Characterization of the High Affinity Glucose Transport System in *Pseudomonas putida*

by

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CHARACTERIZATION OF THE HIGH AFFINITY GLUCOSE TRANSPORT SYSTEM IN *Pseudomonas putida*

BY

HONG TONG

A Practicum submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

MASTER OF SCIENCE

Abstract

Glucose transport in *P. putida* appears to be very similar to that for *P. aeruginosa*, including a low affinity oxidative pathway and an inducible high affinity phosphorylative pathway. In this study, the latter pathway was further examined. Inducible glucose uptake in *P. putida* was determined using standard filter binding assays. We found that glucose uptake by whole cell followed saturation kinetics (Km=5.5 μM, Vmax=17.5 nmol/min/mg protein), was temperature dependent, and was inhibited by gluconate, galactose, and fructose. Filter binding assays also revealed that shock fluid from glucose induced cells bound glucose. This activity was attributed to a 43,700 Da glucose binding protein (GBP). This protein was partially purified and found to have a specific glucose binding activity of 9.3 nmol/min/mg protein.

The presence of the glucose binding protein (GBP) gene in *P. putida* was verified by Southern blotting and probing with synthetic oligonucleotides derived from the amino acid sequence of the putative glucose binding protein of *Pseudomonas aeruginosa*. A 3.0-kilobases *Kpn*I-*Xho*I fragment of the *P. putida* chromosome containing the GBP gene was cloned into the multicopy plasmid pPZ375. The vector contains a unique *Kpn*I and *Xho*I sites for cloning and a selectable gene encoding ampicillin resistance. A glucose transport mutant of *P. aeruginosa*, strain PAO PFB362a transformed with the chimeric plasmid GR7 containing the *P. putida* GBP gene regained the ability to grow on glucose as verified by assaying the ability of whole cells to incorporate [^{14}C] glucose. Polyclonal sera specific for *P. putida* GBP was used to screen Western immunoblots of shock fluid from GR7, demonstrating the presence of GBP. This gene was sequenced and was found to be 1332 bases long. From this sequence, the mature protein

was found to have a molecular mass of 43,700 and substantial homology to the *P. aeruginosa* glucose binding protein.

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List of Abbreviations

braB, the gene encoding sodium-coupled carrier

braC, the gene encoding LIVAT-BP

braD, E, F, G, the gene required for LIV

GBP, glucose binding protein

glpD, G3P dehydrogenase

glpK, glycerol kinase

glpT, glycerol specific inducible transport system

LivH, the gene encoding the ennergy coupling component of branched chain amino acid transport system of *E. coli*

LIV I, the high affinity transport system of branched-chain amino acids

LIV II, the low affinity transport system of branched-chain amino acids

LIVAT-BP, leucine-, isoleucine-, and valine-binding protein

Man-BP, mannitol-binding protein

OprB or D1, glucose-selective porin

oprP, the gene encoding oprP

oprP, phosphate-selective porin

PBP, phosphate-binding protein

phos, the gene encoding PBP

Chapter 1 Introduction and Literature Review

1.1 Introduction

Pseudomonas putida is a bacterial species in the Gram-negative family Pseudomonadaceae. *Pseudomonas putida* is a member of the Pseudomonas fluorescens branch, a group of organisms that also includes *P. aeruginosa*. The outer membranes of *P. putida* and related pseudomonads contain a number of proteins which are homologous biochemically, immunologically and genetically to those described in *P. aeruginosa*. Pseudomonads are aerobic rods and motile by means of a single flagellum. Temperature requirements are extremely variable for this genus, ranging from 4°C, or lower, to 43°C. They are chemooganotrophs and produce their energy by respiration. They utilize many kinds of carbohydrates like glucose, 2-ketogluconate, geraniol, L-valine, β-alanine, and D- or L-arginine (Doudoroff and Palleroni, 1975).

Gram-negative bacteria have a complex cell surface, consisting of an outer membrane, the cell wall or peptidoglycan, and a cytoplasmic membrane (Nikaido and Vaara, 1985). The compartment between the outer and the inner cell membranes is known as the periplasm. The outer membrane constitutes an important barrier against a variety of assaults, such as enzymatic and detergent attack, and antibiotic entry (Nikaido and Vaara, 1985). Small solutes cross the outer membrane by way of water-filled channels (porins or substrate- specific channels) and diffuse readily through the peptidoglycan, a rigid layer permeable to most molecules. The cytoplasmic membrane, on the other hand, is impermeable to most water-soluble compounds unless a special transport system is provided.

These transport systems (permeases) can be broadly divided into two classes depending on whether they use the electrochemical ion gradient or substrate level phosphorylation for energization(Ames *et al*,. 1990). Electrochemical ion gradient-energized permeases are shock-resistant systems, usually composed of a single, very hydrophobic cytoplasmic membrane protein that acts as a symport or an antiport, utilizing an ion or a proton gradient. In contrast, periplasmic permeases, which are energized by substrate level phosphorylation, have a complex composition. These transport systems are also referred to as shock-sensitive permeases because they are inactivated during osmotic shock (Neu and Heppel, 1965). In all periplasmic permeases studied, osmotic shock releases an essential component, i.e. a soluble protein, that binds the transported solute with high affinity. Periplasmic transport systems are used by a numerous gram-negative organisms including the psedomonads.

My interest lies in the study of one of these systems, the high affinity glucose transport system of *P. putida* and the comparison of this system to the well characterized *P. aeruginosa* equivalent. In this study, I have identified, purified, and characterized a glucose-binding protein (GBP) of *P. putida* and described an inducible high affinity system for active transport of glucose. I also cloned, sequenced, and demonstrated the expression of the structural gene for GBP.

1.2 General structure and function of periplasmic binding proteins

Periplasmic solute-binding proteins of gram-negative bacteria and homologous, external membrane-bound lipoproteins of gram-positive bacteria are generally known to participate in two related recognition phenomena: chemoreception and transmembrane transport (Furlong, 1987; Gilson *et al.*, 1988; Higgins, 1990). In gram-negative bacteria, these proteins bind specific

substrates to move them from the inner surface of the outer membrane to the outer surface of the inner membrane. The hydrophillic solute-binding domains of these proteins interact with integral cytoplasmic membrane proteins following a conformational change induced by solute binding. Solute binding is known to induce a substantial conformational change in the periplasmic binding proteins of gram-negative bacteria, which facilitates interaction with the transmembrane chemoreception or transport protein(s) (Miller *et al.*, 1983). Recent evidence suggests that in addition to their roles in transport and chemoreception, some solute-binding proteins function in the initiation of sensory transduction pathways (Ames *et al.*, 1992 and Bourret *et al.*, 1991). They apparently detect signals in the external environment and transmit these signals via their cognate transmembrane transport proteins or other transmembrane proteins to cytoplasmic constituents. Cytoplasmic proteins then signal a change in state, usually by altering the rates of specific gene expression.

The periplasmic binding proteins are the most thoroughly analyzed of these transport components. They are monomeric with molecular weights varying between about 25,000 and 56,000. Several are stable to heat. They have high affinity for their substrates. They undergo a conformational change upon binding of substrates and have two functionally and genetically separable active domains.

The binding affinity (K_D) of these proteins range between 0.1 and 1 μM for sugar substrates and around 0.1 μM for amino acids. Binding proteins undergo a conformational change upon binding of substrate, as has been measured in the case of the histidine-, maltose-, arabinose-, ribose-, galactose-, glucose-, glutamine-, and leucine-isoleucine-valine-binding proteins by a variety of methods (Ames 1986; Furlong, 1987; Meenakshi *et al.*, 1994). Since substrate binding

to these proteins induces a conformational change, it is likely to be an essential aspect of their mechanism of action.

X-ray crystallographic studies of several binding proteins have shown that they are organized into two globular domains (lobes) forming a cleft connected by a flexible hinge. The binding site for the substrate is located in the concave region between the two lobes. The molecule is flexible, the cleft becoming somewhat narrower upon binding of substrate (Mao *et al.*, 1982, Newcomer *et al.*, 1981), thus 'trapping' the substrate deep within the protein. Once the substrate is trapped in the liganded form of the protein, it is likely that this more stable form requires an external stimulus to release the substrate, presumably supplied by the interaction with the membrane-bound transport components.

Thus, the first step in the action of these permeases involving the liganding of the substrate to the periplasmic protein in the periplasm results in the formation of the actual transport substrate, the liganded binding protein (Prossnize *et al.*, 1989). The result of this first step is that the solute to be transported is presented in a concentrated form to the membrane-bound complex, as a consequence of the high binding affinity and very high concentration (in the order of mM) of the binding protein. For example, the histidine permease of *E. coli* was shown to be around 10 μM. This value is considerably higher than that measured for the transport process as a whole (apparent Km=6 nM). This disparity is accounted for by the high periplasmic concentration of the binding protein and the relative amounts of the binding protein versus the membrane complex (Prossniz *et al.*, 1989).

The number of the membrane-bound components of these transport systems is between two and four, as determined for several permeases by genetic or recombinant DNA techniques

(Ames, 1986). Two of the components (when the total number is three or four) are usually very hydrophobic (Bell *et al.*, 1986; Froshauer and Beckwith, 1984). In the case of the histidine permease, HisQ and HisM proteins are accessible to protease digestion from both the outer and the inner surface of the cytoplasmic membrane, thus indicating that they span the membrane.

A total of eight different binding protein structures, representing about a third of the entire family of transport receptor proteins from Gram-negative bacteria, have been determined and refined to resolutions of better than 1.9 Å (Quiocho, 1991 and Yao *et al.*, 1994). The structures determined previously are binding proteins with specificities for arabinose/galactose/fucose, glucose/galactose, maltodextrins, leucine/isoleucine/valine, leucine, sulfate, and phosphate from *E. coli* (Quiocho, 1991). The glucose/galactose-binding protein and maltodextrin-binding protein (MBP) are also primary components of chemotaxis. All these proteins share the following structural features. (1) They have similar overall tertiary structures, consisting of two similar globular lobes separated by a deep cleft. (2) The ligand is bound and sequestered within the cleft. (3) Hinge-bending motion between the two lobes allows access to and from the ligand-binding site. (4) Molecular recognition and binding of the different ligands are achieved principally by hydrogen-bonding interactions (Yao, 1994).

All these common structural features are in many ways essential for function. Both domains in concert with the hinge-binding motion, are used in binding and sequestering the substrate in the cleft and in docking to either of two sets (one for active transport and another for chemotaxis) of protein components bound in the cytoplasmic membrane. All the structures of the binding protein-ligand complexes show one domain to be more heavily involved in the ligand binding than the other domain(Sack *et al.*, 1989).

The actual protein-substrate interaction may occur through hydrogen bond formation, as has been shown for the glutamine-binding protein by NMR spectroscopy (Shen *et al.*, 1985). There has been no evidence that binding proteins undergo covalent modification except for a report that an arginine-binding protein can be phosphorylated by ATP while transporting arginine (Celis, 1984).

1.3 General structure and function of porins

In order to enter the cytoplasm of the Gram-negative bacterial cell, an extracellular hydrophilic molecule must pass through both the outer and inner membranes of the cell envelope. Passage through the outer membrane is normally accomplished via hydrophilic proteinaceous pores, called porins. Among these proteins are the OmpF and OmpC porins (Benz, 1988) which are fairly nonspecific with respect to their substrate specificities. The gross physicochemical parameters of the solute obviously influence its rate of penetration through these channels, but these channels do not appear to contain specific ligand-binding sites.

Specific channels also produce water-filled channels, which contain stereospecific binding sites. The presence of specific binding sites has important consequences. The diffusion of the solutes of a specific class is accelerated when the solute concentration is low, but it is slowed down when the concentration is high, producing saturation-type kinetics very similar to Michaelis-Menten enzyme kinetics. This behaviour is very different from that of the non-specific porin channel, where the solute diffusion rate increased proportionally to the solute concentration on one side of the membrane if the concentration on the other side is zero. An example is the phage lambda receptor protein, or LamB protein, of *Escherichia coli*, which produces a specific channel for the diffusion of maltose and maltodextrins (Benz *et al.*, 1986, Luckey *et al.*, 1980).

Most porins from enteric bacteria studied so far, including the OmpF, OmpC, and PhoE porins of *E. coli*, exist as tight, trimeric complexes that are not dissociated even in sodium dodecyl sulphate (SDS), unless the protein is denatured by heating.

A more widespread property of porins and specific channels appears to be the predominance of β-structure (Nau and Konisky, 1989). The porin PhoE exists as a tightly assembled trimer, and each of the subunits produces a water-filled channel. The polypeptide chain of a subunit traverses the membrane 16 times as antiparallel-β-strands. One of the external loops folds back into the channel and produces a significant narrowing of the channel. This construction seems ideally suited for the physiological function of porins, i.e. to produce a narrow channel to exclude toxic compounds, yet to maximize the influx of nutrients by minimizing the friction between solute molecules and the walls of the pore (Weiss *et. al.*,1991).

In comparing the sequences of various related porin genes, it is remarkable that short deletions (or insertions) always seemed to have occurred in the externally exposed parts of the molecule. Undoubtedly these sequences exposed on the bacterial outer surface are undergoing an extremely rapid evolutionary change, because they are the targets of recognition by components of the host immune systems as well as phages and bacteriocins found in the environment.

Porins form large (0.6 to 2.3 nm diameter), transmembrane, water-filled channels. The size of the channel largely determines the exclusion limit of the outer membrane for hydrophilic compounds and most porins demonstrate little chemical (as opposed to ion or charge) selectivity for different substrates (Nikaido and Vaara, 1985). The interior of a porin channel contains charged amino acids lining part of the channel (Hancock *et.al.,* 1986) and it is the number and the position of these charges relative to the most constricted portion of the channel which appear to be the strongest determinants of the ion selectivity of the channel (Hancock *et al.*, 1986). Porin

channels can be either cation or anion selective, but generally this selectivity is weak for small ions like K^+ and Cl^- (ranging from threefold anion selective to 40-fold cation selective (Benz *et al.*, 1985). These ion selectivities are reflected in the sieving properties of the porins for antibiotics of different charges. For example the cation-selective OmpF channel favours the passage of zwitterionic over anionic-lactams, whereas for the anion-selective PhoE channel this situation is reversed (Nikaido *et al.*, 1983).

Most porin proteins form simple aqueous channels which confer a molecular sieving function on the outer membrane. However, a few porin proteins demonstrate substrate specificity. The well-characterized *P. aeruginosa* OprP and *E. coli* LamB proteins have been shown to function in vivo in phosphate (Poole and Hancock, 1986) and maltose-maltodextrin (Ferenci *et al.*, 1980) transport, respectively, across the outer membrane. Both channels demonstrate binding sites with affinities of around 0.2 to 0.3 mM for their respective substrates and it has been suggested that they have channel structures that are quite different from those of general diffusion porins like OmpF and OmpC (Benz *et al.*, 1986 and Hancock *et al.*, 1986). The PhoE porin, while having many similarities to the general diffusion porins (Benz *et al.*,1985), has been shown to function in polyphosphate uptake (Dargent *et al.*, 1986 and Ferenci *et al.*, 1980).

Not all molecules cross outer membranes via porin proteins. Excreted proteins, transforming DNA, and bacteriocins are all too large to pass through the channels of porins, and the mechanisms of passage of these macromolecules are inadequately understood (Hancock, 1984). In addition, two alternative (nonporin) uptake pathways have been proposed, the self-promoted pathway for polycation (e.g., polymyxin and aminoglycoside antibiotics) uptake and the hydrophobic pathway for uptake of hydrophobic substances (Nikaido, 1976; Nikaido and

Nakae, 1979).

1.4 The glucose transport systems of *Pseudomonas aeruginosa*

An active transport system is used by *Pseudomonas aeruginosa* to take up glucose, as first suggested by Eagon and Phibbs (1971) and confirmed by different experimental approaches (Midgley and Dawes, 1973; Guymon and Eagon, 1974; Hunt and Phibbs, 1983). There are two mechanisms by which *P. aeruginosa* may transport glucose. One is the low affinity pathway (Km=1-2 mM) (Hancock and Carey, 1980) which oxidizes glucose extracellularly to gluconate and 2-ketogluconate, prior to uptake of these substrates into the cytoplasm. Subsequently, these products are converted to 6-phosphogluconate and can enter the Entner-Doudoroff pathway (Cuskey and Phibbs, 1985, Cuskey *et al.*, 1985). The second pathway is a high affinity phosphorylative pathway (Km=7-8 μM) (Guymon and Eagon, 1974; Midgley and Dawes, 1973) which transports glucose directly into the cytoplasm followed by intracellular phosphorylation to glucose-6-phosphate which then enters the Entner-Doudoroff pathway (Cuskey and Phibbs, 1985 and Cuskey *et al.*, 1985).

The high affinity glucose transport system is induced by growth on glucose, fructose, mannose, pyruvate plus galactose, 2-deoxyglucose, and methyl-α-glucoside but repressed by growth on gluconate, succinate, acetate, and citrate (Cuskey and Phibbs, 1985; Stinson *et al.*, 1976; Eagon and Phibbs, 1971). It is saturable, energy and temperature dependent which suggests that enzyme-like carriers are involved in the active transport system (Eagon and Phibbs, 1971). Two proteins are produced during induction of this system (Cuskey and Phibbs, 1985), the outer membrane porin protein (OprB)(Hancock and Carey, 1980) and the periplasmic glucose binding protein (GBP)(Stinson *et al.* 1977).

The glucose-selective porin protein, OprB, from *P. aeruginosa* has been shown to be coinduced with the high affinity glucose transport system (Hancock and Carey, 1979 and Mizuno and Kageyama, 1978). The monomeric molecular weight of OprB is 45-46 kilodaltons (Hancock and Carey, 1979; Hancock and Carey, 1980; Trias *et al.*, 1987). It functions in the membrane as an oligomer. Chemical cross-linking studies reveal that its native state in the membrane is as a trimer (Angus and Hancock, 1983). When reconstituted into LPS-phospholipid vesicles, this protein caused a flux of several radiolabelled carbohydrates out of the prepared vesicles (Hancock and Carey, 1980). Sugars with exceptional ability to enter into proteoliposomes for their size included D-glucose, β-form preferred over α, and D-xylose (Trias *et al.*, 1987). The permeation rates of sugars through OprB were not directly proportional to the molecule weight of the substrate being transported. A binding site for glucose and several other carbohydrates was demonstrated in *P. aeruginosa* OprB by Wylie *et al.* (1993). *P aeruginosa* OprB bound glucose with a Ks of 380 mM. Single-channel-conductance measurements indicated that *P. aeruginosa* forms small constricted channels (25ps) for OprB (Wylie *et al.*, 1993). The constriction in the channel of substrate-selective porins is believed to be related to the presence of a binding site for a given substrate within the pore of these proteins (Wylie *et al.*, 1993).

