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Expression of the *Escherichia coli pfkA* Gene in *Alcaligenes eutrophus* and in Other Gram-Negative Bacteria

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The *Escherichia coli pfkA* gene has been cloned in the non-self-transmissible vector pVK101 from hybrid plasmids obtained from the Clarke and Carbon clone bank, resulting in the plasmids pAS300 and pAS100; the latter plasmid also encoded the *E. coli tpi* gene. These plasmids were transferred by conjugation to mutants of *Alcaligenes eutrophus* which are unable to grow on fructose and gluconate due to the lack of 2-keto-3-deoxy-6-phosphogluconate aldolase activity. These transconjugants recovered the ability to grow on fructose and harbored pAS100 or pAS300. After growth on fructose, the transconjugants contained phosphofructokinase at specific activities between 0.73 and 1.83 U/mg of protein, indicating that the *E. coli pfkA* gene is readily expressed in *A. eutrophus* and that the utilization of fructose occurs via the Embden-Meyerhof pathway instead of the Entner-Doudoroff pathway. In contrast, transconjugants of the wild type of *A. eutrophus*, which are potentially able to catabolize fructose via both pathways, grew at a decreased rate on fructose and during growth on fructose did not stably maintain pAS100 or pAS300. Indications for a glycolytic futile cycling of fructose 6-phosphate and fructose 1,6-bisphosphate are discussed. Plasmid pAS100 was also transferred to 14 different species of gram-negative bacteria. The *pfkA* gene was expressed in most of these species. In addition, most transconjugants of these strains and of *A. eutrophus* exhibited higher specific activities of triosephosphate isomerase than did the corresponding parent strains.

Alcaligenes eutrophus relies on a strictly respiratory energy metabolism. This bacterium is therefore unable to grow on fructose or gluconate in the absence of terminal electron acceptors like oxygen or nitrate. Despite its respiratory energy metabolism, typical fermentation enzymes (30) like an NAD-dependent lactate dehydrogenase (35–37) and an unspecific NAD(P)-dependent alcohol dehydrogenase (38) are derepressed if the cells are incubated under conditions of restricted oxygen supply (29), and the respective fermentation products are excreted into the medium (41). In addition, reduced pyridine nucleotides are reoxidized during the formation of poly-β-hydroxybutyrate (31) and to some extent during the evolution of molecular hydrogen, which is catalyzed by NAD-dependent hydrogenase (20). It is not known why *A. eutrophus* cannot take advantage of the fermentation enzymes and grow under anaerobic conditions. Since the cells grow well in the absence of molecular oxygen with nitrate as a terminal electron acceptor (25), oxygenases are apparently not involved in the anabolic metabolism of *A. eutrophus*.

One reason for this organism's failure to grow anaerobically by fermentative energy generation may be the low ATP yield during fructose degradation via the Entner-Doudoroff pathway as compared to the Embden-Meyerhof pathway (4, 17). If the latter pathway could be established in *A. eutrophus*, the ATP yield would be twice as high. As phosphofructokinase is the only enzyme required to complete the Embden-Meyerhof pathway in *A. eutrophus*, recombinant DNA techniques were applied to establish this enzyme. Easy success was promised by the availability (i) of the *pfkA* gene encoding phosphofructokinase 1 from *Escherichia coli* on hybrid plasmids (32, 39, 40), (ii) of the wide-host-range cloning vector pVK101 (19), which is suitable as a cloning vector in *A. eutrophus* (C. Hogrefe, Ph.D. thesis, University of Göttingen, Göttingen, Federal Republic of Germany, 1984), and (iii) of mutants of *A. eutrophus* H16 lacking the ability to grow on fructose and gluconate due to the lack of active 2-keto-3-deoxy-6-phosphogluconate (KDPG) aldolase (5).

MATERIALS AND METHODS

Bacterial strains. Strains of *E. coli*, *A. eutrophus*, and other bacteria used in this study are listed in Tables 1 and 2. *A. eutrophus* HF172 has been previously designated as strain F34 (5).

Growth conditions. *E. coli* was grown either in complex Luria-Bertani (LB) medium (21) or in M9 mineral salts medium (21) which contained 0.2% (wt/vol) mannitol, 50 μg of proline per ml, and 2 μg of thiamine per ml at 37°C. *A. eutrophus* and the other gram-negative bacteria were grown either in a complex medium of nutrient broth (NB) (0.8% wt/vol) or in a mineral salts medium (28) at 30°C. For heterotrophic growth, the mineral salts medium was supplemented with filter-sterilized solutions of the carbon source as indicated. For autotrophic growth, the gas atmosphere contained a mixture of H_2, CO_2, and O_2 in a ratio of 8:1:1 (vol/vol). Growth experiments were performed in sidearm flasks which contained liquid medium to about 15% of the flask volume. Growth was monitored with a Klett-Summerson colorimeter equipped with a 520- to 580-nm filter; 100 Klett units was equivalent to an optical density of about 1 measured with a PL 4 Zeiss spectrophotometer at 436 nm.

Enzyme assays. For the determination of enzyme activities, washed cells were disrupted by sonication (1 min/ml of suspension) by using an MSE (150 W) ultrasonic disintegrator with a probe of 9.5 mm in diameter. Unbroken cells and membrane particles were removed by 60 min of centrifugation at 100,000 × *g*. Enzyme activities in the supernatant were determined at 30°C by following the oxidation of NADH at 365 nm. One unit of enzyme activity was defined as the transformation of 1 μmol of substrate per min. Specific activity was measured in units per milligram of protein.

