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Cloning of Genes Specifying Carbohydrate Catabolism in *Pseudomonas aeruginosa* and *Pseudomonas putida*

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A 6.0-kilobase *Eco*RI fragment of the *Pseudomonas aeruginosa* PAO chromosome containing a cluster of genes specifying carbohydrate catabolism was cloned into the multicopy plasmid pRO1769. The vector contains a unique *Eco*RI site for cloning within a streptomycin resistance determinant and a selectable gene encoding gentamicin resistance. Mutants of *P. aeruginosa* PAO transformed with the chimeric plasmid pRO1816 regained the ability to grow on glucose, and the following deficiencies in enzyme or transport activities corresponding to the specific mutations were complemented: *glcT1*, glucose transport and periplasmic glucose-binding protein; *glcK1*, glucokinase; and *edd-1*, 6-phosphogluconate dehydratase. Two other carbohydrate catabolic markers that are cotransducible with *glcT1* and *edd-1* were not complemented by plasmid pRO1816: *zwf-1*, glucose-6-phosphate dehydrogenase; and *eda-9001*, 2-keto-3-deoxy-6-phosphogluconate aldolase. However, all five of these normally inducible activities were expressed at markedly elevated basal levels when transformed cells of prototrophic strain PAO1 were grown without carbohydrate inducer. Vector plasmid pRO1769 had no effect on the expression of these activities in transformed mutant or wild-type cells. Thus, the chromosomal insert in pRO1816 contains the *edd* and *glcK* structural genes, at least one gene (*glcT*) that is essential for expression of the glucose active transport system, and other loci that regulate the expression of the five clustered carbohydrate catabolic genes. The insert in pRO1816 also complemented the *edd-1* mutation in a glucose-negative *Pseudomonas putida* mutant but not the *eda-1* defect in another mutant. Moreover, pRO1816 caused the expression of high specific activities of glucokinase, an enzyme that is naturally lacking in these strains of *Pseudomonas putida*.

Pseudomonas aeruginosa metabolizes carbohydrates mainly through the Entner-Doudoroff pathway (for review, see reference 14). Carbohydrates are catabolized to the key intermediate, 6-phosphogluconate (6PG) which is converted to glyceraldehyde-3-phosphate and pyruvate through the actions of the inducible enzymes, 6-phosphogluconate dehydratase (EDD) and 2-keto-3-deoxy-6-phosphogluconate (KDPG) aldolase (EDA). Glucose can be converted to 6PG by either the direct oxidative or phosphorylative routes (Fig. 1). The direct oxidative pathway involves the sequential oxidation of periplasmic glucose to gluconate and 2-ketogluconate via membrane-bound glucose and gluconate dehydrogenases, followed by the transport and catabolism of these compounds to 6PG in the cytoplasm. In the phosphorylative pathway, glucose is transported into the cell by an active transport system that requires a periplasmic glucose-binding protein (GBP). Intracellular glucose is phosphorylated by a glucokinase (GLK) and subsequently oxidized to 6PG by glucose-6-phosphate dehydrogenase (ZWF). The phosphorylative pathway is the sole route of glucose catabolism in denitrifying cells due to the lack of glucose dehydrogenase activity in cells grown under anaerobic conditions (9, 10).

Recent mapping data have shown that gene loci that specify the expression of glucose active transport (*glcT*), ZWF (*zwf*), EDA (*eda*), and EDD (*edd*) are tightly clustered in the 52- to 53-min region of the *P. aeruginosa* PAO chromosome (3, 21). In this report, we describe the cloning of a portion of this region of the chromosome. One recombinant plasmid, pRO1816, was shown to contain the structural genes for EDD and GLK and possibly for the GBP component of the glucose transport system. We also report on the transformation and expression of the cloned *P. aeruginosa* DNA fragment in mutant strains of *P. putida*. (S. M. Cuskey, J. A. Wolff, R. H. Olsen, and P. V. Phibbs, Jr., Abstr. Annu. Meet. Am. Soc. Microbiol., 1984, D73, p. 63).

