minicells were isolated and labeled with [^{35}S]methionine according to a modification of the protocol of Clark-Curtis and Curtis (10) as described by Eggink et al. (15). The labeled proteins were separated on an 8% (wt/vol) denaturating acrylamide gel and subsequently analyzed by fluorography as described by Wensink and Witholt (48).

Nucleotide sequencing. A nested set of unidirectional deletions of pAA101 was constructed by using exonuclease III and mung bean nuclease, essentially as described by Henikoff (24). Dideoxy sequencing reactions were done with T7 DNA polymerase, with unlabeled primers and fluorescein-labeled ATP (45, 50). Nucleotide sequencing was done with the Automated Laser Fluorescent DNA sequencer (Pharmacia, Uppsala, Sweden). The nucleotide sequence data were compiled and analyzed by using the programs supplied in the PC/GENE software package (Intelligenetics, Mountain View, Calif.).

Tree construction. An alignment of PFK proteins was constructed by using ClustalW (42) and will be made available on request. The programs supplied in the PHYLIP 3.5c package were used to analyze the phylogenetic relationships among the PFK proteins (18). Distance matrices were calculated with PROTDIS by using Dayhoff's PAM 001 matrix (13). A phylogenetic tree was subsequently constructed via the neighbor-joining method (38) as implemented in the NEIGHBOR program. A maximum-parsimony tree was constructed with the program PROTPARS (jumble option selected; value = 10). The reliability of the branches of the phylogenetic tree was tested via bootstrapping (18) by using SEQBOOT and CONSENSE (500 replicates).

Preparation of cell extracts and enzyme assays. *E. coli* cells containing the appropriate plasmids were grown on LB medium at 37°C. Growth was monitored by measuring the optical density of the culture at 660 nm in a 1-cm-diameter cuvette on a Hitachi U-1100 spectrophotometer. At an A_{660} of 0.5, IPTG (1 mM) was added to induce transcription from the *lac* promoter and growth was allowed to continue for 4 h. Cells were harvested via centrifugation at 7,000 × *g* for 15 min at 4°C and resuspended in 50 mM Tris-HCl (pH 7.5). Preparation of cell extracts and determination of PFK activity was done as described previously (2). Protein concentrations were determined according to the method of Bradford (5) by using the Bio-Rad (Hercules, Calif.) protein determination kit with bovine serum albumin as the standard.

Nucleotide sequence accession number. The nucleotide sequence presented in this paper was entered into GenBank under accession number U32177.

RESULTS

Design of *pfp*-specific probes. We previously purified the PP_i-PFK from *A. methanolica* and determined the amino-terminal amino acid sequence (2). On the basis of the obtained amino-terminal amino acid sequence, two degenerated oligonucleotides which corresponded to the segments DCPGLNA VIRAVV (P38) and KGIEAHGWEIVGF (P37) of the PFK protein of *A. methanolica* were designed (see Fig. 3). Since both probes are based on the amino terminus of PP_i-PFK, it is very likely that they will hybridize to the same restriction fragments of *A. methanolica* chromosomal DNA. Southern hybridization experiments showed that this was indeed the case (data not shown). We therefore concluded that the probes specifically hybridized to the *pfp* gene.

Cloning the *pfp* gene from *A. methanolica*. Total plasmid DNA was isolated from two pools of the *A. methanolica* genomic library and digested with restriction enzymes. The *pfp* probes hybridized to restriction fragments from both pools, indicating that the *pfp* gene is present in the *A. methanolica* genomic library. One of the genomic library pools was diluted, plated on LB plates containing kanamycin, and replica plated. The colonies were scraped off, and total plasmid DNA was isolated and tested for hybridization with the *pfp* probes. This procedure was repeated until a single colony which contained a plasmid with a 12.3-kb insert was identified. Further subcloning reduced the size of the DNA fragment containing the

