Cloning, Expression, and Characterization of Pyrophosphate-
Dependent Phosphofructokinase Gene from
Porphyromonas gingivalis

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Abstract: We have cloned, sequenced, and expressed the gene encoding a pyrophosphate (PPi)-dependent-
phosphofructokinase (PFK) (EC 2.7.1.90), designated PgPFK, from Porphyromonas gingivalis 381. The
PgPFK gene consisted of 1,650 bp which coded for a protein of 550 amino acids with a calculated molecular
mass of 61,044 Da. The deduced amino acid sequence of PgPFK exhibited a significant similarity to the 62
kDa Ppi-PFKs from Borrelia burgdorferi and Treponema pallidum PFKβ-subunit. Most of the amino acid
residues might not be assumed to be involved in substrate binding in other Ppi-PFKs were conserved in PgPFK.
Recombinant PgPFK was purified to homogeneity and characterized. The purified enzyme was found to be
enzymatically active when it was expressed in Escherichia coli and was not regulated by classical effectors
of ATP-dependent PFKs (ATP-PFKs) (EC 2.7.1.11) such as ATP, ADP, or fructose 2,6-bisphosphate like
the known Ppi-PFKs. Reverse transcriptase polymerase chain reaction analysis revealed that the PgPFK
gene was expressed in P. gingivalis. Phylogenetic analysis showed that the long sequence type group of Ppi-
PFKs including PgPFK formed a coherent cluster like the ATP-PFK family. These results suggest that
PgPFK is one of the most typical of Ppi-PFK functioning in P. gingivalis. Moreover, it seems that this
organism might not have any fructose 1,6-bisphosphatase (FBPase). From these results we propose that
PgPFK serves as a FBPase in the gluconeogenic direction.

Key words: Porphyromonas gingivalis, Phosphofructokinase, Pyrophosphate

Introduction

Adult periodontitis is a chronic inflammatory
disease induced by a microbial infection that is the
major cause of the tooth loss in the adult popu-
lation1. Among a variety of bacterial species associat-
ed with the initiation and progression of peri-
donitis, Porphyromonas gingivalis, a gram-negative
anaerobe, is known to be an important pathogen in
severe manifestations of the disease1,2,3. P. gingivalis
has been shown to express a variety of factors which
may play significant roles in pathogenicity,
including adhesins, proteases, endotoxins, and
cytotoxins3. It is reported that P. gingivalis is an
asaccharolytic organism and dependent on
nitrogenous substrates for energy4,5. Although
sugars such as glucose can be utilized by this organ-
ism, these compounds are not converted to meta-
bolic end products, but rather are used for the
biosynthesis of intracellular macromolecules6,7. How-
ever, the detailed mechanism of synthesis
remains to be determined.

Phosphofructokinase (PFK) phosphorylates
fructose 6-phosphate (F6-P) to fructose 1,6-bis-
phosphate (F1,6-BP) and thus occupies a key
position in glycolysis7. Two major types of PFK
have been described with respect to phosphoryl
donor specificity. The more widespread ATP-
dependent PFK (ATP-PFK) utilizes ATP and
catalyzes an irreversible catabolic reaction. How-
ever, another form, pyrophosphate (PPI)-dependent PFK, which utilizes inorganic PPI is found in some bacteria, protists, and all green plants. PPI-PFK catalyzes a reversible reaction of low-free energy change and can function in both glycolysis and gluconeogenesis. PPI-PFKs have recently been characterized from a number of important pathogens including *Giardia lamblia*, *Entamoeba histolytica*, *Toxoplasma gondii*, *Borrelia burgdorferi*, and *Trichomonas vaginalis*.

However, limited information regarding PPI-PFKs in oral bacteria is currently available. In addition, to our knowledge, there is no genetic study of the PFKs in oral bacteria.

Our ultimate goal is to develop therapeutic agents for periodontal disease by interfering with vital energy metabolism in bacteria without affecting the metabolism of the human host. From this perspective, PPI-PFK would be one of candidates.

In the present study, we have isolated a gene encoding a PPI-PFK (PgPFK) in *P. gingivalis* 381 in the upstream region of the *P. gingivalis* endopeptidase (PgPepO) gene which we have previously reported. Here, we report the cloning, expression, and characterization of a PFK, designated PgPFK, and indicate a possible role in the metabolism of this organism.

