

Pyruvate-Phosphate Dikinase of Oxymonads and Parabasalia and the Evolution of Pyrophosphate-Dependent Glycolysis in Anaerobic Eukaryotes†

Claudio H. Slamovits and Patrick J. Keeling*

Canadian Institute for Advanced Research, Botany Department, University of British Columbia, 3529-6270 University Boulevard, Vancouver, British Columbia V6T 1Z4, Canada

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In pyrophosphate-dependent glycolysis, the ATP/ADP-dependent enzymes phosphofructokinase (PFK) and pyruvate kinase are replaced by the pyrophosphate-dependent PFK and pyruvate phosphate dikinase (PPDK), respectively. This variant of glycolysis is widespread among bacteria, but it also occurs in a few parasitic anaerobic eukaryotes such as *Giardia* and *Entamoeba* spp. We sequenced two genes for PPDK from the amitochondriate oxymonad *Streblospio trichomonas* and found evidence for PPDK in *Trichomonas vaginalis* and other parabasalia, where this enzyme was thought to be absent. The *Streblospio* and *Giardia* genes may be related to one another, but those of *Entamoeba* and perhaps *Trichomonas* are distinct and more closely related to bacterial homologues. These findings suggest that pyrophosphate-dependent glycolysis is more widespread in eukaryotes than previously thought, enzymes from the pathway coexists with ATP-dependent more often than previously thought and may be spread by lateral transfer of genes for pyrophosphate-dependent enzymes from bacteria.

Adaptation to anaerobic metabolism is a complex process involving changes to many proteins and pathways of critical function to the cell, but it is found in several distantly related eukaryotic lineages (25, 26), raising questions of how it evolved. Some of these anaerobic protists were previously thought to be ancient lineages that retained anaerobic metabolism as a primitive, premitochondrial trait, but extant eukaryotic anaerobes are now nearly all known to have evolved from mitochondrion-bearing ancestors (8, 10, 15), so the acquisition of anaerobiosis-related traits is most likely secondary.

Embden-Meyerhof-Parnas glycolysis is the starting point of the core carbon metabolism in eukaryotes. In aerobic eukaryotes, glycolysis is a minor source of energy, since ca. 95% of ATP production comes from subsequent tricarboxylic acid cycle reactions and oxidative phosphorylation. In contrast, anaerobic organisms rely almost exclusively on glycolysis and fermentation for ATP production, so optimizing the energy output from glycolysis may be subject to strong selective pressure (21, 22). One significant variation of the standard glycolytic pathway that has been described in some anaerobic protists is pyrophosphate-dependent glycolysis, which uses pyrophosphate (PP_i) instead of ATP as a phosphate donor (21). This version of glycolysis has been best studied in *Entamoeba histolytica* (29, 30). In this parasite, two key glycolytic enzymes, phosphofructokinase (PFK) and pyruvate kinase (PK), have been replaced by the PP_i-dependent versions, PP_i-phosphofructokinase (PPFK) and pyruvate-phosphate dikinase

(PPDK), respectively (for a comparison of these reactions, see reference 21). Energy efficiency seems to be the chief reason for adopting a PP_i-dependent glycolysis, since PP_i-dependent glycolysis can in theory yield five ATP molecules instead of the two yielded by standard glycolysis. This increase of 2.5-fold can be crucial under severe energy-limiting conditions (21, 22).

PPDK catalyzes the interconversion between phosphoenolpyruvate (PEP) and pyruvate. Unlike the reaction catalyzed by PK, this reaction has a small ΔG and is reversible, so it can proceed either in the catabolic or the gluconeogenic directions (34). This highly versatile enzyme has been appointed to many functions in different eukaryotes, although its coexistence in some cells with the ATP-dependent enzyme PK makes it difficult to address its role (21, 26). Involvement of PPDK in glycolysis has been shown or suggested in *Giardia* (28), *Entamoeba* (30), and *Phytomyxa* (18). PPDK also works in the opposite direction in chloroplasts of C4 plants, where it catalyzes the regeneration of PEP in the stroma of leaf mesophyll cells (6). A cytoplasmic isoenzyme of unclear function also exists in C3 and C4 plants, and it has been suggested that it may have a glycolytic role, especially in oxygen-starved tissues (22). Trypanosomatids target PPDK to glycosomes where it fulfills a thermodynamic role of pyrophosphate recycling in place of pyrophosphatase (1).