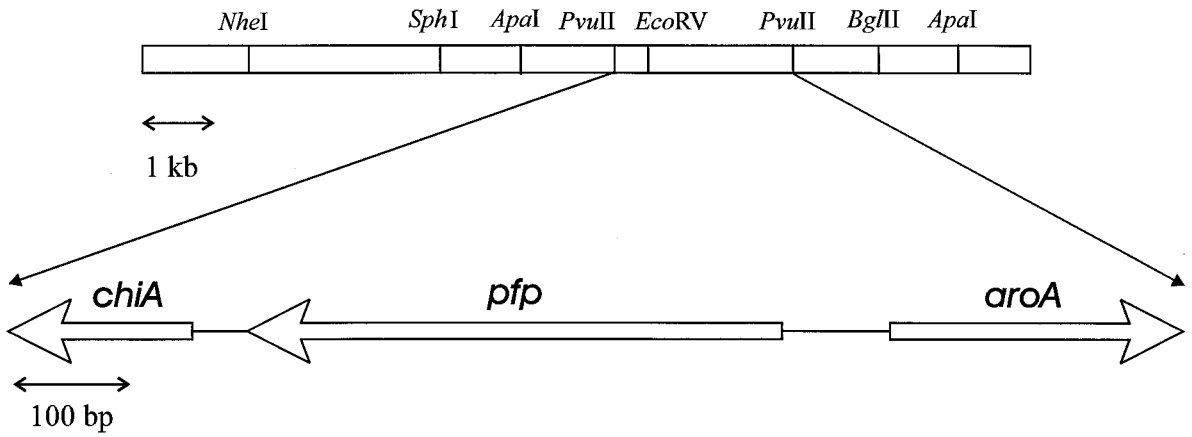

FIG. 1. Restriction map of the DNA fragment containing the *A. methanolica pfp* gene. The top bar displays the 12.3-kb DNA fragment isolated from the genomic library of *A. methanolica*. The bottom bar shows the 2.3-kb *Pvu*II fragment that was sequenced in both directions and the positions of the *aroA*, *pfp*, and *chiA* genes.

sequences hybridizing to the *pfp* probe to a 2.3-kb *Pvu*II fragment.

The 2.3-kb *Pvu*II fragment encodes a 45-kDa protein. In order to determine the number and molecular weights of the proteins encoded on the cloned DNA fragments, a minicell-producing *E. coli* strain was transformed with pAA601, containing a 6-kb *Apa*I fragment, and pAA101, which harbors a 2.3-kb *Pvu*II fragment (Fig. 1). Newly synthesized polypeptides were labeled with [^{35}S]methionine and were analyzed on denaturing acrylamide gels before being subjected to fluorography (Fig. 2). Two unique proteins with molecular masses of 40 and 45 kDa were present in minicells harboring pAA601, whereas only the latter protein was present in minicells containing pAA101. The subunits of the previously purified PP$_i$-PFK of *A. methanolica* each have a molecular mass of 45 kDa (2), which strongly suggests that the 2.3-kb *Pvu*II fragment contains the complete *pfp* gene.