Materials and methods

1. Bacterial strains and growth conditions

*P. gingivalis* strain 381, *P. gingivalis* ATCC 33277, *P. gingivalis* W 83, *Prevotella intermedia* ATCC 25611 were kindly provided by Dr. K. Ishihara, Tokyo Dental College, Japan. *Actinobacillus actinomycetemcomitans* Y 4 and *Streptococcus sobrinus* AHT were kindly provided by Dr. T. Koga, Kyushu University, Japan. The organisms were grown anaerobically as described previously. *Escherichia coli* JM109 and *E. coli* BL21 (DE3)/pLysS (Novagen) were used in subcloning and expression experiments. All *E. coli* strains were grown on Luria–Bertani (LB) agar plates or in LB broth (Difco) in the presence of appropriate antibiotics (ampicillin, 50 µg/ml; chloramphenicol, 34 µg/ml).

2. Construction of a genomic library and screening

Genomic DNA from *P. gingivalis* 381 was isolated as previously described. Standard procedures for recombinant DNA manipulations were carried out as described by Sambrook et al. For construction of the genomic library, *P. gingivalis* chromosomal DNA was digested with *EcoRV* and ligated to pBluescript II SK (-) (Stratagene), that had been cut with the corresponding restriction enzyme and treated with bacterial alkaline phosphatase. The oligonucleotide probe (~800 bp) was labeled with a digoxigenin kit (Boehringer) according to the manufacturer’s instructions. Colony hybridization was performed as previously described. Positive clones were detected with the reagents and procedures of the same kit.

3. DNA sequencing

Plasmid DNA from the pBluescript clones obtained above was prepared with the Wizard Miniprep system (Promega) and sequenced using the dideoxy method described by Sanger et al. with a dye terminator sequencing kit (Applied Biosystems) together with a synthetic oligonucleotide primer. The sequence was determined with an Applied Biosystems model 373 S automated DNA sequencer. The nucleotide sequences were analyzed with a computer software package DNA Strider, version 1.2. Amino acid homology searches and comparisons were done with GENETYX-Mac software (Software Development) and the BLAST network services of DDBJ. Sequence alignments were optimized with the CLUSTAL W program. The programs supplied in the PHYLIP software package were used to analyze the phylogenetic relationships among the PFK proteins. A phylogenetic tree was subsequently constructed using the neighbor-joining method.

4. Expression and purification of PgPFK gene in *E. coli*

The open reading frame (ORF) in pTA21 was subcloned in the T7-based bacterial expression plasmid pET 21a (Novagen) as follows. The open reading frame was amplified by the following primers: TA110 (5’-GGAGTTGGAGCTCATGGCAAAGAGTG-3’) and TA111 (5’-GGAGAAGGAAG-
CTTTCAGCGATCGTTCTTTTTCC-3′) (the SacI and HindIII sites, respectively, are underlined). Amplification of the DNA fragments was carried out with these primers in standard PCR buffer that included 2 mM MgCl₂, 200 μM (each) dATP, dTTP, dCTP, and dGTP, and 2.5 U of Pfu polymerase (Stratagene). The thermal cycle parameters were: 94°C for 1 min (denaturation), 52°C for 2 min (annealing), and 72°C for 1 min 50 sec (extension), for 35 cycles. In addition, time delays of 2 min at 94 and 72°C were incorporated at the beginning and end, respectively. The PCR product was restricted with SacI and HindIII and cloned into the same sites of pET 21a as described previously. The resulting clone, pET 21a–PgPFK, was confirmed by DNA sequencing and introduced into E. coli BL21 (DE3)/pLysS. Growth of the transformant, induction with isopropyl-1-thio-β-D-galactopyranoside (IPTG), and lysis with lysozyme were carried out essentially as described previously. Sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE) analysis of proteins was performed with 16% acrylamide (acrylamide/bisacrylamide ratio, 30:0.4) gel.