In analyzing the inhibition of the high affinity glucose transport system at two concentrations of [^{14}C]glucose (0.8 μM and 70 μM), Wylie *et al.* (1993) found that when 70μM [^{14}C]glucose was used, glucose, maltose, galactose, and xylose showed a significantly higher level of inhibition compared to other substrates with glucose having the highest inhibitory capacity. Galactose and maltose showed levels of inhibition statistically indistinguishable from each other. Xylose showed lower level of inhibition than galactose and maltose but higher than fructose,

ribose, or arginine. With 0.8 μM[^{14}C]glucose only unlabelled glucose showed a significantly different level of inhibition in comparison to other substrates. No inhibition was observed for any other substrate. The results from the higher concentration of [^{14}C]glucose are those expected based on the reported high degree of in vitro specificity of GBP for glucose (Stinson *et al.*, 1977). In contrast, the results from the lower concentration of [^{14}C]glucose are similar to earlier in vivo results and correlate with the substrate specificity of OprB. Wylie *et al.* (1993) concluded that the high affinity glucose transport system of *P. aeruginosa* as a whole is highly specific for glucose when examined as an intact system in vivo. This is expected due to the specificity of GBP for glucose. OprB does have affinity for other sugars in addition to glucose (Saravolac *et al.*, 1991 and Trias *et al.*, 1988).

The second component of the high affinity glucose transport system is the periplasmic glucose-binding protein (GBP). Its activity, not its level of production, is inhibited by ten fold concentrations of glucose, galactose, or glucose-1-phosphate (Stinson *et al.*, 1976). This may be a regulatory mechanism of the system or simply may reflect a low dissociation constant for the protein-substrate complex, Kd=0.35 μM (Stinson *et al.*, 1977).

The molecular weight of GBP from *P. aeruginosa* is 44.5 kilodaltons and it functions as a monomer, binding one molecule of glucose per molecule of GBP. An amino acid profile shows high levels of alanine, glutamic acid, glycine, and low levels of sulphur-containing amino acids. Stinson *et al.*(1977) isolated a mutant strain, BM 723, which lacks glucose dehydrogenase activity as well as GBP activity. This strain is defective in both membrane transport and glucose chemotaxis, thus implicating GBP as an important factor in glucose chemotaxis. This function is similar to the maltose (Hazelbauer, 1975) and galactose (Hazelbouer and Adler, 1971) transport

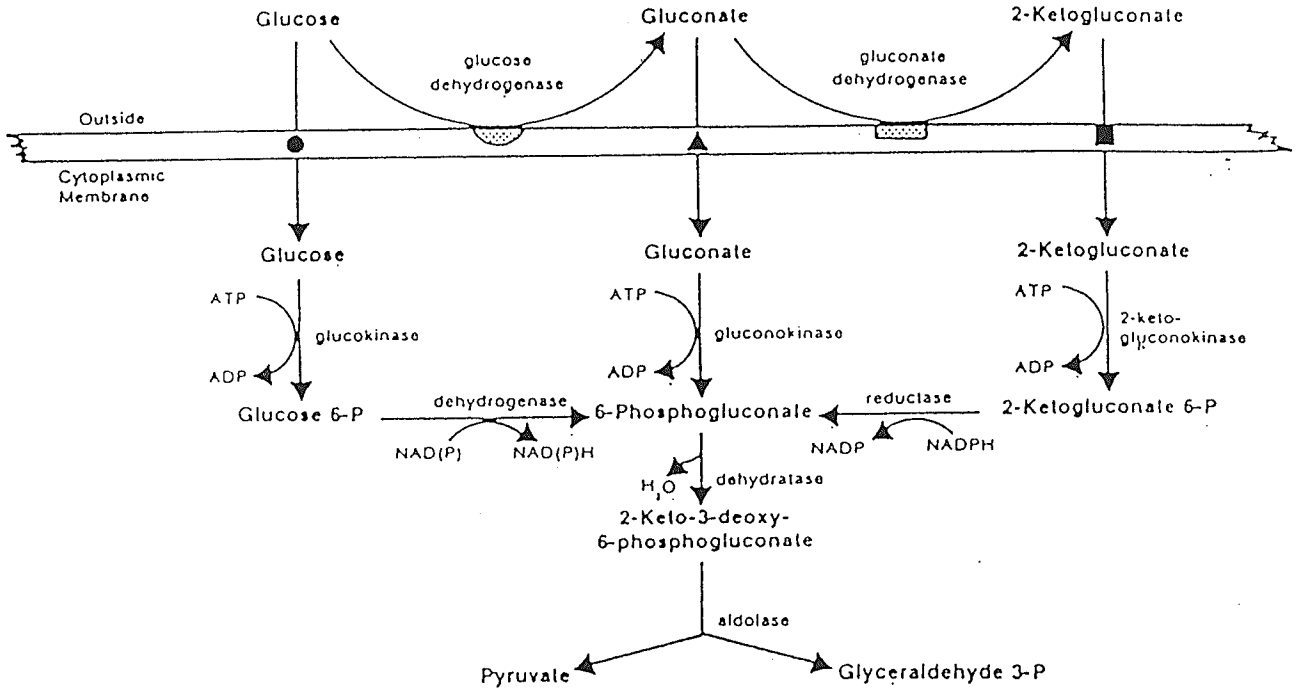
systems in *E. coli* and the ribose transport system in *S. typhimurium* (Askamit and Koshland, 1972).

The gene for the *P. aeruginosa* GBP has been located and is near several other genes involved in glucose catabolism (Cuskey and Phibbs, 1985). It is at the 52-53' region of the chromosome (Cuskey *et al.*, 1985). After being isolated on a 6.0 EcoRI fragment of DNA, the proposed gene was shown to reconstitute GBP expression and activity in glucose defective strains PFB360 and PFB362 (Cuskey and Phibbs, 1985, Cuskey *et al.*, 1986). The strain MB723 (Stinson *et al.*, 1976) was not reconstituted with this DNA fragment leading Cuskey *et al.* (1985) to suggest that perhaps it contains a functional GBP but lacks some other component of the high affinity glucose transport system. The gene *gltB* encoding GBP is located 5' to *glk* and *zwf*, the genes encoding glucokinase and 6-phosphoglucose dehydrogenase, respectively. These gene products are required for the transformation of internalized glucose to glucose-6-phosphate and subsequently, to 6-phosphogluconate. The latter is believed to control the expression of the associated operon (Temple *et al.*, 1990). Additionally, a glucose-negative *P. putida* mutant MAP1, which was naturally deficient in glucokinase, produced this enzyme when transformed with the plasmid containing cloned *P. aeruginoda* DNA fragment (Cuskey *et al.*, 1985).

1.5 The glucose transport systems of *Pseudomonas putida*

P. putida, P. fluorescens, and *P. aeruginosa* have been grouped together (Stainer *et al.*, 1966), on the basis of common physiological characters, as comprising the aerobic fluorescent pseudomonads. All of these bacteria are known to transport glucose by common pathways (Agbanyo and Taylor, 1985 and Lessie and Phibbs, 1983). Fig.1 shows alternative peripheral pathways for glucose catabolism in *P. aeruginosa* (Hunt and Phibbs, 1981). By previous

Figure 1. Alternative peripheral pathways for glucose catabolism by *P. aeruginosa* (reproduced from Hunt and Phibbs, 1981.)


Glucose
Gluconate
2-Ketogluconate
glucose dehydrogenase
gluconate dehydrogenase
Outside
Cytoplasmic Membrane
Glucose
Gluconate
2-Ketogluconate
ATP
ADP
glucokinase
gluconokinase
2-keto-gluconokinase
Glucose 6-P
dehydrogenase
NAD(P)
NAD(P)H
6-Phosphogluconate
reductase
NADP
NADPH
2-Ketogluconate 6-P
H_2O
dehydratase
2-Keto-3-deoxy-6-phosphogluconate
aldolase
Pyruvate
Glyceraldehyde 3-P

biochemical studies, it was suggested that the first part of the pathway for glucose and gluconate degradation in *P. putida* consists in the conversion of glucose into gluconate-6-phosphate via gluconate and 2-ketogluconate (Vicente *et al.*, 1973). Direct phosphorylation of gluconate must be considered as a minor shunt that cannot account for significant growth on this substrate. *P. putida* has been reported to utilize glucose primarily via oxidation to 2-ketogluconate (Deley, 1960; Frampton and Wood, 1961; and Vicente and Canovas, 1973). There are some divergences between *P. aeruginosa* and *P. putida.* The former developed a highly inducible pyridine nucleotide-dependent glucose-6-phosphate dehydrogenase activity (Lessie and Neidhardt, 1967); the latter gained an equally active system to oxidize glucose to gluconate by means of an oxygen-dependent dehydrogenase. *P. putida* cells have two forms of glucose dehydrogenase: a thermostable form bound to the membrane and a labile form which is soluble (Vicente and Canovas, 1973). Vicente *et al.* (1974) showed that glucose and gluconate uptake obeyed saturation kinetics: the apparent Km for glucose was 6 mM and that for gluconate was 0.5 mM. It was found that glucose and gluconate are parallel inhibitors for their uptake (Vicente *et al.*, 1975). Strains selected for their inability to transport glucose were found to be deficient in gluconate uptake. The reverse was also true: mutation affecting gluconate entry also blocked the uptake of glucose. Another discrepancy is that gluconate induced glucose transport in *P. putida* (Vicente *et al.*, 1974) but not in *P. aeruginosa* (Guymon *et al.*, 1974). These results suggest that a common carrier is involved in the uptake of both glucose and gluconate by *P. putida* cells. Similar to *P. aeruginosa*, the uptake of glucose and gluconate by *P. putida* cells was found to be inducible, obeyed saturation kinetics and could be specifically blocked by genetic lesions. These properties are commensurate with active transport mediated by an enzyme-like carrier.

Similar to *P. aeruginosa*, one important protein in the outer membrane of *P. putida* for glucose transport is the OprB. It was demonstrated that the glucose-inducible, organic acid-repressible OprB of *P. putida* has glucose-specific pore-forming activity (Saravolac *et al.*, 1991). There is a functional homology between OprB from *P. aeruginosa* and *P. putida*. OprB behaves as a glucose-specific porin in the outer membrane and may form an important part of the high affinity glucose uptake system of *P. putida* (Saravolac *et al.*, 1991). Consistent with this, OprB appears to be coregulated with the high-affinity glucose transport system observed in *P. putida*, as indeed it is in *P. aeruginosa* (Guymon *et al.*, 1974 and Hancock *et al.*, 1980). OprB from *P. putida* and *P. aeruginosa* showed heat-modifiable mobility on SDS-PAGE. Heat modifiability has been attributed to the high β-sheet content of outer membrane proteins (Hancock *et al.*, 1990). Although *P. aeruginosa* and *P. putida* have minimal DNA homology and differences in apparent monomer molecular weights, the OprB proteins have similar amino acid compositions and similar SDS-PAGE mobilities in the heat-unmodified form (Saravolac *et al.*, 1991). Several pseudomonad species carrying the OprB gene were identified (Wylie *et al.*, 1994). Of the species examined, only members of rRNA Group I produced a protein similar to *P. aeruginosa* OprB. Immunological and genetic analysis suggests that *P. putida* and *P. chlororaphis* OprB are most closely related to *P. aeruginosa* OprB. Reconstitution experiments with black lipid membranes showed that OprB of *P. putida* formed small, cation-selective pores which bound glucose (Ks=110 mM) and other carbohydrates (Saravolac *et al.*, 1991). However, the binding site of OprB appeared to be distinct from that of the equivalent carbohydrate specific porin of *E. coli*, the maltodextrin-specific porin LamB.

The most profound difference between LamB and OprB was in the sugar-binding

characteristics. The binding affinity of LamB increases as the size of the maltodextrin increases from 2 to 5 glucose units and then remains constant for maltodextrins with 6 and 7 glucose units (Benz *et al.*, 1986 and Benz *et al.*, 1987). In contrast, although OprB bound glucose and maltose with affinities similar to those measured for LamB, it bound maltotriose more poorly than maltose and maltotetraose extremely poorly (Saravolac *et al.*, 1991). The binding site for OprB must be much shorter, with binding site corresponding to 2 glucose molecules, than LamB. There are also some differences in the specificity of these two channels. For example, LamB binds galactose 2.5-fold better than glucose and binds sucrose almost as well as maltose (Benz *et al.*, 1987), whereas OprB has three fold preference for glucose over galactose and a 13-fold preference for maltose over sucrose (Saravolac *et al.*, 1991). OprB induction within the family Pseudomonaceae showed *P. aeruginosa* and *P. putida* OprB facilitate the diffusion of xylose and maltose in addition to glucose (Wylie *et al.*, 1994). However, of several carbohydrates, only glucose can support the growth of *P. aeruginosa* and *P. putida* and OprB function in these species would be expected to be restricted to glucose uptake (Wylie *et al.*, 1994).

1.6 Other transport systems of the pseudomonads

Some other transport systems in *P. aeruginosa* and *P. putida* have been identified. These include the transport of inorganic phosphate, phosphorylethanolamine, mannitol, and amino acids in *P. aeruginosa* and fructose and succinate in *P. putida*.

Phosphate can be transported in *P. aeruginosa* by different mechanisms, the low and the high affinity transport systems. They both obey Michaelis-Menten kinetics. The Km of the low affinity transport system is 12.0 μM while that of the high affinity system is 0.46 μM (Lacoste *et al.*, 1981). These systems differ in their sensitivity to osmotic shock, their specificity for other

phosphate-containing compounds and their energy requirements. The high affinity phosphate transport system of *P. aeruginosa* is induced in growth conditions with low phosphate concentrations (5.0 mM) which results in the induction of the *pho* regulon. Genes included in this regulon encode the outer membrane porin protein, *oprP*, the periplasmic binding protein, *phoS*, as well as others required for regulation of the system (Filloux *et al.*, 1988). The outer membrane protein OprP has a monomeric molecular weight of 48 kDa. Chemical cross-linking studies show that it functions as a trimer in the outer membrane (Angus and Hancock, 1983). It forms a 0.6 nm pore which is anion specific and is saturable, thus likely contains a binding site (Hancock and Benz, 1986). The Kd for phosphate is 0.3 mM, 100 fold higher than the Kd for chloride ion, which is 40 μM, suggesting that the binding site is specific for inorganic phosphate (Siehnel *et al.*, 1990). The high affinity phosphate transport system of *P. aeruginosa* is sensitive to osmotic shock indicating the presence of a periplasmic phosphate-binding protein (PBP) (Poole and Hancock, 1984). PBP is essential for transport as mutants which lack it are also deficient in phosphate transport. PBP binds one molecule of phosphate per binding protein with a Kd of 0.34 μM. Some compounds compete for its binding site such as arsenate, pyrophosphate, and polyphosphates up to 15 units (Poole and Hancock, 1984).

The transport system of o-phosphorylethanolamine is a binding protein-dependent transport system with a Km of transport between 25 and 55 μM. It is independent of the phosphate transport system and is not inhibited in the presence of phosphate, phosphatidylserine, phosphatidylcholine, glycerol-3-phosphate, 2-aminophosphonic acid, or ethanolamine. Initial characterization of the o-phosphorylethanolamine-binding protein shows that it binds one mole of phosphate per protein molecule with a Kd of 1.2 μM. It has a molecular weight of 38 kDa and a pI of 5.35 (Domora *et al.*, 1987).

Hoshino and Kose (1990) have described the two mechanisms in *P. aeruginosa* which can transport the branched chain amino acides leucine, isoleucine, and valine. The high affinity transport system, LIV I, is a binding protein-dependent transport system which does not require sodium, compared to the low affinity system, LIV II, which requires a sodium-coupled carrier encoded by the gene, *braB*. The LIV I can transport not only the branched chain amino acids, but also alanine and threonine. The binding protein, LIVAT-BP is encoded by the *braC* gene which is 1.1 kb in length. The *P. aeruginosa* LIVAT-BP deficient mutant, braC310, has shown wild type activity after addition of the cloned DNA fragment. The LIVAT-BP amino acid sequence shows 51% identity to both the LIV and leucine-specific binding proteins from *E. coli* K12 (Hoshino and Kose, 1989). Since the cloning of the LIVAT-BP gene (*braC*), it has been found that a 4.0 kb piece of DNA located 3' of *braC* shows four open reading frames which have been designated *braD, E, F*, and *G*. The gene *braD* and *braE* gene products are both hydrophobic and produce proteins of 307 and 417 amino acids. The *braF* gene product is a 255 amino acid with a putative ATP-binding site. The *braF* gene product shows 67% homology with *livH* gene product, the energy coupling component of the high affinity branched chain amino acid transport system of *E. coli.* The *braG* gene product is 233 amino acids and also contains a putative ATP-binding site. All of these proteins are components of the cytoplasmic membrane (Hoshino and Kose, 1990).

An inducible mannitol-binding protein (Man-BP) in *P. aeruginosa* has been described (Eagon and Phibbs, 1982). Intracellularly, mannitol is broken down to produce fructose-6-phosphate which can be converted for use in the Entner-Doudoroff pathway. Radiochromatographic evidence has been presented for inducible fructokinase activity during

growth on either mannitol or fructose but not glucose (Phibbs *et al.*, 1978). Man-BP has a molecular weight of 37 kilodaltons and has been purified and released from a closely associated enzyme, mannitol dehydrogenase, the molecular weight 85 kilodaltons. It has a pI of 8.3 and a Kd of 2.3 μM. At equimolar concentrations, mannose, fructose, and sorbitol show inhibition of this system (Eagon and Phibbs, 1982).

Vicente *et al.* (1974) showed that fructose transport was not apparently affected in a number of *P. putida* strains with deranged activity of a common glucose-gluconate uptake system, indicating the existence of an independent fructose uptake system. Fructose uptake by glucose-gluconate uptake mutants was induced by fructose and obeyed saturation kinetics (apparent Km=0.3 mM). The entry of fructose into *P. putida* cells appears to be mediated also by the glucose-gluconate uptake system, as shown by the ability to accumulate fructose of wild type cells grown on glucose, a substrate that induces the glucose-gluconate uptake system but not the fructose uptake system. In addition, fructose was found to be an inducer of the glucose-gluconate uptake system.

