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Gluconeogenic Mutations in *Pseudomonas aeruginosa*: Genetic Linkage between Fructose-bisphosphate Aldolase and Phosphoglycerate Kinase

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Mutants of mucoid *Pseudomonas aeruginosa* defective in fructose-bisphosphate aldolase (FBA), NADP-linked glyceraldehyde-3-phosphate dehydrogenase (GAP) or 3-phosphoglycerate kinase (PGK) were unable to grow on gluconeogenic precursors like glutamate, succinate or lactate. The *gap* and *pgk* mutants could grow on glucose, gluconate or glycerol, but *fba* mutants could not. This suggests that the metabolism of glucose or gluconate does not require either PGK or NADP-linked GAP but does require the operation of the aldolase-catalysed step. For gluconeogenesis, however, all three steps are essential. Recombinant plasmids carrying genes for FBA, PGK, GAP or phospho-2-keto-3-deoxygluconate aldolase (EDA) activities were constructed from a genomic library of mucoid *P. aeruginosa* selecting for complementation of deficiency mutations. Analysis of their complementation profile indicated that one group of plasmids carried *fba* and *pgk* genes, while another group carried *eda*, 6-phosphogluconate dehydratase (*edd*) and glucose-6-phosphate dehydrogenase (*zwf*) genes. The *gap* gene was not linked to any of these markers. Partial restoration of FBA activity in spontaneous revertants of Fba$^-$ mutants was accompanied by a concomitant loss of PGK activity. These experiments indicate a linkage between the *fba* and *pgk* genes on the *P. aeruginosa* chromosome.

INTRODUCTION

The group of bacteria represented by the genus *Pseudomonas* utilizes carbohydrates predominantly by the Entner–Doudoroff pathway (Lessie & Phibbs, 1984). Fig. 1 shows a scheme of this and related pathways in *Pseudomonas aeruginosa*. Mutations affecting enzymes of sugar degradation pathways have been investigated in *P. aeruginosa* (Blevins *et al.*, 1975; Phibbs *et al.*, 1978). The genes *zwf*, *edd* and *eda*, which encode the enzymes glucose-6-phosphate dehydrogenase (ZWF, EC 1.1.1.49), 6-phosphogluconate dehydratase (EDD, EC 4.2.1.12), and phospho-2-keto-3-deoxygluconate aldolase (EDA, EC 4.1.2.14) are clustered on the chromosome (Roehl *et al.*, 1983). In contrast, the gluconeogenic pathway in this organism has not been studied to this extent. Experiments using *Pseudomonas putida* have raised questions on the role of the Embden–Meyerhof pathway in gluconeogenesis (Ruiz-Amil *et al.*, 1969; Aparicio *et al.*, 1971).

In the course of a study on the biosynthesis of alginic acid in a mucoid strain of *P. aeruginosa*, we had isolated several mutants, each affected in a single enzyme of the Embden–Meyerhof pathway (Banerjee *et al.*, 1983, 1985). These mutants were unable to grow on gluconeogenic precursors such as succinate or glutamate and included lesions in the following three enzymes: NADP-linked glyceraldehyde-3-phosphate dehydrogenase (GAP) (NADP-linked; EC

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Abbreviations: EDA, phospho-2-keto-3-deoxygluconate aldolase; EDD, 6-phosphogluconate dehydratase; FBA, fructose-bisphosphate aldolase; GAP, glyceraldehyde 3-phosphate dehydrogenase; PGK, 3-phosphoglycerate kinase; ZWF, glucose-6-phosphate dehydrogenase.