5. Purification of PgPFK

The crude enzyme extract from the sonicate of the induced cells was applied to a column of Mono SP (HR 5/5: Pharmacia) equilibrated with 50 mM Tris–HCl buffer (pH 7.5). The PPI-PFK activity was eluted in a stepwise fashion using increasing concentrations of NaCl (0, 0.1, 0.2, 0.3, 0.4, 0.5, 1.0 M) in 50 mM Tris–HCl buffer (pH 7.5). A fraction containing PPI-PFK activity was pooled and dialyzed overnight against 50 mM Tris–HCl buffer (pH 7.5). The contents of the dialysis bag were subjected to a column of Mono Q (HR 5/5: Pharmacia) pre-equilibrated with 50 mM Tris–HCl buffer (pH 7.5). The PPI-PFK activity was eluted in stepwise fashion using increasing concentrations of NaCl (0, 0.1, 0.2, 0.3, 0.4, 0.5, 1.0 M) in 50 mM Tris–HCl buffer (pH 7.5). The active fraction was dialyzed and subjected to a Mono SP column again as described above. The active fraction was subjected to HPLC using TSK-gel G 3000 SWxl (TOSOH) pre-equilibrated with 25 mM Tris–HCl buffer (pH 7.5), which was also used for determination of the molecular mass of PgPFK. For molecular standards, the MW-Marker kit was used (Oriental Yeast). The purified enzyme was frozen and stored at −80°C.

6. RT-PCR analysis

Total RNA was prepared from P. gingivalis 381 using the RNeasy Mini Kit (Qiagen) according to the manufacturer’s protocol. Before the solution was used for reverse transcriptase polymerase chain reaction (RT-PCR), contaminating DNA was eliminated by digestion with RNase-free DNase (DNase I, Gibco BRL). Reverse transcription was performed with SuperScript II RNase H– reverse transcriptase (Gibco BRL). Relevant primers for RT-PCR are shown in Fig. 1: TA 41 (5′-AGAGGAGGATCCTTACCAAACGACTACAC-3′) and TA 111 (5′-GGAGAAGAGGCTTTACGCGATCGTTCTTTTCC-3′). Primers TA 41 and TA 111 were used to synthesize cDNA in the PgPFK gene region. The reaction was carried out at 42°C for 60 min. The cDNA was used for PCR amplification directly with the following sets of primers: for the PgPFK region, primers TA 110 and TA 111. The thermal cycle parameters were: 94°C for 1 min (denaturation), 52°C for 2 min (annealing), and 72°C for 3 min (extension), for 35 cycles. A final extension step was performed at 72°C for 10 min. Control reactions were performed by omitting the reverse transcriptase.

7. Enzyme assays

The phosphorylating reaction, called “forward reaction”, was assayed in a reaction mixture with 50 mM Tris–HCl buffer (pH 7.5), 5 mM MgCl₂, 0.15 mM NADH, 10 mM F 6-P, 1.0 U of aldolase per ml, 5 U of triosephosphate isomerase per ml, 0.85 U of α-glycerol phosphate dehydrogenase per ml, and 1.0 to 10 µg of enzyme. The reaction was started by the addition of 1 mM sodium PPI. The synthesis of F 1,6-BP from F 6-P was measured by the disappearance of NADH at 340 nm. However, the dephosphorylating reaction, named “reverse reaction”, was assayed in a reaction mixture with 50 mM Tris–HCl buffer (pH 7.5), 5 mM MgCl₂, 0.4 mM NADP, 2 mM F 1,6-BP, 1.75 U of glucose 6-phosphate isomerase per ml, 1.75 U of glucose 6-phosphate dehydrogenase per ml, and 1.0 to 10 µg of
enzyme. The reaction was started with 5 mM 
K$_2$HPO$_4$. The synthesis of F 6-P from F 1,6-BP 
was measured by the increase of NADPH at 340 nm. 
One unit of activity in the forward and reverse 
reactions was defined as the amount of enzyme 
producing 1 μmol of F 1,6-BP or F 6-P per min at 
37°C, respectively.

The GenBank accession number for the 
nucleotide sequence of the PgPFK gene reported in 
this paper is AB039836.

Results

1. Nucleotide and amino acid sequence analysis

A clone containing a 7.0-kb EcoRV fragment of 
the genomic DNA of P. gingivalis 381 was selected 
by colony hybridization with an oligonucleotide 
probe (~800 bp EcoRI-KpnI fragment within plasmid 
pAL2)[29]. The sequencing strategy and a 
restriction map are shown in Fig. 1. We have deter-

mined the nucleotide sequence of a 6,835 bp DNA 
fragment (pTA 21). The total sequence contained 
four complete ORFs (ORF 1, ORF 2, ORF 3, and 
ORF 4). ORF 1 upstream of ORF 2 exhibited signif-