Induced succinate uptake and transport by *P. putida* have been described by Dubler *et al.* (1973). The uptake and transport are active processes resulting in intracellular succinate concentration. Uptake was studied with the wild-type strain *P. putida* P2 and transport with a mutant strain deficient in succinate dehydrogenase activity. Addition of succinate, fumarate, or malate to the growth medium induces both processes above the basal level. Induction is dependent on protein synthesis and subject to catabolite repression. When extracts of induced and noninduced wild-type cells are assayed for succinate dehydrogenase, fumarase, and malate dehydrogenase, only malate dehydrogenase increased in specific acticity. Kinetic parameters are:

basal uptake (cells grown on glutamate) Km=11.6μM, Vmax=0.32 nmoles per min mg dry cell mass; induced transport (cells grown on succinate plus NH_4Cl) Km 12.5 μM, Vmax=5.78 nmoles per mg dry cell mass. The similarities in the Km values for induced and basal uptake could indicate that induced transport represents an increased synthesis of the basal transport system (Dubler *et al.*, 1973).

1.7 Purpose

The purpose of this thesis was to determine whether the high affinity glucose transport system exists in *P. putida*; if does, which protein is responsible for this system; what the characteristics of this protein are; and which structural gene encodes this protein. In this study, I have identified, purified, and characterized a glucose-binding protein of *P. putida* and described an inducible high affinity system for transport of glucose. I also cloned, sequenced, and expressed the structural gene for the *P. putida* glucose binding protein.

Chapter 2 Materials and Methods

2.1 Chemicals

D-Glucose-U-[^{14}C] (2.9 mCi/mmol), D-Fructose-U-[^{14}C] (302.00 mCi/mmol), and D-Glycerol-U-[^{14}C] (147.00 mCi/mmol) were purchased from Du Pont Canada Inc., Markham, Ontario, Canada. All other compounds were from local distributors and were analytical grade.

2.2 Organisms and growth conditions

Bacterial strains and plasmids used in this study are listed in Table 1. To induce the high affinity glucose transport system, *P. putida* and *P. aeruginosa* were grown to mid log phase in 5 ml of Luria-Bertani (LB) broth at 28°C on a rotary shaker, transferred 1 ml to 50 ml basal medium 2 (BM2) minimal medium (Hancock and Carey, 1979), which contained per liter: K_2HPO_4, 7 g; KH_2PO_4, 3g; $(NH_4)_2SO_4$, 1g; $Mg_2SO_4.7H_2O$, 0.03g; $Fe_2SO_4.7H_2O$, 0.01 g; and glucose, 3.5 g. One ml of second culture was transferred to 100 ml of the same medium and grown to late log phase (16-18 hours). For transformation and expression experiments, antibiotics were added to media at the following concentrations: ampicillin, 50 μg/ml for *E. coli* strains; ampicillin, 50 μg/ml and carbenicillin, 500 μg/ml for *P. putida* and *P. aeruginosa*.

2.3 Measurement of substrate uptake

The transport of glucose by *P. putida* was measured at room temperature (22°C) and 28°C. Cells for whole cell uptake were harvested by centrifugation (speed at 7,000 x g) and resuspended in BM2 to an absorbance of 0.236 at 660 nm (=0.13 mg/ml dry weight). A series of 10-ml Erlenmeyer flasks containing a total volume of 1 ml of BM2: 0.1 mg of cells (dry weight), 2.9 mCi/mmol of D-glucose-U-[^{14}C], 302.00 mCi/mmol of [^{14}C] fructose, or 147 mCi/mmol of [^{14}C]

Table 1. Bacterial strains and plasmids

Bacterium or plasmid	Genotype or phenotype[c]	Derivation or source (reference)
P. putida	wild type	ATCC 12633
P. putida (MAP-1)	*edd*-1[a]	Vicente *et al.*, 1973
P. aeruginosa (PAOI H103)	wild type	Hancock and Carey,1979
P. aeruginosa (PFB362a)	*glcT*1[b]	PFB311, Glc$^-$, EMS Cuskey *et al.*, 1985
E. coli (NM522)	*sup*E *thi* Δ(*lac-pro*AB)*hsd5* F'[*pro*AB$^+$*lac*^{Iq} *lac*Z ΔM15	UMCC
Plasmids		
pM13	Amp^r ColE1	
ppZ375	Ampr, Broad host range derivation of pGEM-3Z$^+$	Paul V. Phibbs
pTZ19R	Amp^r ColE1	(Pharmacia)

[a] edd-1 indicates 6-phosphogluconate dehydratase
[b] glcT1 describes a deficiency in glucose transport.
[c] abbreviations: Glc, glucose utilization; EMS, ethylmethanesulfonate mutagenesis.
Abbreviation: ATCC, American Type Cuulture Collection; UMCC, University of Manitoba Culture Collection; Italics, genes as indicated; Amp^r,ampcillin resistant.

glycerol to yield final concentrations as indicated in the text and figures. After 20 s of incubation at room temperature, the uptake was terminated by dilution of 100 µl of the cellular suspension with 20 volumes of BM2, pipetting onto a Millipore filter membrane (0.45 µm) (Millipore [Canada] Ltd.), filtered instantaneously by vacuum, and immediately washed with another 20 volumes of BM2. The vacuum was kept on for 30 min, the filter was removed from the suction apparatus while still under vacuum and transferred to a vial containing 10 ml of scintillation fluid (Ecolume, ICN Biochemicals Inc., Irvine, California). Radioactivity was measured in a Beckman LS-230 Liquid scintillation system (Beck Instruments Canada Inc., Mississauga, Ontario).

2.4 Binding protein extraction

GBP cell free shock fluids were isolated using the cold shock procedure of Hoshino and Kageyama (1980) as modified by Poole and Hancock (1986). Cells were subjected to two rounds of hot/cold shock (30°C / 0°C) in the presence of 50 mM Tris HCl (pH 8.0) containing 0.2 M $MgCl_2$. The suspension was centrifuged at a speed of 7,000x g and the resulting supernatant containing the periplasmic proteins was concentrated 20 fold with a 50 ml Amicon concentrator (Model 52, Diaflo, Amicon Div. W.R. Grace & Co., Danvers, Mass.). The concentrated material was dialyzed at 4°C against 50 mM Tris HCl, pH 7.5, for 20 h.

2.5 Assay of binding activity

Nitrocellulose filtration assays were used to measure binding activity of GBP preparation. Shock fluids were diluted to an absorbance of 1.70 at 280 nm, incubated at 30°C for 5 min with 28 nM ^{14}C-D-glucose (2.9 MBq/ml, DuPont Canada Inc.) and filtered through 0.45 µM Millipore nitrocellulose filters. Filters were washed twice with 1 ml of 50 mM Tris HCl (pH 7.3) before they were dried and counted by liquid scintillation spectrometry.

2.6 Purification of glucose binding protein

1. Gel-Filtration chromatography. GBP-containing shock fluid was subjected to gel filtration on a Sephadex G-200 column (3 x 65 cm) as previously described by (Stinson *et al.*,1977). Shock fluids (4 ml of a 10 mg protein/ml concentration) were loaded on columns which had previously been equilibrated with TM buffer (10 mM Tris-HCl, pH 7.5, containing 1 mM $MgCl_2$). Fractions (2 ml) were collected and 0.2 ml of every second fraction was assayed for glucose-binding activity as described above. Active fractions were pooled and concentrated 10 fold by Amicon PM-10 ultrafiltration and dialyzed overnight against TM buffer. Samples were run on SDS-PAGE and stained with Coomassie Brilliant Blue R-250 to verify their purity, as described below.

2. Electroelution. The electroelution of protein was performed using the method described by Hunkapiller *et al.*(1983). The protein band in the gel was cut out after running on SDS-PAGE. The gel slice was filled into glass tubes. The entire module was placed into the buffer chamber. The lower buffer chamber was filled with 600 ml of buffer and the upper buffer chamber was filled with 100 ml of buffer . Elution was done at 8-10 mA/glass tube. The eluted sample was verified by running on SDS-PAGE.

2.7 Sodium dodecyl sulphate-polyacrylamide gel and urea gel electrophoresis

Glucose binding proteins were analyzed by SDS-PAGE with the 11% gel system described by Lugtenberg *et al.* (1975) with a 5% stacking gel. Gels were stained using Coomassie Brilliant Blue R-250 as described by Maized (1966) omitting the fixing step. Samples (10 µl) were solubilized at 100°C for 10 min prior to loading in the presence of 0.12% (w/v) SDS and 5% (v/v) 2-mercaptoethanol.

Urea gel electrophoresis was performed as described by Mizushima *et al.* (1975) using 18% (w/v) acrylamide and 8 M urea in the running gel. The stacking gel, gel stain, and the solubilization of protein samples are same as SDS-PAGE.

2.8 Determination of molecular weight of GBP

SDS-polyacrylamide gel electrophoresis was used for this experiment. Bovine serum albumin (69,300), ovalbumin (45,800), carbonic anhydrase (28,700), β-lactoglobulin (18,200), and lysozyme (15,400) were used as molecular-weight standards. After running GBP samples, the gel was stained with Coomassie brilliant blue. The electrophoretic mobilities were plotted against the logarithm of the known polypeptide chain molecular weight. A smooth curve was obtained which was used for determining GBP molecular weight.

2.9 Genetic DNA isolation

Genomic DNA was prepared from *P. putida* by the procedure of Ausubel *et al.* (1989) with the following changes. Cultures (200 ml) were grown at 28°C for 3 hours and the final isolates were resuspended in 100 µl TE buffer. The bacterial cell wall was disrupted with SDS and proteinase K. CTAB/NaCl (10% hexadecyltrimethylammonium bromide, 0.7 M NaCl) (Sigma Chemical Co., St. Louis, Missouri) was then added and centrifuged to bind and remove cell wall debris, denatured protein, and polysaccharide while leaving nucleic acids in solution. Several alcohol washes were performed to remove any CTAB complexes before the resulting DNA was precipitated with isopropanol and resuspended in TE buffer (pH 7.5).

2.10 DNA digestion and electrophoresis

Restriction enzymes used were from Pharmacia (Pharmacia Canada Inc., Dorval, Quebec, Canada) and digestions were performed according to the manufacturers' instructions. The reaction volume was 20 µl which consisted of 1 µl (10 units) enzyme, 2 µl 10X buffer, 5 µg DNA, and

water to bring the volume to 20 µl. The reactions were incubated for 1 to 1.5 hrs at 37°C when the recombinant DNA was being digested. The reactions were stopped with 4 µl stop buffer (0.25% bromophenol blue; 0.25% xylene cyanol FF; 15% Ficoll [Type 400, Pharmacia] in water) and then loaded onto agarose gel. Electrophoresis was carried out in TBE buffer (0.5 mM Tris, 0.5 mM H_3BO_3, 0.5 mM EDTA) using 0.7% (w/v) agarose gels which contain appropriate amount of ethidium bromide so that the DNA bands could be visualized under ultraviolet light. The BRL 1 Kb DNA ladder (containing fragments of DNA which ran molecular weights ranging from 12.2 to 0.30 kb) was used as a standard for determining fragment sizes.

2.11 Southern blotting and hybridization

Southern blots were prepared using Zeta-probe Blotting membranes (Bio-Rad Laboratories, Richmond, California) according to the manufacturer's instructions for alkaline blotting. Pre-hybridization was done for 30 min at 42°C with shaking in hybridization solution [5X SSC containing 0.75 M Na_3citrate, 20 mM sodium phosphate (pH 7.0), 0.5% SDS, 5X Denhardt's solution, 100 µg/ml denatured herring sperm DNA] and hybridization was carried out for 16-18 hours at 42°C with shaking in the same solution with radiolabelled probe added. The probes (15 pmol) were end-labelled with [γ-^{32}P]ATP (370 MBq/ml, Dupont Canada Inc., Markham, Ontario, Canada). The procedure used was that of Ausubel *et al.* (1989) using T_4 polynucleotide kinase (Pharmacia Canada Inc., Dorval, Quebec, Canada). After hybridization, filters were washed three times at 42°C for 15 min in 5X SSC, and finally 1X SSC. An additional 15 min wash was carried out for the blot probed with probe A and probe B (oligonucleotides derived from the N-terminal amino acid sequence og P. aeruginosa GBP) at 45°C in 1XSSC. Autoradiography was done for 20-24 hours at -70°C using Kodak diagnostic film, X-OMAT XRP-1 (Eastman Kodak Company, Rochester, New York, USA).

2.12 Preparation of partially digested DNA

To obtain the DNA fragment whose size is appropriate for insertion into a plasmid vector, partial digestion of genomic DNA was required. 20 µl (120 µg) genomic DNA from *P. putida* was digested with the restriction enzyme *Sal*I for 1 hour. This digestion was incubated on ice for 5 min and 100 µl 7.5 M ammonium acetate and 675 µl of 100% ethenol were added. After incubation for 30 min at -20°C, the digested DNA was centrifuged for 15 min at 4°C. The supernatant was removed and the pellet was dried under vacuum for 15 min. The pellet was resuspended in 100 µl STE buffer (10 mM Tris-HCl, 1 mM EDTA, and 150 mM NaCl, pH 7.0) and stored at -20°C. Sucrose solutions were made in a buffer containing 1 M NaCl, 20 mM Tris.HCl (pH 7.5), and 5 mM EDTA. Ten ml 10-40% sucrose density gradient was prepared in a Beckman polyallomer tube (BECKMAN Inc., Missisauga, Ontario, Canada). Digested DNA was heated at 65°C for 5 min to dissociate aggregates, cooled to 20°C for 10 min, and loaded on the gradient. The loaded gradient was centrifuged at 25,000 rpm for 24 hours in SW-41 rotor. After centrifuging, the bottom of the centrifuge tube was punctured and 200 µl fractions were collected. 20 µl of every third fraction were taken and mixed with 10 µl of H_2O and 5 µl of gel-loading dye (0.25% bromophenol blue; 0.25% xyleme cyanol FF; 15% Ficoll [Type 400, Pharmacia] in water). Analysis was done by electrophoresis through a 0.7% agarose gel.

2.13 Transformation of *E. coli* NM522 and selection of recombinants

The gradient fractions containing 4-5 kb DNA were purified with ethanol and were ligated with plasmid pTZ, pM13, or pPZ375 digested with appropriate enzymes. The ligation was incubated for 14-16 hours at 14°C. *E. coli* NM522 cells were made competent using a $CaCl_2$ treatment. Competent cells were then transformed with ligated DNA by a heat shock technique (Ausubel, *et al.*, 1989). The selection of recombinant is based on the marker gene, β-galactosidase

gene (lacZ), in the vector plasmids. The lacZ gene permits detection of recombinant by blue/white selection of bacterial colonies on plates containing both ampicillin and 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal)/isopropylthio-β-D-galactoside (IPTG). Colonies containing a plasmid with an intact lacZ gene are blue, white colonies containing a recombinant plasmid with an interrupted lacZ gene are white.

2.14 Screening for the GBP gene

Colony blots of recombinant *E. coli* NM522 were screened with oligonucleotide probes derived from the N-terminal amino acid sequence of GBP of *P. aeruginosa* (Sly *et al.*, 1990). The two probes were synthesized by the Regional DNA Synthesis Laboratory, University of Calgary, Calgary, Alberta. Two oligonucleotide probes were as follows:

probe A: dGGC GA[AG] GT[CG] GA[AG] GT[CG] CT[CG] CAC TCC

probe B: dACC [AT][GC]C GC[TCG] CAC GA[AG] AAG GAC

15 pmol of the oligonucleotides were 5'end labelled with [γ-^{32}P] ATP using T4 polynucleotide kinase (Ausubel *et al.*, 1989). Lysis of bacteria on filters was done in 0.5 M NaOH/1.5 M NaCl (Woods, 1984) and hybridizations were performed at 55°C.

2.15 DNA sequencing

DNA sequencing was determined by the dideoxy-chain method of Sanger *et al.* (1977) using the ^{T7}Sequenase™ Kit (Pharmacia, Uppsala, Sweden) or the Sequenase™ version 2.0 DNA sequencing Kit (USB, Cleveland, Ohio). Appropriate restriction fragments were subcloned into pM13. Compressions were resolved by substituting 7-deaza dGTP for dGTP (USB, Cleveland, Ohio). DNA templates for sequencing were prepared from the double-stranded plasmids as described by Hattori and Sakaki (1986). We could not consistently sequence some regions from

double-stranded preparetions, therefore, single stranded preparations of DNA were also used (USB, Clevland, Ohio). Nucleotide sequence analysis was performed with the PC/Gene program and Seqaid program.

2.16 Western immunoblotting

Following SDS-PAGE, proteins were transferred to nitrocellulose using a Bio-Rad Trans-Blot Cell (Bio-Rad Laboratories, Mississauga, Ontario, Canada) and assayed for antibody binding as described by Burnette (1981) and Towbin *et al.* (1979). Dilution of 1/200 (50 µl of rabbit polyclonal antibody to 20 ml of 1% skim milk/PBS) was used.

2.17 Antibody production

Rabbit anti-GBP antibodies were produced in female New Zealand White rabbits by subcutaneous injection of *P. putida* GBP (4 mg) in 2 ml phosphate-buffered saline (PBS) mixed with 2 ml Freund's Complete Adjuvant (Difco Laboratories Inc., Detroit, Michgan USA) followed by boost injections after four to six weeks with 1 mg GBP in 1 ml PBS mixed with 1 ml Freund's Incomplete Adjuvant (Ausubel *et al.*, 1989). GBP used in this procedure was isolated by electroelution.

2.18 Recombinant plasmid expression

The recombinant plasmids containing the putative GBP (pGR7) structural gene was examined for expression in GBP deficient mutant strain PFB362a derived from *P. aeruginosa* and glucose transport negative mutant strain MAP-1 derived from *P. putida.* The genomic DNA fragment containing GBP gene was ligated in a multicopy plasmid, pPZ375 which is a broad-host-range vector, between the restriction sites *Xba*I and *Kpn*I. The transformation of recombinant DNA to the above strains was performed by the method which is specific for pseudomonas

transformation and uses $MgCl_2$ instead of $CaCl_2$ for making competent cells (Olsen *et al.*, 1982). The expressed protein was visualized in SDS PAGE and western blots of protein samples prepared by osmotic shock fluid procedure. C^{14}-glucose uptake assays were performed by the procedure described previously.

Chapter 3 Results

3.1 Time course for induced glucose uptake

P. putida was induced for glucose transport by growth on glucose as previously described (Vicente *et al.*, 1973). Cells were resuspended in BM2 (without glucose), after washing twice with BM2, to an absorbance of 0.20 at 600 nm. Cells were prewarmed at 30°C for 10 min prior to initiation of the glucose incorporation assays. 120 µls of [^{14}C]-glucose (2.9 mCi/mmol) were added to 40 mls of cell solution to yield final concentration at 0.1 mM. Triplicate aliquots (1 ml) were removed at 1, 2, 4, 6, 10, 16 minutes and filtered onto 0.45 µM filters. Filters were washed immediately with 4 ml of BM2 containing 20 mM glucose. As is demonstrated by Fig. 2, the uptake of [^{14}C] glucose by induced cells was rapid, extensive, and saturable. This graph is a representative experiment of all uptake experiments hence error bars are not indicated.