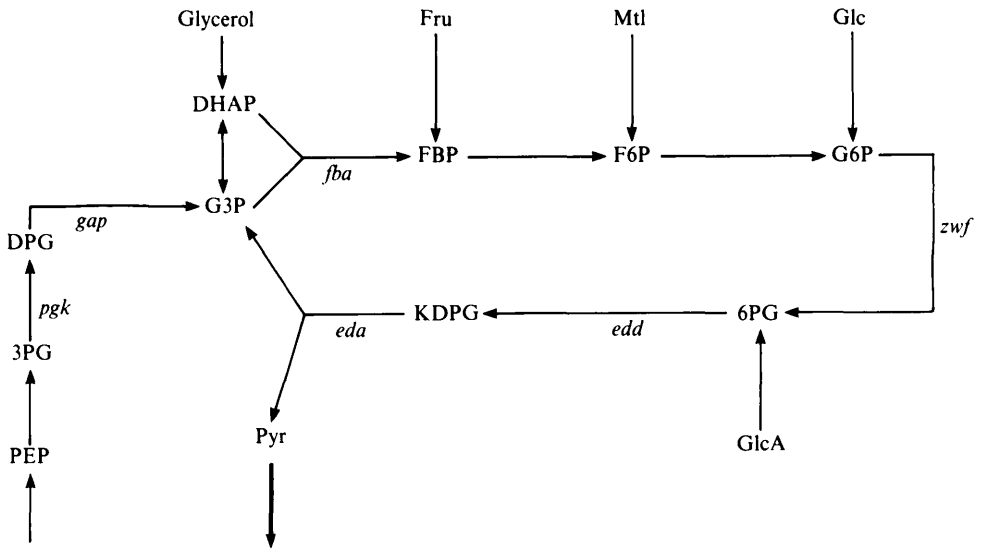

Fig. 1. Pathways for sugar degradation and synthesis in *P. aeruginosa*. Mutations used in this work are indicated in italics. Fru, fructose; Mtl, mannitol; Glc, glucose; GlcA, gluconate; 6PG, 6-phosphogluconate; KDPG, 2-keto-3-deoxy-6-phosphogluconate; Pyr, pyruvate; PEP, phosphoenolpyruvate; 3PG, 3-phosphoglycerate; DPG, 1,3-diphosphoglycerate; G3P, glyceraldehyde 3-phosphate; DHAP, dihydroxyacetone phosphate; FBP, fructose 1,6-bisphosphate; F6P, fructose 6-phosphate; G6P, glucose 6-phosphate.

1.2.1.13), 3-phosphoglycerate kinase (PGK, EC 2.7.2.3) and fructose-bisphosphate aldolase (FBA, EC 4.1.2.13) (structural genes *gap*, *pgk* and *fba* respectively). This report describes biochemical characters of these mutants, and a close linkage of the genes *pgk* and *fba*. We also confirm the clustering of the genes of the Entner–Doudoroff pathway enzymes on the chromosome of *P. aeruginosa*.

METHODS

Bacterial strains and plasmids. These are described in Table 1. FRD1 is a mucoid *P. aeruginosa* isolated from sputum of a cystic fibrosis patient. *P. aeruginosa* FRD7 and 8821 are two His^- auxotrophs derived from mucoid isolates of cystic fibrosis origin. *P. aeruginosa* strain 8822 is a spontaneous non-mucoid (Alg^-) derivative of the mucoid (Alg^+) strain 8821. Strain 8830 is a stable alginate-producing organism derived from strain 8822 (Ohman & Chakrabarty, 1981; Darzins & Chakrabarty, 1984). The isolation and characterization of the carbohydrate-negative mutants of mucoid *P. aeruginosa* used in this study have been described previously (Banerjee *et al.*, 1983, 1985). Mutant 1504 was chosen from among 20 independent isolates, while mutant 1518 was the only one found to have lost the NADP-linked GAP activity. Among four aldolase mutants, three isolates (FBA1, FBA2 and FBA4) were chosen, as their reversion patterns indicated that they were not likely to be phenocopies.