TABLE 1. Bacterial strains used in this study

Strain	Plasmid(s)	Relevant characteristics[a]	Reference or source[b]
Alcaligenes eutrophus			
H16	pHG1	Wild type, autotrophic, prototrophic	ATCC 17699, DSM 428 (42)
HF172	pHG1	KDPG aldolase-negative mutant of H16, Fru$^-$, Glu$^-$, autotrophic, prototrophic	DSM 539 (5)
C145	pHG1	KDPG aldolase-negative mutant of H16, Fru$^-$, Glu$^-$, autotrophic, prototrophic	B. Bowien
N9A	pHG3	Wild type, autotrophic, prototrophic	DSM 518
Escherichia coli			
DF1020	None	Deletions in *pfkA* and *pfkB*, Man$^-$, auxotrophic for proline and thiamine	CGSC 6194
HB101	pVK101	Km^r Tc^r (encoded by pVK101)	19
S17-1	None	*tra* genes of plasmid RP4 are integrated into the chromosome, auxotrophic for proline and thiamine	34
C600	pGE42	Contains the *pfkA* gene	32
JA200	F	None	CGSC 6059 (8)
JA200	F, pLC16-4	pLC16-4 contains the *pfkA* and *tpi* genes	39, 40

[a] Abbreviations: Fru, fructose; Glu, gluconate; Man, mannitol; negative superscript, failure to utilize the corresponding substrate for growth.
[b] ATCC, American Type Culture Collection; DSM, Deutsche Sammlung von Mikroorganismen; CGSC, *E. coli* Genetic Stock Center. B. Bowien, Ph.D. thesis, University of Göttingen, Göttingen, Federal Republic of Germany, 1970.

The activity of 6-phosphofructokinase (EC 2.7.1.11) was determined in 100 mM Tris hydrochloride (pH 8.2) containing 10 mM $MgCl_2$, 2 mM NH_4Cl, 1 mM fructose 6-phosphate, 0.2 mM NADH, 1 U of fructose 1,6-bisphosphate aldolase per ml, 3 U of triosephosphate isomerase per ml, and 1 U of glycerol 3-phosphate dehydrogenase per ml. The reaction was started by the addition of 1 mM ATP (13). For the determination of triosephosphate isomerase (EC 5.3.1.1) activity, the procedure described by Bergmeyer (2) was slightly modified. The assay was performed in 50 mM triethanolamine hydrochloride (pH 7.4) in the presence of 0.38 mM NADH and 5.5 U of glycerol 3-phosphate dehydrogenase per ml. The reaction was started by the addition of 1.25 mM DL-glyceraldehyde 3-phosphate. The activity of KDPG aldolase (EC 4.1.2.14) was determined in 45 mM triethanolamine hydrochloride (pH 7.6) containing 0.25 mM NADH and 20 U of L-(+)-lactate dehydrogenase per ml by the method of Blackkolb and Schlegel (4). The reaction was started by the addition of 1 mM KDPG.

Transformation. For transformation, *E. coli* DF1020 and S17-1 were grown in LB medium at 37°C. Competent cells were prepared and transformed by using the calcium chloride procedure described by Maniatis et al. (21).

Conjugation. Matings of *A. eutrophus* and other gram-negative bacteria (recipients) with *E. coli* S17-1 harboring hybrid plasmids (donor) were performed on solidified NB medium as described by Friedrich et al. (15). After 20 h of incubation at 30°C, the cells were washed from the agar and plated onto mineral salts medium containing succinate (0.2%, wt/vol) and tetracycline (12.5 μg/ml).

Isolation of plasmid DNA. Preparative isolation of plasmids pVK101, pLC16-4, pAS100, and pAS300 was performed by the method of Birnboim and Doly (3) and included centrifugation to equilibrium in ethidium bromide-containing cesium chloride density gradients (21). Plasmid pGE42 was isolated from *E. coli* by the method described by Colman et al. (10) after amplification of plasmids in the presence of chloramphenicol during growth in LB medium. For a rapid plasmid screening procedure, crude lysates were prepared by the method of Casse et al. (7) with the modifiications mentioned previously (15).

Analysis of plasmid DNA. Crude lysates were separated by electrophoresis in horizontal slab gels containing 0.8% (wt/vol) agarose in TBE buffer (89 mM Tris hydrochloride, 89 mM boric acid, 2.5 mM disodium EDTA, pH 8.5). Electrophoresis was done at 150 V and 80 mV for 6 h.

Isolated plasmid DNA was incubated with *Bgl*II, *Eco*RI, *Pst*I, *Hin*dIII, or *Sal*I alone; with *Bgl*II and *Eco*RI, *Bgl*II and *Pst*I, *Eco*RI and *Pst*I, *Bgl*II and *Sal*I, and *Bgl*II and *Hin*dIII; or with *Bgl*II, *Eco*RI, and *Pst*I. Digestions were carried out under the conditions described by Maniatis et al. (21). DNA restriction fragments were separated in TBE buffer in horizontal slab gels containing 0.5, 0.8, or 2.0% (wt/vol) agarose or in vertical polyacrylamide gels containing 3.5, 8.0, or 20.0% (wt/vol) acrylamide. The conditions used for separation followed the recommendations of Maniatis et al. (21).