MATERIALS AND METHODS

Bacterial strains and growth media. Bacterial strains and plasmids used in this study are listed in Table 1. Strain PRP444 (*glcK1*) was isolated after transduction of strain PFB14 (*pyc-2 glcK1*) with the generalized transducing phage G-101 propagated on *P. aeruginosa* PAO1 essentially as previously described for phage F116L (20). Strain PRP444 was selected by growth on mannitol minimal medium. The retention of the *glcK1* phenotype was confirmed by demonstrating the inability of strain PRP444 to grow anaerobically with nitrate and with glucose as the sole carbon source (9) and by enzyme analysis.

All pseudomonads were routinely cultured either in basal salts medium (BSM) (5) or in complex medium (LNG) that contained the following (grams per liter): tryptone, 10; yeast extract, 5; and sodium chloride, 5. Stock solutions of carbon sources were sterilized by autoclaving or by filtration (glucose) and were added to sterile BSM to a final concentration of 20 mM. When added for the inductions of enzyme or the glucose transport system, glucose was present at a final concentration of 10 mM. Amino acid supplements were used

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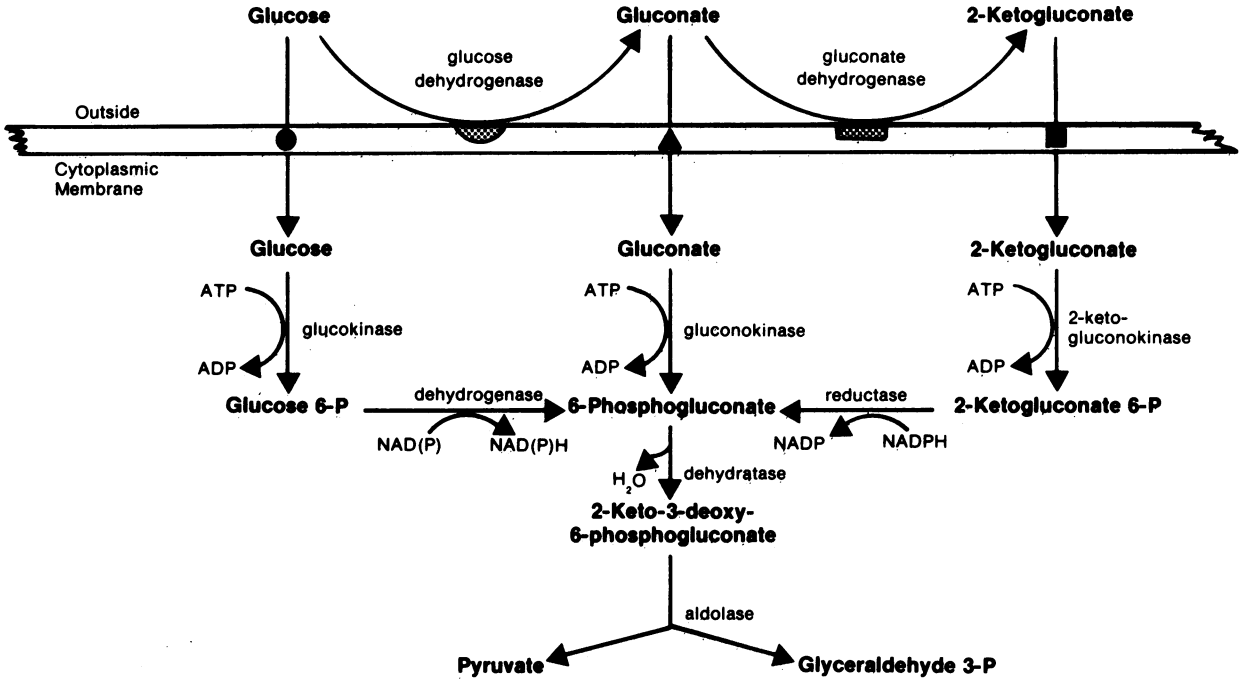

FIG. 1. Alternative peripheral pathways for glucose catabolism in *Pseudomonas aeruginosa*. Reproduced from previous communication (9) by permission of the publisher.

at 10 μg/ml. Antibiotics were added to media at the following concentrations: gentamicin, 10 μg/ml for *P. putida* or 25 μg/ml for *P. aeruginosa*; kanamycin, 500 μg/ml; carbenicillin, 500 μg/ml; streptomycin, 500 μg/ml. Solid media contained 1.5% agar.

