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Isolation of Dicarboxylic Acid- and Glucose-Binding Proteins from *Pseudomonas aeruginosa*

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Inducible binding proteins for C_4-dicarboxylic acids (DBP) and glucose (GBP) were isolated from *Pseudomonas aeruginosa* by extraction of exponential-phase cells with 0.2 M $MgCl_2$ (pH 8.5) and by an osmotic shock procedure without affecting cell viability. DBP synthesis was induced by growth on aspartate, α-ketoglutarate, succinate, fumarate, malate, and malonate but not by growth on acetate, citrate, pyruvate, or glucose. Binding of succinate by DBP was competitively inhibited by 10-fold concentrations of fumarate and malate but not by a variety of related substances. GBP synthesis and transport of methyl α-glucoside by whole cells were induced by growth on glucose or pyruvate plus galactose, 2-deoxyglucose, or methyl α-glucoside but not by growth on gluconate, succinate, acetate, or pyruvate. The binding of radioactive glucose by GBP was significantly inhibited by 10-fold concentrations of glucose, galactose, and glucose-1-phosphate but not by the other carbohydrates tested. The binding of glucose by GBP or succinate by DBP did not result in any chemical alteration of the substrates.

In recent years, considerable attention has been focused on the role of periplasmic binding proteins in the transport of nutrients by gram-negative bacteria. Evidence has accumulated that implicates these proteins as substrate recognition components in a number of active transport systems (4, 15, 18, 29). Several binding proteins have also been shown to undergo substrate-induced conformational changes, a phenomenon that may be involved in the function of these proteins in transport (4, 6, 34).

Binding proteins are easily isolated from enteric bacteria by the osmotic shock procedure of Neu and Heppel (27); however, attempts to isolate similar transport components from the surfaces of unrelated bacteria have been largely unsuccessful. Recently, Cheng et al. (7) demonstrated that alkaline phosphatase could be released from the periplasm of *Pseudomonas aeruginosa* by extraction with 0.2 M $MgCl_2$ without affecting cell viability. In this report, we describe the isolation of inducible C_4-dicarboxylic acid- and glucose-binding proteins (DBP and GBP) from *P. aeruginosa* by using slight modifications of this extraction procedure.

MATERIALS AND METHODS

Organism and cultural conditions. *P. aeruginosa* MB-720 (derived from ATCC 15692) was grown in a mineral salts medium containing 150 mM K_2HPO_4-KH_2PO_4 (pH 6.5), 15 mM $(NH_4)_2SO_4$, 10 μM $FeSO_4$, 1 mM $MgSO_4$, 100 μM $CaCl_2$, and an appropriate carbon source (15 to 40 mM). Cultures (400 ml in 2-liter flasks) were incubated on a New Brunswick gyratory shaker at 30°C until late logarithmic growth, usually 16 to 18 h. Growth was followed by measuring turbidity with a Klett-Summerson photoelectric colorimeter (no. 66 filter). Cultures with optical densities between 250 and 300 Klett units (2×10^9 cells/ml; 0.7 mg of cell dry weight per ml) were harvested by centrifugation at 22°C. For studies concerning alkaline phosphatase, bacteria were grown in the inorganic phosphate-deficient medium described by Cheng et al. (8).

Binding protein extraction. In early studies, a modification of the method described by Cheng et al. (7) for the isolation of alkaline phosphatase from *P. aeruginosa* was used for the extraction of binding proteins. Packed cells were rapidly suspended (1.4 mg of cell dry weight per ml) in 0.2 M $MgCl_2$-0.05 M tris(hydroxymethyl)aminomethane-hydrochloride buffer, pH 8.5 (extraction buffer). Cell suspensions were incubated at 22°C for 1 h with constant stirring and then centrifuged for 20 min at 16,300 $\times$ *g*. The clear supernatant fluids containing periplasmic proteins were filtered on membrane filters (0.45-μm pore size; Millipore Corp.) and concentrated 20-fold by ultrafiltration (Amicon Corp.; Diaflo UM-10 filter). The concentrated material was dialyzed at 4°C against 1 mM $MgCl_2$-10 mM tris(hydroxymethyl)aminomethane-hydrochloride buffer, pH 7.5 (TM buffer), for 24 h.

