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Enzymes of Glucose and Methanol Metabolism in the Actinomycete *Amycolatopsis methanolica*

A. M. C. R. ALVES,[1] G. J. W. EUVERINK,[1] H. J. HEKTOR,[1] G. I. HESSELS,[1] J. VAN DER VLAG,[1] J. W. VRIJBLOED,[1] D. HONDMANN,[2] J. VISSER,[2] AND L. DIJKHUIZEN[1]*

Department of Microbiology, University of Groningen, 9751 NN Haren,[1] and Department of Genetics, Agricultural University, 6703 HA Wageningen,[2] The Netherlands

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The actinomycete *Amycolatopsis methanolica* was found to employ the normal bacterial set of glycolytic and pentose phosphate pathway enzymes, except for the presence of a PP_i-dependent phosphofructokinase (PP_i-PFK) and a 3-phosphoglycerate mutase that is stimulated by 2,3-bisphosphoglycerate. Screening of a number of actinomycetes revealed PP_i-PFK activity only in members of the family *Pseudonocardiaceae*. The *A. methanolica* PP_i-PFK and 3-phosphoglycerate mutase enzymes were purified to homogeneity. PP_i-PFK appeared to be insensitive to the typical effectors of ATP-dependent PFK enzymes. Nevertheless, strong N-terminal amino acid sequence homology was found with ATP-PFK enzymes from other bacteria. The *A. methanolica* pyruvate kinase was purified over 250-fold and characterized as an allosteric enzyme, sensitive to inhibition by P_i and ATP but stimulated by AMP. By using mutants, evidence was obtained for the presence of transketolase isoenzymes functioning in the pentose phosphate pathway and ribulose monophosphate cycle during growth on glucose and methanol, respectively.

Actinomycetes are important bacterial producers of secondary metabolites. There is a strong interest in the genetics of secondary-metabolite biosynthesis, with most studies concentrating on these pathways and their control. Many secondary metabolites are initially derived from intermediates of the central pathways of primary metabolism. Little is currently known, however, about the enzymes and regulation of, for instance, glucose metabolism in actinomycetes. This is mostly because of a general lack of physiological studies on primary metabolism in actinomycetes (21). We have initiated such studies with the actinomycete *Amycolatopsis methanolica* (8), belonging to the family *Pseudonocardiaceae* (42), which includes many species producing bioactive compounds, e.g., the antibiotics rifamycin and erythromycin. *A. methanolica* is one of the few methanol-utilizing gram-positive bacteria known (10, 12, 17). Methanol oxidation via formaldehyde and formate to carbon dioxide results in energy generation (5, 17). Carbon assimilation starts by formaldehyde fixation via the ribulose monophosphate (RuMP) cycle (9, 17). This cycle involves the specific enzymes hexulose-6-phosphate synthase (HPS) and hexulose-6-phosphate isomerase (HPI), the glycolytic enzymes 6-phosphofructokinase (PFK) and fructose-1,6-bisphosphate (FBP) aldolase (9), and various enzymes also involved in the related pentose phosphate pathway (Fig. 1) (12).

The identity, properties, and regulation of enzymes involved in glucose and methanol metabolism in *A. methanolica* were examined in this study.

MATERIALS AND METHODS

Microorganisms and cultivation. Wild-type *A. methanolica* NCIB 11946, its maintenance, and the procedures followed for cultivation in batch cultures, harvesting of cells, and growth measurements have been described previously (8–11). Carbon-limited chemostats (working volume, 1 liter) were run at a constant temperature of 37°C and pH 7.0, which was controlled by automatic adjustment with 2 M NaOH. The medium contained (per liter) the following: KH_2PO_4, 1.0 g; $(NH_4)_2SO_4$, 1.5 g; $MgSO_4$, 0.2 g; and a trace element solution (41), 0.2 ml. Measurements were made with steady-state cultures.

Various actinomycetes, including *A. methanolica* (NCIB 11946), *Amycolatopsis orientalis* (K99; JCM 4235), *Amycolatopsis mediterranei* (K98; JCM 4789), *Amycolatopsis azurea* (K114; J. Lacey, Rothamsted Experimental Station, Harpenden, United Kingdom), *Amycolata saturnea* (A195; DSM 43195), *Faenia rectivirgula* (F1; ATCC 33515), *Nocardia asteroides* (N8; R. Hütter, ETH, Zürich, Switzerland), *Streptomyces coelicolor* A3(2) (LBGA 3170), and *Streptomyces griseus* (NCTC 6961), were grown in YEME medium (23) and screened for PP_i-dependent PFK (PP_i-PFK) and/or ATP-dependent PFK (ATP-PFK) activity (see below).

Mutant isolation. Mutants were derived from wild-type *A. methanolica* or a plasmid-cured derivative, strain WV2 (41a). Transketolase mutants were derived from the wild-type strain by diepoxyoctane treatment (10, 13). In the screening procedure, glucose mineral medium was supplemented with aromatic amino acids, each to a final concentration of 1 $mg \cdot liter^{-1}$, and pinpoint colonies were further characterized. Mutants of strain WV2 unable to grow on methanol (500 mM) gelrite (15 $g \cdot liter^{-1}$) plates but positive on glucose agar were selected following UV irradiation. Glucose kinase-negative mutants of the wild-type strain were isolated among the spontaneous 2-deoxyglucose (2DOG)-resistant colonies that appeared after incubation for 5 to 7 days at 37°C on lactate, glycerol, or arabinose (10 mM) agar with 2DOG (100 mM). Approximately 100 2DOG-resistant colonies were further purified and tested for growth on glucose (20).