3.2 Effect of various substrates on glucose uptake by whole cells

The effect of various substrates on glucose uptake by whole cells was examined. The reaction mixtures consisted of 0.1 mg (dry weight) induced cells resuspended in 0.8 ml of BM2. After adding 0.1 ml of 100 µM or 1mM testing substrates (the final concentration is 10 µM or 100 µM), 0.1 ml of 10µM [^{14}C]-glucose (2.9 mCi/mmol) (the final concentration is 1 µM) was added to the reaction mixtures immediately. After the reaction mixtures were incubated for 30 s (initial rate) and 5 min (steady-state phase) at room temperature, the reaction was terminated by dilution of 100 µl of the cellular suspension with 20 volumes of BM2, pH7. Table 2 shows that [^{14}C]-glucose uptake was strongly inhibited by glucose, gluconate, galactose, and fructose. The inhibition by these three sugars is more than 50%. The gucose uptake was reduced more than 30% by L-arabinose, D-glucosamine, lactose, and maltose. Pyruvate, mannose, succinate, and

Figure 2. Uptake of [^{14}C] glucose by *P. putida* when grown on 20 mM glucose. External glucose concentration was 0.1 mM. Uptake at 22°C. Each point is the mean of triplicate determinations. This graph is a representative of three independent experiments.

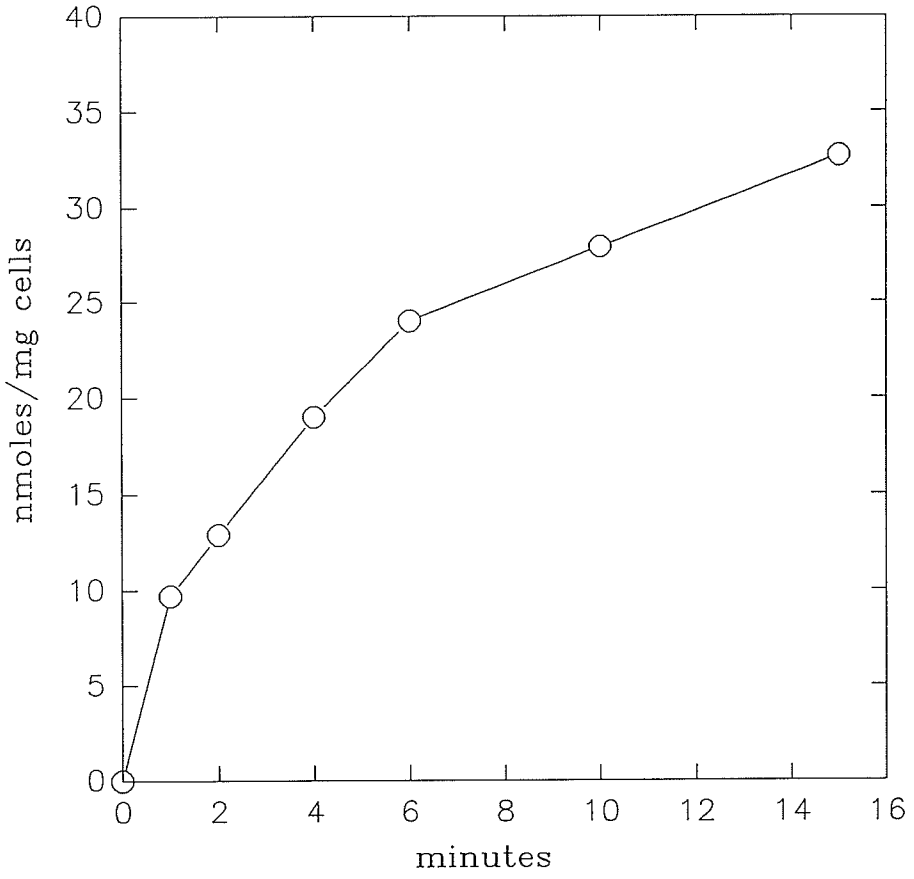

40
35
30
25
20
15
10
5
0
nmoles/mg cells
0
2
4
6
8
10
12
14
16
minutes

Table 2. Effect of various substrates on the uptake of glucose by *P. putida* [a, b]

Substrates added		Inhibition (%) [c] 30 s	Inhibition (%) 5 min
None		0	0
Glucose	10 μM	81 ± 1	76 ± 2
	100 μM	98 ± 2	99 ± 3
Gluconate	10 μM	63 ± 2	45 ± 3
	100 μM	57 ± 3	72 ± 2
Galactose	10 μM	35 ± 2	64 ± 2
	100 μM	43 ± 3	68 ± 3
Fructose	10 μM	66 ± 2	54 ± 3
	100 μM	80 ± 2	59 ± 1
Pyruvate	10 μM	-	13 ± 2
	100 μM	3 ± 1	16 ± 2
L-Arabinose	10 μM	37 ± 2	22 ± 2
	100 μM	46 ± 2	43 ± 2
D-Glucosamine	10 μM	24 ± 2	31 ± 2
	100 μM	30 ± 2	35 ± 2
Mannose	10 μM	15 ± 2	17 ± 2
	100 μM	0	11 ± 2
Lactose	10 μM	31 ± 1	38 ± 3
	100 μM	42 ± 3	53 ± 2
Succinate	10 μM	11 ± 2	-[d]
	100 μM	-	-
Maltose	10 μM	35 ± 3	31 ± 2
	100 μM	21 ± 3	36 ± 3
Mannitol	10 μM	-	-
	100 μM	-	28 ± 2
Arginine	10 μM	17 ± 2	21 ± 3
	100 μM	0	27 ± 3

[a] 1 μM [^{14}C] glucose was used in this experiment.
[b] Standard deviations are calculated for n=3.
[c] The percent of inhibition of binding activity by the subtrates.
[d] Not determined.

mannitol, on the other hand, did not significantly inhibit the glucose uptake.

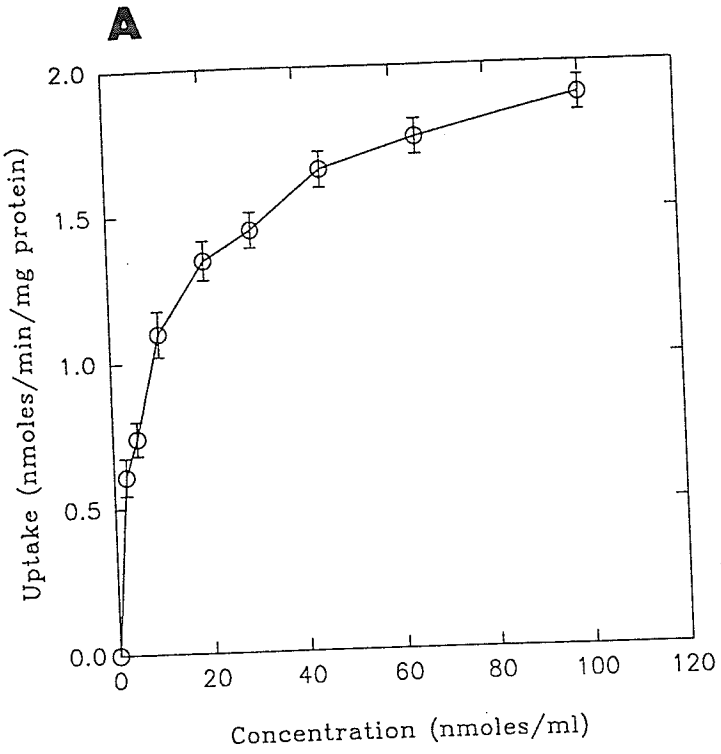
3.3 Kinetics of uptake of glucose

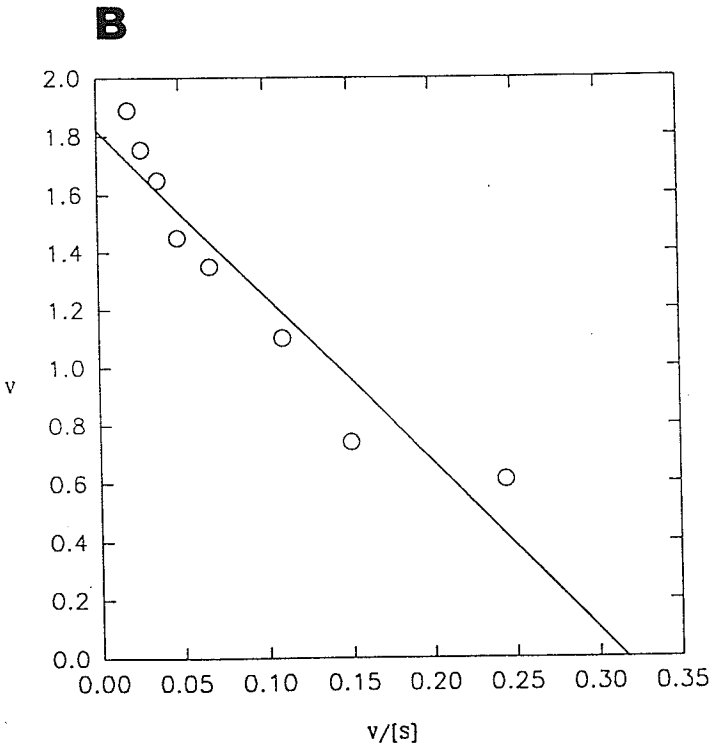
The uptake of glucose by whole cells of *P. putida* exhibited typical Michaelis-Menton kinetics (Fig. 3. and Fig. 4). In Figure 3 and Figure 4, there is a clear indicaton of the low affinity pathway (higher Vmax) in addition to the high affinity pathway. Lines deviating at high [S] (more high [S] in Figure 3 than in Figure 4) suggests the inducibility of the high affinity system. Plotting glucose uptake versus glucose concentration results in a Km and Vmax for basal uptake of 5.5 μM and 1.8 nmoles per min per mg dry weight, respectively, while for induced uptake these values are 5.5 μM and 17.5 nmoles/min/mg dry cell weight. These results are in close agreement with Km values for glucose transport by high affinity system reported for several strains of *P. aeruginosa* (Guymon and Eagon, 1974, Midgley and Dawes, 1973), but are different from the result reported by Vincente *et al.* (1975) in which the Km for glucose uptake by low affinity system of *P. putida* is 6 mM.

3.4 Effect of temperature on uptake

The purpose of this experiment was to determine whether temperature could affect the glucose uptake system. With cells grown at 28°C, the Vmax for glucose uptake was 17.5 nmol/min/mg when the uptake was performed at room temperature, 22°C (during winter). When the room temperature was changed to 28°C (during summer), the Vmax for glucose uptake was increased to 72.5 nmol/min/mg (Fig. 5). The Km for [^{14}C]-glucose uptake did not change significantly. This result is similar to the result reported for one strain of *P. fluorescens* by Lynch and Franklin (1978).

Figure 3. Kinetics of glucose transport by the uninduced cells of *P. putida* grown on LB medium as a function of exogenous glucose concentration. Incubation time, 20 s. Uptake was done at 22°C. (A) A total of three separate experiments were used to obtain the average values with the error bars indicating the calculated standard deviations. (B) Eadie-Hofstee plot indicating the determination of Vmax and Km values for glucose uptake. Data are representative of three experiments same as in (A). (C) is a Lineweaver-Burk plot of the data.


A
2.0
1.5
1.0
0.5
0.0
Uptake (nmoles/min/mg protein)
0
20
40
60
80
100
120
Concentration (nmoles/ml)


B
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
v
0.00
0.05
0.10
0.15
0.20
0.25
0.30
0.35
v/[s]

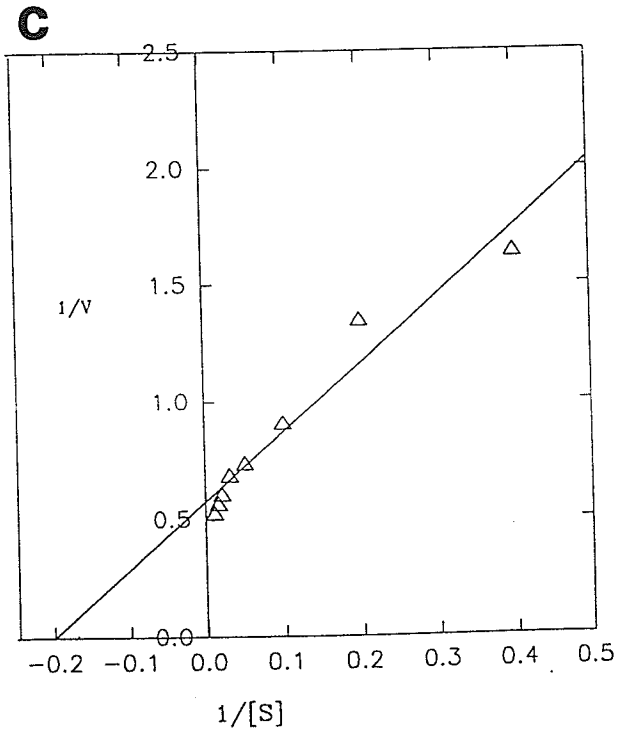
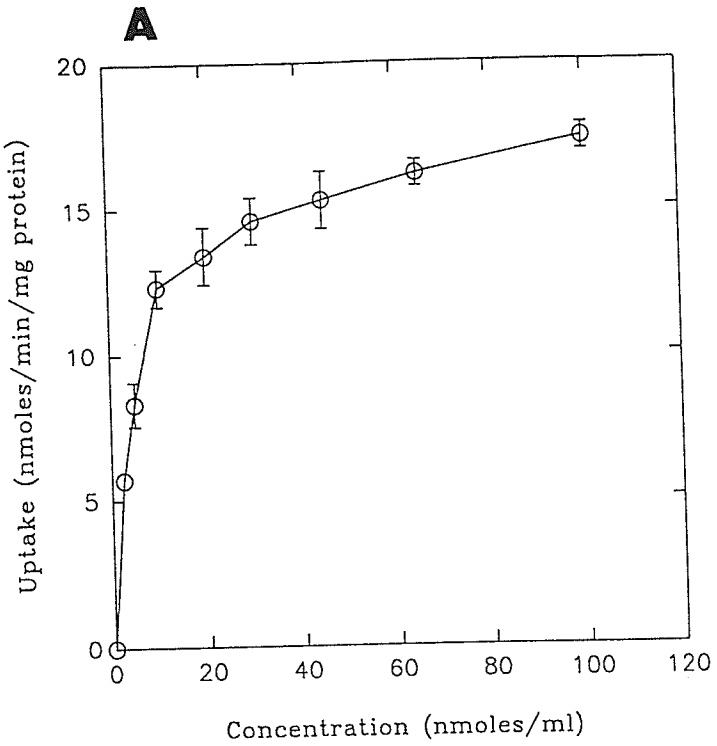
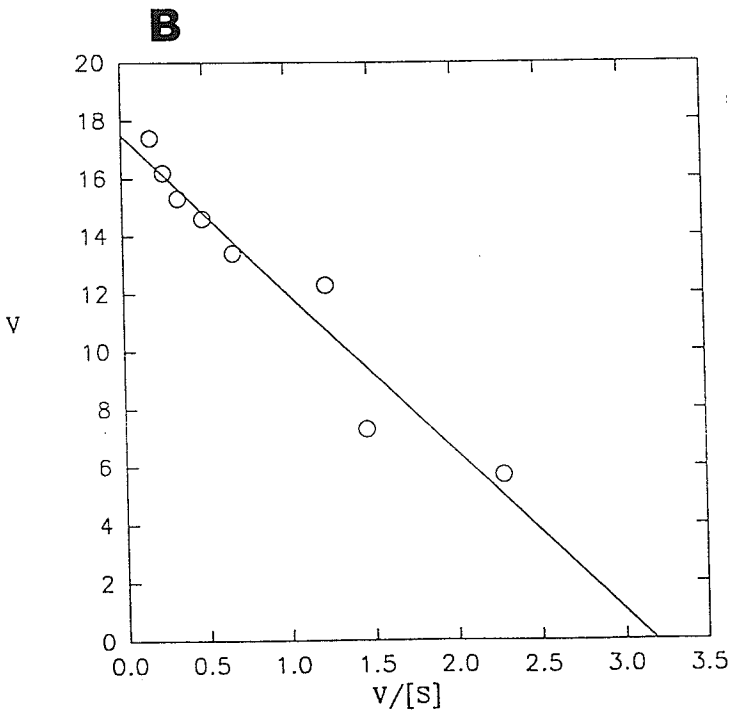

C
2.5
2.0
1.5
1.0
0.5
0.0
1/v
−0.2
−0.1
0.0
0.1
0.2
0.3
0.4
0.5
1/[S]

Figure 4. Kinetics of glucose transport by induced cells of *P. putida* as a function of exogenous glucose concentration. Incubation time, 20 s. Uptake at 22°C. (A) A total of three separate experiments were used to obtain the average values with the error bars indicating the calculated standard deviations. (B) Eadie-Hofstee plot indicating the determination of Vmax and Km values for glucose uptake. Data are representative of three experiments, same as (A). (C) is a Lineweaver-Burk plot of the data.