In later studies, the extraction procedure described below was routinely used since it was less time consuming and more reproducible, and re-

sulted in higher yields of binding protein. Packed log-phase cells were suspended in extraction buffer (14 mg of cell dry weight per ml) and incubated at 22°C for 30 min with constant stirring. The cells were collected by centrifugation and rapidly suspended at the same concentration in distilled water. After an additional 30 min of mixing at 22°C, the suspension was centrifuged and the "shock fluid" was removed and combined with the magnesium extract. After dialysis against TM buffer for 18 to 20 h, the proteins were precipitated with ammonium sulfate (0 to 95% saturation). The precipitates were dissolved in a small volume of TM buffer and dialyzed against the same buffer overnight. Extracted cells were stored frozen at −18°C.

Assay of binding activity. The binding activity of crude magnesium shock extracts was determined by equilibrium dialysis (1, 19, 30). Dialysis bags (Union Carbide, 8 mm in diameter) were filled with 0.3 ml of extract (0.03 to 0.90 mg of protein per ml), which was dialyzed for 20 to 22 h at 4°C against 5 ml of TM buffer containing 0.02% sodium azide and radioactively labeled substrate (2 μM). Binding activity was linear within these protein concentrations. Specific radioactivities of the substrates were: acetate, 55.3 mCi/mmol; fumarate, 3 mCi/mmol; glucose, 100 mCi/mmol; β-hydroxybutyrate, 11.8 mCi/mmol; malate, 35 mCi/mmol; and succinate, 10.5 mCi/mmol. Upon completion of dialysis, 0.1-ml samples were removed from each solution for liquid scintillation counting in a Nuclear-Chicago Mark II spectrometer. One unit of binding is equivalent to 1 nmol of substrate bound. Specific activities are expressed in units per milligram of protein.

Substrate uptake. The uptake of methyl α-glucoside (αMeGlc) by *P. aeruginosa* cells was determined using a modification of the procedures of Guymon and Eagon (13) and Midgley and Dawes (25). Bacteria were harvested from log-phase cultures by centrifugation and washed and resuspended in mineral medium (2.0 mg of cell dry weight per ml). A 0.5-ml portion was added to a 10-ml Erlenmeyer flask containing 0.1 ml of chloramphenicol (2.0 mg/ml) and 0.3 ml of the mineral salts medium, and the mixture was incubated at 30°C on a New Brunswick water-bath shaker (200 rpm) for 15 min before addition of 0.1 ml of 50 mM ^{14}C-labeled αMeGlc (0.7 mCi/mmol). At various intervals during the incubation, 0.05-ml samples of the cell suspension were withdrawn and the cells were collected and washed by membrane filtration (Millipore Corp.). The filters were then transferred to scintillation vials and counted in a Nuclear-Chicago Mark II spectrometer. Uptake of substrate is expressed as nanomoles per minute per milligram of cell dry weight. The rate of uptake was calculated during the linear period between 0 and 3 min.

Cell extracts. The cell pellets obtained after the extraction of binding proteins were suspended in ice-cold TM buffer (200 mg [wet weight]/ml) and disrupted ultrasonically (Bronwill Corp.). The crude extracts were centrifuged at 27,000 × *g* for 15 min at 4°C to remove unbroken cells. The supernatant fluids were removed and separated into soluble and particulate fractions by centrifugation at 105,000 × *g* for 1 h. The resulting pellets were resuspended in an appropriate volume of TM buffer.

Enzyme assays. Glucose dehydrogenase (EC 1.1.99.a) was assayed spectrophotometrically using the procedure of Hauge (14). Glucokinase activity was determined by the method of Hylemon and Phibbs (16). Succinate dehydrogenase (EC 1.3.99.1), fumarase (EC 4.2.1.2), and malate dehydrogenase (EC 1.1.1.37) activities were determined according to Dubler et al. (9). Succinate thiokinase (EC 6.2.1.5) and malic enzyme (EC 1.1.1.40) were assayed according to Lo et al. (20). Alkaline phosphatase activity was determined at room temperature by monitoring the hydrolysis of *p*-nitrophenyl phosphate (12) with a Zeiss PMQ II spectrophotometer.