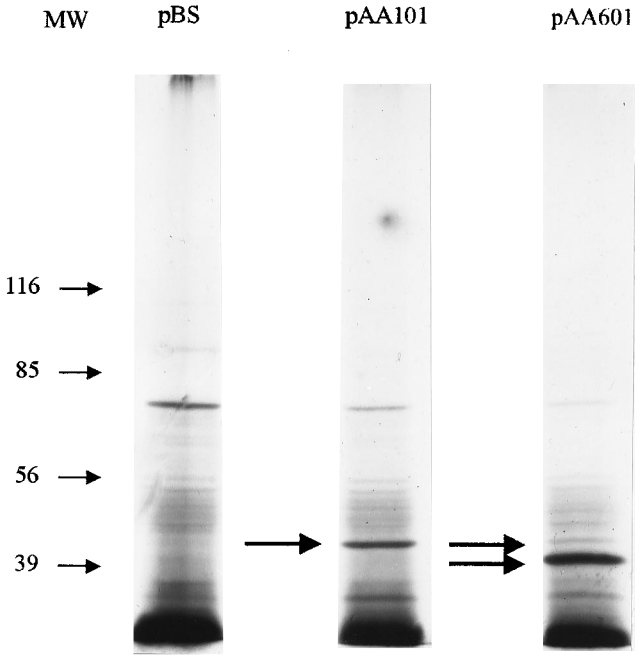

FIG. 2. Proteins synthesized by *E. coli* minicells containing the recombinant plasmids pAA101 and pAA601. The minicell-producing *E. coli* P678.54 was transformed with the recombinant plasmids and with pBluescriptKS$^+$ as a control plasmid. The purified minicells were labeled with [^{35}S]methionine, and the polypeptides were analyzed by denaturing polyacrylamide gel electrophoresis followed by fluorography. Numbers refer to the molecular weight (MW) markers (in thousands).

Expression of the *pfp* gene in *E. coli*. Further confirmation of expression of the *pfp* gene in *E. coli* was obtained by determining the PP$_i$-PFK activity in the crude extracts of *E. coli* DH5α transformed with pAA101 and pAA108, before and after induction with IPTG (Table 2). *E. coli* cells harboring pAA101, which contains the *pfp* gene in the same orientation as the *lac* promoter, had a PP$_i$-PFK activity 100-fold higher than did *E. coli* cells transformed with pAA108, which has the *pfp* gene in the opposite orientation. This shows that expression of the *pfp* gene in *E. coli* is dependent on the *lac* promoter. PP$_i$-PFK activity was absent in *E. coli* containing pBluescript. We therefore conclude that the *pfp* gene is located on the 2.3-kb *Pvu*II fragment.

DNA sequence of the *A. methanolica pfp* gene. The nucleotide sequence of the 2.3-kb *Pvu*II fragment containing the *pfp* gene was determined in both directions by the dideoxy method (50) by using unlabeled primers and fluorescein-labeled ATP (45). The nucleotide sequence contains an open reading frame (ORF), ORFA, of 340 codons, starting with an ATG codon at position 806 which is preceded by a plausible ribosome binding site (Fig. 3). The deduced amino-terminal sequence of the protein encoded by ORFA is identical to the amino-terminal amino acid sequence of PP$_i$-PFK of *A. methanolica* (2). Furthermore, a search of the nonredundant nucleotide database at the National Center for Biotechnology Information with the deduced amino acid sequence of ORFA as the query sequence, using BlastX, revealed extensive similarities to PFK proteins.

TABLE 2. Heterologous expression of the gene encoding PP$_i$-PFK of *A. methanolica* in *E. coli* DH5α

Plasmid	PFK activity[a]	
	Before induction	After induction
pAA101	<1	210
pAA108	<1	2
pBluescriptKS$^+$	<1	<1

[a] Enzyme activity is measured in nanomoles per minute per milligram of protein.