A
Uptake (nmoles/min/mg protein)
Concentration (nmoles/ml)
0
5
10
15
20
0
20
40
60
80
100
120


B
V
V/[S]
0
2
4
6
8
10
12
14
16
18
20
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5

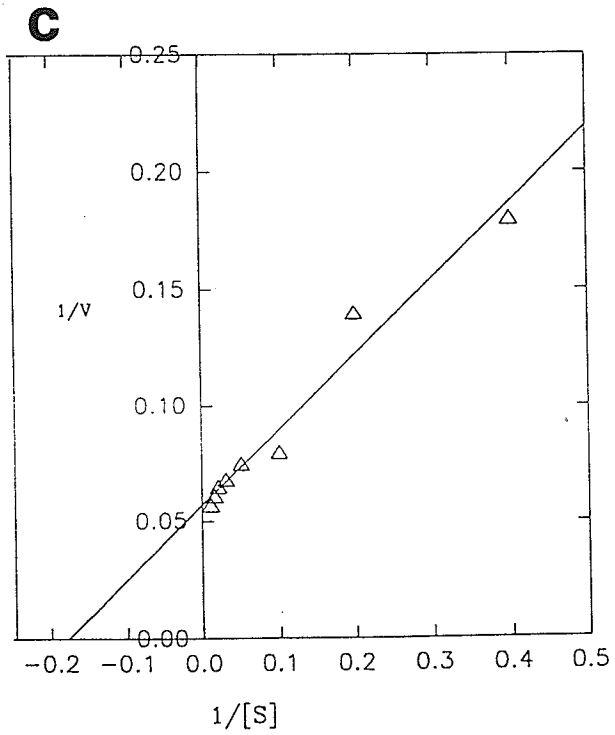
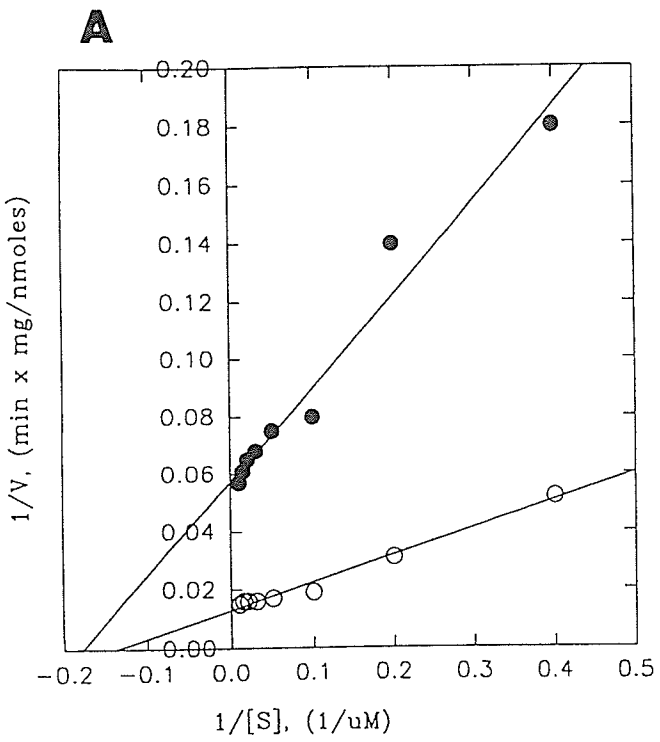
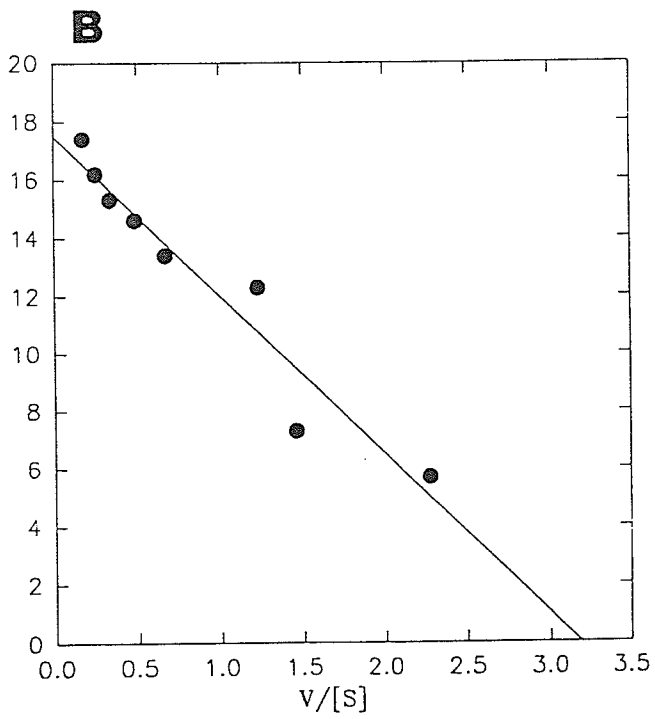
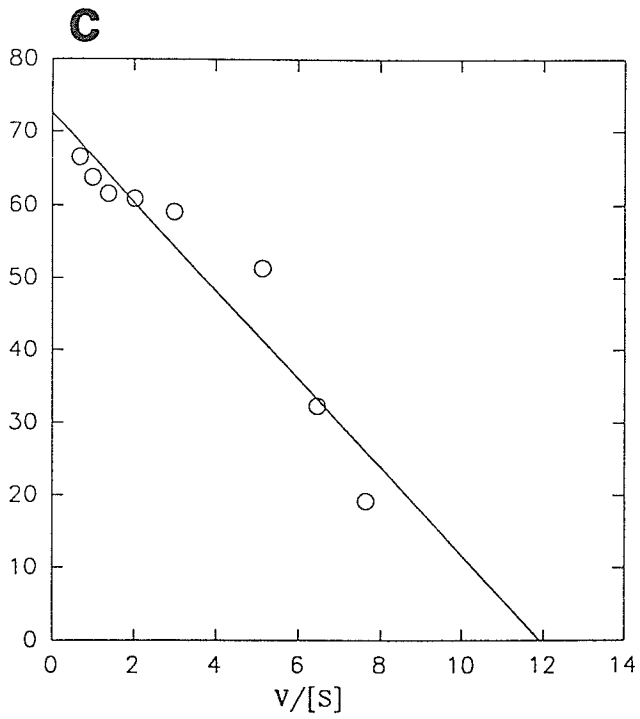

C
1/V
1/[S]
0.00
0.05
0.10
0.15
0.20
0.25
−0.2
−0.1
0.0
0.1
0.2
0.3
0.4
0.5

Figure 5. Effect of temperature on glucose uptake by *P. putida*. (A) is double-reciprocal plots of induced glucose uptake at 22°C (•) and 28°C (○). (B) and (C) are Eadie-Hofstee plots indicating the determination of Vmax and Km values for glucose uptake at 22°C (•) and 28°C (○). Data are the means of three independent determinations.


A
0.20
0.18
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
1/V, (min x mg/nmoles)
−0.2 −0.1 0.0 0.1 0.2 0.3 0.4 0.5
1/[S], (1/uM)


B
20
18
16
14
12
10
8
6
4
2
0
0.0 0.5 1.0 1.5 2.0 2.5 3.0 3.5
V/[S]

V


C
80
70
60
50
40
30
20
10
0
0 2 4 6 8 10 12 14
V/[S]

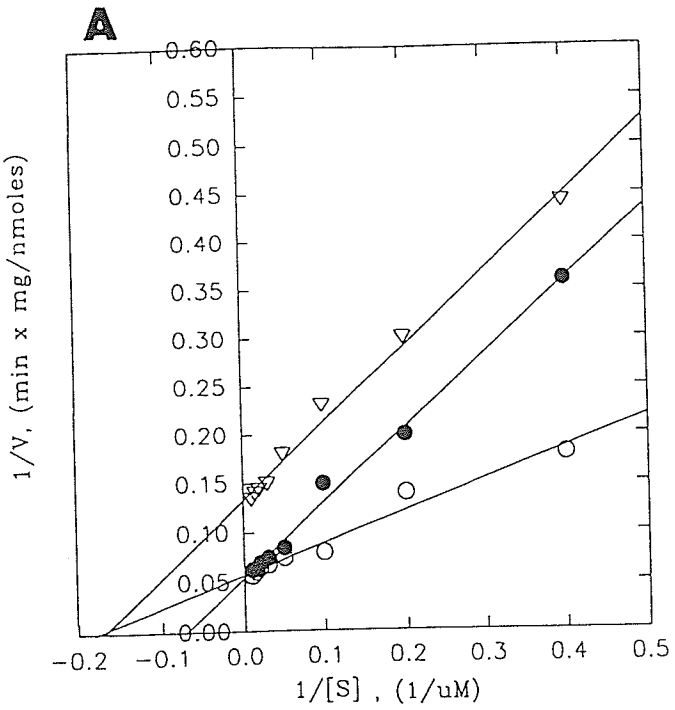
3.5 Uptake inhibition

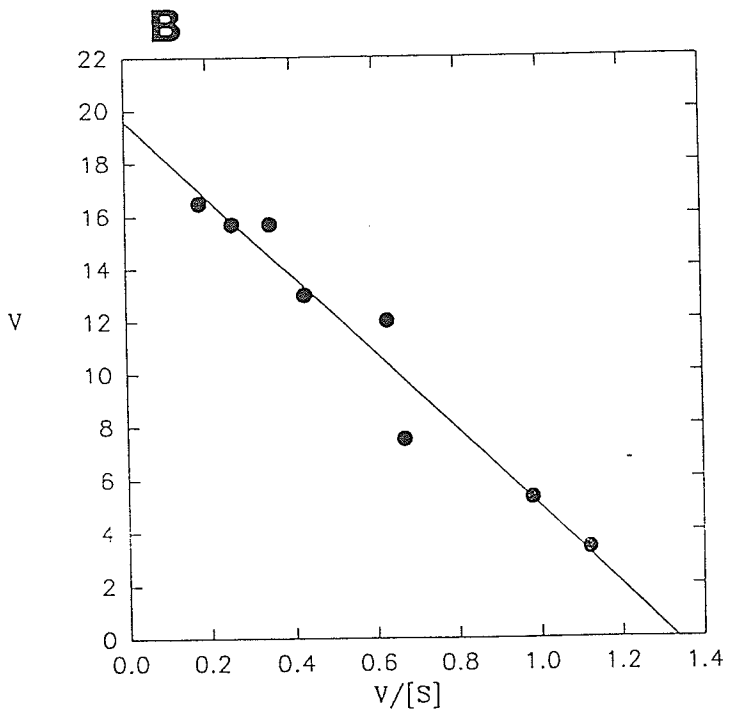
Figure 6 and Figure 7 show the plots of 1/v against 1/[S] obtained from various inhibition experiments. When an inhibitor galactose (Fig.6) or gluconate (Fig.7) is present, the Km remains unchanged but Vmax is decreased. Therefore, galactose and gluconate are noncompetitive inhibitors for glucose in its induced uptake. The Km is not changed in the presence of these sugars. But the Vmax for glucose uptake changes in the presence of galactose from 17.5 to 8.3 nmoles/min/mg (22°C) and in the presence of gluconate from 72.5 to 26.0 nmoles/min/mg (28°C). We also found that fructose is a competitive inhibitor of induced glucose uptake (Fig. 6). In the presence of fructose, the Km is increased from 5.5 to 14.7 μM, but Vmax is not changed.

3.6 Fructose and glycerol uptake by *Pseudomonas putida*

To understand if fructose transport system or glycerol transport system of *P. putida* is dependent on glucose transport system or not, [^{14}C] fructose or [^{14}C] glycerol uptake was examined. [^{14}C] fructose (10 μM and 30 μM) uptake by *P. putida* grown on 20 mM glucose was not apparent. However, when the cells were grown on 20 mM fructose and uptake of [^{14}C] fructose (10 μM) was followed, the uptake of fructose was significant (Fig.8). This result is consistent with the report by Vicente *et al.* (1975) indicating an independent fructose uptake system in *P. putida*. Glucose is unable to induce this system. Eagon and Phibbs (1971) also showed the same result in *P. aeruginosa*. Figure 9 shows that glycerol uptake by *P. putida* was induced by growth on glycerol, but not on glucose. This result is similar to the result reported by Tsay *et al.* (1971). It was demonstrated that in *P. aeruginosa*. The transport of glycerol was shown to be genetically controlled and to be dependent on induction by glycerol.

Figure 6. Inhibition of glucose uptake by fructose and galactose by *P. putida*. (A) is a Lineweaver-Burk plots of induced glucose uptake (o), inhibition of glucose uptake by fructose (•), and inhibition of glucose uptake by galactose (▽) at 22°C. (B) and (C) are Eadie-Hofstee plots indicating the determination of Vmax and km values for inhibition of glucose uptake by fructose (•) and galactose (▽). [^{14}C]-glucose concentration was 10 µM. The concentration of inhibitors was 10mM. Each point is the mean of triplicate determinations and is a representative of three independent experiments.


A
1/V, (min x mg/nmoles)
0.60
0.55
0.50
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
−0.2
−0.1
0.0
0.1
0.2
0.3
0.4
0.5
1/[S] , (1/uM)


B
V
22
20
18
16
14
12
10
8
6
4
2
0
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
V/[S]

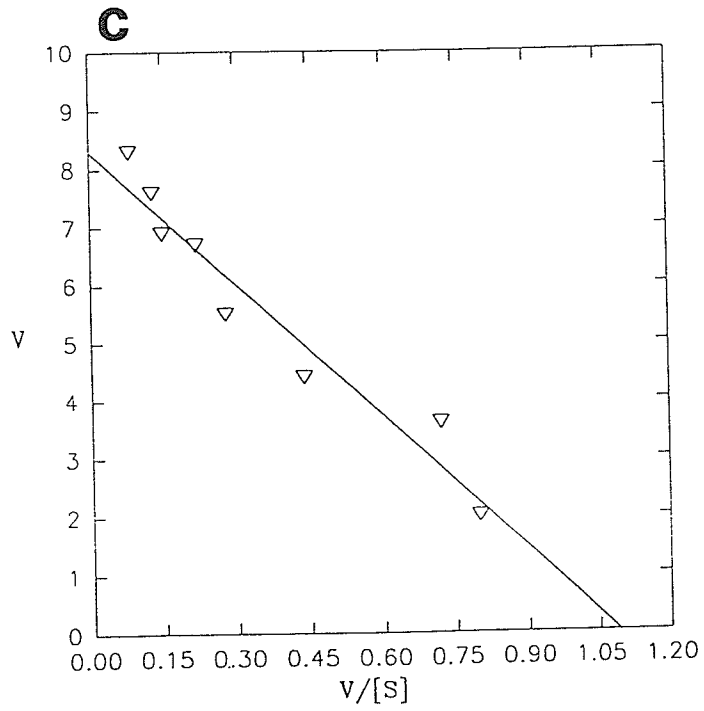
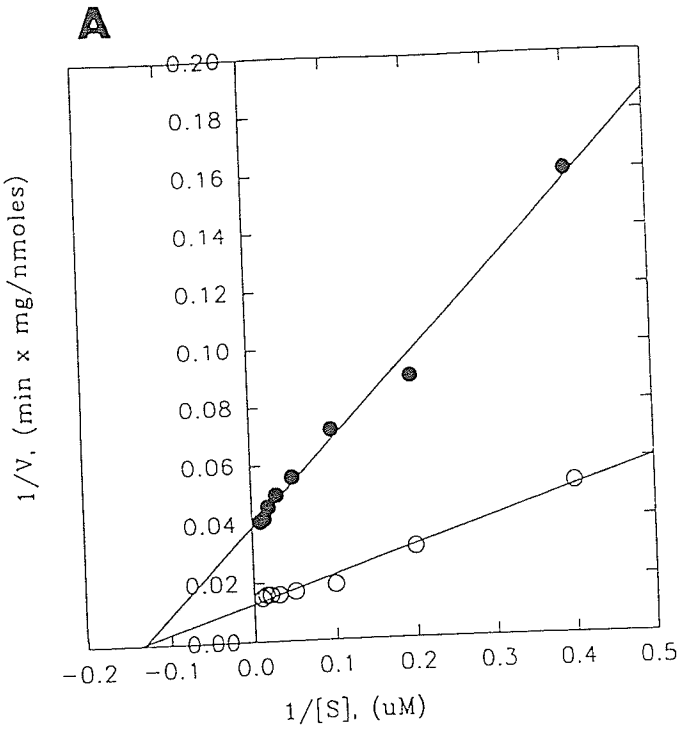

C
V
10
9
8
7
6
5
4
3
2
1
0
0.00
0.15
0.30
0.45
0.60
0.75
0.90
1.05
1.20
V/[S]

Figure 7. Inhibition of glucose uptake by gluconate by *P. putida*. (A) is a Lineweaver-Burk plots of induced glucose uptake (o) and inhibition of glucose uptake by gluconate (•) at 28°C. (B) is a Eadie-Hofstee plot indicating the determination of Vmax and Km values for inhibition of glucose uptake by gluconate. [^{14}C]-glucose concentration was 10 µM. Gluconate concentration was 10 mM. Each point is the mean of triplicate determinations and is representative of three independent experiments.


A
0.20
0.18
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
1/V, (min x mg/nmoles)
−0.2
−0.1
0.0
0.1
0.2
0.3
0.4
0.5
1/[S], (uM)

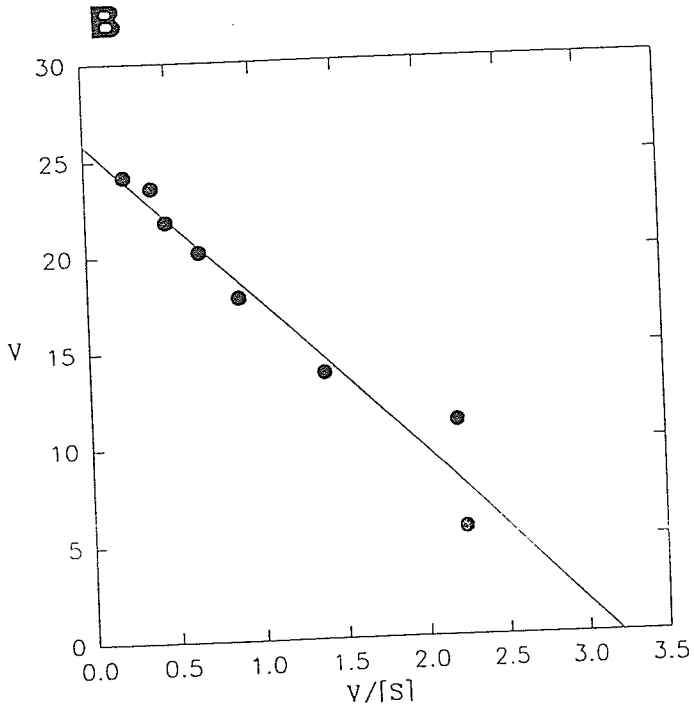

B
30
25
20
V
15
10
5
0
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
V/[S]

Figure 8. Uptake of [^{14}C] fructose by *P. putida* after growth on 20 mM glucose (•) and 20 mM fructose (o). External [^{14}C] fructose concentration was 10 µM. Uptake at 22°C. Each point is the mean of triplicate determinations and is a representative of three independent experiments.