Other assays. Protein was measured by the method of Lowry et al. (24), with crystalline bovine serum albumin as a standard.

Cell dry weight was determined with distilled water-washed cells dried to constant weight.

Chemicals. [1,4-^{14}C]succinic acid (10.5 mCi/mmol), D-[U-^{14}C]glucose (200 mCi/mmol), and [1-^{14}C]fumaric acid (3 mCi/mmol) were purchased from ICN Pharmaceuticals, Inc. Methyl α-D-[U-^{14}C]glucopyranoside (3 mCi/mmol), [U-^{14}C]malic acid (35 mCi/mmol), D(−)-3-hydroxy[3-^{14}C]butyric acid (11.8 mCi/mmol), and [U-^{14}C]sodium acetate (55 mCi/mmol) were purchased from Amersham/Searle Corp. Chloramphenicol and 2-deoxy-D-glucose were purchased from Sigma Chemicals. Unlabeled methyl α-D-glucoside was obtained from Pfanstiehl Laboratories, Inc. All other compounds were from local distributors and were analytical grade.

RESULTS

Extraction of binding proteins and the effect on cell viability. The work of Cheng et al. (7, 8) has clearly shown that alkaline phosphatase is a periplasmic protein and can be selectively removed from cells by extraction with 0.2 M $MgCl_2$. We have confirmed these results (Table 1) and have also demonstrated that the $MgCl_2$ extraction procedure can be utilized to extract a GBP and a DBP from glucose- or succinate-grown cells. These extraction procedures did not affect cell viability (Table 1).

DBP. The specificity of the DBP from succinate-grown *P. aeruginosa* cells was examined by equilibrium dialysis (Table 2). Of the number of radioactive ligands tested, binding was detected with succinate, malate and fumarate, but not with acetate, β-hydroxybutyrate or glucose. The quantities of C_4-dicarboxylic acids bound were very similar, suggesting that a common binding protein for dicarboxylic acids was present in the extract.

The specificity of DBP was also investigated by measuring the ability of unlabeled compounds to compete with the binding of [^{14}C]-succinate. Of the substances tested, only unla-

TABLE 1. *Extraction of binding proteins and alkaline phosphatase from P. aeruginosa and the effect on cell viability*[a]

Treatment	Growth on:								
	Peptone			Succinate			Glucose		
	Viability (CFU/ml)	Apase[b]		Viability (CFU/ml)	DBP		Viability (CFU/ml)	GBP	
		Units	Sp act		Units	Sp act		Units	Sp act
1 mM Mg^{2+} + 0.01 M Tris, pH 7.5	2.0×10^{10}	0		2.1×10^{9}	0		2.0×10^{10}	0	
0.2 M Mg^{2+} + 0.05 M Tris, pH 8.5	2.1×10^{10}	40	0.28	2.2×10^{9}	1.3	0.61	2.1×10^{10}	2.9	0.55
Water (after 0.2 M Mg^{2+} extraction)	2.2×10^{10}	1.6	0.01				2.1×10^{10}	1.7	0.30

[a] Log-phase bacteria were suspended in 1 liter of each treatment solution (14 mg of cell dry weight/ml of peptone- or glucose-grown cells; 1.4 mg of cell dry weight/ml of succinate-grown cells) for 30 min at 22°C and then centrifuged. The resulting wash fluids were assayed for activity. Prior to centrifugation a sample of each cell suspension was serially diluted and plated on nutrient agar. CFU, Colony-forming units.

[b] One unit of alkaline phosphatase (Apase) is that activity liberating 1 μmol of *p*-nitrophenol per min under defined conditions.

TABLE 2. *Specificity of DBP*[a]

Substrate	Amt bound (nmol/mg of protein)
Succinate	1.55
Fumarate	1.61
Malate	1.69
Acetate	0
DL-β-Hydroxybutyrate	0
Glucose	0.1

[a] Binding proteins were extracted from succinate-grown cells. Assays were performed under standard conditions with 2 μM of the indicated ^{14}C-labeled compounds as substrate and 150 μg of cell extract protein. Specific radioactivities of the substrates are listed in Materials and Methods.