Preparation of extracts and enzyme assays. Washed cell suspensions were disrupted in a French pressure cell at 1,000 MPa. Unbroken cells and debris were removed by centrifugation at 40,000 × *g* for 20 min at 4°C. Following desalting through PD 10 Pharmacia columns, the supernatant, containing 2.5 to 5.0 mg of protein $\cdot ml^{-1}$, was used for enzyme assays

* Corresponding author. Mailing address: Department of Microbiology, University of Groningen, Kerklaan 30, 9751 NN Haren, The Netherlands. Phone: 31.50.632153. Fax: 31.50.632154. Electronic mail address: L.Dijkhuizen@biol.rug.nl

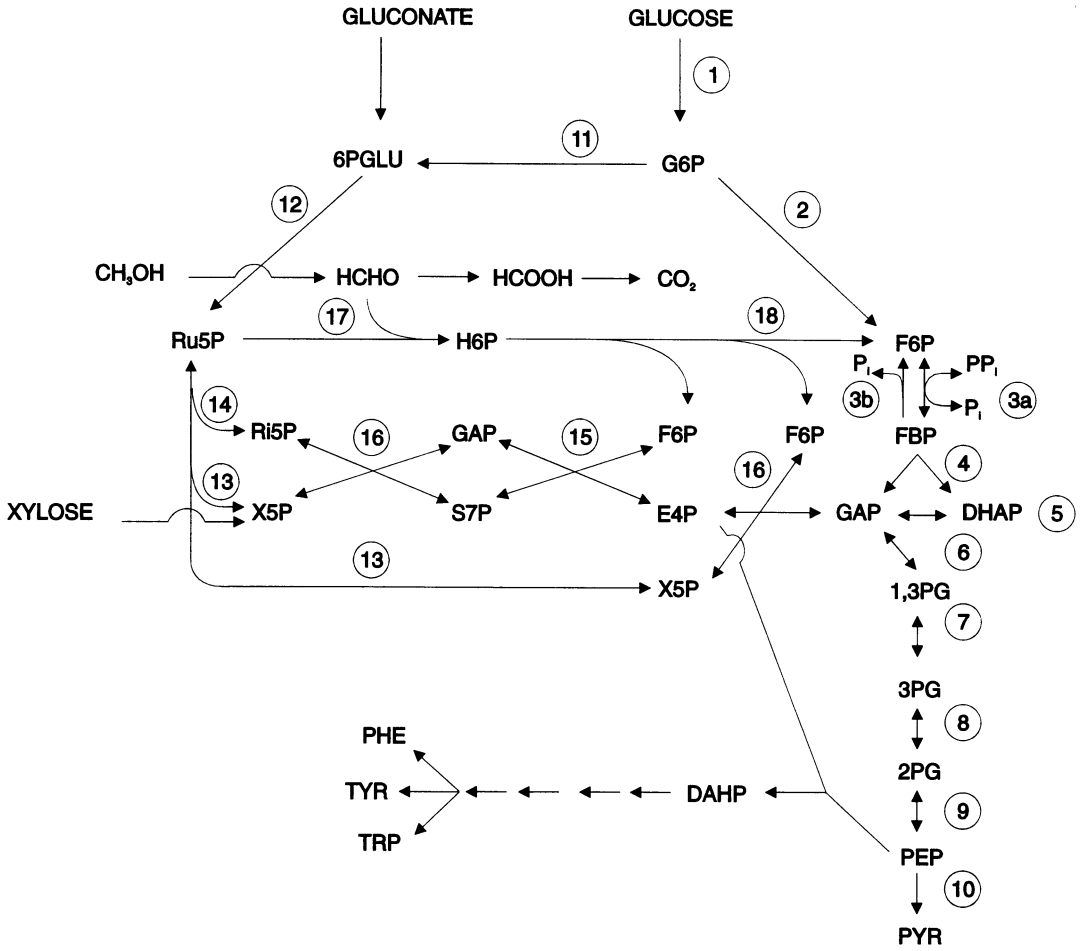

FIG. 1. Schematic representation of the pathways for glucose, gluconate, xylose, and methanol metabolism and biosynthesis of aromatic amino acids in *A. methanolica*. The circled numbers refer to the enzyme activities shown in Table 1.

at 37°C. Triton X-100 (0.01%) was added to all assay mixtures containing NADH in order to reduce endogenous NADH consumption. All enzyme assays were performed in triplicate; data presented are averages with a standard deviation of less than 10%.

FBP aldolase (EC 4.1.2.13) (with 1.0 mM $CoCl_2$ replaced by 100 mM KCl), glucose-6-phosphate (G6P) dehydrogenase (EC 1.1.1.49) (with 40% glycerol added to stabilize the enzyme), 6-phosphogluconate (6PG) dehydrogenase (EC 1.1.1.44) (with 40% glycerol added to stabilize the enzyme), 6PG dehydratase (EC 4.2.1.12) plus 2-keto-3-deoxy-6-phosphogluconate aldolase (EC 4.1.2.14), and fructose-1,6-bisphosphatase (FBPase; reaction was started with FBP) (EC 3.1.3.11) were assayed as described previously (40). Hexokinase (EC 2.7.1.1) (7), G6P isomerase (EC 5.3.1.9) (16), 3-phosphoglycerate (3PGA) kinase (EC 2.7.2.3) (40% glycerol was added to stabilize the enzyme) (1), enolase (EC 4.2.1.11) (37), 3PGA mutase (PGM) (2,3PGA dependent [EC 2.7.5.3] or independent [EC 2.7.5.5]) (43), pyruvate kinase (reaction was started with ADP) (EC 2.7.1.40) (25), triosephosphate isomerase (TIM) (EC 5.3.2.1) (25), HPS and HPI (9), and transaldolase (EC 2.2.1.2), transketolase (EC 2.2.1.1), ribulose-5-phosphate (Ru5P) 3-epimerase (EC 5.1.3.1), and Ribose-5-phosphate (Ri5P) isomerase (EC 5.3.1.6) (29) were assayed according to published methods.