At present, studies on the molecular evolution of PP_i-linked glycolysis have focused almost exclusively on PFK and PPF (3, 27). These proteins are homologous, and the gene family has had a complex evolutionary history including multiple events of duplication, loss, and lateral gene transfer (3, 27). In contrast, the phylogeny of PPDK has not been extensively explored. Previous studies using limited taxon sampling addressed the relationships of certain eukaryotic PPDKs, but whether eukaryotic enzymes originated in common or independently has not been clear (it has been shown that a plant

* Corresponding author. Mailing address: Canadian Institute for Advanced Research, Botany Department, University of British Columbia, 3529-6270 University Boulevard, Vancouver, British Columbia V6T 1Z4, Canada. Phone: (604) 822-4906. Fax: (604) 822-6089. E-mail: pkeeling@interchange.ubc.ca.

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cluster is separated from other eukaryotes [17, 28]). In addition, it is not known whether PPK and PFP are always found together, or if some organisms use one PP_i-dependent enzyme in glycolysis but not the other. In *Giardia* and *Entamoeba* both PP_i-dependent enzymes are used, but in *Trichomonas vaginalis*, PP_i-specific PFK and ATP-specific PK have been found, but no PPK activity has been detected (24). Interestingly, neither PK nor PPK activities have been detected in the related *Tritrichomonas fetus* (14, 21).

One group of amitochondriates of which we know relatively little are the oxymonads. These anaerobes are found almost exclusively in association with animals and are generally not available in cultivation. Oxymonad molecular and biochemical data are scarce because they are typically part of a complex community of eukaryotes from which it is difficult to determine which genes or proteins come from which species. We have used the recent documentation of a noncanonical genetic code in one oxymonad, *Streblomastix strix*, to circumvent this problem (16). *S. strix* is found exclusively in the hindgut of the Pacific damp-wood termite *Zootermopsis angusticollis*, an environment it shares with at least six species of parabasalia (37). From this environment we isolated PPK genes and used the noncanonical genetic code to identify *S. strix* homologues. We also identified other protist PPK genes and, comparing them to *T. vaginalis* genomic data identified the first parabasalian PPK genes, overturning the notion that *T. vaginalis* lacks this enzyme. A comprehensive phylogenetic analysis of PPK shows several independent clades of eukaryotes, overall suggesting at least some lateral gene transfer may have occurred.

MATERIALS AND METHODS

cdNA library construction and EST sequencing. Termites were collected from a rotten log in Point Grey, Vancouver, Canada. The whole hindgut content of about 60 individuals of *Z. angusticollis* from a single colony was collected and total RNA was extracted using standard TRIZOL procedure (Invitrogen). A cdNA library was constructed (Amplicon Express) and 5,337 clones were sequenced from the 5' end. Expressed sequence tags (ESTs) were trimmed for vector and quality, and assembled into 2,595 clusters by PEPdb (<http://amoebidia.bcm.umontreal.ca/public/pepdb/agrm.php>).