icant homology with the peptidyl-t-RNA hydrolase 
of B. burgdorferi (36% identical residues) and ORF 3 
had a similarity to RNA polymerase of Bacillus 
subtilis (30% identical residues). ORF 4 did not show 
any significant similarities to known proteins. 
ORF 2 contained 1,650 bp encoding a putative 
peptide of 550 amino acids with a calculated 
molecular mass of 61,044 Da and an estimated pl of 
5.52. A significant potential ribosome-binding site 
was not found. There were no convincing motifs for 
transcription or translation signals recognized in 
the flanking regions. The overall G+C content of 
PgPFK gene (54%) agreed closely with that esti-

mated for the chromosomal DNA from P. gingivalis 
strains (46 to 48%)[30]. The deduced amino acid 
sequence corresponding to ORF 2 was compared to 
all proteins in the SwissProt database with the 
GENETYX-Mac program (Software Development). A significant degree of sequence homology 
was found between the ORF 2 and 62 kDa B. burg-
dorferi PFK (50% identical residues)[31], Treponema 
pallidum PFKβ-subunit (52% identical residues)[32], 
60 kDa E. histolytica PFK (49% identical resi-
dues)[11], and G. lamblia PFK (46% identical resi-
dues)[33]. Therefore, the ORF was named PgPFK. 
Furthermore, to determine the overall sequence 
similarities of the PgPFK with known PFKs and to 
identify structural features correlated with its PPi 
dependence, we aligned PgPFK with 15 sequences of 
enzymes. The sequence dataset was comprised of 11 
sequences of PPi-PFKs and 3 sequences of ATP-

PFKs. We selected a well-conserved sequence frag-

ment corresponding to positions 58 to 297 of the 
PgPFK sequence. The result of multiple-sequence 
alignment analysis within all PFKs includi
PgPFK showed the existence of conserved motifs GGDX, PXTIDXD, and MGR as summarized by Mertens. Of 11 amino acids that bind F 6-P/F 1,6-BP in the E. coli PFK, 6 were conserved in the PgPFK (Thr-200, Asp-202, Asp-204, Met-247, Gly-248, and Arg-249).

2. Phylogenetic analysis
A phylogenetic tree of ATP- and PPI-PFKs was constructed by the neighbor-joining method in order to examine their relationships (Fig. 2). We confirmed that the PPI-PFKs were classified into four groups as reported by Mertens et al. In this phylogeny, group II consisted of the long sequence type PFKs including G. lamblia PFK, 60 kDa E. histolytica PFK, T. pallidum PFKβ-subunit, 62 kDa B. burgdorferi PFK, and PgPFK reported here. This group appeared to form a coherent cluster as in the case of ATP-PFKs.

3. Purification of recombinant PgPFK
The purification of recombinant PgPFK is summarized in Table 1. The enzyme was purified 32.3 fold with a final specific activity of 277.7 U/mg, and the overall yield of the activity was 40.2%. The molecular mass of PgPFK was estimated by gel filtration to be about 120 kDa. On SDS-PAGE, the purified enzyme revealed a single band with a molecular mass of ~60 kDa, which is in good agreement with the calculated molecular mass of the predicted amino acid sequence (61,044 Da) (Fig. 3). These results suggest that PgPFK is a homodimer of identical subunits, which is similar to other long sequence type PPI-PFKs from B. burgdorferi (62 kDa), E. histolytica (60 kDa), and G. lamblia.

4. Substrate specificity
PgPFK showed high specificity for its substrates (PPI, F 6-P, and F 1,6-BP). As shown in Table 2, in the forward reaction, PPI could not be replaced by ATP or ADP, and glucose 6-phosphate was not used as a substrate. In the reverse reaction, fructose 2,6-bisphosphate (F 2,6-BP) was not used as the substrate. The activity of PgPFK in both directions did depend on the presence of Mg2+ ions, as also shown by inhibition in the presence of EDTA (Table 2).

5. pH dependence and thermal stability
The pH dependence of PFK activity is shown in Table 2. PgPFK was active in a narrow pH range (7.5-7.8) in both directions. Thermal stability test revealed that the purified PgPFK was thermostable, with a half-life longer than 0.5 h at 65°C in Tris buffer (Table 2).

6. Transcription of the PgPFK gene in P. gingivalis
RT-PCR analysis was used to demonstrate transcription from PgPFK and its flanking regions. PCR with primers TA 110 and TA 111 produced a 1.6-kb product that corresponded with the size of the PgPFK gene (Fig. 4). There was no product observed in PCR from total RNA preparations that were not first reverse transcribed, suggesting that the RT-PCR products were derived from mRNA but not from contaminating chromosomal DNA.