Media and growth conditions. Growth phenotypes of *P. aeruginosa* mutants (Banerjee *et al.*, 1983, 1985) are shown in Table 2. *Escherichia coli* AC80 harbouring recombinant plasmids were maintained on plates of L agar (Miller, 1972) containing 25 μg tetracycline (Tc) ml^{-1}. Tc^R *P. aeruginosa* mutant strains were selected on synthetic medium (Brammar & Clarke, 1964) containing 50 mM carbon source, 0·2 mM-histidine and 75–100 μg Tc ml^{-1}. Selected isolates were maintained on the same medium containing 25 μg Tc ml^{-1}.

Metabolite estimation. The methods were as described by Banerjee *et al.* (1985).

Isolation and manipulation of DNA. Total *P. aeruginosa* DNA from strain 8830 was isolated according to the method of Marmur (1961) and purified by CsCl/ethidium bromide ultracentrifugation. Chromosomal DNA was dissolved in TE buffer (10 mM-Tris/HCl, 1 mM-EDTA, pH 8·0) and stored at −20 °C until required. Plasmid DNA was isolated by a modification (Chatterjee *et al.*, 1981) of the procedure of Casse *et al.* (1979) and purified as described above. Recombinant plasmids were detected by the miniscreening method of Holmes & Quigley (1981). Agarose gel electrophoresis procedures have been previously described (Darzins & Chakrabarty, 1984). Restriction endonucleases were purchased from New England Biolabs. Digestion reactions were carried out under conditions specified by the supplier.

Table 1. *Bacterial strains*

Strain	Plasmid	Genotype/ phenotype	Enzyme deficiency	Reference
P. aeruginosa				
FRD1		Wild-type		Ohman & Chakrabarty (1981)
FRD7		*his* Alg$^+$		
8821		*his-1* Alg$^+$		Darzins & Chakrabarty (1984)
8822		*his-1* Alg$^-$ (stable)		
24		*zwf*	ZWF	
13		*edd*	EDD	
1		*eda*	EDA	Banerjee *et al.* (1983)
1518		*gap*	GAP	
1504		*pgk*	PGK	
FBA1, FBA2 FBA4		*fba*	FBA	Banerjee *et al.* (1985)
E. coli				
AC80		*thr leu met-1 hsdR hsdM*		Chakrabarty *et al.* (1978)
HB101	pLAFRI	*recA hsdM hsdR pro leu*		Friedman *et al.* (1982)
HB101	pCP13	*recA hsdM hsdR pro leu*		Figurski & Helinski (1979)
HB101	pRK2013	*recA hsdM hsdR pro leu*		

Table 2. *Growth patterns of P. aeruginosa mutants*

Growth was observed for 2–3 d at 37 °C on a shaker. Mtl, mannitol; Glc, glucose; GlcA, gluconate; Suc, succinate; Glu, glutamate; Fru, fructose. ++, Good growth; +, slow growth; ±, very slow growth; −, no growth.

	Growth on							
Strain	Mtl	Glc	GlcA	Suc	Glu	L Broth	L Broth −Glc	L Broth −Glc+Fru
1518 (*gap*)	++	++	++	−	±	+	±	+
1504 (*pgk*)	++	++	++	−	−	+	−	±
FBA2, FBA4 (*fba*)	±	±	−	−	−	±	±	++
FBA2R*	++	++	++	−	−	+	−	++
FBA4R†	++	++	++	++	++	+	+	++
24 (*zwf*)	−	++	++	++	++	++	++	ND
13 (*edd*)	−	−	−	++	++	−	++	ND
1 (*eda*)	−	−	−	++	++	−	++	ND

ND, Not determined
* Gluconate revertant of mutant FBA2.
† Glutamate revertant of mutant FBA4.

Preparation of the P. aeruginosa genomic libraries. The construction of the clone banks in the broad host range cosmid vectors pLAFRI and pCP13 have been described previously (Darzins & Chakrabarty, 1984). Clone banks were preserved at −20 °C in 3 ml portions which contained equal volumes of the stationary-phase culture and glycerol.