Characterization of *S. strix* PPK. *S. strix* sequences were recovered from EST sequences by scanning BLASTX outputs to identify putative protein coding sequences containing in-frame TAA and TAG stop codons. Two truncated ESTs with high identity scores to PPK were recovered according to this criterion. The C-terminal portion of both was acquired by 3'RACE (Ambion) using specific primers and total hindgut RNA. Most of the 5' portion of the gene was obtained by reverse transcription-PCR (RT-PCR) amplification using a degenerate primer designed from a conserved block located at about 90 amino acids from the start of the *G. intestinalis* PPK and gene-specific primers designed from the EST sequences. In all cases, PCR products were cloned by using TOPO cloning system (Invitrogen). Sequences were assembled with Sequencher, and the sequence was confirmed to come from a single gene by amplifying from DNA using primers at the ends of the assembled contig. Parabasalian PPK and PFP sequences from the EST data were identified by comparing them to the *T. vaginalis* genome project. *T. vaginalis* preliminary sequence data was obtained from The Institute for Genomic Research (TIGR; <http://www.tigr.org>, database).

Sequence and phylogenetic analyses. Deduced amino acid sequences of new and publicly available PPK sequences were aligned by using CLUSTALX and edited manually using McCLade. Several ongoing eukaryotic genome and EST projects were also searched, from which the *T. vaginalis* (TIGR) and *T. pseudonana* (genome.jgi-psf.org/thaps1/thaps1.home.html) were retrieved. Sequences were aligned by using CLUSTALX and adjusted with McCLade. Gaps and ambiguously aligned residues were excluded. This resulted in a matrix of 54 sequences with 713 positions. Maximum-likelihood trees were built by using PhyML (<http://atgc.lirmm.fr/phyml/>) with the WAG model of amino acid substitutions and eight categories of rates, with gamma and proportion of invariant

sites estimated from the data. Support of the nodes was estimated by using 100 bootstrap replicates.

RESULTS AND DISCUSSION

Identification of PPK genes from the oxymonad *S. strix* and parabasalian symbionts. From an EST sequence survey of the eukaryotic fauna from the hindgut of the termite *Z. angusticollis*, we identified three ESTs matching the PPK family and other related PEP-utilizing enzymes. Two sequences contained several in-frame, TAA and TAG stop codons, identifying them as belonging to the flagellate *S. strix*, which has an alternate genetic code where TAA and TAG encode glutamine (Q) instead of translational stops (16). One of these sequences was extended by using a combination of RT-PCR with degenerate primers and 3' RACE so that 88% of the gene was ultimately characterized. Attempts to recover the extreme 5' end of the gene by 5' RACE were unsuccessful, probably due to the highly complex nature of the sample and the large size of the gene (the *G. intestinalis* homologue is 2,655 bp). These procedures resulted in a nucleotide sequence encoding an open reading frame of 780 codons. The second *S. strix* sequence differed only slightly at the nucleotide level, indicating that at least two gene copies or alleles of this gene exist in this *S. strix* population and so was not characterized further. Since *ppdk* belongs to a gene family with at least three functionally differentiated clusters, we wanted to identify which group the *S. strix* sequences belong to. Phylogenetic analyses of the *S. strix* gene, including members of the PPK, water dikinase, and glucan dikinase groups of the family, clearly revealed that the *S. strix* sequences belong to the PPK family (not shown).

The third EST we identified was not highly similar to the *S. strix* genes and did not contain in-frame stop codons. The other major eukaryotic constituents of this environment are several species of parabasalia, which are thought not to contain PPK (24). To determine whether this PPK could come from a parabasaliid, we performed extensive BLAST searches against NCBI databases and found 13 ESTs from *T. vaginalis* with high similarity to PPK. Twelve partially overlapping ESTs assembled to form an open reading frame corresponding to the last 531 amino acids of the PPK. The remaining EST encompassed a nonoverlapping stretch from positions 25 to 286 (according to *C. symbiosum* PPK numbering). Searching the *T. vaginalis* genome project at TIGR, we found gene predictions that perfectly matched these ESTs. Interestingly, the *T. vaginalis* project has at least two PPK sequences, a finding consistent with previous findings that the *T. vaginalis* genome contains multiple copies of several genes for metabolic enzymes. Comparing the *T. vaginalis* PPKs to our EST data showed that the two were very similar, so we infer that this sequence belongs to one of the six species of parabasalia present in *Z. angusticollis* (37), and tentatively named it "parabasalian endosymbiont." The sequence of this EST was extended by RT-PCR to 1,680 bp encoding 653 amino acids.