Growth conditions, extract preparation, and enzyme assays. Crude cell extracts used in enzyme assays were prepared from cells that were cultured in 400 ml of BSM containing 20 mM lactate with or without glucose (10 mM). Cultures were incubated aerobically with rotary shaking (200 rpm) at 37°C for *P. aeruginosa* or 30°C for *P. putida*. Late-exponential-phase cells were harvested by centrifugation and washed once in BSM. The washed cells were suspended in 5.0 ml of 0.1 M Tris-hydrochloride (pH 8.0) and disrupted by ultrasonic oscillation (12). Crude soluble supernatant fractions of cell extracts were collected after centrifugation at 105,000 × *g* for 2 h at 4°C and employed in spectrophotometric assays for enzyme activities. Previously described methods (12) were used for determining activities of ZWF (EC 1.1.1.49) and GLK (EC 2.7.1.2). The combined activity of the Entner-Duodoroff pathway enzymes, EDD (EC 4.2.1.12) and EDA (EC 4.1.2.14), was measured as the rate of pyruvate formation from 6PG as described previously (11). Enzyme specific activities are expressed as nanomoles of product formed per minute per milligram of protein. Protein concentrations were determined by the method of Lowry et al. (15) with bovine serum albumin as standard.

Uptake activities. Cells to be assayed for glucose uptake activity were cultured in BSM containing lactate (20 mM) with and without glucose (10 mM). The cells were harvested during the mid-log phase of growth, washed once in an equal volume of BSM, and suspended in carbon-free BSM to a Klett reading of 175 (Klett-Summerson colorimeter equipped with a red no. 66 filter). The cell suspensions (about 0.4 mg of protein per ml) were incubated at 37°C with shaking for at least 15 min before uptake assays were begun. [*U*-^{14}C]glucose uptake activity was determined by a previously described membrane filtration technique (19) and is expressed as nanomoles of [*U*-^{14}C]glucose accumulated per minute per milligram of protein.

GBP assay. Cells to be assayed for GBP activity were grown in BSM containing lactate (20 mM) with and without glucose (20 mM). Late-log-phase cells were harvested and washed once with an equal volume of BSM. The periplasmic GBP was isolated by using the cold shock procedures of Hoshino and Kageyama (8), and [*U*-^{14}C]GBP activity was assayed by using the membrane filtration method of Stinson et al. (22, 23). One unit of GBP activity is equivalent to the amount of periplasmic protein required to cause the binding of 1 pmol of glucose to the filter. Specific activities were corrected for protein-independent membrane binding and are expressed as units per milligram of protein.

DNA preparation, restriction endonuclease cleavage, ligation, and transformations. Plasmid pRO1769 was used as cloning vector in these experiments. The plasmid was derived from pRO1614 (17) and contains genes coding for resistance to streptomycin and multiple resistances to gentamicin, tobramycin, and kanamycin. The antibiotic resistance genes originated from plasmid pRO11 purified from a *P. aeruginosa* clinical isolate. (R. H. Olsen, G. Zylstra, and J. Punch, unpublished data).

Plasmid and PAO1 chromosomal DNA were isolated from cells treated with lysozyme-EDTA-sodium dodecyl sulfate as described previously (17). The DNA was purified by ethanol precipitation and isopycnic centrifugation in a cesium chloride-ethidium bromide density gradient (6). DNA was stored in TE buffer (10 mM Tris, 1 mM EDTA [pH 8.0]) at −20°C and thawed slowly in ice water when used.

DNA cleavage with restriction endonucleases was performed as recommended by the supplier (Bethesda Research Laboratories, Rockville, Md.). Generally, DNA (100 to 250 μl) was incubated with the appropriate restriction endonuclease (2 to 6 μl) for 60 min at 37°C. The reaction was stopped by incubating the reaction mixture at 70°C for 10