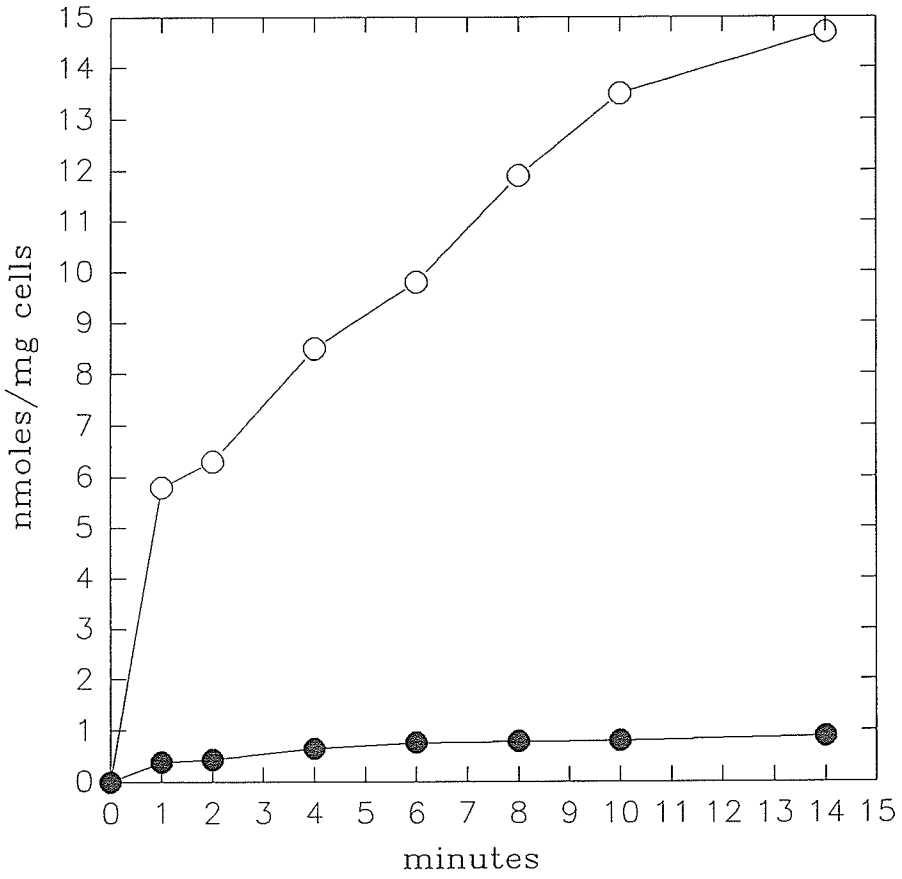
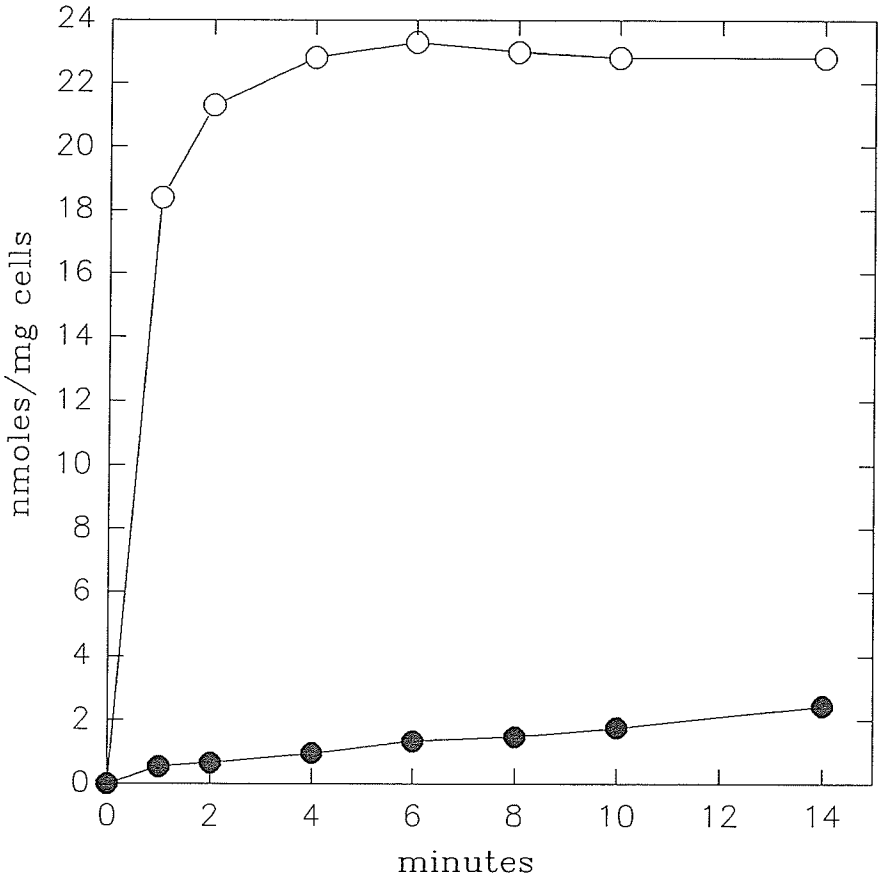

nmoles/mg cells
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
minutes

Figure 9. Uptake of [^{14}C] glycerol by *P. putida* after growth on 20mM glucose (●) and 20 mM glycerol (○). External [^{14}C] glycerol concentration was 10 µM. Uptake at 22°C. Each point is the mean of triplicate determinations and is a representative of three independent experiments.


24
22
20
18
16
14
12
10
8
6
4
2
0
nmoles/mg cells
0
2
4
6
8
10
12
14
minutes

3.7 Purification of glucose-binding protein

To determine if the uptake of glucose by *P. putida* was due in part to a periplasmic glucose binding protein, as was the case with *P. aeruginosa*, I attempted to purify this protein. Osmotic shock fluid was obtained from induced *P. putida* cells as described in Materials and Methods. The dialyzed osmotic shock fluid (4 ml, 10 mg protein/ml) was subjected to gel filtration on a Sephadex G-200 column (3 by 65 cm) that had previously been equilibrated with TM buffer. Fractions (2 ml) were collected and 0.2 ml of every second fraction was assayed for glucose-binding activity as described in Materials and Methods. The samples from every second fraction were also run on SDS-PAGE. From the SDS-PAGE, every sample had a 43,700 Da band (putative GBP). Fractions from 34 to 42 contained the most GBP. The highest glucose binding activity also existed in fractions 32-42. Therefore fractions 32 to 42 were pooled. A profile of the chromatography is shown in Figure 10. GBP was also isolated by SDS-PAGE electroelution. Both gel filtrated GBP and electroeluted GBP were concentrated by ultrafiltration through a PM10 filter, and dialyzed overnight against TM buffer.

After the GBP samples were run on SDS-PAGE and urea gel, it was found that GBP was one band on SDS-PAGE but separated into three bands on urea gel (Fig.11). Therefore, our GBP preparation was only partially purified.

3.8 Molecular weight determination of glucose-binding protein

The molecular weight of the glucose-binding protein determined by SDS-polyacrylamide gel electrophoresis was 43,700 (Fig.12). This is similar to the *P. aeruginosa* (Stinson *et al.*, 1977) and a common MW of many binding proteins (Tam and Saier, 1993).

Figure 10. Elution profile of the glucose binding protein from a Sephadex G-200 column. Shock fluids (4 ml, 40 mg of protein) were applied to a Sephadex G-200 column (3.0 x 65 cm) and eluted with TM buffer (10 mM Tris-HCl, pH 7.5, containing 1 mM $MgCl_2$). 0.2 ml aliquots of each fraction (2ml) were assayed with [^{14}C] glucose as substrate. Active fractions were pooled and dialyzed. Symbols: -•- absorbance at 280 nm; -▲- ^{14}C -glucose binding activity.

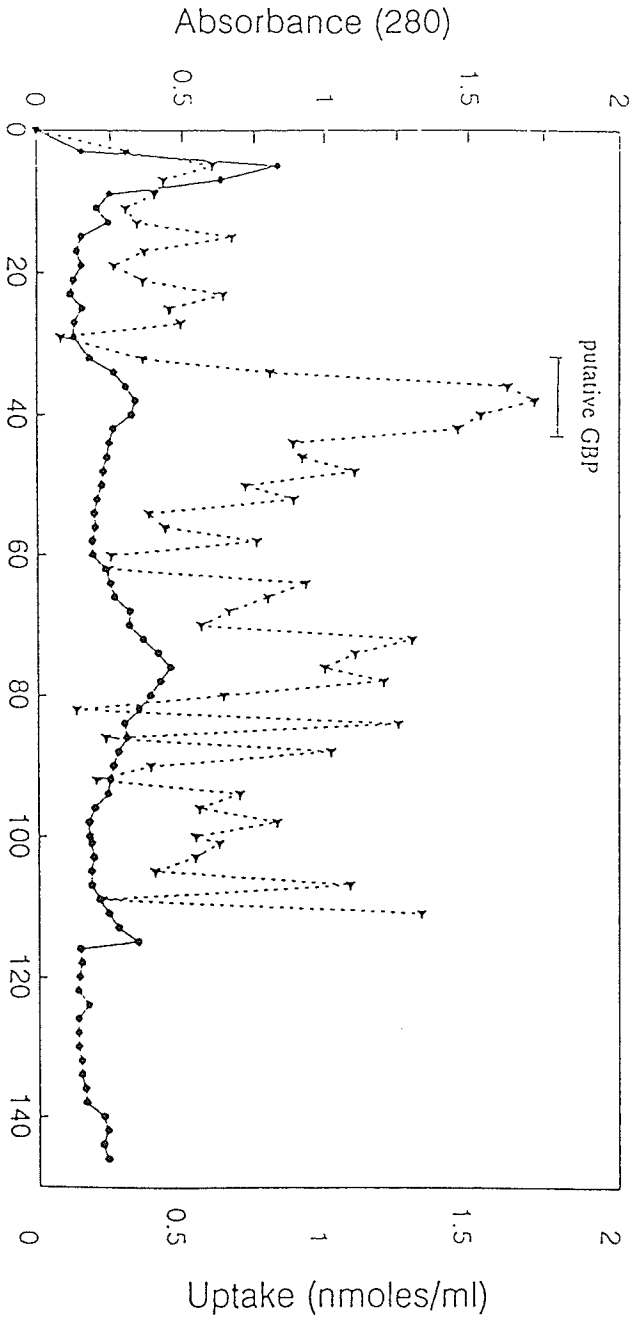

Absorbance (280)
0
0.5
1
1.5
2
0
20
40
60
80
100
120
140
putative GBP
Uptake (nmoles/ml)

Figure 11. SDS-PAGE and urea gel of shock fluids and partially purified GBP from *P. putida*. Panel A is the Coomassie Brilliant Blue stained SDS-electrophotogram. Lane 1) shock fluid from *P. putida*; lane 2) electro-eluted GBP. Panel B is the Coomassie Brilliant Blue stained partially purified GBP urea electrophotogram. All samples were solubilized at 95°C prior to electrophoresis. Molecular weights are as indicated $x10^3$ daltons.

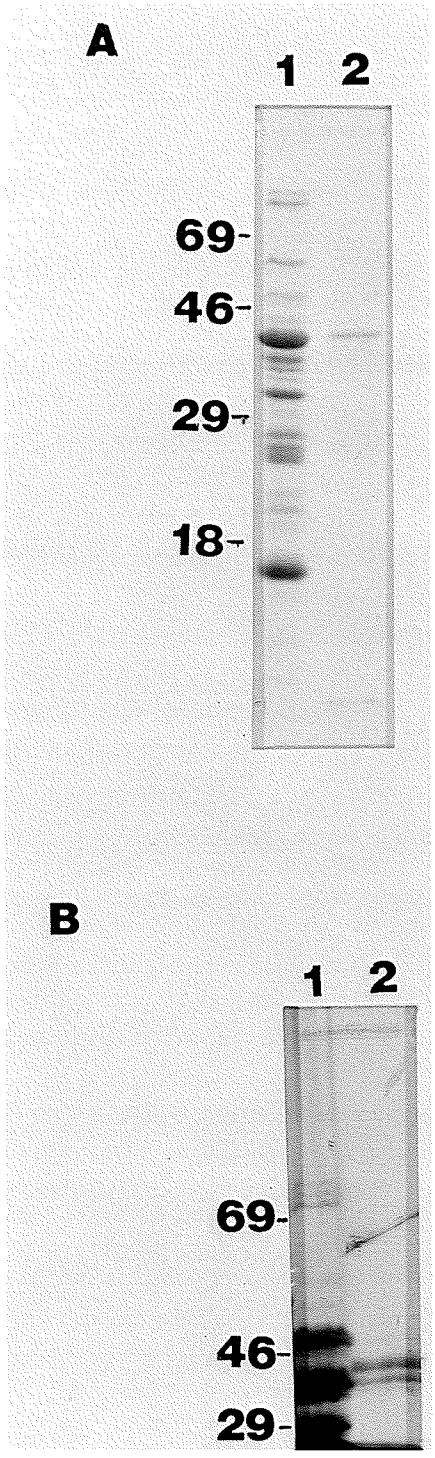
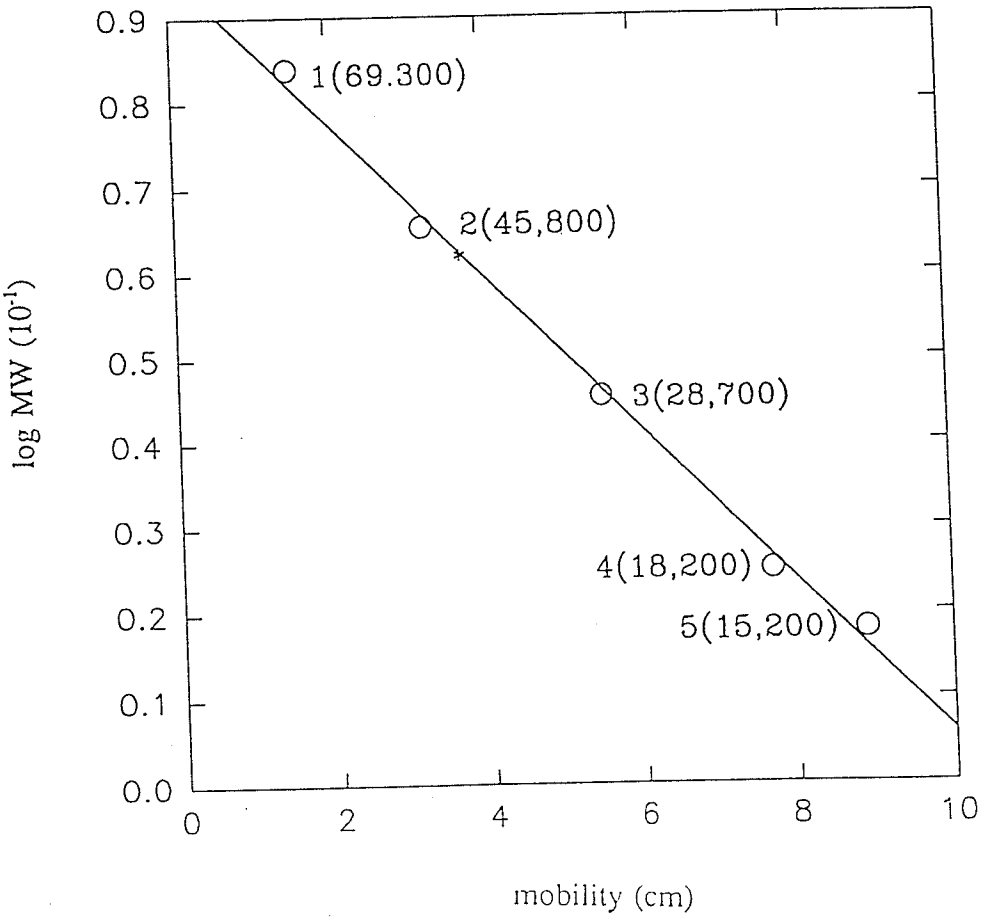

A
1
2
69
46
29
18
B
1
2
69
46
29

Figure 12. GBP molecular-weight determination. Mobility signifies protein migration during SDS-polyacrylamide gel electrophoresis. 1, Bovine serum albumin; 2, Ovalbumin; 3, Carbonic anhydrase; 4, β-lactoglobulin; 5, Lysozyme; *, GBP.


0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
log MW (10^{-1})
1(69.300)
2(45,800)
3(28,700)
4(18,200)
5(15,200)
0
2
4
6
8
10
mobility (cm)

3.9 Effect of various substrates on glucose binding activity of the osmotic shock fluid and GBP

The *P. putida* glucose binding assays were performed as described previously only in these experiments glucose-induced osmotic shock fluids and purified GBP were used. The results for glucose binding of shock fluid and GBP are shown in the Table 3 and 4, respectively. Gluconate, galactose, and fructose show more than 50% inhibition of ^{14}C-glucose binding. L-arabinose, glucosamine, and lactose also reduced ^{14}C-glucose binding by approximately 30%. Compared to whole cell glucose uptake (Table 2), the results from three different levels of purification of GBP (whole cells, osmotic shock fluid, and purified protein) are very similar, indicatively the dominant role GBP plays in this uptake.

3.10 Isolation of the GBP gene of *P. putida*

One of the directions of the laboratory is to study the structure and function of the protein components of the *P. aeruginosa* and *P. putida* high affinity glucose transport system. In order to further examine the *P. putida* GBP, the structural gene was cloned and sequenced. A *P. putida* genomic library was generated in *E. coli* NM522. Screening of the *P. putida* genomic library in *E. coli* NM522 was conducted using oligonucleotides probe A and probe B derived from the N-terminal sequence of purified GBP of *P.aeruginosa* (Sly *et.al.*, 1990). Figure 13 shows the results of *P. putida* genomic DNA hybridized with probe A and probe B. An approximately 4.5 kb DNA fraction from *Sal*I partially digested genomic DNA was selected for ligation with plasmid pTZ19R which was digested by the same enzyme. The ligation mixture was transformed to *E. coli* NM522. Eight hundred colonies were screened. Three positive colonies which contained

Table 3. Effect of various substrates on glucose binding activity of the shock fluid [a, b]

Substrates added		Inhibition (%) 30 s	Inhibition (%) 5 min
None		0	0
Glucose	10 μM	83 ± 3	87 ± 3
	100 μM	79 ± 4	89 ± 2
Gluconate	10 μM	77 ± 2	67 ± 2
	100 μM	80 ± 2	53 ± 1
Galactose	10 μM	58 ± 3	75 ± 3
	100 μM	61 ± 2	77 ± 3
Fructose	10 μM	72 ± 2	57 ± 2
	100 μM	81 ± 2	83 ± 3
Pyruvate	10 μM	12 ± 2	6 ± 2
	100 μM	16 ± 2	5 ± 1
L-Arabinose	10 μM	41 ± 1	32 ± 1
	100 μM	52 ± 2	21 ± 2
D-Glucosamine	10 μM	20 ± 2	0
	100 μM	15 ± 2	11 ± 2
Mannose	10 μM	15 ± 2	17 ± 1
	100 μM	0	11 ± 2
Lactose	10 μM	31 ± 2	38 ± 2
	100 μM	42 ± 2	53 ± 2
Arginine	10 μM	19 ± 2	-[d]
	100 μM	11 ± 1	-

[a] activity was measured by 0.2 mg of protein with 1 μM [^{14}C] glucose.
[b] standard deviations are calculated for n=3.
[c] The percent of inhibition of binding activity by the substrates.
[d] Not determined.