Conservation of key structural features in *S. strix* and *T. vaginalis* PPKs. Certain enzymes can change their substrate specificity with a few substitutions at catalytically active sites. A clear example is PFK, which has been shown to have switched from ATP to PP_i specificity several times independently (3, 23, 27). This transformation requires only a few substitutions that

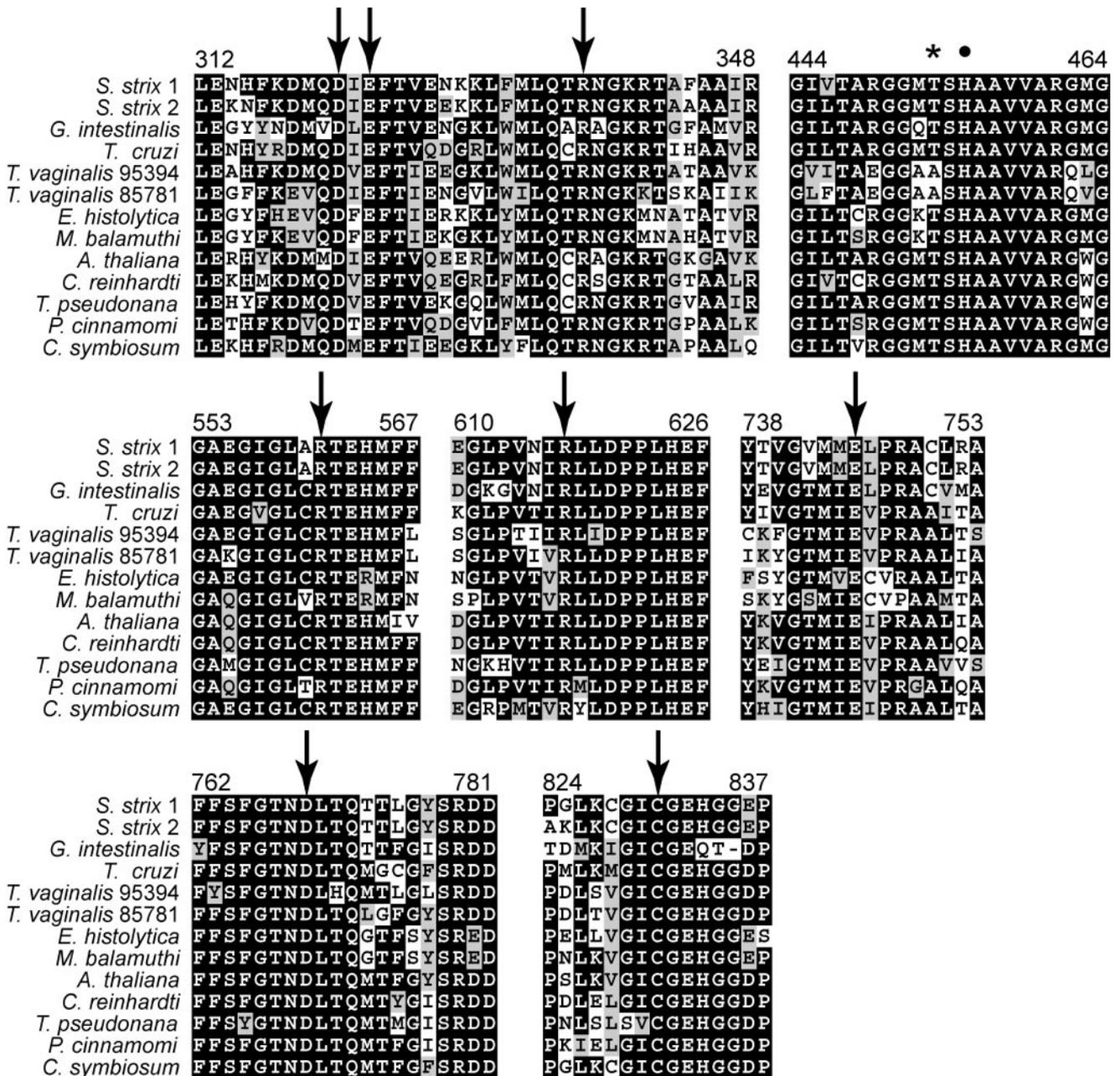


FIG. 1. Sequence alignment of PPDKs from several eukaryotes and *C. symbiosum* showing conservation of important sites for catalytic function. Numbers follow the residue position in *C. symbiosum*. Arrows indicate positions that have shown to be involved in nucleotide and substrate binding. The dot shows the active site histidine, and the asterisk indicates the site involved in regulation of PPDK activity in plants (see the text).

control the entry of ATP into the active site, so that the position of a sequence in a phylogeny does not necessarily predict its function for these enzymes (3). Although functional assays are needed to ultimately demonstrate the actual substrate specificity, careful mapping of the critical positions are usually helpful to predict the substrate specificity, provided that sufficient information on the structure and physicochemical properties of the enzyme exists. Although no cross-substrate specificity has been reported between the ATP and PP_i pyruvate kinases, we examined the *S. strix* and *T. vaginalis* sequences and looked for conservation of the residues involved in substrate