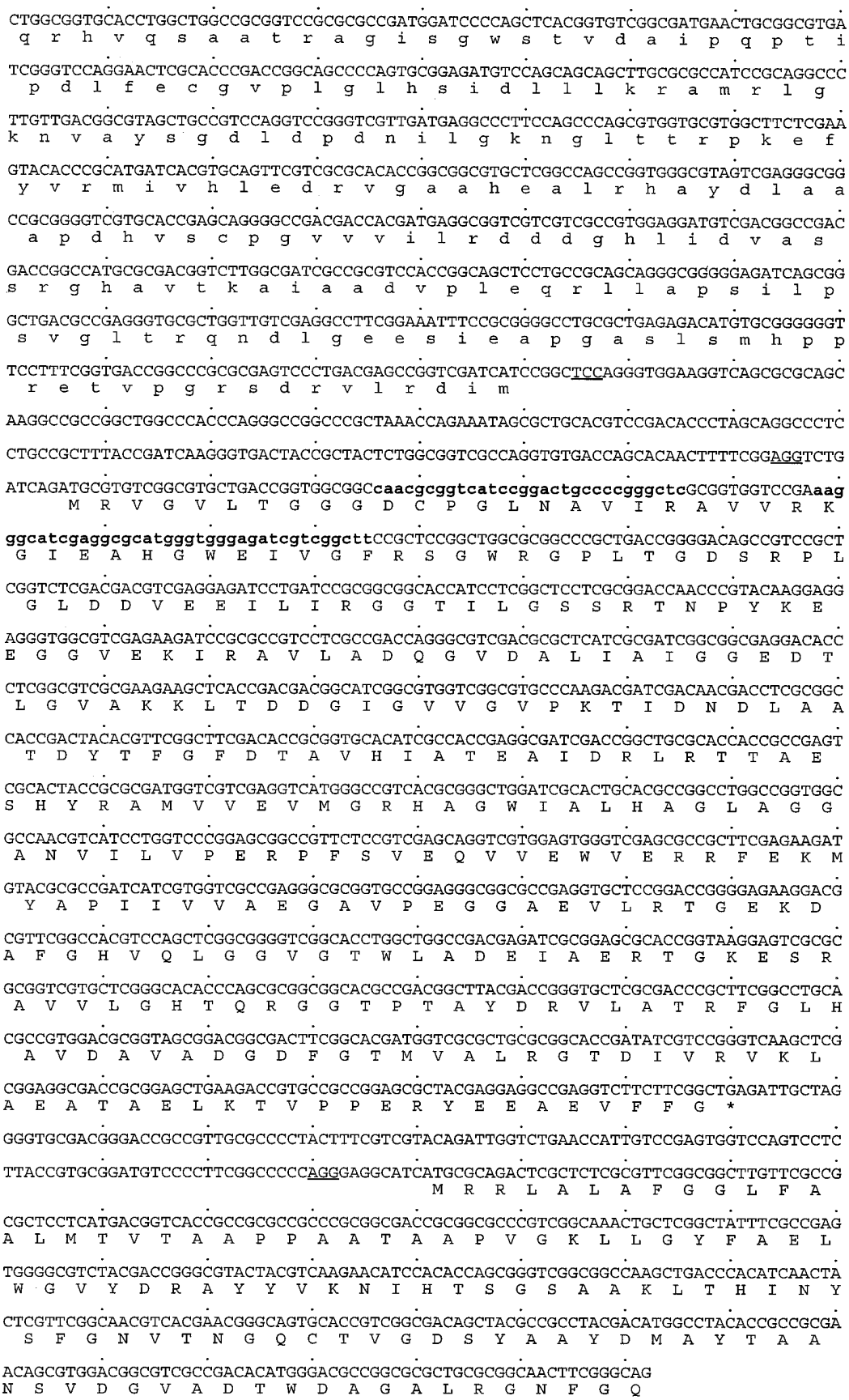

FIG. 3. Nucleotide sequence of the 2,302-bp *Pvu*II fragment containing the complete *pfp* gene and the partial *aroA* and *chiA* genes. The oligonucleotides used in the Southern hybridization experiments are shown in lowercase boldface characters, and putative ribosome binding sites preceding the *pfp*, *aroA*, and *chiA* genes are underlined. The translated amino acid sequence is shown beneath the nucleotide sequence in one-letter code. The amino acid sequence of AroA was derived from the reverse complement and is indicated by lowercase characters.