PFK was assayed in a reaction mixture with 50 mM Tris-HCl buffer (pH 7.5), 5 mM $MgCl_2$, 1 mM KCl, 3 mM NH_4Cl, 5 mM dithiothreitol, 0.15 mM NADH, 10 mM fructose-6-phosphate, 0.9 U of FBP aldolase, 5 U of TIM, 0.85 U of α-glycerol-3-phosphate (G3P) dehydrogenase, and limiting amounts of extract. The reaction was started by the addition of 5 mM sodium PP_i (for PP_i-PFK, EC 2.7.1.90) or 5 mM ATP (for ATP-PFK, EC 2.7.1.11). ATP and PP_i solutions were adjusted to assay pH values before use. The reverse PP_i-PFK reaction was assayed in a reaction mixture with 50 mM Tris-HCl buffer (pH 7.5), 5 mM $MgCl_2$, 1 mM KCl, 3 mM NH_4Cl, 5 mM dithiothreitol, 0.4 mM NADP, 2.5 mM FBP, 1.75 U of G6P isomerase, 1.75 U of G6P dehydrogenase, and limiting amounts of extract. The reaction was started with 5 mM K_2HPO_4. GAP dehydrogenase (EC 1.2.1.12) was assayed in a reaction mixture with 50 mM Tris-HCl buffer (pH 8.5), 5 mM ATP, 0.15 mM NADH, 2 U of 3PGA kinase, and limiting amounts of extract. The reaction was started with 10 mM 3PGA.

Screening of dyes for purification of PP_i-PFK. Screening of dyes suitable for affinity chromatography was done as described previously (22). Of a collection of 96 different dyes, Levafix blue G-3A was chosen for the purification of PP_i-PFK. Rapid screening for PP_i-PFK activity in the eluents of the dye columns was possible by using an assay based on color change,

i.e., by adding G3P oxidase to the assay mixture, resulting in formation of H_2O_2, which could be detected with horseradish peroxidase and *o*-dianisidine. The assay mixture (0.25 ml) contained 50 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 1 mM KCl, 3 mM NH_4Cl, 0.3 mM NADH, 10 mM sodium PP_i, 10 mM fructose-6-phosphate, and excess amounts of the coupling enzymes FBP aldolase, G3P dehydrogenase, and TIM. After 15 min of incubation at 37°C, excess G3P oxidase, horseradish peroxidase, and *o*-dianisidine were added. The formation of a brown precipitate was followed by eye.

Purification of PP_i-PFK. Glucose (50 mM)-grown cells were harvested from a batch culture of *A. methanolica* WV2 at the end of the exponential growth phase. In step 1, a crude extract (25 g of cells [wet weight]) was prepared in 50 mM Tris-HCl (pH 7.5) (buffer A) as described above. A few crystals of DNase I, grade II (bovine pancreas), were added, and the crude extract was incubated on ice for at least 10 min. In step 2, a 2% (wt/vol) freshly prepared pH-adjusted solution of protamine sulfate was slowly added to a final concentration of 0.2% (wt/vol). The mixture was stirred (15 min at room temperature) and centrifuged (15 min at 40,000 × *g*). In step 3, supernatant from step 2 was applied to a Levafix blue G-3A–Sepharose CL-4B (22) column (30 ml) equilibrated with buffer A. The column was washed with 3 column volumes of buffer A, and PP_i-PFK was eluted with buffer A with 5 mM fructose-6-phosphate (flow rate, 3 ml · min^{-1}; 4-ml fractions). In step 4, protein from step 3 (90 ml) was applied to a Mono Q (HR 5/5) column equilibrated with buffer A. Protein was eluted with a linear gradient of 0 to 0.8 M NaCl in buffer A (flow rate, 1 ml · min^{-1}; 1.5-ml fractions). Fractions with the greatest PP_i-PFK activities were pooled, dialyzed against buffer A with 40% glycerol, and stored at −20°C.

Purification of PGM. In step 1, a crude extract (25 g of cells [wet weight]) of strain WV2 was prepared as described above. In step 2, protein was applied to a Q-Sepharose fast-flow anion-exchange column (1.6 by 10 cm) equilibrated in buffer A. Bound protein was eluted with a linear gradient of 0 to 0.5 M KCl in buffer A (flow rate, 4 ml · min^{-1}; 4-ml fractions). In step 3, pooled fractions (24 ml) from step 2 were adjusted to 1.7 M $(NH_4)_2SO_4$, and precipitated protein was removed by centrifugation (10,000 × *g* for 10 min). The supernatant was applied to a butyl-Sepharose fast-flow hydrophobic interaction column (1 by 10 cm) equilibrated in buffer A containing 1.7 M $(NH_4)_2SO_4$. Bound protein was eluted with a linear gradient of $(NH_4)_2SO_4$ (100 to 0%) in buffer A (flow rate, 4 ml · min^{-1}; 4-ml fractions). Pooled fractions were dialyzed against buffer A. In step 4, protein from step 3 (46 ml) was applied onto a Resource Q anion-exchange column (5 mm by 1 cm) equilibrated in buffer A. Bound protein was eluted with a linear gradient of 0 to 0.5 M KCl in buffer A (flow rate, 2 ml · min^{-1}; 1-ml fractions). In step 5, pooled fractions from step 4 were concentrated to 1.5 ml with a Microsep ultrafiltration device (cutoff molecular weight, 10,000; Filtron, Breda, The Netherlands) and applied onto a Superdex 200 gel filtration column (XK 16/60), which was equilibrated and eluted with buffer A containing 0.15 M KCl (flow rate, 1 ml · min^{-1}; 1-ml fractions). In step 6, pooled fractions from step 5 were adjusted to 1.0 M $(NH_4)_2SO_4$ and applied to a phenyl-Superose hydrophobic interaction column (HR 5/5) equilibrated in buffer A containing 1.0 M $(NH_4)_2SO_4$. Bound protein was eluted with a linear gradient of $(NH_4)_2SO_4$ (100 to 0%) in buffer A (flow rate, 0.5 ml · min^{-1}; 0.5-ml fractions). Fractions with the greatest PGM activities were pooled, dialyzed against buffer A, concentrated (see above), adjusted to 45% glycerol, and stored at −20°C.