interaction and enzymatic function. We aligned these with PPDKs from the protists *Giardia*, *Entamoeba*, *Trypanosoma*, along with maize and *C. symbiosum*. The latter has been subject to detailed mutagenesis and structural analysis, from which the positions essential for catalytic function have been identified (4, 19, 20, 32, 33, 35, 36, 38). All but two positions identified as catalytically important in *C. symbiosum* are included in the known *S. strix* sequences, and every one is conserved in *S. strix* and *T. vaginalis* sequences, including the reactive center His455 (Fig. 1). One notable difference found between the parabasalian and other PPDK enzymes is two residues up-

stream of the catalytic His458, where a threonine is conserved in all but the parabasal (Fig. 1 and Fig. S1 in the supplemental material). In C4 plants, this threonine is directly involved in a particular form of light-dependent regulation by a bifunctional protein kinase/phosphatase (5, 6). Briefly, nocturnal inactivation of the enzyme is achieved by ADP-dependent phosphorylation of Thr453 by the PDK regulatory protein (RP), whereas the opposite (dephosphorylation) is catalyzed by RP in light conditions. Although this residue is not directly involved in catalysis, and site-directed mutagenesis has shown that some substitutions of Thr453 have little or no negative effect on activity, it is an important potential control point (5, 7). The *T. vaginalis* and the "parabasal symbiont" sequences have an alanine at this position (Fig. 1 and Fig. S1 in the supplemental material), and experiments of mutagenesis in maize indicate that activity is lost when bulky or strongly charged residues such as phenylalanine, tyrosine, and the acidic amino acids replace threonine, but not when serine and the aliphatic valine are assayed in place of Thr453 (5, 7). Although functional characterization is ultimately needed, the evidence suggests that the Thr-Ala substitution seen in *T. vaginalis* would likely not impede PDK function.