Mating conditions and selection of recombinant plasmids. The clone bank in *E. coli* was mated with each *P. aeruginosa* mutant (Table 1) on a Millipore HAO 45 μm filter in the presence of *E. coli* HB101 containing the helper mobilizing plasmid pRK2013 (Ruvkun & Ausubel, 1981). Filters were placed on L broth or modified L broth (Table 2) and incubated for 6 h at 37 °C. The filters were then washed with 3 ml phosphate buffered saline and vortexed, and 50–100 μl of the suspension was spread onto minimal agar plates containing Tc and various carbon sources. Tc^R *P. aeruginosa* exconjugants appearing within 48–72 h were picked up and checked for growth on permissive and non-permissive plates containing Tc. Each clone able to grow on both types of media was then

tested for the appropriate enzyme activity. Representative clones in which specific enzyme activity reappeared through the introduction of a recombinant plasmid were chosen for further analysis. These recombinant plasmids were mobilized back into *E. coli* (as described above) for preservation. *P. aeruginosa* exconjugants were cured of plasmid DNA by several passages on Tc-free medium.

Enzyme assays. Cell suspensions in either 50 mM-potassium phosphate, 0·5 mM-EDTA, 0·2 mM-DTT, pH 7·5, or 50 mM-Tris, 1 mM-EDTA, 0·5 mM DTT, pH 7·6, were treated with toluene (10 μl ml^{-1}) initially for 1 min on a vortex mixer and then for 10 min at 37 °C on a shaker. These cell suspensions were used for assaying all enzymes except NADP-linked GAP which was estimated in cell-free extracts. Assay mixtures were as described by Banerjee *et al.* (1983, 1985). Reactions were conducted in 1 ml cuvettes either in a Gilford 2600 spectrophotometer at 340 nm or fluorometrically. Chemicals were purchased from Sigma.

RESULTS

Growth characteristics of gluconeogenic mutants

All three mutants described here were obtained by selecting for their inability to grow on glutamate as sole carbon source (Banerjee *et al.*, 1983, 1985). They were also unable to grow on succinate, lactate or LB medium (Miller, 1972) from which glucose was omitted (Table 2). The growth phenotypes of the *pgk* and *gap* mutants were similar in that they grew on each of the following as sole carbon source: fructose, mannitol, glucose, gluconate or glycerol. The *fba* mutants were, however, unable to grow on gluconate or glycerol and their growth on either fructose, mannitol or glucose was very slow. Fig. 2 illustrates the growth kinetics of these mutants in permissive and non-permissive media. An interesting feature is that the loss of either NADP-linked GAP or PGK did not at all impair the growth on gluconate; furthermore, the presence of a non-permissive substrate such as glutamate did not affect the growth rate of these mutants with a permissive substrate like gluconate. The *gap* mutant grew slowly on glutamate unlike the *pgk* strain. This was presumably due to the presence of an NADP-linked GAP in the *gap* mutant (Banerjee *et al.*, 1985).

Metabolite profile in mutants

Addition of glucose or gluconate to a preinduced culture of wild-type *P. aeruginosa* led to intracellular accumulation of phosphorylated metabolites (Banerjee *et al.*, 1983, 1985). The levels of these phosphorylated compounds, viz. fructose 1,6-bisphosphate and triose phosphates, in strains 1504 (*pgk*) and 1518 (*gap*) were very similar to those seen in the wild-type strain FRD1 (Banerjee *et al.*, 1985). In the mutant lacking FBA on the other hand, incubation with fructose or mannitol, but not gluconate, caused fructose 1,6-bisphosphate to accumulate to approximately 2 μmol (g wet cells)$^{-1}$; in contrast, the level of triose phosphates was higher with gluconate than with fructose or mannitol (data not shown).