TABLE 3. *Effect of various substances on succinate binding*[a]

Inhibitors added	% Inhibition	
	20 μM	200 μM
L-Malate, -fumarate, or -succinate	90–92	100
D-Malate	20	48
Methylsuccinate	16	35
Acetate	8	18
L-Aspartate	<1	50
α-Ketoglutarate	<1	36
Pyruvate	<1	32
Malonate	<1	24
Isocitrate	<1	23
Oxaloacetate	<1	23
Citramalate	<1	<1
Citrate	<1	<1
DL-Tartrate	<1	<1
DL-β-Hydroxybutyrate	<1	<1
DL-Lactate	<1	

[a] Assays were performed under standard conditions with 2 μM [^{14}C]succinate (10.5 mCi/nmol) in the presence of the indicated nonlabeled compounds. Each reaction mixture contained 150 μg of protein. Binding activity of DBP in the absence of inhibitor was 4,110 cpm/150 μg of protein.

beled succinate, fumarate, and malate were strong competitive inhibitors at 10-fold excess (Table 3). At higher concentrations (100-fold excess), a variety of other substances exhibited significant affinities for the DBP.

To ascertain whether binding activity could have been due to dicarboxylate-metabolizing enzymes, the periplasmic extract was assayed for succinate dehydrogenase, succinate thiokinase, fumarase, malate enzyme, and malate dehydrogenase. As can be seen in Table 4, their activities were not detected. Furthermore, thin-layer radiochromatography of [^{14}C]succinate after incubation with the DBP preparation showed no chemical alteration of the substrate.

Induction of DBP synthesis. Induction of DBP was observed when *P. aeruginosa* was grown on aspartate, fumarate, α-ketoglutarate, malate, malonate, and succinate. Much smaller quantities were detected when the organism was grown on acetate, citrate, pyruvate, and glucose (Table 5).

GBP. The specificity of the GBP was investigated by measuring the ability of unlabeled substances to compete with the binding of [^{14}C]glucose (Table 6). At 10-fold concentrations, only unlabeled glucose, galactose, and glucose-1-phosphate were significant competitors. At 100-fold excess, a variety of other

TABLE 4. *Enzymatic activity of DBP preparations and whole-cell extracts*

Enzyme	Sp act[a]	
	Whole-cell extract	Periplasmic extract
Succinate dehydrogenase	56	ND[b]
Succinate thiokinase	12	ND
Fumarase	15	ND
Malate dehydrogenase	63	ND
Malic enzyme	16	ND

[a] One unit of enzyme is defined as that quantity that caused a spectrophotometric change of 0.01 optical density unit/min under standard assay conditions. Specific activities are expressed as units of enzyme activity per milligram of protein.

[b] ND, No activity detected.

TABLE 5. *Production of DBP by P. aeruginosa grown on various carbon sources*[a]

Substrate	Total units	Sp act (units/mg of protein)
Fumarate	0.9	0.32
Malate	0.9	0.42
Succinate	1.4	0.52
L-Aspartate	1.1	0.22
α-Ketoglutarate	1.1	0.24
Malonate	1.1	0.44
Acetate	0.2	0.06
Citrate	0.1	0.04
Pyruvate	0.4	0.12
Glucose	0.2	0.07

[a] Bacteria were grown on 1.6 liters of mineral medium containing 15 mM of the indicated carbon sources and harvested in late exponential phase (200 Klett units). Binding proteins were isolated as previously described. DBP assays were performed with 2 μM [^{14}C]succinate and 150 μg of protein. One unit of binding is equivalent to 1 nmol of substrate bound.

sugars and glucose derivatives had significant affinity for the binding protein.

To eliminate the possibility that the observed binding was due to the presence of glucose-specific enzymes, the crude periplasmic extract was assayed for glucokinase and glucose dehydrogenase activities. No enzymatic activity was detected (Table 7). Also, [^{14}C]glucose examined by thin-layer radiochromatography after incubation with GBP was unchanged.