Discussion
In the present study, we cloned and analyzed a phosphofructokinase gene, PgPFK gene, from P. gingivalis 381. To date, although several PPI-PFKs have been characterized in oral bacteria including Streptococcus salivarius, Actinomyces naeslundii, and Bacteroides species, there has not been any PFK gene isolated from oral bacteria yet. In this study, comparison of the sequence of PgPFK with the sequences of other proteins clearly indicated that PgPFK had a significantly greater homology to the PPI-dependent PFKs than to ATP-dependent PFKs. In general, PPI-PFKs are known to be further classified into two types based on molecular size and susceptibility to F 2,6-BP: Type I and Type II. The type I enzyme, which is a homopolymer with a subunit molecular mass of 40-60 kDa, is independent of F 2,6-BP. Type II enzymes are present in higher plants and Euglena gracilis together with ATP-PFK activity, and stimulated by F 2,6-BP. Based on these diagnostic criteria, we propose that PgPFK may be classified as the Type I group of PPI-PFK enzymes. Interestingly, very recently, PFK from P. intermedia, which is a common oral bacteria and implicated as a periodontal pathogen, was reported to be a Type I enzyme, like PgPFK.

Based on crystallographic studies of bacterial ATP-PFKs as well as site-directed mutagenesis studies of both ATP- and PPI-PFKs, several motifs...
Fig. 2. Phylogenetic tree of ATP- and PPI-PFKs

The tree is based on distance analysis (neighbor-joining method) of sequences of PFKs in the EMBL and SwissProt data banks. Sequence regions (positions 58 to 297 of PgPFK) that show unequivocal similarity were included.

Abbreviations: only ATP-PFK group: D. d: Dictyostelium discoideum; D. m: Drosophila melanogaster; H. c: Haemonchus contortus; H. s: Homo sapiens; S. m: Schistosoma mansoni; E. c, E. coli; B. s, B. stearothermophilus; L. d, Lactobacillus delbrueckii; L. l: L. lactis; S. t, Streptococcus thermophilus; B. m, Bacillus macquariensis; C. a, Clostridium acetobutylicum; group II (long sequence type): G. l, G. lambia; 62 kDa B. b; 62 kDa B. burgdorferi; P. g, P. gingivalis; T. pβ, T. pallidum β-subunit; 60 kDa E. h; 60 kDa E. histolytica; P. β, potato β-subunit; R. cβ, Ricinus communis β-subunit; R. ca, R. communis α-subunit; P. α, potato α-subunit; group I: N. f, N. fowleri; T. v, T. vaginalis; group III: 47.6 kDa E. h, 47.6 kDa E. histolytica; T. b, T. brucei; 48 kDa B. b, 48 kDa B. burgdorferi; T. pPFK, T. pallidum PFK; group IV: T. t, T. tenax; M. l, Mycobacterium laprae; A. m, A. methanolica; S. c, S. coelicolor; uncertain group: P. f, Propionibacterium freudenreichii.
and amino acid residues within those motifs have been shown to be crucial for catalysis and substrate binding in PFKs. The sequences GGDD (positions 170 to 173 in PgPFK) and PKTIDGD (positions 198 to 204 in PgPFK) are found in almost all determined PFKs. In general, PPI-PFKs have motifs, PKTIDND [N was replaced by G-203 in PgPFK, as in the case of T. pallidum PFK β-subunit (G-215), and 60 kDa E. histolytica PFK (G-205)] and GGDD, while ATP-PFKs have predominantly PGTIDND and GGDG motifs. The sequence MGR (positions 247 to 249 in PgPFK) is found in all PPI-PFKs and also in most ATP-PFKs. Furthermore, mutagenesis has recently shown that the arginine residue of this tripeptide is important for the activity of Naegleria fowleri PPI-PFK.

The biological function of PPI-PFK is currently unknown. It has been suggested that the PPI-PFKs are more suited to a role in anaerobic metabolism than is its ATP-dependent counterparts. It has also been reported that in contrast to the virtually irreversible reaction catalyzed by the ATP-PFK, phosphorylation by PPI is reversible. Furthermore, it is suggested that this enzyme may function to equilibrate pools of hexose and triosephosphates.