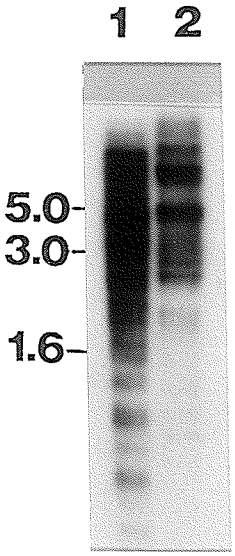
Table 4. Specificity of glucose-binding protein [a, b]

Substrates added		Inhibition (%) 30 s	Inhibition (%) 5 min
None		0	0
Glucose	10 μM	74 ± 2	93 ± 2
	100 μM	93 ± 1	95 ± 1
Gluconate	10 μM	92 ± 1	90 ± 1
	100 μM	90 ± 1	76 ± 2
Galactose	10 μM	89 ± 1	94 ± 2
	100 μM	87 ± 1	82 ± 1
Fructose	10 μM	49 ± 3	89 ± 2
	100 μM	63 ± 2	90 ± 1
Pyruvate	10 μM	36 ± 1	5 ± 1
	100 μM	24 ± 1	-[d]
Glucosamine	10 μM	32 ± 1	11 ± 1
	100 μM	27 ± 1	44 ± 1
Arabinose	10 μM	24 ± 2	0
	100 μM	45 ± 2	51 ± 2
Arginine	10 μM	9 ± 1	-
	100 μM	15 ± 1	-

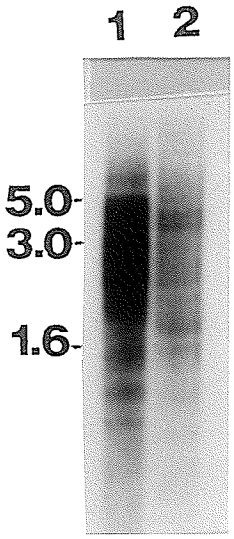
[a] activity was measured by 20 μg of GBP with 1 μM [^{14}C] glucose.
[b] Standard deviations are calculated for n=3.
[c] The percent of inhibition of binding activity by the substrates.
[d] Not determined.

Figure 13. Southern blots (I and II) probed with radiolabelled oligonucleotides deduced from the N-terminal amino acid sequence of *P. aeruginosa* GBP. Genomic DNA isolated from *P. aeruginosa* H103 and *P. putida* was digested with restriction enzyme *SalI* (I) and probed with end-labelled oligonucleotide probe A (I) and probe B (II). Lane 1), *P. aeruginosa* H103 DNA; lane 2), *P. putida* DNA. Molecular weight are as indicated $x10^3$ kilobases.

I



II



inserts of approximately 4 kb were isolated and designated them pGR1, pGR2, and pGR3. Plasmid pGR2 was selected for further analysis. The restriction endonuclease map of the 4 kb DNA fragment is shown in Figure 14. Southern-blot hybridization analysis demonstrated the GBP gene is located between restriction sites *Xho*I and *Sma*I. In order to sequence this structure gene, *Pst*I-*Pst*I (0.7 kb), *Xho*I-*Sma*I (2.6kb), and *BamH*I-*BamH*I (2.1 kb) fragments were then subcloned into plasmid pM13 and designated as pGR4, pGR5, and pGR6. The *Xho*I-*Kpn*I fragment, containing the GBP gene, was subcloned into broad host range plasmid pPZ375 and designated as pGR7.

3.11 Nucleotide sequence of GBP gene

The sequencing strategy for the GBP gene is shown in Fig. 15. Sequence was determined from the *Sma*I site to the *Xho*I site of pGR2. Greater than 95% of the sequence information was obtained from both strands, and all of the DNA fragments were overlapped using fragments generated at other restriction sites. The alignment results of GBP gene of *P. putida* and oligonucleotides probe A and probe B deduced from the N-terminal amino acid sequence of the *P. aeruginosa* GBP gene were shown in Figure 16. The identities are 91.7% for probe A and 85.7% for probe B. The final sequence of the GBP gene is shown in Figure 17. An open reading frame of 1332 bp was revealed by Seqaid program analysis, encoding 443 amino acids, with the initiation codon (ATG) at position 136 and the termination codon (TGA) at position 1467. The mature N-terminus of the protein is encoded by residues begins at nucleotide 261, which is the first nucleotide of probe A to align with *P. putida* GBP gene. The molecular weight of the deduced protein was 43,657. This result agreed with the molecular mass of the product from *P. putida* by SDS-PAGE. Codon usage and G+C composition (65.1%) was similar to that

Figure 14. Restriction enzyme map of *P. putida* genomic DNA containing the GBP gene and the regions of different subclones. The location of the GBP gene is bold. Arrow indicates the direction of translation.

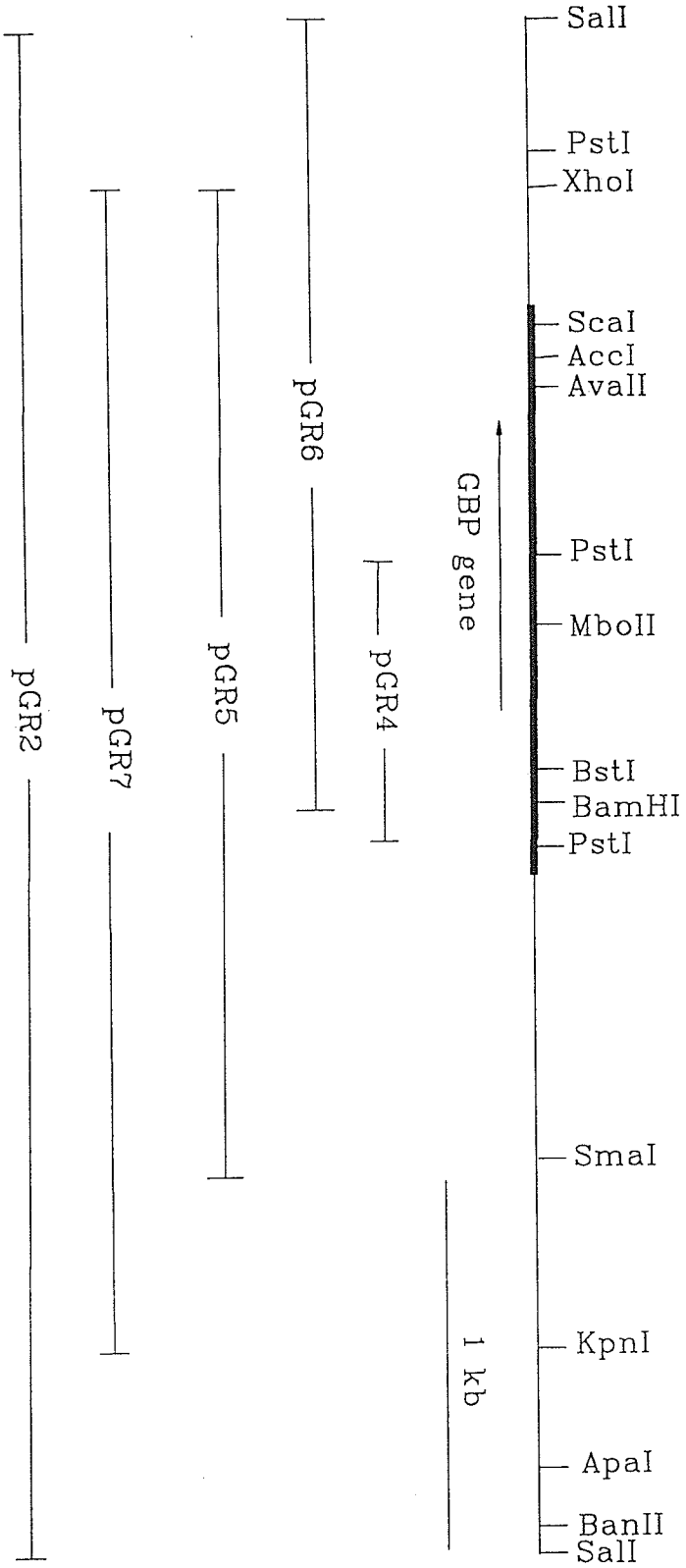

SalI
PstI
XhoI
ScaI
AccI
AvaII
PstI
MboII
BstI
BamHI
PstI
SmaI
KpnI
ApaI
BanII
SalI
GBP gene
1 kb
pGR2
pGR7
pGR5
pGR6
pGR4

Figure 15. Sequencing strategy for the GBP gene. The arrows denote sequencing directions (5' to 3') and lenghs of sequences determined by the dideoxy-chain termination method.

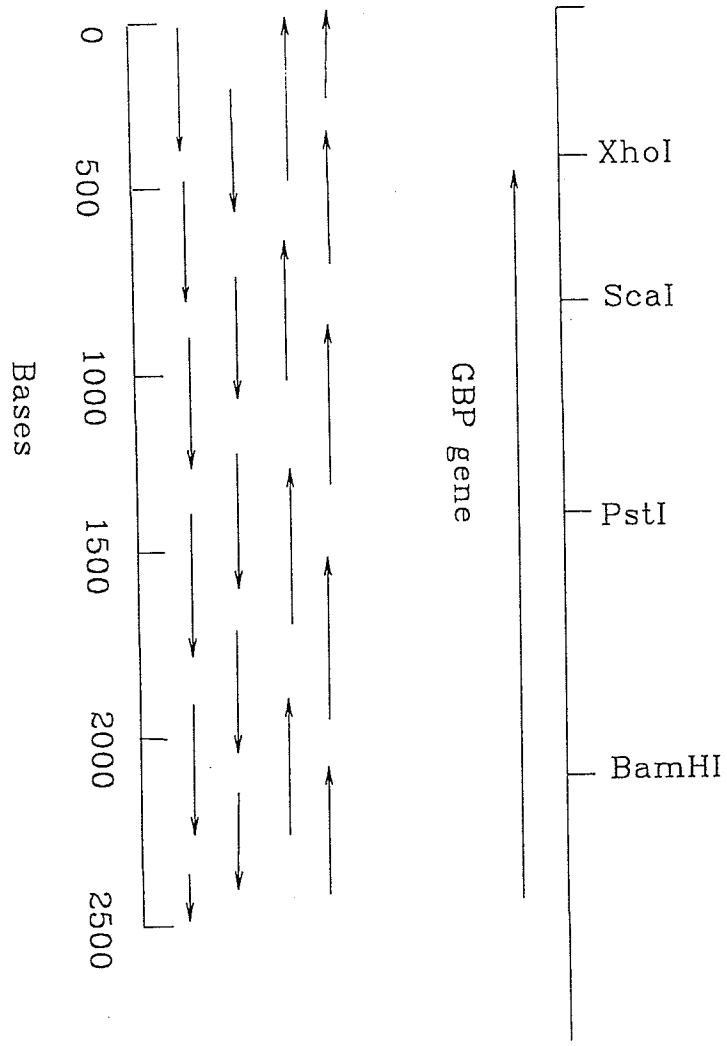

0
500
1000
1500
2000
2500
Bases
GBP gene
XhoI
ScaI
PstI
BamHI

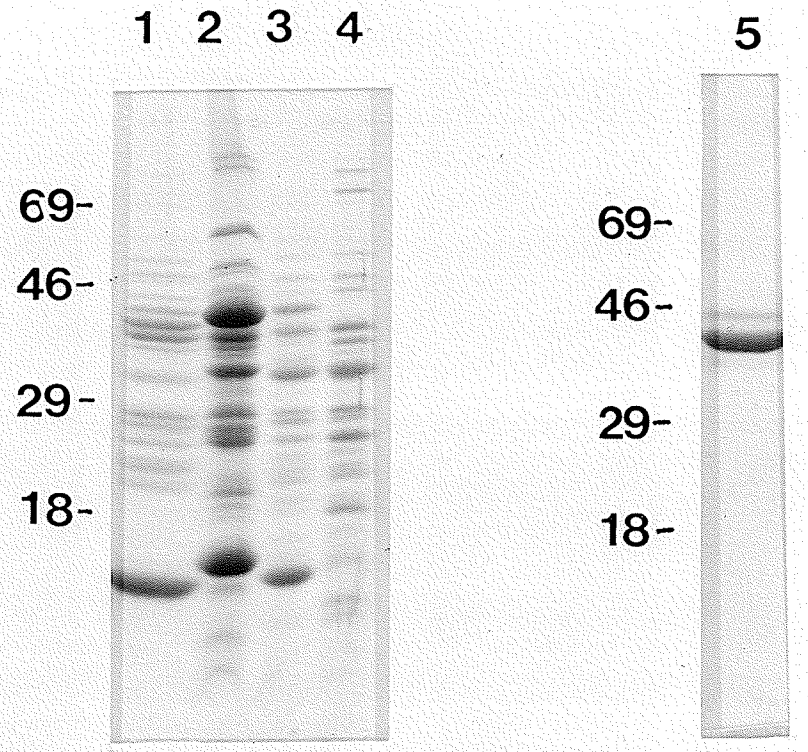
Figure 16. The alignment results of GBP gene of *P. putida* and oligonucleotides probe A and probe B deduced from the N-terminal amino acid sequence of the *P. aeruginosa* GBP gene.

P. putida GBP gene - GGCCAGGTCGGTGGTGCTGATGGCCAGCAGGTCACCGAACAGGT
P. aeruginosa probeA- GGCGAGGT-GGAGGTGCTG----------CAC---------

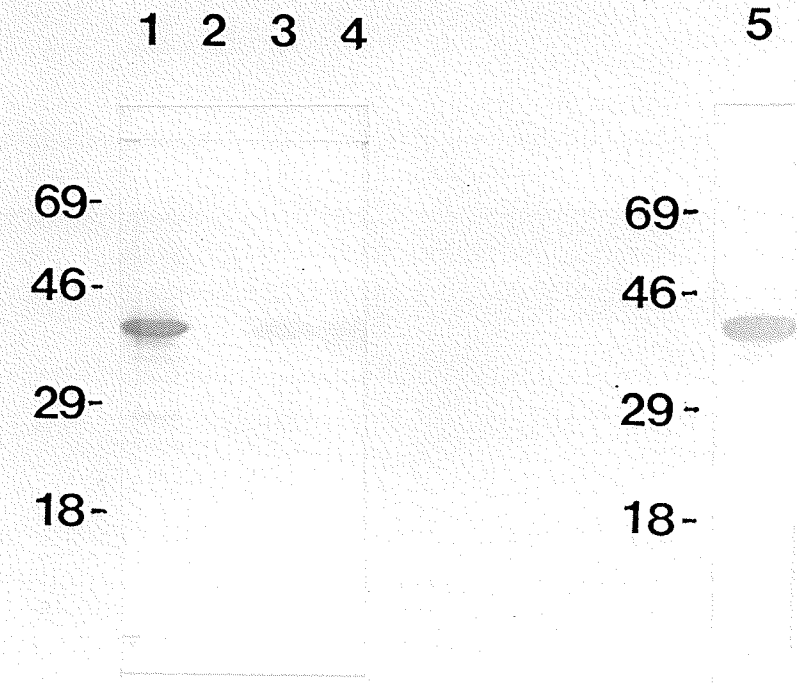
P. putida GBP gene - GGCCAGGTCGGTGGTGCTGATGGCCAGCAGGTCACCGAACAGGT
P. aeruginosa probeB- A----------CCAGC-GCTCAC-GAAAAGG-

Figure 17. Nucleotide sequence of the *P. putida* GBP gene. The signal sequence is dashed-underlineed The putative Shine-Dalgarno sequence is boxed. The potential ρ-independent terminator is bold.

A



B



determined for other *Pseudomonas* gene (West and Iglewski, 1988). There is a sequence indicative to a ribosome-binding site (the Shine-Dalgarno sequence) (AGGA) upstream of the initiation codon (ATG). Inspection of the amino acid sequence of the GBP reveals that this protein is rich in glutamic acid, alanine, aspartic acid, and glycine. This result is similar to the *P. aeruginosa* GBP and LIVATBP (Table 5).

3.12 The expression of GBP gene in glucose-transport mutant

To determine if the above described, cloned GBP gene expressed the GBP protein we wanted to demonstrate GBP expression in a mutant strain. We, however, did not have a appropriate *P. putida* strain, hence used a *P. aeruginosa* glucose transport mutant. Mutant strain PFB362a from *P. aeruginosa* 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 *glc T1* in glucose transport activity which blocks utilization via the phosphorylative route (Stephen *et al.* 1985). Plasmid GR7 (2.6 kb DNA insert in which the *P. putida* GBP gene was located) ligated with plasmid pPZ375 was transformed into PFB362a. Osmotic shock fluids were obtained from PFB362a with GR7 cells grown in BM2 plus 20 mM glucose supplemented with 50 μg/ml ampicillin and 500 μg/ml carbenicillin for the selection of cells with GR7. After SDS-PAGE, western-blotted shock fluid samples were probed with GBP specific polyclonal serum. A protein band from PFB362a with plasmid GR7 shock fluid was observed by both SDS-PAGE and western-blotting analysis (Fig. 18, lane 3 in panel A and lane 3 in panel B). Since the difference of the amino acid compositions of *P. putida* GBP and *P. aeruginosa* GBP (Table 5), there must be a certain difference of amino acid sequences between

Table 5. Comparison of predicted amino acid compositions of some binding proteins from *P. putida* and *P. aeruginosa*

Amino acid	No. of residues/100 amino acid residues (%)		
	P. putida GBP	*P.aeruginosa* GBP[a]	*P. aeruginosa* LIVATBP[b]
Ala	13.9	14.7	13.0
Cys	2.0	0.8	0.6
Asp+Asn	7.2	11.2	10.2
Glu+Gln	14.9	13.0	7.8
Phe	1.2	4.5	4.3
Gly	11.7	10.1	10.4
His	6.5	0.9	1.2
Ile	1.2	2.9	6.1
Lys	0.5	7.1	8.4
Leu	8.7	7.3	8.4
Met	0.5	0.3	2.0
Pro	5.2	5.2	4.3
Arg	9.2	2.1	2.6
Ser	3.2	5.4	2.9
Thr	3.2	4.1	5.8
Val	7.2	6.6	8.1
Trp	2.0	3.2	0.3
Tyr	1.7	1.4	2.9

[a] Stinson *et al.* (1977);
[b] Hoshino and Kose (1989).