Growth properties of the revertants

The frequencies of spontaneous reversion in the glutamate medium for the various mutants were as follows: 1518 (*gap*), 1×10^{-6}; 1504 (*pgk*), 7×10^{-10}; 1603 (another *pgk* mutant), 1×10^{-6}; FBA2 (*fba*), 1×10^{-8}; FBA4 (another *fba* mutant), $2{\cdot}5 \times 10^{-9}$. The revertants of *fba* mutants were particularly interesting. Reversion of FBA2 on gluconate or glycerol produced colonies which were unable to grow on glutamate or on other gluconeogenic precursors. However, when the *fba* mutants were reverted on glutamate, the revertants were able to grow on other substrates. The revertants of *pgk* and *gap* mutants, in contrast, could grow on all gluconeogenic compounds.

Enzyme levels in the mutants and their revertants

Table 3 summarizes some of the glycolytic enzyme activities in the *pgk*, *gap* and *fba* mutant strains, and in revertants of *fba* mutants. The mutant strain 1504 was defective in PGK activity, while the mutant strain 1518 had greatly reduced NADP-linked GAP activity; the NADH-linked GAP was not much affected. The level of NADPH-linked GAP was not increased in the glutamate revertants of the *gap* mutant strain, presumably due to the synthesis of a labile enzyme (data not shown).

The revertants of *fba* mutants revealed an unexpected feature (Tables 2 and 3). The gluconate

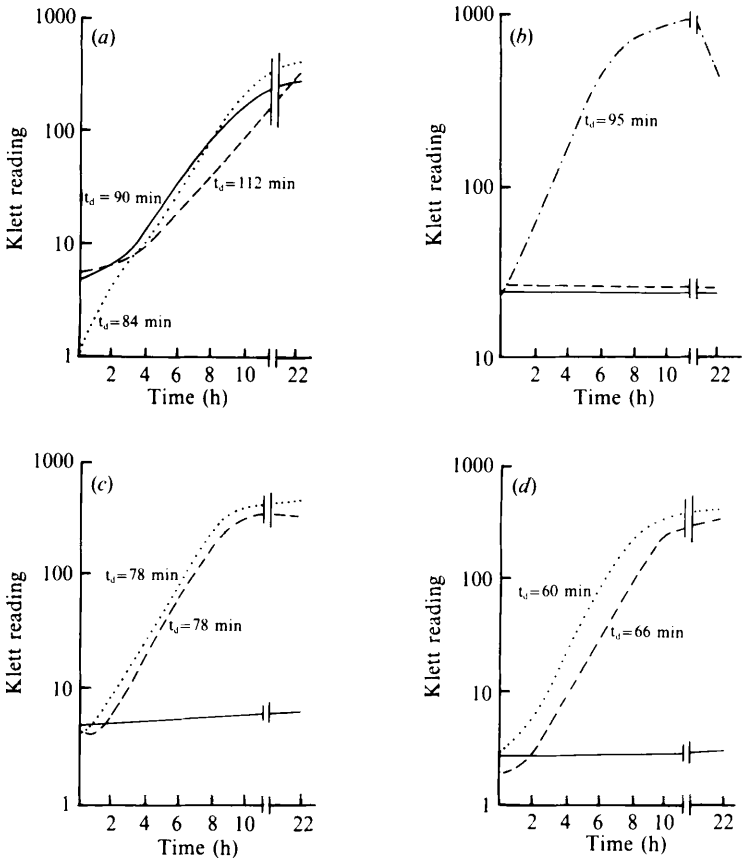

Fig. 2. Growth kinetics of gluconeogenic mutants of *P. aeruginosa*. Growth was at 37 °C on a rotary shaker; turbidity was measured periodically in a Klett-Summerson colorimeter using a green (540 nm) filter. (*a*) FRD1, wild-type strain of mucoid *P. aeruginosa*; (*b*) *fba* mutant; (*c*) *gap* mutant; (*d*) *pgk* mutant, strain 1504. ———, Glutamate (50 mM) in minimal medium (Brammar & Clarke, 1964); – – –, gluconate (50 mM); ·····, gluconate (50 mM) and glutamate (50 mM) in minimal medium; —·—·—, L broth with fructose (5 mM) but lacking glucose. The doubling times (t_d) are indicated.

or glycerol revertants of FBA2 which could not grow on glutamate had partially recovered FBA activity, but completely lost PGK activity. The activities of NADPH-linked GAP, enolase and pyruvate kinase were also increased in these revertants. In contrast, the glutamate revertants of FBA4 had no detectable aldolase activity, which may be due to synthesis of an unstable enzyme; however, the activities of PGK and the other enzymes remained almost unchanged in these revertants. A very similar enzyme profile was seen in several other independent revertants of the FBA2 mutant.