Phylogeny and evolution of PDK. Although they share many metabolic similarities, eukaryotic microbes with PP_i -linked glycolysis are not all closely related to one another (26). This was noted some time ago (25, 26), and it was debated whether their metabolism was retained from an ancient ancestor or was a derived adaptation to anaerobic environments. Current data seem to favor the latter interpretation (3). PFK has been extensively sampled (3, 23, 27) and is now known to have a very complex history due to a combination of early duplications and multiple independent losses, as well as some lateral gene transfer events. The phylogeny of PDK has not been explored in great detail, so we analyzed PDK sequences from the three domains of life to determine whether the eukaryotic homologues share a common ancestor. A preliminary analysis, including about 100 sequences of purported PDKs and another member of the extended gene family (water dikinase), confirmed the monophyly of PDK enzymes (not shown). PDK genes were subsequently analyzed alone to allow more positions to be included. The overall level of resolution for this phylogeny is not very high, but several nodes are well supported. The maximum-likelihood tree topology (Fig. 2) is generally not consistent with the known relationships within and among different groups of bacteria, archaea, and eukaryotes. For the most part this is probably due to lack of resolution in the phylogeny, but there may also be a case for some ancient duplication events and the incongruent distribution of taxa in some of the well-supported nodes suggests that at least one event of lateral gene transfer probably also occurred (Fig. 2).

PDK is broadly represented in eubacteria, although its representation varies from group to group. For example, PDK is scarce among proteobacteria in general but ubiquitous in the alpha subdivision. In archaea, PDK is restricted to a few groups from both crenarchaeotes and euryarchaeotes, even though more than 40 complete genomes are known. This, and the fact that the archaeal sequences do not cluster together are suggestive of lateral acquisition from eubacteria (Fig. 2). Some bacterium-only groupings in the PDK tree

contain unrelated species, but most of these nodes have low bootstrap support (<50), making it impossible to infer anything about their evolution, although it is noteworthy that some groups such as firmicutes, actinobacteria, and proteobacteria show discontinuous distribution (Fig. 2).

Eukaryotic PDK sequences do not form a monophyletic group. The largest eukaryotic clade comprises plant and green algae, as well as *Phytophthora* and *Thalassiosira* sequences. This grouping is well supported by all reconstruction methods used. *Phytophthora* and *Thalassiosira* are heterokonts, which diverged from the green lineage deep in eukaryotic history. Two explanations for this observation are possible: plant and heterokont PDK derive from an ancestral gene in early eukaryotes that was lost elsewhere, or they were independently acquired from related sources. It is tempting to postulate some link to plastid evolution, given its involvement in photosynthesis in some plants and that the diatom is also photosynthetic and plastid-related genes have been found in *Phytophthora* (2). No cyanobacteria has yet been shown to possess a related PDK gene, however, and none of these genes encode plastid-targeted proteins, so this link is perhaps coincidence. The sequences from the trypanosomatid parasites *T. cruzi* and *T. brucei* branch together, as expected, but show no well-supported association to any other organism in the present sample. The trypanosomatid PDKs are glycosomal and used to recycle pyrophosphate accumulated from various biosynthetic reactions (1). A controversial hypothesis that trypanosomatids evolved from a plastid-harboring ancestor (13) suggested that PDK was acquired from the phototrophic endosymbionts (12). However, the trypanosomatid PDK shows no association with the plant-algal cluster as would have been expected, much like other genes proposed to be endosymbiont-derived (9).

Among amitochondriate eukaryotes, the only relationship that is consistently found by different reconstruction methods is the branch uniting *Giardia* and *Streblomastix*. This node is moderately supported by bootstrap (69%); however, adding the PDK from a sister-lineage to oxymonads, *Trimastix pyriformis* (A. J. Roger, unpublished data), this value increases to 76% (not shown). Common ancestry between the *Giardia* and *Streblomastix* sequences is consistent with the hypothetical eukaryotic supergroup Excavata (11, 31). The PDK from *T. vaginalis*, which is also an excavate, does not branch with these sequences but rather with actinobacteria and *Thermus* spp. with moderate bootstrap support. Finally, PDK from the amitochondriate amoebae *E. hystolytica* and *M. balamuthi* branch within a strongly supported clade containing the gamma-proteobacterium *Methylococcus* and the crenarchaeon *Pyrobaculum*. Overall, there is no support for a single origin of amitochondriate PDK, although the *G. intestinalis* and *S. strix* genes likely branch together, and we cannot rule out a common origin with the *T. vaginalis* gene; the PDK genes from amoebae have an origin apparently independent from those of other eukaryotes.