TABLE 1. Bacterial strains and plasmids

Bacterium or plasmid	Genotype or phenotype[a]	Derivation or source[b] (reference)
P. aeruginosa (ATCC 15692 = ATCC 17503)		
PAO1	Prototroph	Holloway[c] (7)
PAO1838	*eda-9001 met-9020*	Matsumoto[d] (21)
PFB2	*edd-4*	(2)
PFB9	*edd-1*	(2)
PFB14	*pyc-2 glcK1*	(18)
PFB57	*edd-8*	Lacks multiple components of direct oxidative pathway (2)
PFB98	*zwf-1*	(19)
PFB103	*zwf-2*	(19)
PFB362	*glcT1*	Two-step Glc$^-$ derivation from PFB57[e] (this study)
PRP444	*glcK1*	PFB14, Transduced with G101 to Mtl$^+$ (this study)
MB723	*trp gdh glcT*	Stinson[f] (23)
P. putida (ATCC 12633)		
MAP-1	*edd-1*	Wheelis[g] (25)
MAP-2	*eda-1*	Wheelis (25)
Plasmids		
pRO1614	Cb^r Tc^r	(17)
pRO1769	Sm^r Gm^r	(This study)
pRO1807	Gm^r	(This study)
pRO1816	Gm^r	(This study)

[a] Genes: *eda*, 2-keto-3-deoxy-6-phosphogluconate aldolase; *edd*, 6-phosphogluconate dehydratase; *zwf*, glucose-6-phosphate dehydrogenase; *pyc*, pyruvate carboxylase; *glcK*, glucokinase; *glcT*, glucose transport; *gdh*, glucose dehydrogenase; *met*, methionine requirement; *trp*, tryptophan requirement. Phenotypes: plasmid-encoded resistance to carbenicillin (Cb), tetracycline (Tc), streptomycin (Sm), and gentamicin, kanamycin, and tobramycin (Gm).

[b] Abbreviations: Glc$^-$, lack of growth on glucose; Mtl$^+$, growth with mannitol as the sole carbon source.

[c] B. W. Holloway, Monash University, Clayton, Victoria, Australia.

[d] H. Matsumoto, Shinshu University, Matsumoto, Japan.

[e] PFB362 is an EDD$^+$ spontaneous revertant of PFB57 that retains parental multiple defects in the glucose oxidative pathway. It is Glc$^-$ because of a new mutation (*glcT1*) in glucose active transport (3).

[f] M. W. Stinson, State University of New York, Buffalo, N.Y.

[g] M. L. Wheelis, University of California, Davis, Calif.

min. Digested DNA to be ligated was incubated for 10 h at 17°C with T4 DNA ligase-bovine serum albumin-dithiothreitol as recommended by the supplier (Bethesda Research Laboratories). The ligation mixtures were used for transformations.

P. aeruginosa strains were transformed by using a previously described modification (17) of the procedure of Mercer and Loutit (16). *P. putida* strains were transformed by using a previously described modification (17) of the method of Davis et al. (4).

Recombinant plasmids were visualized in agarose gel electropherograms of DNA samples prepared by the rapid lysis procedure of Gonzalez et al. (5a), a modification of the procedure of Hansen and Olsen (6).

Agarose gel electrophoresis. Plasmids and their restriction endonuclease cleavage products were analyzed by agarose (0.7%) gel electrophoresis with 40 mM Tris–2 mM EDTA buffered to pH 8.0 with acetic acid or with 89 mM Tris-borate–2 mM EDTA (pH 8.0). DNA bands were visualized in UV light after staining with ethidium bromide (0.5 μg/ml).

Chemicals and reagents. Pyridine nucleotides, commercial coupling enzymes, enzyme substrates, and other reagents used in enzyme assays were of the highest purity available from either P-L Biochemicals, Inc., Milwaukee, Wis., or Sigma Chemical Co., St. Louis, Mo. Antibiotics were purchased from Sigma Chemical Co. Radiolabeled glucose was purchased from New England Nuclear Corp., Boston, Mass. Other chemicals were of reagent grade purity, purchased from J. T. Baker Chemical Co., Phillipsburg, N.J., Fisher Scientific Co., Pittsburg, Pa., or Sigma Chemical Co. Tryptone, yeast extract, and agar were purchased from Difco Laboratories, Detroit, Mich.