Cosmid cloning of P. aeruginosa gluconeogenic and related genes

Cloning of selected anabolic genes involved in gluconeogenesis and other metabolic genes was carried out with an *Eco*RI- and *Hin*dIII-generated genomic library of strain 8830, using the broad-host-range cosmid vectors, pLAFRI and pCP13, respectively. Tc^R exconjugants of the *fba*, *gap*, *pgk* and *eda* mutants were selected on minimal glutamate (*fba*, *gap*, *pgk*) and mannitol (*eda*) agar plates. Clones isolated from each mating were purified on the same Tc-containing non-permissive media and among the many exconjugants in each group one was selected for further analysis. The Tc^R determinants of the *P. aeruginosa* exconjugants were mobilized back into *E. coli* AC80 and then reintroduced into the respective *P. aeruginosa* mutants. The cloned

Table 3. *Enzyme activities in mutants and revertants*

Cultures were grown overnight on minimal medium (Brammar & Clarke, 1964) with carbon sources added as follows: strains FRD1 (wild-type), 1504 (*pgk*), 1518 (*gap*) and FBA2R – 50 mM-gluconate; FBA4R – 50 mM-gluconate, 50 mM-glutamate and 10 mM-fructose. Pyruvate kinase was estimated in extracts without EDTA; other enzymes were estimated in extracts containing EDTA and no Mg^{2+}.

	Activity mU (mg protein)$^{-1}$					
Enzyme	Wild-type	*pgk*	*gap*	FBA2	FBA2R*	FBA4R†
Fructose-1,6-bisphosphate aldolase	21	14	17	0	2·3	0
Glyceraldehyde 3-phosphate dehydrogenase						
NADPH-linked	170	242	1·5	179	358	195
NADH-linked	18	6	9	ND	ND	ND
3-Phosphoglycerate kinase	190	1	200	195	0	183
Phosphoglycerate mutase	17	20	29	4	6	13
Enolase	52	64	82	46	109	50
Pyruvate kinase	65	90	72	56	204	68

ND, Not determined
* Gluconate revertant of the *fba* mutant FBA2.
† Glutamate revertant of the *fba* mutant FBA4.

Table 4. *Complementation of different metabolic mutations in P. aeruginosa by recombinant plasmids*

		Complementation* by:				
Strain	Mutation	pAD100	pAD101	pAD150	pAD250	pPB1
24	*zwf*	–	–	–	+	–
13	*edd*	–	–	–	+	–
1	*eda*	–	–	–	+†	–
1518	*gap*	–	–	–	–	+†
1504	*pgk*	+	+	+†	–	–
FBA1	*fba*	+†	+†	+	–	–

* +, Positive complementation in respect of both enzyme activity and Tc^R; –, complementation only in respect of Tc^R, but not for enzyme activity.
† Indicates the gene originally cloned in the plasmid.

sequences contained in the isolated recombinant plasmids were able to restore both growth on the non-permissive media and enzyme activity (Table 4). The Tc^R determinants carried in the Gap^+, Pgk^+ and Eda^+ clones were designated as pPB1, pAD150 and pAD250 respectively. Two independent plasmids, pAD100 and pAD101, carried the *fba* mutation.