Recently, the rml genes, products of which catalyze the anabolism of dTDP-d-l-rhamnose from d-glucose-1-phosphate, have been isolated from P. gingivalis. In this report, it is suggested that dTDP-d-l-rhamnose may be involved in synthesis of the lipopolysaccharides or glycoconjugates which are essential for cell viability of P. gingivalis. However, it is well known that PPI-PFK in organisms without ATP-PFK not only functions as an important glycolytic enzyme, but may also play important roles in other metabolic pathways. Considering that P. gingivalis depends on nitrogenous substrates instead of carbohydrates for energy production, it is possible that the gluconeogenic pathway is one way to produce macromolecules including cell-surface polysaccharides.

### Table 1 Purification of recombinant PgPFK

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Total protein (mg)</th>
<th>Total activity (U)</th>
<th>Specific activity (U/mg)</th>
<th>Yield (%)</th>
<th>Purification (fold)</th>
<th>Activity ratio (F 1, 6-BP/F 6-P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>crude extract</td>
<td>17.2</td>
<td>179.0</td>
<td>8.6</td>
<td>100.0</td>
<td>1</td>
<td>1.4</td>
</tr>
<tr>
<td>Mono SP (I)</td>
<td>10.3</td>
<td>140.3</td>
<td>14.4</td>
<td>78.4</td>
<td>1.7</td>
<td>1.5</td>
</tr>
<tr>
<td>Mono Q</td>
<td>2.96</td>
<td>122.1</td>
<td>42.7</td>
<td>68.2</td>
<td>5.0</td>
<td>1.4</td>
</tr>
<tr>
<td>Mono SP (II)</td>
<td>0.28</td>
<td>72.0</td>
<td>277.7</td>
<td>40.2</td>
<td>32.3</td>
<td>1.2</td>
</tr>
</tbody>
</table>

* : PPI-PFK activity was measured with F 1, 6-BP as the substrate
# : Activity ratio was expressed as the ratio of activity with F 1, 6-BP and F 6-P as the substrate.

![Fig. 3 SDS-PAGE](16% polyacrylamide) analysis of the fractions of the following purification steps of PgPFK: molecular mass markers (lane M), the crude enzyme extract (lane 1), the enzyme preparation eluted from Mono SP (lane 2), the enzyme preparation eluted from Mono Q (lane 3), the purified PgPFK (~4 μg) (lane 4). Proteins were stained with Coomassie blue R-250.
Table 2 Substrate specificity for PgPFK in the forward and reverse reaction

<table>
<thead>
<tr>
<th></th>
<th>Forward reaction</th>
<th>Reverse reaction</th>
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<tbody>
<tr>
<td></td>
<td>Conc (mM)</td>
<td>%Activity</td>
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<tr>
<td><strong>Divalent ion</strong></td>
<td></td>
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<tr>
<td>Mg$^{2+}$</td>
<td>5</td>
<td>100.0</td>
</tr>
<tr>
<td>Zn$^{2+}$</td>
<td>5</td>
<td>5.7</td>
</tr>
<tr>
<td>Mn$^{2+}$</td>
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<td>7.3</td>
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<tr>
<td>Ba$^{2+}$</td>
<td>5</td>
<td>10.1</td>
</tr>
<tr>
<td><strong>Phosphoryl donor</strong></td>
<td></td>
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<tr>
<td>PPI</td>
<td>1</td>
<td>100.0</td>
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<tr>
<td>ATP</td>
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<td>1.0</td>
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<tr>
<td>ADP</td>
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<td>1.4</td>
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<tr>
<td>AMP</td>
<td>1</td>
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<tr>
<td><strong>Substrate</strong></td>
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<tr>
<td>F6-P</td>
<td>10</td>
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<tr>
<td>G6-P</td>
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<td>0.3</td>
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<tr>
<td>G1-P</td>
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<td>0.5</td>
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<tr>
<td>F1-P</td>
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<td>0.5</td>
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<tr>
<td><strong>Effector</strong></td>
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<tr>
<td>ATP</td>
<td>0.1</td>
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<tr>
<td>F2,6-BP</td>
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<td>102.4</td>
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<td>1</td>
<td>98.3</td>
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<td></td>
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<tr>
<td><strong>pH range</strong></td>
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<tr>
<td></td>
<td>7.5-7.8</td>
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<tr>
<td><strong>Thermal stability</strong></td>
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<tr>
<td></td>
<td>(half-life at 65°C)</td>
<td>0.5 h</td>
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</table>

*: For the determination of the effects of effectors, F6-P (10 mM) and PPI (1 mM) were used in the forward reaction, on the other hand, in the reverse reaction, F1,6-BP (2 mM) and K$_2$HPO$_4$ (5 mM) were used as the substrate under standard assay conditions as described in Materials and methods, respectively.