Induction of GBP synthesis and glucose uptake. The glucose transport system of *P. aeruginosa* has been reported to be induced by galactose and αMeGlc during growth on pyruvate or glycerol (13, 16). Galactose is apparently not used by *P. aeruginosa* as a carbon source for growth. The transport system is repressed by acetate and various tricarboxylic acid cycle intermediates (13, 25, 26, 28). Maximal production of GBP was observed when cells were grown on glucose or pyruvate plus galactose, 2-deoxyglucose, or αMeGlc (Table 8). Small amounts of GBP were detected in cells grown on acetate, gluconate, pyruvate, or succinate. The induction of GBP synthesis correlated closely with transport activity of whole cells. Cells induced for GBP also demonstrated maximal uptake of ^{14}C-labeled-αMeGlc. Conversely, conditions that did not induce GBP failed to induce αMeGlc uptake. A low level of transport activity was, however, detected in

TABLE 6. *Effect of various substances on glucose binding*[a]

Inhibitors added	% Inhibition	
	20 μM	200 μM
Glucose	97	100
Galactose	23	70
Glucose-1-phosphate	28	42
Glucose-6-phosphate	4	41
Methyl α-glucoside	3	40
Gluconate	<1	36
Glucosamine	<1	20
2-Deoxyglucose	<1	19
Fructose	<1	15
Mannose	<1	15
Fucose	<1	<1
Arabinose	<1	<1
N-acetylglucosamine	<1	<1
Lactose	<1	<1

[a] Assays were performed under standard conditions with 2 μM [^{14}C]glucose (100 mCi/mmol) in the presence of the indicated nonlabeled compounds. GBP extract was used at a level of 200 μg of protein per reaction mixture. Binding activity of GBP in the absence of inhibitor was 15,723 cpm/200 μg of protein.

TABLE 7. *Enzymatic activity of GBP preparations and whole-cell extracts*[a]

Cell extract	Sp act (units/mg of protein)	
	Glucose dehydrogenase	Glucokinase
Whole cell, soluble	0.017	0.145
Whole cell, particulate	0.060	0.009
Mg shock fluid		

[a] One unit of glucose dehydrogenase is expressed as that quantity of enzyme that oxidizes 1 μmol of glucose per min at room temperature. One unit of glucokinase activity is that amount of enzyme that reduces 1 μmol of nicotinamide adenine dinucleotide phosphate per min at room temperature.

[b] ND, No detectable activity.

TABLE 8. *Transport of αMeGlc and production of GBP by P. aeruginosa grown on various carbon sources*[a]

Growth substrate	GBP		Uptake (nmol/min/mg)
	Units	Units/mg	
Glucose	1.5	0.59	13
Pyruvate	0.1	0.03	4.8
2-Deoxyglucose + pyruvate	1.0	0.21	11
αMeGlc + pyruvate	1.2	0.57	10
Galactose + pyruvate	0.8	0.20	13
Gluconate	0.15	0.06	5
Acetate	0.1	0.05	4.5
Succinate	0.12	0.08	4.5

[a] Bacteria were grown in 1.6 liters of mineral medium containing 20 mM of the indicated carbon sources. Non-metabolizable compounds (2-deoxyglucose, αMeGlc, and galactose) were added at a 10 mM concentration in the presence of 20 mM pyruvate. GBP was isolated and assayed as previously described. Transport activities of untreated cells were determined as in Materials and Methods.

noninduced cells, apparently a result of facilitated diffusion since αMeGlc was not accumulated above the external concentration. This basal transport activity has not been detected by other workers (13, 16, 25), presumably because of less sensitive assay procedures.

DISCUSSION

Periplasmic binding proteins for glucose and for dicarboxylic acids were readily removed from *P. aeruginosa* cells by extraction with 0.2 M Mg^{2+} followed by osmotic shock. The release of periplasmic proteins occurred without detectable losses in cell viability. Analysis of crude extracts indicated that cytoplasmic or cytoplasmic membrane enzymes were not released by this treatment.