TABLE 2. Expression of the *E. coli pfkA* and *tpi* genes in various gram-negative bacteria[a]

Strain	Reference or source[b]	Sp act (U/mg of protein) of:	
		Phosphofructokinase	Triosephosphate isomerase
Alcaligenes hydrogenophilus M50	24	<0.01	0.39
Alcaligenes hydrogenophilus M50(pAS100)	This study	0.82	0.78
Alcaligenes eutrophus CH34	22	<0.01	0.63
Alcaligenes eutrophus CH34(pAS100)	This study	<0.01	3.66
Alcaligenes eutrophus JMP222	11	<0.01	0.68
Alcaligenes eutrophus JMP222(pAS100)	This study	1.37	2.24
Alcaligenes faecalis	CIP 62.31	<0.01	0.38
Alcaligenes faecalis(pAS100)	This study	0.19	0.58
Alcaligenes paradoxus	ATCC 17713, DSM66	<0.01	0.32
Alcaligenes paradoxus(pAS100)	This study	1.32	0.14
Pseudomonas palleronii	ATCC 17724, DSM 63	<0.01	0.75
Pseudomonas palleronii(pAS100)	This study	1.23	1.04
Pseudomonas oxalaticus	26	<0.01	0.36
Pseudomonas oxalaticus(pAS100)	This study	1.23	1.05
Pseudomonas facilis	ATCC 17695, DSM 550	<0.01	0.37
Pseudomonas facilis(pAS100)	This study	0.56	0.24
Aquaspirillum autotrophicum SA32	DSM 732	<0.01	0.32
Aquaspirillum autotrophicum SA32(pAS100)	This study	1.16	0.40
Azotobacter chroococcum	ATCC 9043, DSM 2286	<0.01	0.33
Azotobacter chroococcum (pAS100)	This study	0.20	3.65
Azotobacter vinelandii	ATCC 478, DSM 2289	<0.01	0.29
Azotobacter vinelandii(pAS100)	This study	2.57	4.70
Xanthobacter autotrophicus 7CSF	DSM 2267	<0.01	0.17
Xanthobacter autotrophicus 7CSF(pAS100)	This study	0.02	0.59
Rhizobium meliloti MVII	A. Pühler	0.02	0.25
Rhizobium meliloti MVII(pAS100)	This study	0.43	7.27
Thiobacillus A2	ATCC 25364, DSM 582	0.01	0.78
Thiobacillus A2(pAS100)	This study	0.02	0.70

[a] Cells were grown in NB medium to the early stationary phase, except for cells of *A. chroococcum*, *A. vinelandii*, and *X. autotrophicus*, which were grown in succinate-containing mineral salts medium. The preparation of crude extracts and assay conditions were as described in Materials and Methods.

[b] CIP, Collection de l'Institut Pasteur; ATCC, American Type Culture Collection; DSM, Deutsche Sammlung von Mikroorganismen.

The molecular weight of these fragments was estimated by comparing their migrations in the gels with the migration of standard fragments obtained by digestion of λ DNA with *Hin*dIII, *Eco*RI, or *Pst*I and of pBR322 DNA with *Hae*III.

DNA bands were stained with ethidium bromide and visualized on a UV transilluminator.

DNA ligation. Ligation of restricted plasmid DNA was performed as described by Maniatis et al. (21). Restriction endonucleases were inactivated by heat (10 min, 80°C) before ligation.

Protein determination. Soluble protein was routinely determined by the method of Bradford (6).

Chemicals. Restriction endonucleases, T4 ligase, pBR322 DNA and λ DNA, auxiliary enzymes, and substrates used in enzyme assays were obtained from C. F. Boehringer & Soehne (Mannheim, Federal Republic of Germany). Agarose type V and antibiotics were purchased from Sigma Chemical Co. (St. Louis, Mo.). All complex media were from Difco Laboratories (Detroit, Mich.). Most other chemicals were obtained from E. Merck AG (Darmstadt, Federal Republic of Germany).

RESULTS

Cloning of the *pfkA* gene in pVK101. Plasmid pLC16-4, derived from the Clarke and Carbon gene bank (8), harbors the *E. coli* genes for phosphofructokinase 1 (*pfkA*) and triosephosphate isomerase (*tpi*) (39, 40). Three additional proteins were recognized to be encoded by the chromosomal DNA cloned in this plasmid, and besides *pfkA* and *tpi* two genes were identified (23, 33). This plasmid was isolated from *E. coli* JA200(pLC16-4). It was digested with *Hin*dIII and ligated to pVK101 vector DNA linearized by digestion with *Hin*dIII (Fig. 1). Ligated DNA was transformed into *E. coli* DF1020. This mutant strain has deletions in both genes coding for phosphofructokinase activity and is therefore not able to utilize mannitol as a carbon source for growth (14). Cells were spread on tetracycline-containing LB agar plates, and transconjugants that were able to grow on mannitol and were resistant to tetracycline but sensitive to kanamycin were isolated.