Purification of pyruvate kinase. In step 1, a crude extract (8 g of cells [wet weight]) of strain WV2 was prepared in 50 mM Tris-HCl (pH 8.0)–10% (vol/vol) glycerol–5 mM $MgCl_2$–1 mM dithiothreitol (buffer B), as described above. In step 2, protein was applied to a Q-Sepharose fast-flow anion-exchange column (1 by 10 cm) equilibrated in buffer B, and bound protein was eluted with a linear gradient of 0 to 0.5 M KCl in buffer B (flow rate, 4 ml · min^{-1}; 4-ml fractions). In step 3, pooled fractions were adjusted to 60% $(NH_4)_2SO_4$. Precipitated protein was centrifuged at 30,000 × *g* for 10 min, dissolved in 3 ml of buffer B, and applied to a Superdex 200 gel filtration column (XK 16/60), which was equilibrated and eluted with buffer B containing 0.15 M KCl (flow rate, 1 ml · min^{-1}, 2-ml fractions). In step 4, pooled fractions from step 3 were adjusted to 1.5 M $(NH_4)_2SO_4$ and applied to an alkyl-Superose hydrophobic interaction column (HR 5/5) equilibrated with buffer B containing 1.5 M $(NH_4)_2SO_4$. Bound protein was eluted with a linear gradient of $(NH_4)_2SO_4$ (100 to 0%) in buffer B (flow rate, 0.5 ml · min^{-1}; 1-ml fractions). Fractions with the greatest pyruvate kinase activities were pooled, dialyzed against buffer B with 40% glycerol, and stored at −20°C.

SDS-PAGE. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed (28) with the marker proteins β-galactosidase (molecular weight, 116,400), fructose-6-phosphate kinase (85,200), glutamate dehydrogenase (55,600), aldolase (39,200), TIM (26,600), trypsin inhibitor (20,100), and lysozyme (14,300) (Combithek; Boehringer Mannheim Biochemica). Gels were stained with Coomassie brilliant blue R250.

Estimation of molecular weights. The relative molecular weights of native enzymes were estimated by using a Superdex 200 column with thyroglobulin (molecular weight, 670,000), gamma globulin (158,000), ovalbumin (44,000), myoglobin (17,000), and cobalamin (1,350) as gel filtration standards (Bio-Rad, Richmond, Calif.).

N-terminal amino acid sequence analysis. Purified PP_i-PFK protein was applied to a Pro-spin cartridge (Applied Biosystems) containing a polyvinylidene difluoride membrane. Sequencing was performed on an Applied Biosystems model 477A/120A automated gas phase sequencer equipped with on-line high-pressure liquid chromatography for detection of the phenylthiohydantoin amino acid derivatives (Eurosequence, Groningen, The Netherlands).

N-terminal sequence alignment. N-terminal amino acid sequences were aligned with PALIGN from PCGENE. Conserved amino acids are indicated according to the following scheme: P, A, G, S, T; Q, N; E, D; R, K; I, L, M, V and F, Y, W; C; H.

Kinetic studies. Kinetic parameters were determined at 37°C and pH 7.5 (for PP_i-PFK and PGM) or pH 8.0 (for pyruvate kinase) and were calculated with SigmaPlot for Windows 1.0 (Jandell Scientific Software) by using curve fitting with the Hill or Michaelis-Menten equation. Effectors of PP_i-PFK and pyruvate kinase were tested by adding several metabolites (pH adjusted, 1 mM final concentrations) separately to the assay mixtures with the purified enzymes, using near-K_m concentrations of fructose-6-phosphate (0.4 mM) and PP_i (0.2 mM) for PP_i-PFK and phosphoenolpyruvate (PEP) (0.17 mM; 2.5 mM ADP) and ADP (0.11 mM; 2.5 mM PEP) for pyruvate kinase.

Analytical methods. Methanol and acetate were determined gas chromatographically (18, 26), and glucose was determined with the GOD-Perid method (Boehringer, Mannheim, Federal Republic of Germany). Protein concentrations were determined with the Bio-Rad protein determination kit with bovine serum albumin as a standard (4).

TABLE 1. Specific activities of enzymes of glucose and methanol metabolism in cells of *A. methanolica* grown in chemostat cultures at $D = 0.065\ h^{-1}$

Enzyme[a]	Sp act (U · mg of protein^{-1}) with growth substrate		
	Glucose (10 mM)	Methanol (80 mM)	Acetate (30 mM)
1. Glucokinase			
ATP	0.21	0.09	0.11
GTP	0.34	0.17	0.14
2. G6P isomerase	0.97	0.46	0.76
3a. PP_i-PFK	0.14	0.03	0.02
3b. FBPase	0.17	0.09	0.17
4. FBP aldolase	0.12	0.41	0.28
5. TIM	0.15	0.10	0.09
6. GAP dehydrogenase	0.80	0.67	0.60
7. 3PGA kinase	1.00	0.94	1.30
8. PGM	1.70	0.64	1.90
9. Enolase	0.13	0.19	0.29
10. Pyruvate kinase	0.21	0.09	0
11. G6P dehydrogenase (NADP)	0.09	0.02	0.05
12. 6PG dehydrogenase (NAD)	0.02	0.01	0.03
13. Ru5P epimerase	2.30	2.40	1.50
14. Ri5P isomerase	0.40	1.10	0.22
15. Transaldolase	0.27	0.13	0.15
16. Transketolase	0.21	1.10	0.28
17. HPS	0	7.70	0
18. HPI	0	3.00	0

[a] Numbers refer to the reactions shown in Fig. 1.

RESULTS

Enzymes of glucose metabolism. All enzymes of the glycolytic and pentose phosphate pathways could be measured in extracts of glucose-grown cells of *A. methanolica* (Table 1; Fig. 1). Only a PP_i-PFK could be detected, and there is no biochemical evidence for the additional presence of an ATP-PFK. Addition of fructose-2,6-bisphosphate, a well-known positive effector of ATP-PFK activity in yeasts and higher organisms (and of PP_i-PFK in plants and *Euglena gracilis* [31]), to the assay mixture had no effect. The reverse reaction of PP_i-PFK, conversion of FBP and P_i into fructose-6-phosphate and PP_i, could not be measured accurately in extracts because of the presence of a large amount of interfering FBPase activity; the latter enzyme catalyzes the conversion of FBP into fructose-6-phosphate and P_i. For the same reasons, FBPase activity could not be accurately determined (Fig. 1). During optimization of the assays, it became apparent that extracts had to be subjected to filtration or dialysis to remove inhibitory low-molecular-weight molecules (pyruvate kinase), that 2,3PGA (PGM) had to be added for maximal activity, or that glycerol had to be added to stabilize enzymes (3PGA kinase, NADP-G6P dehydrogenase, and NAD-6PG dehydrogenase). PGM activity was stimulated approximately fivefold by 0.2 mM 2,3PGA. The presence of Mg^{2+} ions was essential for PP_i-PFK and pyruvate kinase activities. Only NADP-dependent G6P dehydrogenase and NAD-dependent 6PG dehydrogenase activities were found (Table 1). No Entner-Doudoroff pathway enzymes were detectable.