Evolution of PP_i -dependent glycolysis in amitochondriate eukaryotes. Three of the main questions surrounding the evolution of PP_i -dependent glycolysis are (i) whether the presence of PFP and PDK in glycolysis are correlated with one another, or if only one or the other may be used; (ii) whether or how analogous PP_i - and ATP-dependent enzymes performing

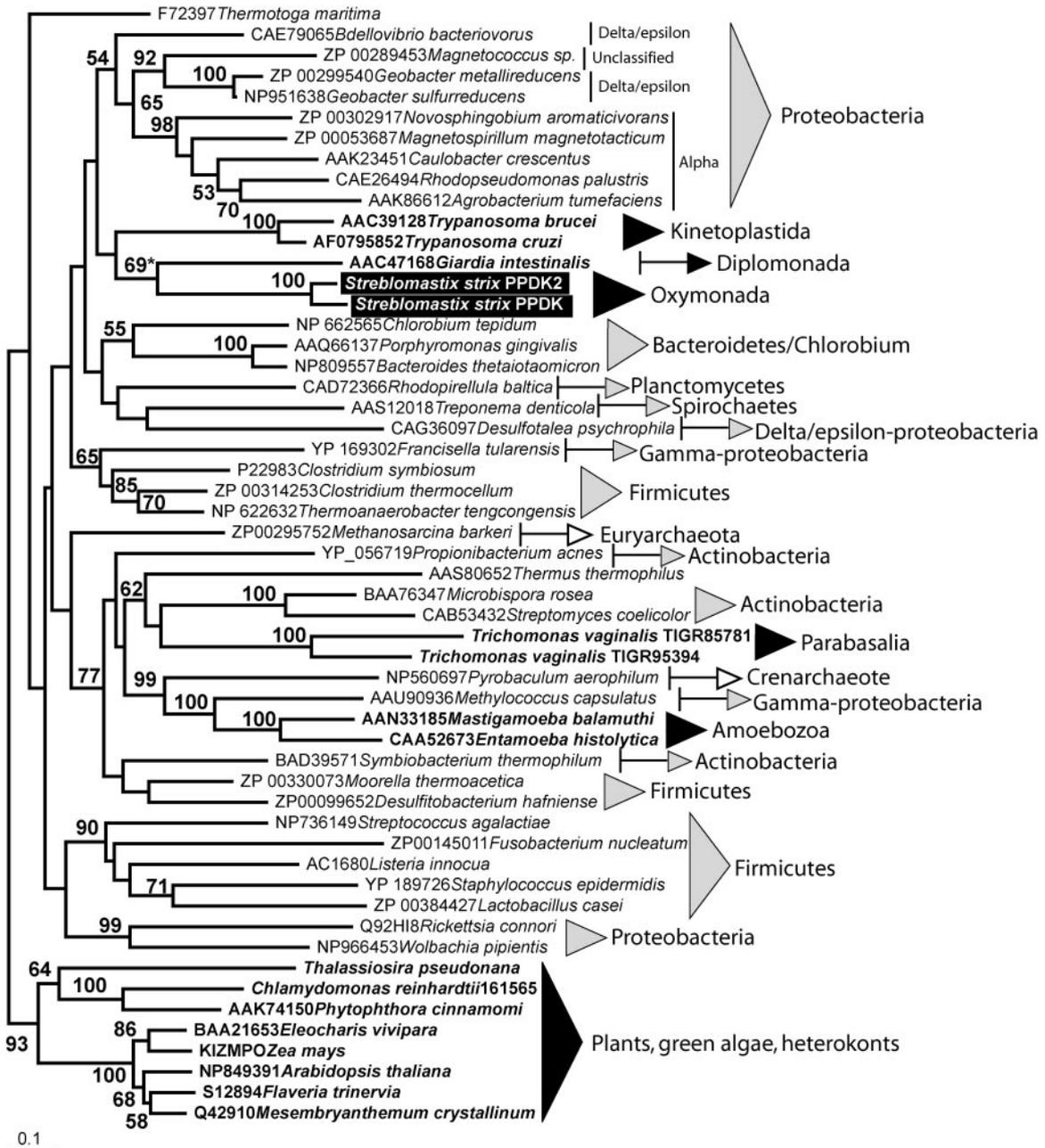


FIG. 2. Maximum-likelihood tree showing the relationships among bacterial, archaeal, and eukaryotic PPDKs. Eukaryotic sequences are in boldface, and the sequences from *S. strix* are marked with black rectangles. Taxonomic groups are indicated with black, gray, and white triangles for eukaryotes, bacteria, and archaea, respectively. Numbers represent bootstrap values (higher than 50%). The bootstrap support for the node uniting *S. strix* and *G. intestinalis* was 69% in the analysis shown but higher (76%) when the unpublished *T. pyriformis* sequence was included (see text).

the same reaction in one organism are functionally differentiated; and (iii) where did the PP_i-dependent enzymes come from and how widespread are they?

Previously nothing was known of oxymonads, and it was thought that parabasalia used PFP but not PPDK. We have now shown that oxymonads and parabasalia both possess PPDK. It is unknown if oxymonads use PFP or PFK to phosphorylate fructose-6-phosphate, or if they also encode PK, but it is possible that their glycolysis is at least partially PP_i depen-

dent. In the case of parabasalia, PPDK was undetected in enzyme assays from *T. vaginalis*, and it was assumed that glycolysis was mixed, such that the phosphorylation of fructose-6-phosphate by PFP was the only PP_i-dependent step. However, *T. vaginalis* and at least one other parabasalian do contain genes for PPDK, further supporting the correlated presence of PFP and PPDK. By analogy to *G. intestinalis*, *E. histolytica*, and now also *T. vaginalis*, we hypothesize that PFP will eventually be found in oxymonads as well. However, *G. intestinalis*, *E.*