Another source of the *pfkA* gene was plasmid pGE42, which had previously been constructed by intramolecular ligation after the digestion of pLC16-4 with *Pst*I (32). This plasmid lacks a considerable portion of the chromosomal *E. coli* DNA, e.g., no intact *tpi* gene is present, and also lacks some of the ColE1 vector DNA originally present in pLC16-4 (32). Plasmid pGE42 was isolated from *E. coli* C600(pGE42) and digested with low concentrations of *Sau*3A. Samples containing a large portion of fragments with a molecular size of approximately 8.5 kilobase pairs, as revealed from electropherograms, were ligated to pVK101 vector DNA linearized by digestion with *Bgl*II. Ligated DNA was transformed into *E. coli* DF1020, and transformants that were able to grow on mannitol and were resistant to tetracycline and kanamycin were isolated.

Two recombinant plasmids designated pAS100 and

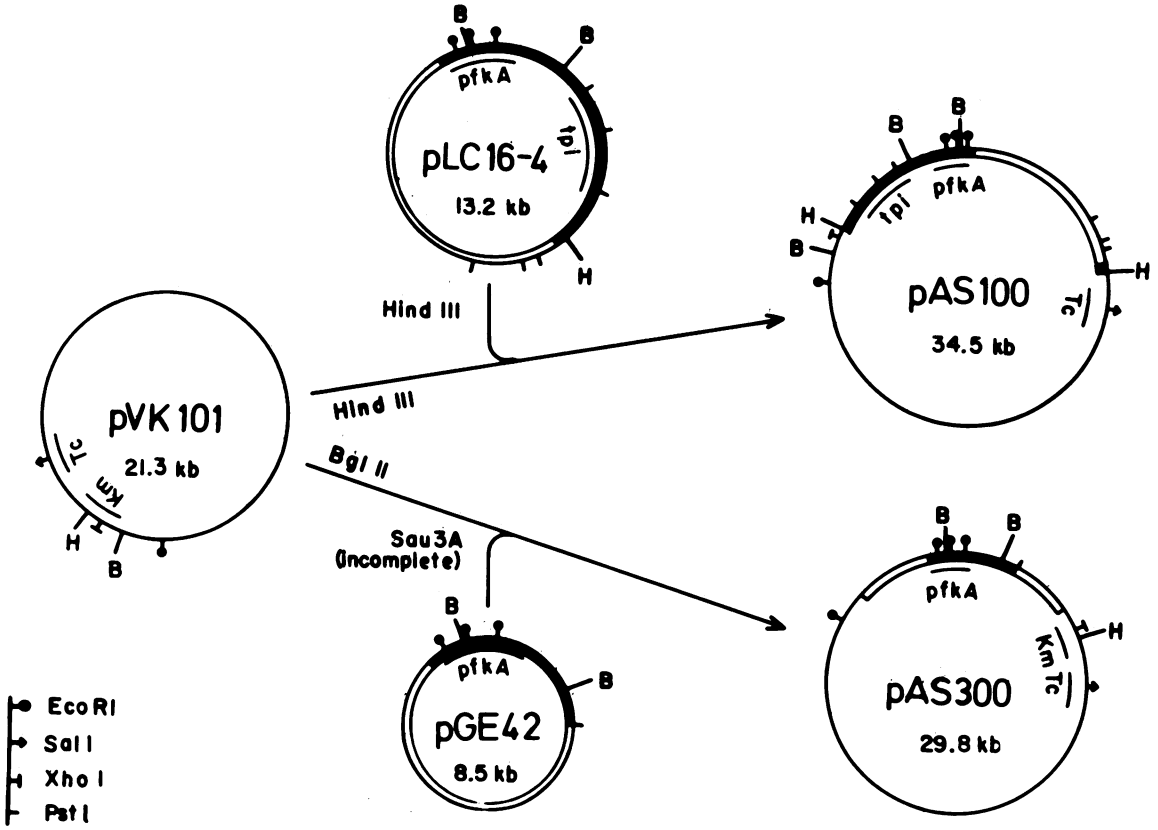

FIG. 1. Construction of plasmids. The thin lines forming the DNA circles represent pVK101 vector DNA. The open bars represent ColE1 DNA, and the solid bars represent *E. coli* chromosomal DNA. The lines inside the DNA circles indicate the approximate locations of the genes for phosphofructokinase 1 (*pfkA*), triosephosphate isomerase (*tpi*), teteracycline resistance (Tc), and kanamycin resistance (Km). The physical maps are an interpretation of the information obtained from digestions of plasmid DNA with restriction endonucleases (see Materials and Methods), taking into consideration previously published data (31). Abbreviations: B, *Bgl*II; H, *Hin*dIII; kb, kilobase pairs.

pAS300, with molecular sizes of 34.5 and 29.8 kilobase pairs, were derived from pLC16-4 and pGE42, respectively. For the construction of restriction cleavage maps, plasmids pLC16-4, pGE42, and pVK101 were digested with five restriction endonucleases which were applied alone or in combination (see Materials and Methods). The DNA fragments generated were separated by electrophoresis and analyzed for molecular size. An interpretation of the results (not shown in detail) is presented in Fig. 1. Three closely adjacent *Eco*RI sites were detected in pLC16-4 and pGE42; upon digestion of either plasmid with *Eco*RI, these sites led to the formation of two small fragments (340 and 470 base pairs [bp]) in addition to one large fragment. One of these *Eco*RI sites mapped about 40 bp from a *Bgl*II site.

In other experiments (not shown in detail) it was demonstrated that the small *Bgl*II fragment (about 2,000 bp) obtained from pGE42 did not code for active phosphofructokinase in *E. coli* DF1020 if cloned into the *Bgl*II site of pVK101.