Both ATP- and GTP-dependent glucose kinase activities could be detected. 2DOG (100 mM) completely inhibited growth on lactate, glycerol, and arabinose agar. 2DOG-resistant colonies appeared spontaneously on these agar plates at a frequency of 2×10^{-5} and displayed either a glucose-negative (37%) or glucose-positive (63%) growth phenotype. Mutants in the first category invariably appeared to have lost both the ATP- and GTP-dependent glucose kinase activities, but this did not affect their ability to grow on fructose as a carbon source. This hexokinase thus plays a key role in glucose metabolism but not in fructose metabolism.

Enzyme activities in cells grown on various substrates. Growth rates of *A. methanolica* on glucose, acetate, and methanol in batch cultures are rather different (0.35, 0.14, and 0.11 h^{-1}, respectively). To exclude effects of growth rate, enzyme activities in cells grown on these substrates were compared following growth in chemostat cultures at a fixed growth rate ($D = 0.065\ h^{-1}$). Most importantly, no pyruvate kinase activity was detectable in acetate-grown cells, whereas PP_i-PFK activity became strongly reduced in methanol- and acetate-grown cells (Table 1). Filtration of extracts to remove inhibitory compounds had no effect, suggesting that the syntheses of at least these two enzymes are regulated.

The glycolytic enzymes FBP aldolase and PFK and the pentose phosphate pathway enzymes function in both glucose and methanol metabolism (RuMP cycle; Fig. 1). Methanol-grown cells possessed significantly higher activities of FBP aldolase, Ri5P isomerase, and transketolase than glucose-grown cells (Table 1). This was not the case, however, for PP_i-PFK, Ru5P epimerase, and transaldolase.

Isolation of transketolase-negative mutants. Attempts to isolate methanol-negative mutants blocked in the RuMP pathway yielded only many HPS- and/or HPI-negative mutants (e.g., strains MM1 and MM2). This could be taken to suggest the presence of isoenzymes for the steps common to both the pentose phosphate pathway and the RuMP cycle, similar to the situation previously observed for transaldolase in *Arthrobacter* strain P1 (30). Among auxotrophic mutants of *A. methanolica*, requiring the three aromatic amino acids tryptophan, tyrosine, and phenylalanine for growth in glucose mineral media, we subsequently identified three transketolase-negative mutants, strains GH2, GH4, and GH5. Transketolase catalyzes two separate steps in the pentose phosphate pathway; its mutational inactivation results in depletion of erythrose-4-phosphate, a precursor for the biosynthesis of the aromatic amino acids (Fig. 1). Strains GH2, GH4, and GH5 were unable to grow on C sources which are metabolized directly via the pentose phosphate pathway (i.e., xylose and gluconate; Fig. 1), even when supplemented with the aromatic amino acids. All three of these mutant strains, however, retained the ability to grow at normal rates in methanol mineral media. These data provide evidence for the presence of a second, methanol-inducible, transketolase isoenzyme in *A. methanolica*.

Presence of PP_i-PFK in actinomycetes. Various actinomycetes were screened for the presence of PP_i- and/or ATP-PFK activity (Table 2). Representatives of the family *Pseudonocardiaceae* (42) were found to possess PP_i-PFK activity, with the exception of *F. rectivirgula*. *Nocardia* and *Streptomyces* strains, on the other hand, possessed only ATP-PFK activity. This suggests that the PP_i-PFK distribution reflects an evolutionary signature.

Purification and characterization of PP_i-PFK. PP_i-PFK was purified to homogeneity (1,500-fold, 76% yield; Table 3). The dye affinity chromatography step, involving biospecific elution of PP_i-PFK with its substrate fructose-6-phosphate, was extremely useful, allowing a 350-fold purification with a yield of 93%. A subsequent Mono Q anion-exchange step yielded pure PP_i-PFK, as judged by SDS-PAGE, eluting from the column at approximately 0.6 M NaCl.

Characterization of pure PP_i-PFK revealed an M_r for the native enzyme of 170,000 with a subunit M_r of 43,000, suggest-

TABLE 2. PP_i-PFK and ATP-PFK activities in cells of various actinomycetes grown in batch cultures on YEME medium

Organism	Activity (U · mg of protein^{-1})	
	PP_i-PFK	ATP-PFK
A. methanolica	0.08	ND[a]
A. orientalis	0.30	ND
A. mediterranei	0.17	ND
A. azurea	0.06	ND
A. saturnea	0.02	ND
F. rectivirgula	ND	0.41
N. asteroides	ND	0.02
S. coelicolor	ND	0.06
S. griseus	ND	0.03

[a] ND, not detectable.

ing a tetrameric structure. The enzyme displayed a rather broad temperature optimum, and at least 90% of total activity was found between 35 and 46°C. The pH optimum was pH 7.5 for both the forward and the reverse reactions. Storage of the purified enzyme at −20°C in buffer with 40% glycerol did not reduce the activity significantly over a period of 12 months.

PP_i-PFK displayed Michaelis-Menten kinetics, and the apparent K_m values for fructose-6-phosphate and PP_i were estimated as 0.40 ± 0.037 and 0.20 ± 0.01 mM, respectively (Fig. 2); those for FBP and P_i (in the reverse reaction) were estimated as 0.025 ± 0.002 and 0.84 ± 0.068 mM, respectively (not shown). Mg^{2+} ions were essential for activity, and the K_m values for $MgCl_2$ were estimated as 0.04 ± 0.004 mM (forward reaction) and 0.77 ± 0.089 mM (reverse reaction). The enzyme showed an absolute specificity for its substrates. Neither ATP, ITP, UTP, nor polyphosphates could substitute for PP_i. Fructose-6-phosphate could not be replaced by fructose-1-phosphate or G6P. The metabolites ATP, ADP, AMP, citrate, α-ketoglutarate, oxaloacetate, 2,3PGA, PEP, and pyruvate (all 1 mM) and fructose-2,6-bisphosphate (0.1 mM) had no effect on enzyme activity.