Cross-complementation of the fba and pgk mutations with plasmids pAD100, pAD101 and pAD150

The *fba*-containing plasmids pAD100 and pAD101 were introduced into the remaining *P. aeruginosa* mutants and only the Tc^R exconjugants of mutant 1504 (*pgk*) were found to grow on glutamate, the non-permissive carbon source. In addition, mutant 1504 harbouring either pAD100 or pAD101 had wild-type levels of PGK activity. The presence of *pgk* in pAD100 and pAD101 was further confirmed by the simultaneous loss of Tc^R and PGK activity when exconjugants were cured of either recombinant plasmid by successive transfer on Tc-free

permissive (gluconate) medium. Conversely, introduction of the *pgk*-bearing plasmid pAD150 into the FBA mutant restored its ability to grow on such non-permissive carbon sources as gluconate and glutamate. The presence of FBA activity in FBA1 and pAD150 exconjugants (Table 4) and its absence in Tc^S cells cured of the plasmid (data not shown) confirmed that FBA activity is indeed plasmid-borne. Plasmids pAD250 (*eda*$^+$) and pPB1 (*gap*$^+$), on the other hand, were unable to restore PGK or FBA activity in mutants lacking these enzymes. Furthermore, neither pAD100, pAD101 nor pAD150 could restore ZWF, EDD, EDA or GAP activity in the respective mutants (Table 4).

Plasmid DNA was isolated and purified from *E. coli* AC80 containing pAD100, pAD101 or pAD150. Digestion of pAD100 and pAD150 with *Eco*RI and digestion of pAD101 with *Hin*dIII and *Eco*RI produced in all three cases a fragment that comigrated with linearized vector DNA and other fragments, of varying size, representing the cloned sequences (data not shown). Using phage λ *Hin*dIII and *Hin*dIII/*Eco*RI fragments as size markers it was determined that approximately 22, 23 and 6·2 kb of cloned DNA were present in pAD100, pAD101 and pAD150, respectively. Since cross-complementation of the FBA1 and 1504 mutants with all three plasmids was observed it was expected that the cloned inserts present on these plasmids should have some sequences in common. In fact, when the *Eco*RI digests of all three plasmids were compared on a 0·7% agarose gel there were several fragments which comigrated and appeared identical (data not shown). Taken together these results demonstrate that, as in *E. coli* K12 (Bachmann, 1983), the *fba* and *pgk* genes in mucoid *P. aeruginosa* are closely linked on the chromosome to within 6·2 kb.

Clustering of zwf, edd and eda genes in mucoid P. aeruginosa

Plasmid pAD250, derived from the gene library of strain 8822, was found to complement the *P. aeruginosa eda* mutant. When pAD250 was subsequently mobilized into the other mutants (*zwf* and *edd*) it was found to confer ZWF and EDD activities in mutants 24 (*zwf*) and 13 (*edd*) respectively (Table 4). With several transfers of these exconjugants on Tc-free permissive media, the enzyme activities in the corresponding mutants were lost along with the Tc^R character. Non-permissive medium (minimal mannitol) or Tc^R selection was, therefore, necessary in order to retain the enzyme activities in the pAD250 exconjugants. These results demonstrate that the activities of these enzymes are indeed plasmid-borne and confirm that in mucoid *P. aeruginosa* these catabolic genes are clustered as has been reported earlier for *P. aeruginosa* strain PAO (Roehl *et al.*, 1983). The clustering of the *zwf*, *eda* and *edd* genes has also been demonstrated in *E. coli* K12 (Bachmann, 1983).

Isolation of the gap gene from P. aeruginosa

Plasmid pPB1, which was isolated from a *Hin*dIII-generated cosmid clone bank of strain 8830, was found to restore the growth of the *gap* mutant on minimal medium containing glutamate as a carbon source. pPB1 also restored NADP-linked GAP activity to mutant 1518, but it could only provide the Tc^R character to the remaining mutants and could not complement the missing enzyme activities (Table 4). These results suggest that the chromosomal fragment contained on pPB1 harboured the *P. aeruginosa gap* gene and none of the genes for the other enzymes tested.