RESULTS

Plasmid pRO1769 (Fig. 2) is a 5.5-kilobase (kb) multicopy vector which carries genes encoding resistance to streptomycin and gentamicin. The plasmid contains a unique *Eco*RI cleavage site which when cleaved inactivates the streptomycin resistance locus. *P. aeruginosa* PAO1 chromosomal DNA and pRO1769 were each cleaved with *Eco*RI, mixed, and ligated, and the ligation mixtures were used to transform strains with known carbohydrate catabolic mutations. Transformants which grew on the carbohydrate selec-

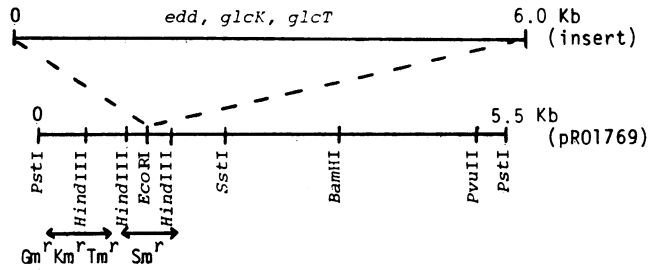

FIG. 2. Physical map of plasmids; plasmid cloning vector pRO1769 with cloned *P. aeruginosa* chromosomal DNA insert in chimeric plasmid pRO1816.

TABLE 2. Expression of glucose transport and GBP activities

Strain (plasmid)	Relevant genotype	Growth conditions[a]	Glucose uptake[b]	GBP activity[c]
P. aeruginosa				
PAO1	Wild type	Lct	2	4
		Lct + Glc	28	458
PAO1(pRO1769)		Lct	<1	ND[d]
		Lct + Glc	25	ND
PAO1(pRO1816)		Lct	10	ND
		Lct + Glc	27	ND
PFB362	*glcT1*	Lct	<1	<1
		Lct + Glc	3	<1
PFB362(pRO1816)		Lct	6	148
		Lct + Glc	19	296
MB723	*trp gdh glcT*	Lct	<1	<1
		Lct + Glc	<1	<1
MB723(pRO1816)		Lct	<1	<1
		Lct + Glc	<1	<1
P. putida				
MAP-1	*edd-1*	Lct	<1	<1
		Lct + Glc	<1	<1
MAP-1(pRO1816)		Lct	<1	<1
		Lct + Glc	<1	<1

[a] Cells were grown in BSM with 20 mM lactate (Lct) or lactate plus 10 mM glucose (Lct + Glc). See the text for additional details.
[b] Glucose uptake activity is expressed as nanomoles of [*U*-^{14}C]glucose accumulated per minute per milligram of protein.
[c] GBP activity is expressed as picomoles of [*U*-^{14}C]glucose bound per milligram of protein in periplasmic extracts.
[d] ND, Not determined.

tion medium and expressed the appropriate antibiotic resistances were assayed for the presence of recombinant plasmids.

Mutant strain PFB362 is unable to utilize glucose by either the oxidative or phosphorylative pathway because of multiple defects in the direct oxidative pathway for glucose utilization and a specific defect (*glcT1*) in glucose transport activity which blocks utilization via the phosphorylative route. Chimeric plasmid pRO1807 containing a 13.9-kb DNA insert was isolated from a glucose-positive transformant of PFB362 that was selected under aerobic culture conditions on glucose-BSM. Strain PFB362(pRO1807) also grew anaerobically on glucose-nitrate-BSM, indicating that the glucose transport lesion was complemented in this strain.

The cloned *Eco*RI fragment in pRO1807 contained three *Eco*RI sites. This plasmid was cleaved, and the two resulting chromosomal DNA fragments (7.9 and 6.0 kb) were subcloned into the *Eco*RI site of vector pRO1769 and transformed into strain PFB362. The smaller of the subcloned fragments, the one in chimeric plasmid pRO1816, retained the ability to complement the glucose-negative phenotype of PFB362.

Glucose transport and GBP production. Glucose transport and periplasmic GBP activities were coinduced by glucose in wild-type strain PAO1, but neither activity was expressed in mutant strain PFB362 (Table 2). Both activities were restored in cells of PFB362 after transformation with pRO1816, and appreciable levels of both activities were expressed in transformed cells grown in the absence of glucose. Elevated levels of transport activity were also observed in noninduced wild-type cells transformed with pRO1816. This effect was not observed in cells that were transformed with the cloning vector, pRO1769. Transformants containing pRO1816 were selected as Gm^r glucose-positive cells and were shown to be Sm^s. Cells transformed with vector pRO1769 were selected simply as Gm^r and shown also to be Sm^r.

Stinson et al. (23) have described a glucose-negative mutant strain (MB723) that is deficient in GBP, glucose transport, and glucose dehydrogenase activities. Cells of MB723 transformed with pRO1816 did not regain the ability to grow on glucose and did not express glucose transport and GBP activities (Table 2). The transformed cells of MB723 exhibited the predicted pattern of antibiotic resistance, and pRO1816 was visualized in agarose gel electropherograms of lysed cell contents (not shown).