The first 40 N-terminal amino acids of the PP_i-PFK protein were determined, and surprisingly low homology was found with the PP_i-PFK enzymes from *Propionibacterium freudenreichii* (25% identity, 55% similarity) (27) and potato (α subunit) (12.5% identity, 35% similarity) (6) (Fig. 3A), but high homology was found with ATP-PFK enzymes, e.g., the *Bacillus stearothermophilus* (61.5% identity, 76.9% similarity) (15) and *Escherichia coli* (51.3% identity, 76.9% similarity) enzymes (19) (Fig. 3B). Polyclonal antibodies against ATP-PFK A from *E. coli* cross-reacted with the *A. methanolica* PP_i-PFK (not shown).

Purification and characterization of PGM. PGM was purified to homogeneity (400-fold, 11% yield) in six steps (Table 4). Affinity chromatography with Procion-Red agarose, as described for PGM of *S. coelicolor* A3(2) (43), could not be

TABLE 3. Purification of PP_i-PFK from glucose-grown cells of *A. methanolica* WV2

Step	Protein (mg)	Total activity (U)	Sp act (U · mg^{-1})	Purification (fold)	Yield (%)
1. Crude extract	1,040	72.8	0.07	1	100
2. Protamine sulfate	1,000	70.0	0.07	1	96
3. Levafix blue	2.7	67.5	25	350	93
4. Mono Q	0.52	55.0	107	1,500	76

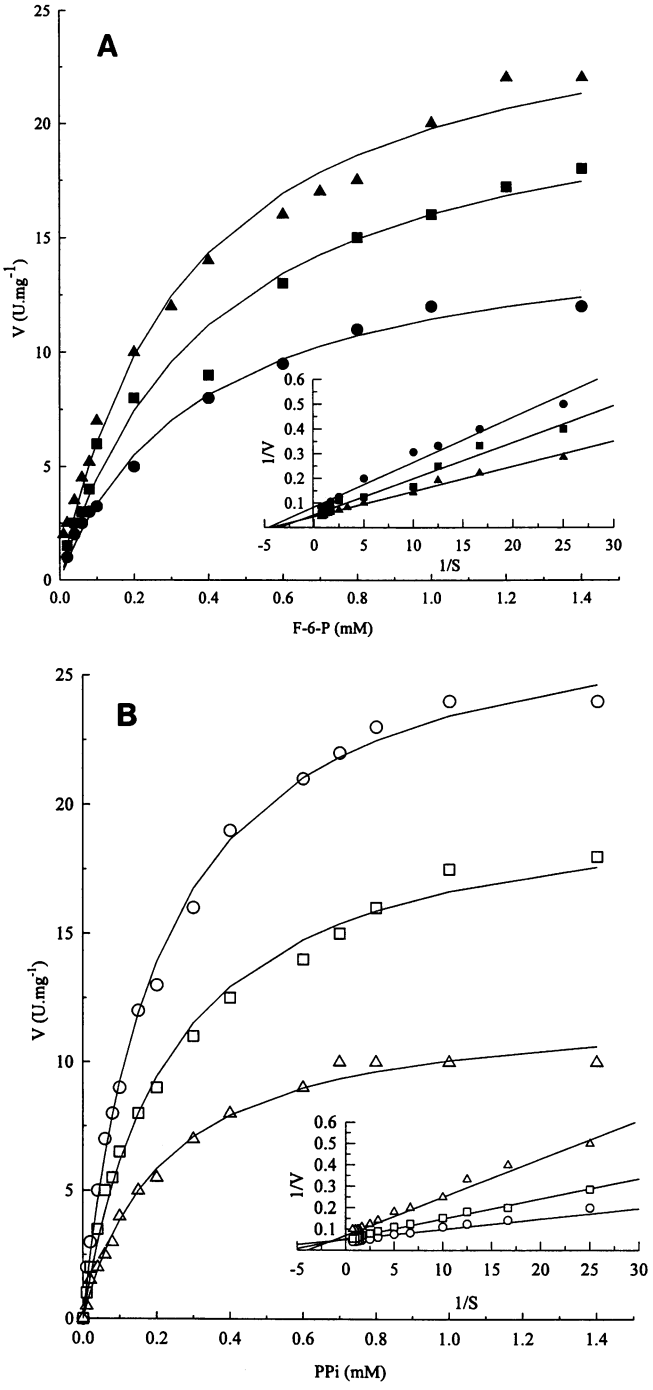

FIG. 2. (A) PP_i-PFK activity versus fructose-6-phosphate (F-6-P) concentration at different PP_i concentrations. The inset shows double-reciprocal plots of enzyme activity (V) versus substrate concentration (S). Activities are expressed in units per milligram of protein. ●, 0.2 mM PP_i; ■, 0.5 mM PP_i; ▲, 1.0 mM PP_i. (B) PP_i-PFK activity versus PP_i concentration at different fructose-6-phosphate concentrations. The inset shows double-reciprocal plots of enzyme activity (V) versus substrate concentration (S). Activities are expressed in units per milligram of protein. △, 0.2 mM fructose-6-phosphate; □, 0.5 mM fructose-6-phosphate; ○, 1.0 mM fructose-6-phosphate.