Transfer of pAS100 and pAS300 into *A. eutrophus*. Plasmids pAS100 and pAS300 were isolated from transformants of *E. coli* DF1020 and transferred to *E. coli* S17-1 by transformation. This strain bears the RP4 plasmid transfer functions integrated in its chromosome and is therefore a mobilizing donor for non-self-transmissible plasmids derived from RP4 (34). Transformant strains of *E. coli* S17-1 were then mated with different strains of *A. eutrophus* H16 as described in Materials and Methods. After the cells were washed from the mating agar, the cell mixtures were plated onto mineral salts medium which contained succinate (0.2%, wt/vol) and tetracycline (12.5 μg/ml). Under these conditions only transconjugants of *A. eutrophus* harboring the hybrid plasmids were able to grow. The growth of wild-type and of *E. coli* S17-1 cells was impaired due to sensitivity towards tetracycline or auxotrophy for proline and thiamine, respectively.

Properties of KDPG aldolase-deficient mutants of *A. eutrophus* harboring pAS100 or pAS300. Both hybrid plasmids were first transferred to mutants of *A. eutrophus* which are deficient in KDPG aldolase and are therefore unable to grow on fructose or gluconate as a carbon source (5). The results obtained with transconjugants of *A. eutrophus* C145, which is a representative mutant strain, are described in detail. Similar results were obtained with 26 independently isolated KDPG aldolase-negative mutants. Three hundred transconjugants of *A. eutrophus* C145 were isolated from succinate-tetracycline plates. All transconjugants were able to utilize fructose as a carbon source for growth, whereas no growth occurred on gluconate-containing mineral agar plates. In addition, all transconjugants were still able to grow chemolithoautotrophically. Twenty of these transconjugants were analyzed for their plasmid content, and each strain was found to harbor a second plasmid in addition to pHG1 (molecular size, 450 kilobase pairs [15]), which was always observed. These additional plasmids resembled pAS100 or pAS300 with respect to their molecular weights (Fig. 2).

Two representative transconjugants, *A. eutrophus* C145(pAS100) and C145(pAS300), were examined in more

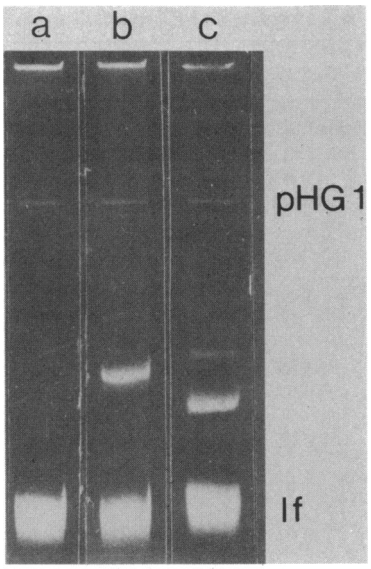

FIG. 2. Agarose gel electrophoresis of plasmid DNA of *A. eutrophus* and of transconjugants. Crude lysates obtained from *A. eutrophus* C145 (lane a), C145(pAS100) (lane b), and C145(pAS300) (lane c) were separated. Lysates were prepared as described previously (7, 15). The positions of pHG1 and of linear DNA fragments (lf) are indicated.

detail. The ability of these strains to grow on fructose was confirmed in liquid cultures (Fig. 3). Whereas the parent strain C145 was unable to utilize fructose or gluconate as a carbon source for growth, strains C145(pAS100) and C145(pAS300) grew well on fructose but did not grow on gluconate. The doubling time of the transconjugants on fructose was about 7 h. In constrast, the wild-type parental strain *A. eutrophus* H16 grew on fructose with a doubling time of 2.5 h.

These results suggested that the *E. coli pfkA* gene is expessed in *A. eutrophus* C145. Crude extracts of cells of strains C145(pAS100) and C145(pAS300), grown on fructose, NB, or other carbon sources, contained phosphofructokinase at high specific activities. KDPG aldolase activity was not detectable (Table 3). Phosphofructokinase activity was not detectable in *A. eutrophus* C145(pVK101). Furthermore, the specific activity of triosephosphate isomerase was two- to threefold higher in strain C145(pAS100) than in strains C145, C145(pAS300), C145(pVK101), or H16, from which the *E. coli tpi* gene is absent (Table 3). These results indicate that the *E. coli tpi* gene is also expressed in *A. eutrophus*. The activities of both enzymes detected in different strains of *E. coli* are shown in Table 3 for comparison.

Both plasmids were also transferred to *A. eutrophus* HF172. All transconjugants isolated from this mutant expressed the *pfkA* gene and probably also the *tpi* gene (Table 3); however, they did not grow on fructose, either in liquid or on solidified medium.

Properties of wild-type strains of *A. eutrophus* harboring pAS100 or pAS300. Both hybrid plasmids were also transferred to two different wild-type strains of *A. eutrophus*. Such transconjugants are potentially able to utilize fructose via two different pathways, and it was of interest to know whether their growth properties on fructose would differ from those of the parent strains. Strain H16 (pAS100) grew significantly more slowly on fructose than did the parental strain H16 or H16(pVK101) (not shown), but the growth rate increased slightly during the time course of the experiment (Fig. 4). The initial growth rate of strain H16(pAS100) resembled that of strain C145(pAS100) on fructose. The increase in growth rate was not visible when strain H16(pAS100) was grown on fructose in the presence of tetracycline. The growth was then similar to that of strain C145(pAS100). The growth of strain C145(pAS100) was not affected by tetracycline.