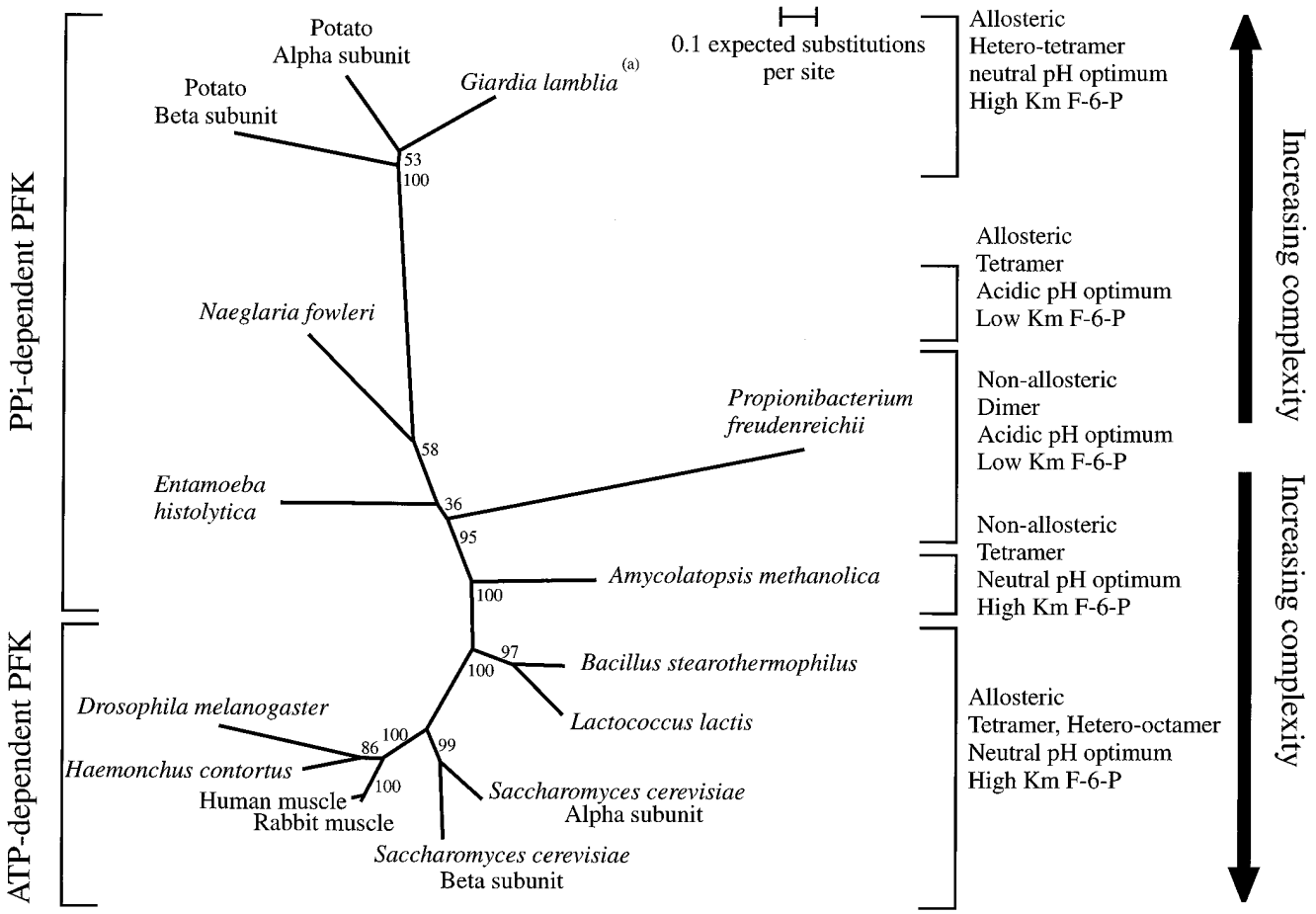

FIG. 4. Phylogenetic relationships of ATP- and PP$_i$-PFKs. The tree is based on a distance analysis of the PFK segments involved in ATP and fructose-6-phosphate binding from *A. methanolica* (residues 1 to 223), *Bacillus stearothermophilus* (residues 2 to 224) (39), *Drosophila melanogaster* (residues 18 to 269) (11), *E. histolytica* (residues 36 to 264) (26), *G. lamblia* (residues 69 to 311) (37), *Haemonchus contortus* (residues 32 to 282) (27), human muscle (residues 16 to 266) (34), *Lactococcus lactis* (residues 2 to 224) (30), *Naegleria fowlerii* (residues 18 to 263) (49), potato α subunit (residues 87 to 328) (7), potato β subunit (residues 42 to 283) (7), *P. freudenreichii* (residues 3 to 268) (21, 28), rabbit muscle (residues 16 to 266) (29), *Saccharomyces cerevisiae* α subunit (residues 206 to 457) (22), and *S. cerevisiae* β subunit (residues 197 to 449) (22). Bootstrap values (in percentages) are based on 500 replicates and are given at each internal node. The structural and biochemical characteristics of the PFK proteins, subunit composition, allosteric behavior, pH optimum of the glycolytic reaction, and the K_m for fructose-6-phosphate ($K_{mF\text{-}6\text{-}P}$), are given next to the brackets. The arrows indicate a trend from nonallosteric, dimeric enzymes with an acidic pH optimum and a low $K_{mF\text{-}6\text{-}P}$ to allosteric multimeric enzymes with a neutral pH optimum and a high $K_{mF\text{-}6\text{-}P}$ (a), The enzyme of *G. lamblia* represents the only exception; this protein has the potato enzyme as its closest relative, whereas it shares the biochemical and structural properties of the enzymes from *E. histolytica* and *P. freudenreichii*.