A

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                                    ..    .   *  .     .     .     .
A. methanolica          (1-40)    M--RVGVLTGGGDCPGLNAVIRAVVRKGIE-AHGWEIVGFRSG
P. freudenreichii       (1-40)    MVKKVALLTAGGFAPCLSSAIAELIKRYTEVSPETTLIGY---
Potato                  (2-41)    DAD-YGIPRELSDLQKLRSHYHPELPPCLQGTTVR--VELRDA
```

B

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                                  ..****.*** **.** **.***...   * *. *   *
A. methanolica          (1-40)    MRVGVLTGGGDCPGLNAVIRAVVRKGIEAHGWEIVGFRSG
E. coli                 (3-41)    KKIGVLTSGGDAPGMNAAIRGVVRSAL-TEGLEVMGIYDG
B. stearothermophilus   (2-40)    KRIGVLTSGGDSPGMNAAIRSVVRKAI-YHGVEVYGVYHG
```

FIG. 3. Alignment of the N-terminal amino acid sequences of the PP_i-PFK enzyme of *A. methanolica* (this study) with those of *P. freudenreichii* (27) and the α subunit of potato (6) (A) and with the ATP-PFK enzymes of *E. coli* (19) and *B. stearothermophilus* (15) (B). *, identical residues; ., conserved residues.

used because *A. methanolica* PGM did not bind to this material. The native M_r of pure PGM was estimated as 36,000 by Superdex 200 gel filtration. SDS-PAGE revealed a single band at an M_r of 28,000, suggesting that PGM has a monomeric structure.

Purified PGM was activated (4.5-fold) by 2,3PGA, as was the case for crude extracts, but 2,3PGA was not essential for activity. The apparent K_a for 2,3PGA was 20.9 ± 2.9 μM (2.5 mM 3PGA), and the apparent K_m for 3PGA was 0.66 ± 0.067 mM (0.2 mM 2,3PGA) (Fig. 4). Similar data have been reported for *S. coelicolor* A3(2) PGM, which was activated 5.1-fold by 2,3PGA, with a K_m of 1.3 mM for 3PGA (43).

Purification and characterization of pyruvate kinase. Pyruvate kinase was purified (253-fold, 14% yield) from glucose-grown cells (Table 5). Pyruvate kinase eluted from the Superdex 200 gel filtration column at a position corresponding to a M_r of 195,000. A subsequent alkyl-Superose hydrophobic interaction step yielded a 99% pure pyruvate kinase preparation, as judged by SDS-PAGE, eluting at approximately 0.7 M $(NH_4)_2SO_4$. The enzyme has a subunit M_r of 55,000, suggesting that the *A. methanolica* pyruvate kinase has a tetrameric structure, as is generally the case for other organisms (14). The optimum conditions for activity were pH 8.0 and 41°C. The temperature optimum was rather broad, and 90% of total activity was found between 38 and 46°C. Storage of the enzyme preparation at −20°C in buffer with 40% glycerol did not reduce the activity significantly over a period of 3 months. Incubation of purified pyruvate kinase at 37°C without PEP resulted in a complete loss of activity in 3 min. Enzyme assays therefore were always started with ADP.

Pyruvate kinase displayed positive cooperativity towards PEP (Fig. 5A), as has been observed for the enzyme from various other sources (14). The $S_{0.5}$(PEP) was 172 ± 9 μM with a Hill coefficient of 1.5 ± 0.1. No cooperativity was observed for ADP (Fig. 5B). The $S_{0.5}$(ADP) was 112 ± 7 μM with a Hill coefficient of 1.1 ± 0.06. Mg^{2+} ions were essential for pyruvate kinase activity; the K_m for $MgCl_2$ was estimated as 2.0 ± 0.2 mM. Various other cations were tested (1 mM concentrations), but compared with $MgCl_2$ (100%), the other cations, i.e., $MnCl_2$ (91%), $CuCl_2$ (82%), $CoCl_2$ (73%), $CaCl_2$ (15%), $FeCl_2$ (9%), and $ZnCl_2$ (6%), were less effective.

A range of possible effectors of pyruvate kinase activity were tested. P_i (10 mM) and ATP (1 mM) caused 92 and 37% (with 0.17 mM PEP and 2.5 mM ADP) or 42 and 41% (with 2.5 mM PEP and 0.11 mM ADP) inhibition, respectively, whereas AMP had no direct effect. Inhibition by P_i, but not that by ATP, could be overcome by adding 1 mM AMP or 2.5 mM PEP to the assay mixture. At 1 mM concentrations G6P, fructose-6-phosphate, ribose-5-phosphate, erythrose-4-phosphate, FBP, 3PGA, 2PGA, 2,3PGA, acetyl coenzyme A, acetate, citrate, α-ketoglutarate, malate, and succinate did not affect pyruvate kinase activity.

TABLE 4. Purification of PGM from glucose-grown cells of *A. methanolica* WV2

Step	Protein (mg)	Total activity (U)	Sp act ($U \cdot mg^{-1}$)	Purification (fold)	Yield (%)
1. Crude extract	747.0	1,584	2.12	1	100
2. Q-Sepharose	54.5	1,723	31.62	15	109
3. Butyl-Sepharose	6.6	740	112.09	53	47
4. Resource Q	0.9	461	512.75	242	29
5. Superdex 200	0.4	315	787.11	371	20
6. Phenyl-Superose	0.2	170	849.15	400	11

DISCUSSION

Glucose metabolism in the actinomycete *A. methanolica* appears to be regulated at the levels of both synthesis (PP_i-PFK and pyruvate kinase) and activity (pyruvate kinase and PGM). The 2,3PGA-activated type of PGM is generally ob-

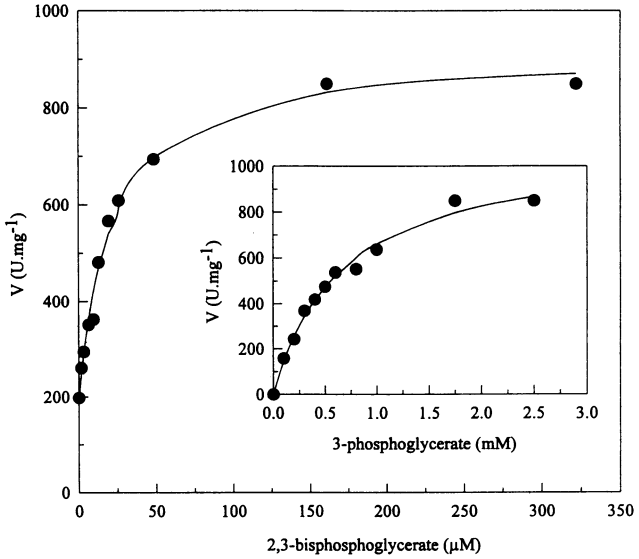

FIG. 4. PGM activity versus 2,3PGA concentration at 2.5 mM 3PGA. The inset shows a plot of PGM activity versus 3PGA concentration at 0.2 mM 2,3PGA. Activity (V) is expressed in units per milligram of protein.