To obtain further information, cells of strain H16(pAS100), grown on fructose in the absence of tetracycline, were harvested in the stationary phase after about 32 h. The cells were plated on NB medium, and colonies were analyzed for their resistance to tetracycline. About 70% of these clones (from 200 analyzed) had lost resistance to tetracycline. Some of these clones were analyzed for their plasmid content. All clones that had become sensitive had lost pAS100 and harbored only pHG1, whereas clones that were still resistant harbored both plasmids. In contrast, all cells of H16(pAS100) and C145(pAS100) grown on fructose in the presence of tetracycline and all cells of C145(pAS100) and H16(pVK101) grown in the absence of tetracycline had retained both pAS100 or pVK101 and pHG1. Similar results were obtained with strains H16(pAS300), N9A, and N9A(pAS100). Transconjugants of the wild type maintained pAS100 stably during growth on NB medium, succinate, and gluconate in the absence of tetracycline. The growth of the transconjugants on these substrates did not differ from that of the corresponding parent strains.

Behavior of transconjugants of *A. eutrophus* under anaerobic conditions. All attempts to obtain anaerobic growth of *A. eutrophus* H16, H16(pAS100), H16(pAS300), N9A, N9A(pAS100), N9A(pAS300), C145(pAS100), or C145(pAS300) on fructose under various culture conditions failed. Anaerobic growth did not occur in liquid or on solidified medium even if the media were supplemented with complex substrates like NB, *Lactobacillus* broth, or Casamino Acids and even after prolonged incubation (6 weeks). Anaerobic growth also did not occur after the medium was freed of oxygen slowly by the cells themselves.

Expression of the *E. coli pfkA* gene in other gram-negative

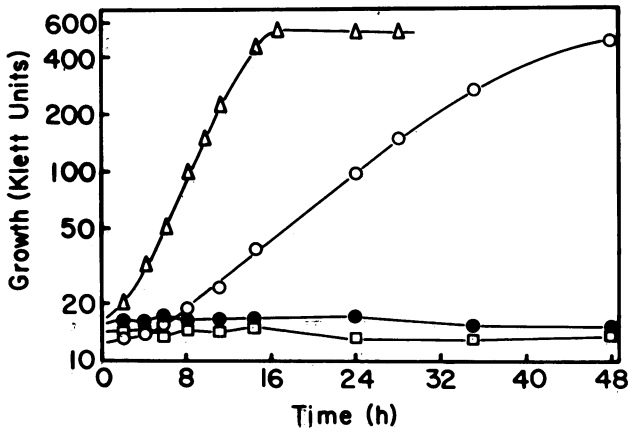

FIG. 3. Growth of wild-type and KDPG aldolase-deficient strains of *A. eutrophus*. *A. eutrophus* H16 (△), C145 (□), and C145(pAS100) (○) were incubated aerobically in 0.5% (wt/vol) fructose in mineral salts medium as described in Materials and Methods. Strain C145(pAS100) was also incubated in 0.5% (wt/vol) gluconate in mineral salts medium (●).

TABLE 3. Expression of the *E. coli pfkA* and *tpi* genes in *A. eutrophus* and *E. coli*

Strain	Growth[a] on:		Growth medium[b]	Sp act (U/mg of protein) of:		
	Fructose	Gluconate		Phosphofructokinase	Triosephosphate isomerase	KDPG aldolase
A. eutrophus H16	++	++	NB	<0.01	1.05	ND[c]
			FMSM	<0.01	0.91	0.13
A. eutrophus C145	−	−	NB	<0.01	0.90	<0.01
A. eutrophus C145(pAS100)	+	−	NB	1.68	2.75	<0.01
			FMSM	1.07	1.61	<0.01
A. eutrophus C145(pAS300)	+	−	NB	1.83	1.22	<0.01
			FMSM	0.73	0.84	<0.01
A. eutrophus HF172	−	−	NB	<0.01	1.10	<0.01
A. eutrophus HF172(pAS100)	−	−	NB	1.07	3.33	<0.01
E. coli DF1020	ND	ND	LB	0.01	ND	ND
E. coli DF1020(pGE42)	ND	ND	LB	27.5	ND	ND
E. coli DF1020(pAS100)	ND	ND	LB	12.6	ND	ND
E. coli S17-1	ND	ND	LB	0.60	5.43	ND
E. coli JA200	ND	ND	LB	0.73	5.06	ND

[a] Growth conditions were as described in Materials and Methods. −, No growth; +, doubling time = 7 h; ++, doubling time = 2.5 h.
[b] Medium used for the cultivation of cells that were used as the source of cell extracts for enzymatic assays. FMSM, Mineral salts medium (28) containing 0.5% (wt/vol) fructose. LB medium was supplemented with 0.5% (wt/vol) glucose.
[c] ND, Not determined.

bacteria. In the preceding work it was shown that the *E. coli pfkA* gene is readily expressed in various strains of *A. eutrophus*. To test for expression in other bacteria, pAS100 was transferred from *E. coli* S17-1 into several gram-negative bacteria (Table 2) by conjugation, as described in Materials and Methods. After mating, the cell mixtures were plated onto succinate-containing mineral salts medium which contained 12.5 μg of tetracycline per ml. Two transconjugants were isolated from each mating. These transconjugants and the parent strains were analyzed for plasmid content and for phosphofructokinase and triosephosphate isomerase activities after growth on NB medium. Strains of *Azotobacter chroococcum*, *Azotobacter vinelandii*, and *Xanthobacter autotrophicus* were examined after growth on succinate-containing mineral salts medium.