Enzyme expression. Purified plasmid pRO1816 was transformed into mutant strains that contained other known defects in carbohydrate catabolism, and enzyme levels were determined in extracts of both noninduced and glucose-induced cells (Table 3). Plasmid pRO1816 complemented three independently isolated *edd* mutations and one *glcK* mutation. The plasmid did not complement two independently isolated *zwf* mutations or the *eda-9001* mutation. The same complementation pattern was observed when the experiment was repeated with cells transformed with pRO1807 (data not shown). When strain PAO1 was transformed with pRO1816, the basal (noninduced) activities of ZWF and GLK and the combined activities of EDD and EDA were raised approximately 5-, 35-, and 25-fold, respectively, over the basal levels detected in nontransformed control cells (Table 3). The induced specific activities of GLK and of EDD and EDA combined (but not ZWF) were also raised in PAO1(pRO1816) by six- and twofold, respectively, compared with those in induced, nontransformed cells. The elevation of the basal levels of these catabolic enzyme activities parallels what was observed with basal levels of glucose transport and GBP activities in cells transformed with pRO1816. The vector pRO1769 had no significant effect on the expression of enzyme activities (Table 3).

EDD-deficient mutants PFB2 (*edd-4*) and PFB57 (*edd-8*) also failed to exhibit normal induction of ZWF, GLK, and EDA, indicating the presence of additional mutations in these strains. Plasmid pRO1816 restored the inducible expression of these enzyme activities and complemented the *edd* mutations of these two strains (Table 3).

Recombinant plasmid expression in *P. putida*. Vicente and Cánovas (24, 25) described a strain of *P. putida* that catabolizes glucose exclusively by the direct oxidative pathway due to natural deficiencies in glucokinase and glucose transport. Plasmid pRO1816 was transformed into derivatives of this strain that contain *edd* and *eda* mutations, and the specific activities of glucose transport, GBP, and certain enzymes were determined (Tables 2 and 4).

pRO1816-transformed cells of mutant strain MAP-1 (*edd-1*) regained the ability to grow on glucose-BSM, and complementation of the *edd-1* mutation was demonstrated by the recovery of EDD activity (Table 4). Although strain MAP-1 is naturally deficient in GLK (24, 25), the glucose-positive transformants also expressed levels of this enzyme activity when grown in the presence or absence of glucose. The GLK specific activities were at least one order of magnitude lower than those measured in transformed *P. aeruginosa* cells (Tables 3 and 4). Both strain MAP-1 and its pRO1816 transformants failed to express detectable activities of GBP and glucose transport (Table 2).

P. putida mutant strain MAP-2 (*eda-1*) was transformed with pRO1816. However, none of the transformants with the appropriate antibiotic resistance pattern (Sm^s Gm^r) would grow in minimal medium containing either lactate alone or lactate with glucose. This apparent toxic effect of pRO1816 in MAP-2 probably results from the accumulation of KDPG, the substrate of EDA, which is known to be very toxic to bacteria (1).

TABLE 3. Effects of vector and chimeric plasmids on enzyme expression in wild-type and mutant strains of *P. aeruginosa*

Strain (plasmid)	Relevant genotype	Growth conditions[a]	Enzyme sp act[b]		
			ZWF	GLK	EDD and EDA
PAO1	Wild type	Lct	8	19	3
		Lct + Glc	159	99	98
PAO1(pRO1769)		Lct	10	24	4
		Lct + Glc	117	67	58
PAO1(pRO1816)		Lct	39	679	84
		Lct + Glc	112	633	205
PFB98	*zwf-1*	Lct	<1	14	4
		Lct + Glc	4	55	190
PFB98(pRO1816)		Lct	2	370	146
		Lct + Glc	2	468	201
PFB103	*zwf-2*	Lct	<1	12	6
		Lct + Glc	8	79	101
PFB103(pRO1816)		Lct	<1	365	75
		Lct + Glc	3	548	244
PRP444	*glcK1*	Lct	11	<1	6
		Lct + Glc	89	<1	63
PRP444(pRO1816)		Lct	61	390	129
		Lct + Glc	162	820	289
PFB2	*edd-4*	Lct	6	22	5
		Lct + Glc	8	32	8
PFB2(pRO1816)		Lct	74	651	171
		Lct + Glc	206	1,088	374
PFB9	*edd-1*	Lct	249	88	3
		Lct + Glc	300	86	4
PFB9(pRO1816)		Lct	52	384	19
		Lct + Glc	190	609	81
PFB57	*edd-8*	Lct	8	13	4
		Lct + Glc	12	19	6
PFB57(pRO1816)		Lct	45	367	110
		Lct + Glc	122	712	197
PAO1838	*eda-9001*	Lct	253	113	6
		Lct + Glc	303	126	8
PAO1838(pRO186)		Lct	239	1,503	8
		Lct + Glc	322	1,462	10