TABLE 5. Purification of pyruvate kinase from glucose-grown cells of *A. methanolica* WV2

Step	Protein (mg)	Total activity (U)	Sp act ($U \cdot mg^{-1}$)	Purification (fold)	Yield (%)
1. Crude extract	495.6	60.8	0.12	1	100
2. Q-Sepharose	19.6	60.2	1.76	14	97
3. Superdex 200	7.6	23.2	8.77	72	38
4. Alkyl-Superose	0.29	9.0	31.1	253	15

served only in vertebrates and yeasts (14), with the single exception of another actinomycete, *S. coelicolor* A3(2) (43). Cofactor-dependent PGM enzymes are active as either monomers, dimers, or tetramers, depending on the enzyme source (14). These enzymes have a subunit size of 27 kDa (14), as is the case in *S. coelicolor* A3(2) (tetramer) (43) and *A. methanolica* (monomer). AMP-activated pyruvate kinase enzymes (type II) similar to the *A. methanolica* enzyme have been found in various organisms, e.g., in *E. coli* (39), *B. stearothermophilus* (36), and *Brevibacterium flavum* (34).

The presence of PP_i-PFK in actinomycetes of the family of the *Pseudonocardiaceae* is rather unexpected. In general this type of PFK enzyme is associated with anaerobic metabolism (31, 32) in *Propionibacterium* spp. (33), various protozoa (32) and amoebas (35), and higher plants (6). These enzymes are rather diverse with respect to subunit size and quaternary structure. The *A. methanolica* enzyme shares with other microorganisms the properties of a PP_i-PFK insensitive to activity regulation by various metabolites. The high N-terminal amino acid sequence homology between the PP_i-PFK of the obligately aerobic bacterium *A. methanolica* and various ATP-PFK enzymes reported in this paper appears exceptional, however, prompting questions about the evolutionary relatedness of these enzymes. The PP_i-PFK of *A. methanolica* may provide a very interesting model system for further comparative studies of the catalytic mechanisms (PP_i versus ATP) and allosteric control of PP_i- and ATP-PFK enzymes. We are currently attempting to clone the PP_i-PFK-encoding gene of *A. methanolica* and to crystallize the protein.

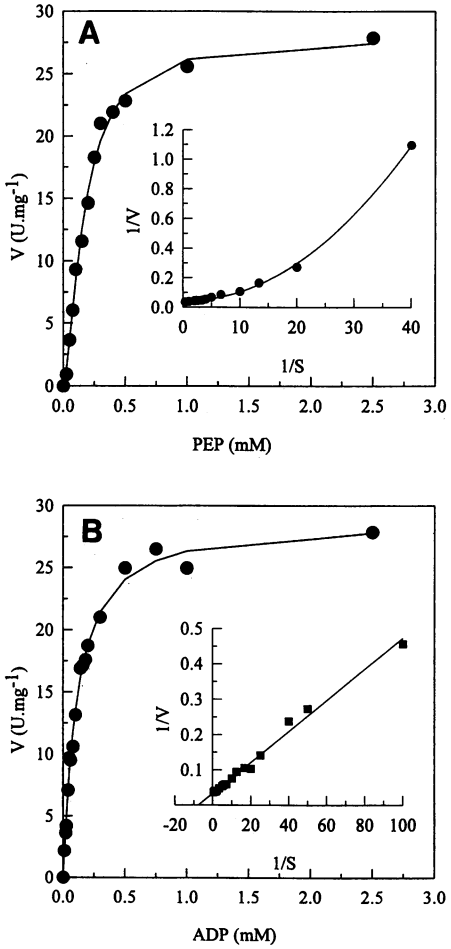

FIG. 5. Pyruvate kinase activity versus PEP concentration at 2.5 mM ADP (A) or versus ADP concentration at 2.5 mM PEP (B). The inset shows double-reciprocal plots of enzyme activity (V) versus substrate concentration (S). Activity is expressed in units per milligram of protein.

The reaction catalyzed by the PP_i-PFK enzyme is readily reversible, in sharp contrast to the essentially irreversible reaction catalyzed by the ATP-PFK enzyme. Accordingly, PP_i-PFK also provides FBP activity for gluconeogenesis. At present we have no explanation for the additional presence and physiological role of a proper FBPase enzyme observed in *A. methanolica* (Table 1; Fig. 1). This enzyme was readily separated from PP_i-PFK upon its purification. Recently, it has been demonstrated (24) that the gene for PP_i-PFK of *P. freudenreichii* complements FBPase deficiency but not ATP-PFK deficiency in *E. coli* mutants. It has been suggested that the intracellular PP_i concentrations in *E. coli* are too low to permit a PFK activity sufficient for glycolysis. The observation of relatively high intracellular concentrations of PP_i in several obligate methanotrophs which employ PP_i-PFK enzymes supports this suggestion (2, 38). The use of PP_i as a phosphate donor thus raises questions about the metabolism of PP_i and its further effects on the regulation of glycolysis in *A. methanolica*.

Growth of methylotrophic and autotrophic bacteria on methanol involves assimilation of one-carbon compounds via the RuMP (Fig. 1) and Calvin cycles, respectively. This generally results in strongly increased levels of those enzymes with a dual role in both the pentose phosphate pathway and the RuMP or Calvin cycle, allowing relatively high rates of one-carbon compound assimilation. For autotrophic bacteria this has been shown to be based on the presence of additional genes encoding these enzymes in the *cbb* gene cluster that become specifically induced when the Calvin cycle is switched on (3). Less information is available about the situation in RuMP cycle methylotrophic bacteria. From mutant and protein purification studies, it has become clear that the methylotroph *Arthrobacter* strain P1 employs a constitutive pentose phosphate pathway transaldolase enzyme during growth on glucose but produces an additional C_1-inducible transaldolase isoenzyme during growth on methylated amines (30). Growth of *A. methanolica* on methanol results in a fivefold increase in transketolase activity; mutant studies suggest that this increase is based on the expression of a second transketolase system.

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