We therefore identified ORFA as the PP$_i$-PFK-encoding gene (*pfp*) from *A. methanolica*. The predicted molecular mass of the 340 amino acids from the Pfp protein is 36,229 Da, which is lower than the molecular mass of the purified PFK subunit. This could be due to the hydrophobic nature of the PFK subunit, which may cause it to migrate anomalously on a denaturing acrylamide gel (30).

Two partial ORFs were detected upstream (ORFB) and downstream (ORFC) from the *pfp* gene (Fig. 3). The deduced amino acid sequence of ORFB is very similar to the sequence of the 3-deoxy-D-arabino-heptulosonate-7-phosphate synthases (DAHP synthases) of *E. coli* (AroG, 52%) (12) and *Corynebacterium glutamicum* (AroF, 52%) (8), which catalyze the first step in the biosynthesis of aromatic amino acids via the shikimate pathway. ORFC is transcribed in the same direction as the *pfp* gene. The protein encoded by ORFC is 70% identical to chitinase proteins from various *Streptomyces* species (19, 43). We therefore tentatively identified ORFB as *aroA*, encoding DAHP synthase, and ORFC as *chiA*, encoding chitinase.

Phylogenetic relationships of PFKs. A previously observed correlation between the presence of PP$_i$-PFK and anaerobic metabolism has led to the suggestion that PP$_i$-PFK proteins arose on various occasions from ATP-dependent enzymes in response to anaerobic growth conditions (33). To test this hypothesis, a phylogeny of PFK proteins was inferred. A striking feature of the primary structures of PFK proteins is the huge difference in length, which makes an alignment of the full-length protein sequences difficult. Only the sequences containing the amino acids which participate in substrate binding (23, 28, 41) were therefore used in constructing a PFK sequence alignment.

Initially, a phylogenetic tree of ATP-PFK proteins was constructed by the neighbor-joining method in order to examine their relationships. The PFK proteins from mammals, yeasts, insects, and bacteria each formed tight clusters (data not shown). Subsequently, two representatives of each cluster were taken and aligned with the sequences of PP$_i$-dependent proteins, after which a phylogenetic tree was constructed by the neighbor-joining method (Fig. 4). The PP$_i$-PFK enzymes form a monophyletic group. The branch leading to the PP$_i$-dependent enzymes is supported by a high bootstrap value (100%). A parsimony analysis resulted in one most parsimonious tree

with the same topology as that of the tree constructed by the neighbor-joining method (data not shown).

DISCUSSION

We have previously purified and characterized the PP_i-PFK of *A. methanolica* (2). The properties of this enzyme are intermediate between those of ATP-dependent and PP_i-dependent enzymes: the use of PP_i and the lack of allosteric regulation are typical for PP_i-dependent enzymes, but the amino-terminal amino acid sequence, the pH optimum, the Michaelis constant for fructose-6-phosphate, and the quaternary structure (tetramer) are similar to those of the ATP-dependent enzymes (20). This paper describes the cloning of the *pfp* gene and the primary structure and phylogenetic position of this unusual PFK enzyme.

The *pfp* gene of *A. methanolica*, encoding the PP_i-PFK, was identified on a 2.3-kb *Pvu*II fragment. The bacterial *pfk* genes encoding the ATP-PFK are usually organized in an operon together with the pyruvate kinase-encoding gene (6, 9, 30, 39). The *pfp* gene is flanked by two ORFs which were tentatively identified as *aroA* and *chiA* on the basis of the high degree of similarity with DAHP synthase- and chitinase-encoding genes, respectively. The *aroA* gene is located upstream and transcribed divergently from the *pfp* gene, indicating that the transcription of the *pfp* gene is initiated in the *aroA-pfp* intergenic region. Since the intergenic region between *aroA* and *pfp* is small (195 bp), the *aroA* and *pfp* promoters may overlap.