Plasmid pAS100 could be transferred into all the strains listed in Table 2. All tetracycline-resistant transconjugants harbored a plasmid which was identical to pAS100 with respect to size. If the parent strains contained plasmids (16), then the plasmid pattern was unaffected by the presence of the new plasmid. Phosphofructokinase activity was detected in nearly all the transconjugants, whereas no activity or only negligible activities were detected in the parent strains. Phosphofructokinase activity was absent or present at only very low levels in transconjugants of *A. eutrophus* CH34, *X. autotrophicus* 7CSF, and *Thiobacillus* strain A2 (Table 2). Most transconjugants also exhibited higher specific activities for triosephosphate isomerase than the corresponding parent strains. Exceptions were transconjugants of *A. paradoxus*, *Pseudomonas facilis*, and *Thiobacillus* strain A2. Transconjugants of *A. eutrophus* CH34 and *X. autotrophicus* 7CSF exhibited 6- and 3.5-fold higher triosephosphate isomerase activities than the parent strains (Table 2).

DISCUSSION

The enzymatic data clearly demonstrate that the *E. coli pfkA* gene is expressed in *A. eutrophus*. The specific activity of phosphofructokinase in transconjugants of *A. eutrophus* varied between 0.73 and 1.83 U/mg of protein, depending on the strain and on the growth conditions. In *E. coli* strains which harbor only the chromosomal genes for phosphofructokinases, the specific activity of this enzyme was between 0.60 and 0.73 U/mg of protein. The much higher activity of

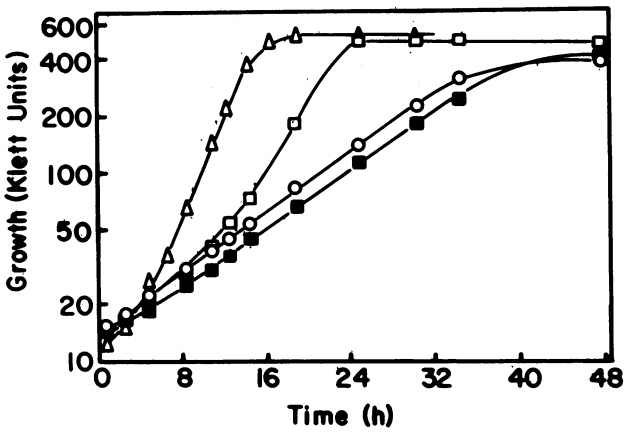

FIG. 4. Growth of various strains of *A. eutrophus*. *A. eutrophus* H16 (△), H16(pAS100) (□ and ■), and C145(pAS100) (○) were incubated aerobically in 0.5% (wt/vol) fructose in mineral salts medium as described in Materials and Methods. The solid symbol indicates the presence of tetracycline (12.5 μg/ml) in the medium.