[a] Growth conditions were as described in footnote *a* of Table 2.
[b] Enzyme specific activity is expressed as nanomoles of product formed per minute per milligram of protein and was determined in the soluble fractions of crude cell extracts. Each value is the average of at least two independent determinations.

TABLE 4. Effects of chimeric plasmid pRO1816 on enzyme expression in *P. putida*

Strain[a]	Relevant genotype	Growth conditions[b]	Enzyme sp act[c]		
			ZWF	GLK	EDD and EDA
MAP-1	*edd-1*	Lct	6	<1	<1
		Lct + Glc	116	<1	<1
MAP-1(pRO1816)		Lct	5	49	3
		Lct + Glc	54	89	50
MAP-2	*eda-1*	Lct	53	<1	<1
		Lct + Glc	223	<1	<1

[a] No growth was detected in strain MAP-2(pRO1816).
[b] Growth conditions were as described in footnote *a* of Table 2.
[c] Enzyme specific activity is expressed as nanomoles of product formed per minute per milligram of protein and was determined in the soluble fractions of crude cell extracts. Each value is the average of at least two independent determinations.

Despite repeated attempts with several different techniques, no plasmid was visualized in DNA extracts of either transformed strain of *P. putida*. We believed that the plasmid was transformed into these strains because of the acquisition of appropriate antibiotic resistance patterns and because of the simultaneous, unselected complementation of the *edd-1* mutation in MAP-1. It is possible that the copy number of the plasmid was greatly reduced in this strain, making the plasmid difficult to detect. Plasmid-bearing *P. putida* cells were sensitive to lower levels of gentamicin than were plasmid-bearing cells of *P. aeruginosa* (data not shown). It is also possible that the plasmid, or some parts of it, may have recombined into the chromosome.

DISCUSSION

We present evidence for the cloning of chromosomal DNA of *P. aeruginosa* PAO which affected the synthesis of the carbohydrate catabolic enzymes, EDD, GLK, ZWF, EDA, and a periplasmic GBP necessary for glucose transport. Recombinant plasmid pRO1816 was constructed with 6.0 kb of chromosomal DNA inserted into the streptomycin resistance locus of the *P. aeruginosa* vector plasmid pRO1769. Complementation data obtained from experiments with both *P. aeruginosa* and *P. putida* mutant strains suggested that the cloned DNA encoded the structural genes for EDD, GLK, and possibly GBP. Plasmid pRO1816 complemented three independently isolated *edd* mutations and one *glcK* mutation in *P. aeruginosa* PAO strains (Table 3) and complemented an *edd* mutation in *P. putida* (Table 4). Additionally, the strain of *P. putida* used in these experiments, which was naturally deficient in GLK, produced this enzyme when transformed with pRO1816 (Table 4).

Mutant strain PFB362 (*glcT1*) is deficient in periplasmic GBP and glucose transport activities. Plasmid pRO1816 complemented the *glcT1* mutation (Table 2) as well as two other independently isolated *glcT* mutations which are tightly linked to the *glcT1* locus in PFB362 (data not shown). However, pRO1816 did not complement the GBP deficiency in another glucose transport-negative mutant, *P. aeruginosa* MB723 (Table 2). Thus, it is unclear whether pRO1816 contains the structural gene for GBP. The mutations in strains PFB362 and MB723 may be in structural or regulatory genes or they may be in loci which encode some other component of glucose transport, causing an apparent loss of GBP. It is evident that, although these two mutant strains share the same phenotype (loss of the GBP and glucose

transport activities), the mutations lie in different genes. By using antibodies to purified GBP, it has been shown that MB723 does not produce a nonfunctional GBP (23). This could be expected if the mutation in this strain was in a regulatory locus. We do not know whether PFB362 produces a nonfunctional GBP.