The action of 0.2 M Mg^{2+} is apparently at the outer cell wall of *P. aeruginosa*. Ingram et al. (17) have shown that this treatment dissociates lipopolysaccharide from these bacteria and that the quantity of lipopolysaccharide released was proportional to the release of the periplasmic enzyme alkaline phosphatase. Extraction with Mg^{2+} avoids problems encountered with the sucrose-ethylenediaminetetraacetic acid (EDTA) procedure of Neu and Heppel (27). Treatment of *P. aeruginosa* with EDTA causes severe damage to both the cell wall and cytoplasmic membrane, resulting in lysis (32). Because of this effect, periplasmic proteins of *P. aeruginosa* have not received as much attention as those of enteric organisms. The presence of an inducible glycerol-binding protein in EDTA shock fluids from *P. aeruginosa* has been reported by Tsay et al. (33). Production of this protein appeared to be requisite for the transport of glycerol by intact cells.

Periplasmic proteins that bind dicarboxylic acids and glucose have also been reported in other bacteria. The periplasmic DBP of *Escherichia coli* was shown by Lo and Sanwal (22) to bind succinate, malate, and fumarate and monocarboxylic acid D-lactate. Two additional binding proteins specific for the dicarboxylic acids were solubilized by detergent extraction of the cytoplasmic membranes of *E. coli* (21). Genetic and biochemical evidence suggested that these membrane proteins cooperate in some unknown way in the transport of succinate in vivo. Similar membrane proteins have also been described in gram-positive bacteria. Fournier and Pardee (11) have detected two presumptive transport components in the membranes of L-malate-induced *Bacillus subtilis*. Binding specificities were demonstrated for malate, fumarate, succinate, and *meso*-tartrate.

A recent study of succinate transport in *P. putida* by Dubler et al. (9) suggests that a common mechanism functions in the uptake of succinate, malate, and fumarate. Transport activity was induced by each of these dicarboxylic acids and by aspartate, although aspartate did not compete with succinate in uptake studies. In the present study, growth of *P. aeruginosa* on aspartate, α-ketoglutarate, and malonate in addition to succinate, malate, and fumarate resulted in maximal production of DBP. The former three acids, however, did not compete with succinate binding by extracts prepared from succinate-, malate-, fumarate-, aspartate-, α-ketoglutarate-, or malonate-grown cells.

A galactose-binding protein, which also has a high affinity for glucose, has been isolated from *E. coli* (1, 2, 5). This protein was shown by Boos and Gordon (5) to be an active component of the β-methylgalactoside transport system, which mediates the translocation of β-methylgalactoside, galactose, glucose, D-fucose, and β-glycerol-galactoside (29).

The transport of glucose by *P. aeruginosa* has not been intensively investigated. Recent studies indicate that two distinct mechanisms may be involved. An active transport system for glucose has been described by Eagon and associates (10, 31), whereas Midgely and Dawes (25) have reported on an alternate mechanism, which involves oxidation of glucose to gluconate by membrane-bound glucose dehydrogenase followed by transport of gluconate through the cell membrane. Membrane vesicles of *P.*

aeruginosa PAO have been reported to lose their ability to transport free glucose (13), suggesting that some component of the glucose transport system, perhaps the GBP, was lost during the preparation of the vesicles. Whereas there is yet very little direct evidence to implicate the GBP of *P. aeruginosa* in transport, it is interesting to note that induction of GBP and glucose transport are co-regulated. Furthermore, we have recently isolated a transport-deficient mutant that appears to lack a functional binding protein.

Recent studies by Berger (3) and Wilson (35) have revealed mechanisms of energy coupling for the active transport of various amino acids and sugars in *E. coli*. Those systems that are osmotic shock sensitive (i.e., associated with periplasmic binding proteins) utilized phosphate-bound energy to drive transport, whereas membrane-bound systems utilized the activated membrane state. In preliminary studies, we have found that the addition of arsenate to starved cells in the presence of gluconate or pyruvate completely inhibited the active uptake of αMeGlc. This observation suggests that glucose transport in *P. aeruginosa* is driven by the energy of adenosine 5'-triphosphate (or a derivative) and belongs to the binding protein-associated transport system.

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