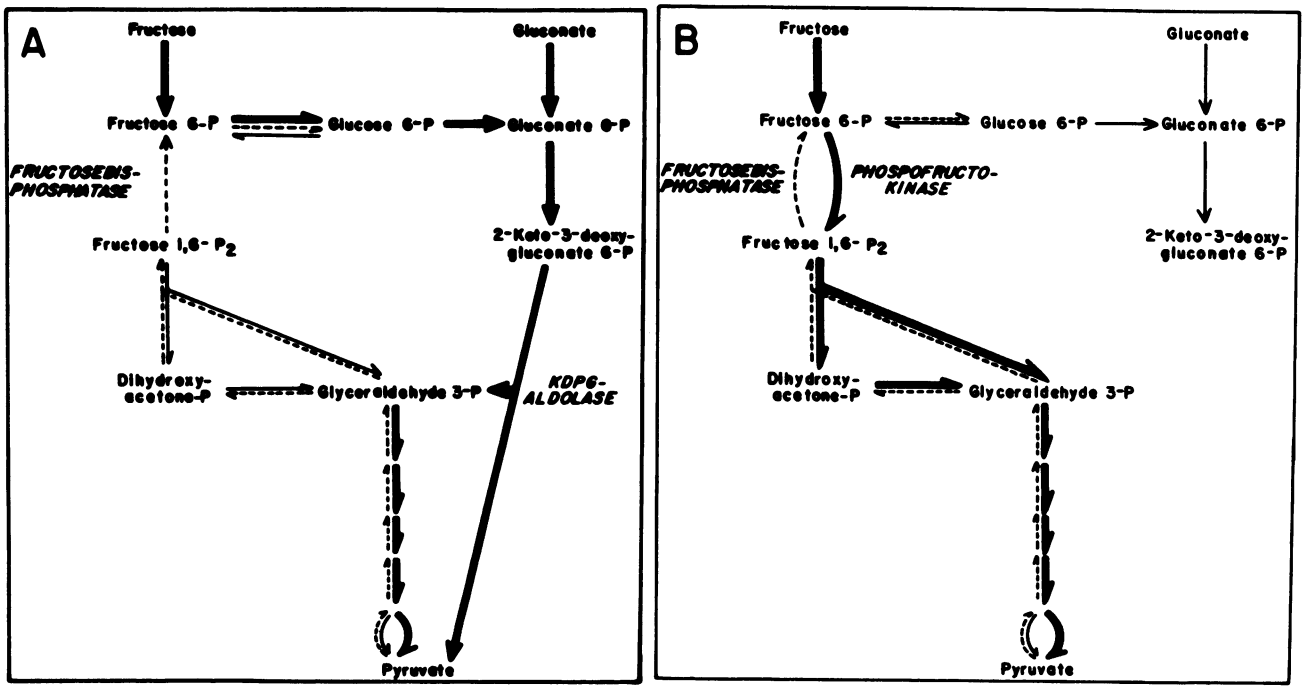

FIG. 5. Pathways of the central carbon metabolism in wild-type strains of *A. eutrophus* (A) and in KDPG aldolase-negative mutants which harbor hybrid plasmids coding for phosphofructokinase (B). Heavy arrows indicate the pathway for the degradation of fructose and gluconate; broken arrows indicate the gluconeogenic pathway; and thin arrows indicate reactions which are potentially available for the cells due to the reversibility of reactions catalyzed by the enzymes present.

phosphofructokinase in *E. coli* DF1020(pGE42) may result from the high copy number with which plasmids containing the ColE1 replicon occur in *E. coli* cells (9). Phosphofructokinase activity was about 50% lower in strain DF1020(pAS100). This may be explained by the joint replicons of RP4 and ColE1 in pAS100; it is known that these plasmids occur in lower copy numbers in *E. coli* (12). Unfortunately, no data concerning the copy numbers of these plasmids and the turnover of the phosphofructokinase protein in *A. eutrophus* are available. It is therefore not possible to compare the transcription rates of the *pfkA* gene in both species. However, the enzymatic data presented here document that the *E. coli pfkA* gene is readily expressed in *A. eutrophus* and in most of the gram-negative bacteria examined in this study.

The data also indicate that the *E. coli tpi* gene is presumably expressed in *A. eutrophus* and in many other bacteria examined here, but this must be confirmed by independent methods, since the genetic information for the formation of triosephosphate isomerase is already present in all these bacteria.

The presence of the *E. coli pfkA* gene and its expression had drastic consequences in *A. eutrophus*. KDPG aldolase-negative mutants, which are not able to utilize fructose or gluconate as a carbon source for growth, recovered the ability to grow on fructose if they harbored a plasmid encoding the *E. coli* gene. Since phosphofructokinase is the only enzyme missing in the glycolytic sequence in *A. eutrophus* (27; Fig. 5), a physiologically functioning Embden-Meyerhof pathway was established in the cells (Fig. 5B). The reaction catalyzed by glucose 6-phosphate dehydrogenase is irreversible; therefore, the transconjugants could not grow on gluconate. Of 27 mutants examined, strain HF172 was the only strain which was unable to grow on fructose after the introduction of the *E. coli pfkA* gene, although the gene was expressed. It is possible that the transport system for fructose is also affected by the mutation in this strain (5).

The presence of the *pfkA* gene and its expression also affected the utilization of fructose in wild-type strains of *A. eutrophus*. Both pathways for the degradation of fructose are present in these strains. All transconjugants of the wild type, e.g., strain H16(pAS100), exhibited a diminished growth rate on fructose which was identical to the growth rate of transconjugants of KDPG aldolase-negative mutants. This result and the loss of the plasmid coding for the *pfkA* gene during growth on fructose indicate that the presence of phosphofructokinase exerts disadvantageous effects during the utilization of fructose in the wild type. The cells can overcome these difficulties by disposing of the genetic information for the formation of phosphofructokinase unless additional selective pressure (e.g., the presence of tetracycline) is imposed on the cells to retain the plasmid. One reasonable explanation for this behavior may be derived from the coexistence of phosphofructokinase and fructose 1,6-bisphosphatase in the cells. The latter enzyme is formed constitutively in *A. eutrophus*, albeit the cells exhibit the highest levels during autotrophic growth (1, 27). The regulation of these enzymes and their kinetic properties may not fit the physiological situation intracellularly in *A. eutrophus* during growth on fructose. The presence of both enzymes may result in a futile cycle involving the reconversion of fructose 1,6-bisphosphate to fructose 6-phosphate. Such a glycolytic futile cycling would result in a waste of energy. Futile cycling apparently does not occur during growth on substrates, such as gluconate or succinate, which require the gluconeogenic formation of glucose 6-phosphate, since growth on these substrates is not affected by the presence of the *pfkA* gene.

Although it was shown in this study that the Embden-

Meyerhof pathway can easily be established in *A. eutrophus*, it was also clearly demonstrated that *A. eutrophus* cannot be transformed from a strictly aerobic into a facultatively anaerobic bacterium by this single step. Therefore, this study provides no answer to the question of what makes the growth of this bacterium strictly dependent on respiratory conditions.

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ADDENDUM

After this manuscript was completed, Hellinga and Evans (18) published the nucleotide sequence of the *pfkA* gene and adjacent regions in pLC16-4. These data confirm the location of the restriction sites for *Eco*RI and *Bgl*II presented here. An additional *Eco*RI site mapping 24 bp from the center *Eco*RI site (18) was not detected in the present study due to the limitations of the methods used. The sequence data also show that the 2,000-bp *Bgl*II fragment cannot code for active phosphofructokinase.

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