The enzymatic and transport functions required for direct phosphorylative catabolism of glucose to glyceraldehyde-3-phosphate and pyruvate were produced at low basal levels in the absence of an inducing compound. In cells transformed with pRO1816, however, a large increase was seen in all of these activities in noninduced cells. In wild-type transformed cells, these activities were increased as follows: glucose transport, 5.5-fold; ZWF, 4.8-fold; GLK, 35.4-fold; EDD and EDA combined, 25.4-fold. Induced levels of GLK and of EDD and EDA combined but not glucose transport or ZWF were also increased in pRO1816-transformed cells as compared with controls with nontransformed cells (Tables 2 and 3).

Plasmid pRO1816 is present at about 30 to 40 copies per cell. It is possible, therefore, that the high level of enzyme synthesis was due to low basal level constitutive expression of 30 to 40 copies of the respective structural genes. This was probably true for much of the large increases seen in GLK, EDD, and, perhaps, glucose transport. However, complementation studies demonstrated that the structural genes for ZWF and EDA were not present on the cloned DNA fragment, necessitating an alternative explanation for the apparent constitutive expression of *zwf* and *eda* genes in pRO1816-transformed cells. It seems likely that the 6.0-kb cloned DNA fragment also contains a site (or sites) involved in the regulation of carbohydrate metabolism. This is supported by data obtained with mutant strains PFB57 (*edd-8*) and PFB2 (*edd-4*). These strains, which are deficient in EDD, are also noninducible for ZWF, GLK, and EDA (2) (Table 3). Transformation by pRO1816 restored the inducibility of these enzymes in these strains.

These data confirm recent mapping data (3) which located the *glcT1* mutation close to a previously described cluster of carbohydrate catabolic genes (21). This cluster comprises, in addition to *glcT1*, the genes coding for ZWF (*zwf*), EDD (*edd*), and EDA (*eda*). The present marker rescue data provide original evidence that a gene specifying glucokinase (*glcK*) and a putative regulatory locus also map in this cluster of carbohydrate catabolic genes. However, we have not yet determined the exact order and orientation of the genes in this region of the chromosome.

It is interesting that *P. aeruginosa* strains with an *edd* or *eda* mutation showed apparent constitutive synthesis of ZWF and GLK. We believe that this is the result of a blockage in the central pathway of carbohydrate metabolism which causes the intracellular accumulation of the putative inducer, 6PG (10, 19, 21). The accumulation of 6PG in strains containing *edd* mutations during growth on lactate (or other gluconeogenic substrates) could cause apparently constitutive synthesis of these inducible enzymes. That a strain with an *eda* mutation, which causes an irreversible (13) accumulation of KDPG, also shows constitutive synthesis of ZWF and GLK suggests a hitherto unsuspected role for KDPG in the induction of these enzymes. This effect is also seen in strains of *P. putida* that contain *edd* or *eda* mutations. KDPG is thought to be the inducer of several carbohydrate catabolic enzymes in *P. putida* (24, 25). In this study, strain MAP-2 (*eda-1*) expressed high basal levels of ZWF. No inducer role has been proposed for 6PG in *P. putida*, and cells of MAP-1 (*edd-1*) did not produce high noninduced levels of ZWF. Interestingly, if pRO1816 contains a regulatory locus which affects the synthesis of ZWF, EDA, and EDD, this locus had no effect in *P. putida*. For example, similar levels of ZWF were observed in both nontransformed and pRO1816-transformed cells of mutant strain MAP-1 (*edd-1*).

To our knowledge, this is the first report of the cloning of a fragment of chromosomal DNA that contains genes specifying carbohydrate catabolism in *Pseudomonas* spp. These results provide strong evidence that the 6.0-kb *Eco*RI fragment contains structural genes for EDD and GLK, as well as one or more genes that affect expression of the glucose active transport system. The cloned fragment may also contain at least one regulatory locus that controls the expression of structural genes that are not present on the cloned DNA fragment (e.g., *eda* and *zwf*). Further study of this interesting region of the PAO chromosome will provide an opportunity to obtain new information on the regulation of gene expression in *P. aeruginosa*.

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