PFK is one of the key enzymes in glycolysis, and its expression level may have a dramatic effect on the intracellular concentrations of glycolytic intermediates such as phosphoenolpyruvate, glyceraldehyde phosphate, and fructose-6-phosphate. DAHP synthase (AroA) catalyzes the first step of the shikimate pathway, for which phosphoenolpyruvate and erythrose-4-phosphate, which is produced from the glycolytic intermediates fructose-6-phosphate and glyceraldehyde phosphate by transketolase, are the substrates. The activity of PP_i-PFK may therefore influence the flux through the shikimate pathway by controlling the substrate levels of DAHP synthase. Overlapping *aroA* and *pfp* promoters may provide the cell with a means to balance the carbon flux through glycolytic and shikimate pathways via the interdependent regulation of the expression levels of DAHP synthase and PP_i-PFK.

The 11 residues which have been implicated in binding of fructose-6-phosphate by the ATP-dependent enzyme of *E. coli* (28, 41) are all conserved in the *A. methanolica* enzyme. Three of 10 residues (Gly-12, Tyr-42, and Arg-73) of the *E. coli* enzyme which are involved in binding of ATP are conserved in the PP_i-dependent enzyme of *A. methanolica*. The Ser-106 and Arg-78 residues of the *E. coli* enzyme are replaced by Thr and Lys in the *A. methanolica* enzyme, which represent conservative substitutions. The conservation of residues involved in ATP binding in the PP_i-dependent enzyme of *A. methanolica* would indicate that these residues are also involved in PP_i binding. Current research focuses on the biochemical characterization of mutant *A. methanolica* PFK proteins in which these residues have been altered.

It has been suggested that the PP_i-PFK is more suited for a role in anaerobic metabolism than is its ATP-dependent counterpart and may have evolved from ATP-PFK on several independent occasions in response to anaerobiosis (33). In this scenario, the *A. methanolica* enzyme would have arisen from a bacterial ATP-dependent enzyme. The phylogenetic analysis of PFK proteins clearly shows that the PP_i-dependent enzymes form a monophyletic group. This contradicts the hypothesis that the PP_i-dependent enzymes evolved many times from ATP-dependent enzymes (33) and supports the view that both types of PFK evolved from a common ancestor.

Since anaerobic metabolism existed before oxygen evolved, the early PFK proteins must have been adapted to anaerobiosis. Interestingly, the simplest PFK proteins, PP_i-dependent homodimeric enzymes lacking allosteric control, are encountered in *P. freudenreichii* (28), *Giardia lamblia* (32), and *Entamoeba histolytica* (26), which have an obligate anaerobic metabolism. The last two species are considered to have branched off from the eukaryotic lineage before *Eucarya* acquired mitochondria (33). The phylogenetic analysis places the enzymes from *P. freudenreichii* and *E. histolytica* in the middle of the tree. Interestingly, there is a strong correlation between the structural and biochemical complexity of the PP_i- and ATP-PFK proteins and the phylogenetic distance between these proteins and those from *P. freudenreichii* and *E. histolytica* (Fig. 4). On the basis of this correlation, we propose that the ancestral PFK resembled the enzymes encountered in *P. freudenreichii* and *E. histolytica* from which the allosteric (hetero)tetrameric PP_i-dependent enzymes and the tetrameric and hetero-octameric ATP-dependent enzymes encountered in plants, bacteria, insects, yeasts, and mammals were derived (Fig. 4). The discovery of an enzyme from *A. methanolica* with molecular and biochemical characteristics which are intermediate between those of bacterial ATP-dependent enzymes and the PP_i-dependent enzymes of the type found in *P. freudenreichii* and *E. histolytica* strengthens this hypothesis.

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