DISCUSSION

Fate of glyceraldehyde 3-phosphate

One feature of the growth properties of mutants lacking the gluconeogenic enzymes of the Embden–Meyerhof–Parnas pathway in *P. aeruginosa* contrasts sharply with that known for similar mutants in enteric bacteria or yeast. In the latter, loss of any of the enzymes prior to the aldolase-catalysed step (in the direction of gluconeogenesis) generates a requirement for two carbon sources, one providing for intermediates 'below' and the other 'above' the blocked reaction (Irani & Maitra, 1977; Lam & Marmur, 1977). In *P. aeruginosa* however, the growth rate of mutants lacking NADPH-linked GAP or PGK was not at all impaired on gluconate

compared to the wild-type strain. This was due not only to the special feature of the Entner–Doudoroff pathway converting a C_6 metabolite directly to pyruvate, but perhaps also to the plurality of metabolic routes available in *Pseudomonas*. The failure of the NADP-linked GAP and the PGK mutants to accumulate triose phosphates or fructose bisphosphate from gluconate reflects this. In *E. coli*, where the glycolytic pathway is the major route of glyceraldehyde 3-phosphate metabolism, the loss of either enzyme causes an accumulation of these metabolites to nearly 10 times the level seen in the wild-type strain (Irani & Maitra, 1977). This suggests that alternative routes for the dissimilation of triose phosphates exist in *Pseudomonas*. One such possibility is the methylglyoxal pathway, whose first enzyme has been demonstrated in *Pseudomonas saccharophila* (Cooper, 1974). The other is a cyclic process converting two molecules of glyceraldehyde 3-phosphate to one of pyruvate and regenerating the triose phosphate with the sequential participation of the following enzymes: triose phosphate isomerase, fructose-1,6-bisphosphate aldolase, fructose-bisphosphatase, phosphoglucose isomerase, glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydratase, and phospho-2-keto-3-deoxygluconate aldolase. In a manner analogous to other biochemical cycles such as the Krebs cycle, this seven-enzyme ensemble would then permit a continuous conversion of glyceraldehyde 3-phosphate to pyruvate. The observation that a double mutant lacking ZWF and enolase does not grow on gluconate, while single mutants do (P. V. Phibbs, Jr, personal communication), is consistent with a necessity for the operation of the cyclic sequence when the flux through the Embden–Meyerhof segment is blocked. Whether the methylglyoxal pathway also operates in *Pseudomonas* as a sugar-degradation route cannot be settled in the absence of mutations affecting the former. The inability of *P. aeruginosa* mutants lacking NADP-linked GAP or PGK activities, to grow on glutamate, succinate or lactate indicates that the Embden–Meyerhof sequence functions as a gluconeogenic rather than as a sugar-degradation pathway.

Linkage of fba and pgk genes

The chromosomal disposition of carbohydrate-catabolizing genes in *Pseudomonas* resembles that found in *E. coli* (Lessie & Phibbs, 1984; Fraenkel & Vinopal, 1973) despite their disparate metabolic characteristics. In both organisms the genes for the EDD, EDA and ZWF activities are tightly linked, although ZWF is a constitutive enzyme in *E. coli*, and inducible in *P. aeruginosa*. The complementation of all three mutations by a single DNA fragment as shown here confirms this. The linkage of the *fba* and *pgk* genes is also apparent from the results in Table 4. Two plasmids, pAD100 and pAD101, selected for FBA activity, complemented the PGK-deficient mutation, while plasmid pAD150, selected for PGK, also restored FBA activity to the *fba* mutant. Although no sub-cloning has been done the minimal length of DNA carrying both these activities resides in a fragment measuring 6·2 kb, a size that could accommodate the structural genes for both of the enzymes (Horecker *et al.*, 1972; Scopes, 1973). The disappearance of PGK activity associated with the restitution of FBA activity on reversion of FBA2 mutant on gluconate also indicated a causal association of these two enzymes. The structural genes *pgk* and *fba* are both located near the 63 min region on the *E. coli* chromosome (Bachmann, 1983).

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