Percentages were calculated by comparison to the activity in the complete standard reaction mixture. Values were given as mean of data obtained from three independent experiments.

**: The pH dependence assay was carried out at 37°C in 500 μl of standard mixture of each direction containing 50 mM buffer: for pH 6.5, 6.8, and 7.0, MES: for 6.8, 7.0, 7.5, 7.8, 8.3, and 8.8, Tris: for 8.1, 8.3, 8.5, and 9.0, glycine-NaOH.

In this study, we confirmed that PgPFK gene was expressed by RT-PCR analysis, however, it remains to confirm whether protein is expressed in this organism. To hypothesize the role of PgPFK, we tried to find fructose 1,6 bisphosphatase (FBPase) in the data from the *P. gingivalis* Genome Project (http://www.forsyth.org/pggp/) and the Institute for Genomic Research (http://www.tigr.org/) based on the *E. coli* FBPase (accession No. P09200), since no FBPase was identified in the genome of *T. pallidum***. However, there was no significant homology found between any of the proteins in the *P. gingivalis* database and the *E. coli*
FBPase. Thus, it seems that *P. gingivalis* may not have any FBPase. From these findings, it is proposed that PgPFK might serve as an FBPase, one of enzymes in the gluconeogenic pathway, in this organism. A detailed analysis will be needed to explore this possibility.

PPi-PFKs are thought to represent a good target for the development of therapeutic agents due to the difference from the ATP-PFKs of the human host. In this respect, it is important that the PPI-PFK gene of *P. gingivalis*, a periodontopathogenic bacteria, is isolated. The inhibition of its metabolism should have either a bacteriostatic effect or, more often, a bactericidal one. Research on genes including PgPFK responsible for *P. gingivalis* metabolism may lead to a new therapy for periodontal disease.

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Porphyromonas gingivalis のピロリン酸依存型
ホスホルクトキナーゼ遺伝子のクローニング、
遺伝子発現および性状解析について

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概要：Porphyromonas gingivalis は、グラム陰性嫌気性桿菌であり、一般に糖分解を行わぬ細菌として知られている。今回 P. gingivalis 381 株におけるピロリン酸依存型ホスホルクトキナーゼ（Ppi-PFK）をコードする遺伝子（PgPFK）のクローニング、塩基配列の決定、および遺伝子発現を行い、その性状解析を行った。PgPFK は 1,650 bp からなり、550 ピロリン酸、分子量 61,044 Da、等電点 5.52、二量体のタンパクであることが明らかとなった。ホモロジー解析の結果、解糖系の key enzyme である PFK のうち、62 kDa Borrelia burgdorferi PFK（50%）、Treponema pallidum β-subunit（52%）、などの Ppi-PFK と高い相補性を示した。ピロリン酸配列を詳細に比較検討したところ、Ppi-PFK に共通して認められるモチーフ（GGDD、TIDX、MGR）および binding sites のほとんどが保存されていることが明らかとなった。また塩基配列をもとに PFK の分子系統樹を作製したところ、PFK は 4 群に分類されることが確認され、PgPFK は 60～62 kDa の分子量をもつ long sequence type に属し、それらは coherent な cluster を形成していることが明らかとなった。PgPFK 遺伝子を組み込んだプラスマド（pET 21a-PgPFK）を構築し、E. coli BL 21（DE 3）株を用いて組み換え PgPFK の発現誘導および精製を行い、得られた精製タンパクの性質を調べた結果、ほかの Ppi-PFK 同様、fructose 6-phosphate から fructose 1,6-bisphosphate への反応は不可逆であること、PFK 活性は Mg⁺依存性であり ATP や fructose 2,6-bisphosphate 等の影響を受けないことが明らかとなった。また RT-PCR 分析を行い、PgPFK 遺伝子が mRNA レベルで発現していることを確認した。

以上の結果から PgPFK が典型的な Ppi-PFK に属し、その性質もいままでに報告されている Ppi-PFK と非常に類似していることが明らかになった。糖新生系の一酵素である fructose 1,6-bisphosphatase が P. gingivalis の遺伝子データベース上に検索されないことから PgPFK がその代謝を果たしている可能性が考えられる。

索引用語：Porphyromonas gingivalis、ホスホルクトキナーゼ、ピロリン酸

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