histolytica, and now also *T. vaginalis* possess both cytosolic PPDK and PK, so these two analogous enzymes clearly can also coexist in the same compartment, raising questions about whether they are functionally distinct. In plants and trypanosomatids PPDK and PK are sequestered in two different compartments, so this is not an issue. It also suggests that other amitochondriates, such as oxymonads, may be found to possess PK as well as PPDK. This growing list of eukaryotes where both kinds of enzyme are found also suggests that a reevaluation of why these enzymes exist in anaerobes and their possible contribution to energy efficiency may be necessary. Biochemical dissection of the role of ATP- versus PP_i-dependent enzymes is reemerging as an urgent task for understanding the metabolism of these organisms.

As for the origin of the PP_i-dependent enzymes, phylogenetic analyses show that ADP-PFK and PP_i-PFP are not mutually exclusive and PFK has been shown to have switched phosphate-donor specificity several times (3, 27); thus, PFP originated several times independently. In contrast, the phylogeny of PPDK is less complex but lacks the resolution needed to conclusively determine whether there is a single origin for the eukaryotic protein. The PPDK of amitochondriates *G. intestinalis*, *S. strix*, and *T. vaginalis* may have originated in common, but this is not clearly supported by the phylogeny, and the support for an independent origin of at least the amoebozoan PPDK is very strong. If several independent lateral gene transfers explain the distribution of PPDK in amitochondriates, then perhaps further sampling of prokaryotes will show this more clearly. Until then, only tentative conclusions can be drawn. Overall, however, analyses of PFP and PPDK suggest that PP_i-linked glycolysis is a derived adaptation to specific environments or lifestyles rather than a primitive eukaryotic trait, although the known distribution of PP_i-dependent enzymes is growing.

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