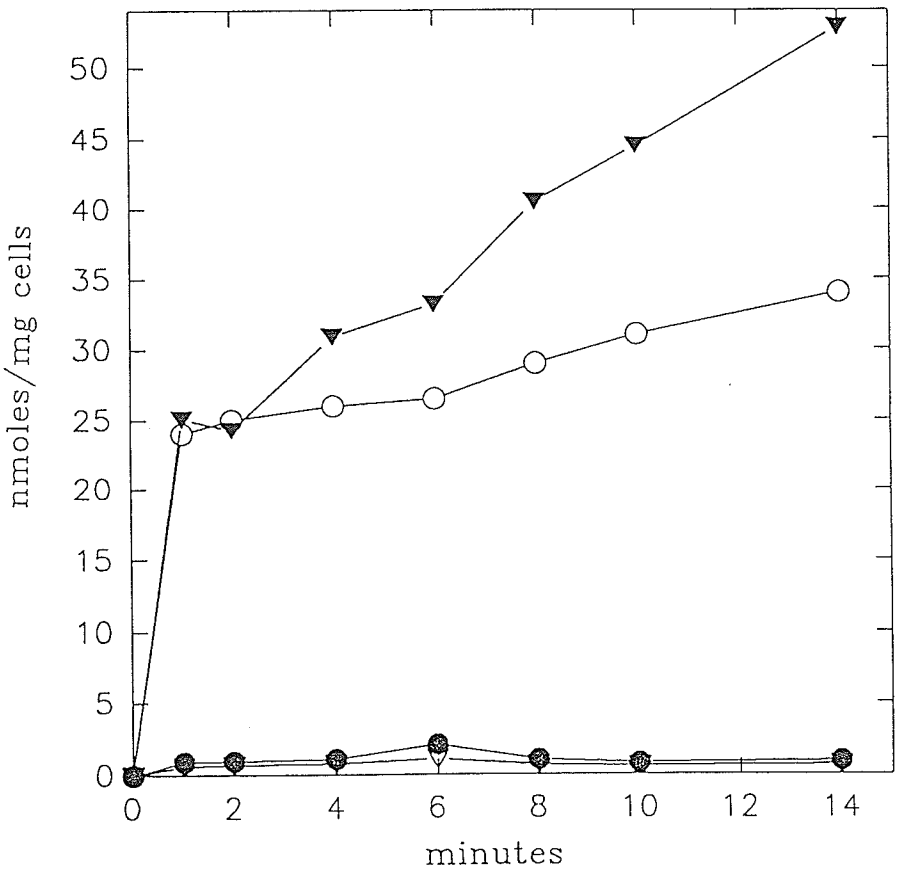
Figure 18. SDS-PAGE and immunoblot of shock fluids from *P. aeruginosa* H103, *P. putida*, mutant PFB362a, mutant PFB362a/pGR7, and *P. putida* GBP. Panel A is the Coomassie Brilliant Blue R-250 stained SDS electrophoretogram. Lane 1) H103 grown on 20 mM glucose; lane 2) *P. putida* grown on 20 mM glucose; lane 3) PFB362a (GR7) grown on 20 mM glucose plus 10 mM lactate; lane 4) PFB362a grown on 20 mM glucose plus 10 mm lactate; lane 5) *P. putida* GBP. Panel B is the immunoblot probed with *P. putida* glucose-binding protein specific polyclonal sera at a 1:100 dilution. Lane 1) *P. putida*; lane 2) PFB362a; lane 3) PFB362a/pGR7; lane4) *P, aeruginosa* H103; lane5), *P. putida* GBP. Samples (10 μl) were solubilized at 95°C for 10 min in the presence of 0.12% SDS ans 5% 2-mereaptoethanol prior to electrophoresis. Molecular weights are as indicated $x10^3$ daltons.

these two GBP. Therefore, the cross-reactivity of the shock fluid of *P. putida* with *P. putida* GBP specific polyclonal serum is stronger than the cross-reactivities of the shock fluids of *P. aeruginosa* and PFB362a/GR7 (Fig.18, panel B). This protein has a molecular weight of 43,657, which was in good agreement with the GBP molecular weight purified from *P. putida* (43,700).

Glucose transport was induced by glucose both in wild-type strain *P. aeruginosa* H103 and the cells of PFB 362a after transformation with GR7. This activity was not expressed by the induced mutant strain PFB362a alone or when it was grown on LB (non-induced state) (Fig. 19).

These results thus indicate that we have indeed cloned structural gene for the *P. putida* GBP and the gene is expressed in *P. aeruginosa*.

Figure 19. Uptake of [^{14}C] glucose by various *P. aeruginosa* strains: H103 grown on 20 mM glucose (▲), PFB362a grown on 20mM glucose plus 10 mM lactate (▽), PFB362a (GR7) grown on LB (•), and PFB362a (GR7) grown on 20 mM glucose plus 10 mM lactate (○). External [^{14}C] glucose concentration was 10 µM. Uptake at 22°C. Each point is the mean of triplicate determinations and is representative of three independent experiments.


nmoles/mg cells
50
45
40
35
30
25
20
15
10
5
0
0
2
4
6
8
10
12
14
minutes

Chapter 4 Discussion

The results presented in this thesis demonstrate the existence of a high affinity system of glucose transport in *P. putida*. The uptake of glucose by specifically induced cells of *P. putida* was found to be temperature dependent, obeyed saturation kinetics, and was reliant on the presence of a periplasmic glucose binding protein.

We found that glucose was transported by *P. putida* by a high affinity system with an apparent Km of about 5.5 μM (Fig.4). This is in close agreement with Km values for glucose transport reported for several strains of *P. aeruginosa* (Guymon and Eagon, 1974, Midgley and Dawes, 1973). Vicente *et al.* (1975) originally reported that glucose appears to be transported by *P. putida* by low affinity systems (Km=6 mM). However, this result was obtained when 100-fold higher concentrations of ^{14}C-glucose were used than we did for our experiments. This group did not report using the lower concentrations hence, they may have missed the detection of a high affinity system. In this thesis, we report the high affinity system of glucose transport by *P. putida*. Therefore, *P. putida*, similar to *P. aeruginosa*, possesses two distinct inducible transport systems: a low affinity pathway and a high affinity pathway.

GBP, like binding proteins described from other periplasmic permeases (Boos, 1974), undergoes a substrate induced conformational change but has no enzymatic activity (Stinson *et al.*, 1976). That is, the substrate is translocated without alteration. This protein was isolated and purified from the periplasm of *P. aeruginosa* (Stinson *et al.*, 1977). It was found to have an average molecular weight of 44,500 daltons, and bound one mole of glucose per mole of GBP with a dissociation constant of 0.35 μM. It was originally believed to be a glycoprotein as it was isolated with sugar associated with it (Stinson *et al.*, 1977). It is more likely the protein was

isolated with tightly bound substrate.

Stinson *et al.* (1977) have isolated a mutant strain, MB723, which lacks glucose dehydrogenase activity as well as GBP activity. This strain did not produce detectable quantities of GBP in pyruvate plus αMeGlc or gluconate, thus suggesting that the binding protein is in some way linked to the glucose transport system. Membrane vesicles of *P. aeruginosa* PAO have been reported to lose their ability to transport free glucose (Guymon and Eagon, 1974), suggesting that some component of the glucose transport system, perhaps the GBP, was lost during the preparation of the vesicles. The studies with whole cells of *P. fluorescens* (Romano *et al.*, 1980) also suggested that the energisation of glucose transport is dependent upon the presence of shock-sensitive glucose binding proteins and phosphate bond energy rather than PMF-dependent glucose-carrier system in the cytoplasmic membrane. Wylie *et al.* (1993) examined specificity of the high affinity glucose transport system of *P. aeruginosa*. It was found that at a concentration of [^{14}C] glucose near the Vmax of the system, inhibition by maltose, galactose, and xylose was detected. This inhibition is similar to that detected in earlier in vivo studies and correlates with the known specificity of OprB, a glucose specific porin of *P. aeruginosa*. At a 100 fold lower level of [^{14}C] glucose, only unlabelled glucose showed a significantly different level of inhibition in comparision to other substrates. This matches the known in vitro specificity of the periplasmic glucose binding protein.

Evidence that the enhanced glucose uptake capacity of *P. putida* grown on glucose results from an induction that is dependent upon protein synthesis is the lack of increased rates of cellular uptake when noninduced cells are exposed to glucose. Vmax value in induced cells was found to be 17.5 nmoles/min/mg, much higher than Vmax value of 1.8 nmoles/min/mg in

noninduced cells (Fig.3 and Fig.4). These results indicate that induced transport represents an increased synthesis of the basal transport system. The specificity of the GBP was investigated by measuring the ability of unlabelled substances to compete with the binding of [^{14}C] glucose (Table 2,3,4). Various sugars were tested by whole cells, osmotic shock fluid, and glucose binding protein of *P. putida*. At 10-fold concentrations, only unlabelled glucose, gluconate, galactose, and fructose were significant competitors. At 100-fold excess, a variety of other sugars had affinity for the binding protein. The results from whole cell, shock fluid and GBP-binding assays are similar, which indicated that glucose uptake was indeed dependent upon GBP. Fructose was a competitive inhibitor of glucose uptake (Fig. 6). This is similar to the conclusion by Vicente *et al.* (1975) that the glucose-gluconate uptake system not only serves to take up glucose and gluconate, but also fructose. This is also in agreement with the result reported by Eagon and Phibbs (1971) that the glucose transport system was also induced by growth of *P. aeruginosa* on either glucose, fructose, or mannitol, but not on succinate. Glucose 6-phosphate is the first intermediate that is common to the catabolism of these three sugars by *P. aeruginosa* (Phibbs and Eagon, 1970). Moreover, formation of glucokinase is also induced by cultivation of *P. aeruginosa* on these three sugars, but not on succinate. Although purely speculative, the induction of the glucose active transport system may be regulated by the level of the intracellular pool of glucose 6-phosphate.

Active transport of fructose was induced when *P. putida* was cultivated on fructose, but not on glucose (Fig. 11).Vicente *et al.*(1975) also got similar results by using another *P.putida* strain A.3.12 and glucose-gluconate uptake mutants. They deduced that transport by the fructose uptake system is mediated by an enzyme-like carrier, as supported by its saturation kinetics, and

its inducibility by fructose. Glucose is also a substrate for the fructose uptake system, but is unable to induce this system, as evidenced by the fact that elevated levels of the fructose uptake system activity were not found in glucose-gluconate uptake system mutants grown on succinate in the presence of glucose. This result is also in agreement with the result from *P. aeruginosa* (Eagon and Phibbs, 1971). Fructose 6-phosphate is the first intermediate to the catabolism of fructose (Phibbs and Eagon, 1970). Similarly, a high level of fructose-phosphorylating activity is induced by cultivation of cells on fructose. Thus, the level of the intracellular pool of fructose 6-phosphate may regulate the induction of the fructose active transport system.

Gluconate were found to be noncompetitive inhibitor of the *P. putida* glucose uptake system. This result also supports the conclusion by Vicente *et al.*(1975). In their experiment, strains selected for their inability to transport glucose were found to be deficient in gluconate uptake. The reverse was also true: mutations affecting gluconate entry also blocked the uptake of glucose. These results demonstrated that a common carrier is involved in the uptake of both glucose and gluconate by *P. putida* cells. There are at least three alternatives to explain how a common carrier could be involved in glucose and gluconate transport: 1) glucose uptake is mediated by a specific system and also by an additional system explicable on the basis that glucose dehydrogenase acts extracellularly to convert glucose into gluconate, which is then transported into the cells; 2) glucose enters into cells after being converted into gluconate as described in 1), but there is no specific glucose transport system; and 3) glucose and gluconate are taken up by an enzyme-like carrier which has affinity for both substrates(Vicente *et al.*, 1975). Therefore, an enzyme-like carrier with affinity for both glucose and gluconate is probably acting in *P. putida* for the uptake of these two substrates. There are remarkable differences

between *P. aeruginosa* and *P. putida* in the transport of glucose and gluconate according to reports about *P. aeruginosa* from other laboratories (Midgley and Dawes, 1973, Guymon and Eagon, 1974). Another discrepancy is that gluconate induced glucose transport in *P. putida* but not in *P. aeruginosa* (Guymon and Eagon, 1974).

We also found interesting results in the experiments of glycerol uptake. Figure 12 shows that active transport of glycerol by *P. putida* was induced by growth on glycerol, but not on glucose. This indicated the existence of an independent glycerol uptake system in *P. putida*. This result was similar to the result from *P. aeruginosa*. In *P. aeruginosa*, glycerol is primarily metabolized through the Entner-Doudoroff pathway (Cuskey *et al.*, 1985). Glycerol is transported into the cell by a specific, inducible transport system (*glpT*). Unlike the case for *E. coli* in which glycerol crosses cytoplasmic membrane by a nonsaturable proteinaceous pore-type mechanism (Heller *et al.*, 1980), in *P. aeruginosa* glycerol transport seems to be mediated by a high-affinity, binding protein-dependent transport system (Siegel and Phibbs,1979). Intracellular glycerol is phosphorylated to *sn*-glycerol-3-phosphate (G3P) by glycerol kinase (*glpK*) and subsequently oxidized to dihydroxy-acetone phosphate by G3P dehydrogenase (*glpD*). Evidence for this pathway rests primarily on the observed specificity of enzyme induction and on the analysis of mutants defective in specific *glp* genes. Herbert and Po (1994) cloned *glpD* gene. The cloned gene was able to complement an *E. coli glpD* mutant.

Once establishing the presence of a shock-dependent high affinity glucose transport system in *P. putida*, the next step was to identify, purify, and analyze the component of the shock fluid mediating this transport, ie. GBP. The periplasmic binding protein for glucose was readily removed from *P. putida* cells by extraction with 0.2 M Mg^{2+} followed by osmotic shock. The

analysis of osmotic shock fluid indicated the release of periplasmic proteins occurred with detectable losses in cell viability and cytoplasmic or cytoplasmic membrane enzymes were not released by this treatment (Stinson *et al.*, 1976). Maximal production of GBP was observed when cells grown on glucose (Fig. 9). Small amounts of GBP were detected in cells grown on acetate, gluconate, pyruvate, or succinate (data not shown). The induction of GBP synthesis correlated closely with the glucose transport activity of whole cells. An active transport system for glucose has been described by Eagon *et al.* (1971). Whereas Midgely and Dawes (1973) 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 (Guymon *et al.*, 1974), suggesting that some component of the glucose transport system, perhaps the GBP, was lost during the preparation of the vesicles.

GBP of *P. putida* was purified and characterized as a protein of 43,700 daltons. The immunoblot analyses showed the osmotic shock fluid from *P. aeruginosa* cross-reacted with anti-GBP antibodies from *P. putida* (Fig. 17) indicating that the structures from both strains have some degree of homology. Stinson *et al.* (1977) purified GBP of *P. aeruginosa* and characterized as a glycoprotein of 44,500 daltons. The presence of a carbohydrate moiety distinguishes the GBP from the binding proteins of *E. coli* and *Salmonella typhimurium*. The latter periplasmic components have been characterized as homogeneous proteins with molecular weights between 22,000 and 42,000 (Boos, 1974). The physiological role of the binding proteins of gram-negative bacterial envelops has been extensively studied in recent years. Biochemical and genetic evidence has strongly implicated a number of *E. coli* binding proteins as chemoreceptors for both active

transport and chemotaxis (Adler, 1975 and Boos, 1974). These membrane phenomena are also closely linked to GBP in *P. aeruginosa* (Sly *et al.*, 1991). The inability of mutant strain MB723 to synthesize GBP was accompanied by severe deficiencies in both glucose transport and chemotaxis (Stinson *et al.*, 1977).

The structural gene for the *P. putida* GBP was cloned and localized on the 2.6 kb *Xho*I-*Sma*I fragment by using the oligonucleotides deduced from the N-terminal amino acid sequence of the *P. aeruginosa* GBP (Sly *et al.*, 1991). Determination of the complete nucleotide sequence of 1.7 kb fragment revealed an open reading frame of 1329 nucleotides that was capable of coding for a 443-amino-acid peptide with a molecular weight of 43,700. The overall G+C content of the GBP gene was 65.1%, which was similar to that (65%) reported previously (Shapiro, 1968) for the average G+C content of the *Pseudomonas* genome. The amino-terminal 40 residues (from Met to the first amino acid of the GBP , aligned with probe A) seems to represent a signal sequence. Blobel and Dobberstein (1975) postulated that secreted proteins possess a transient NH_2-terminal region, termed a signal sequence, that is responsible for initiating the secretion event. It has now been shown, for both eucaryotes and procaryotes, that most proteins destined for secretion are initially translated as precursor forms possessing such an NH_2-terminal extension of some residues (Mckean and Maurer, 1978, Lingappa *et al.*, 1978). As a periplasmic transport component, glucose binding protein must be secreted through the cytoplasmic membrane and therefore should possess a signal sequence.

The comparison of *P. putida* GBP with the GBP and LIVAT BP (leucine-, isoleucine-, and valine-) from *P. aeruginosa* indicate that these periplasmic binding proteins have similar amino acid compositions (Table 5). Thus, although *P. aeruginosa* and *P. putida* minimal DNA

homology (Palleroni et al., 1973), the periplasmic binding proteins appear analogous.

We also present evidence in which the cloning of chromosomal DNA of *P. putida* affected the synthesis of a periplasmic GBP necessary for glucose transport. The cloned *P. putida* GBP gene was examined for its ability to alter glucose transport in *P. aeruginosa*. Recombinant plasmid GR7 containing the cloned *P. putida* GBP structure gene on a multicopy plasmid, pPZ375, was transformed into *P. aeruginosa* and *P. putida* glucose transport defective strains. Mutant strain PFB362a is deficient in periplasmic GBP and glucose transport activities. Plasmid GR7 containing GBP gene from *P. putida* could complete the GBP deficiency in *P. aeruginosa* PFB362a (Fig.18). However, GR7 did not restore glucose uptake in glucose transport-negative mutant, *P. putida* MAP1 (data not shown), which has a disfunctional 6-phosphogluconate dehydratase.

In conclusion, the results presented in this study indicate that *P. putida* utilizes an inducible high affinity transport system to facilitate rapid entry of glucose. Since only a basal level of glucose accumulation was observed in uninduced cells (Fig. 3), induction by glucose is apparently required for transport activity. This transport system was found to be temperature dependent and inhibited by gluconate, galactose, and fructose. Filter binding assays revealed that the shock fluid from glucose induced cells bound glucose. This activity was attributed to a 43,700 Da GBP. The gene responsible for encoding GBP was cloned and sequenced. This gene was successfully expressed in a glucose transport mutant of *P. aeruginosa*.

Further study will include production of *P. putida* GBP mutant strains by transposon or chemical methods to construct genetic insertion mutants; analysis of the structure and function of GBP gene which includes the genetic manipulation of this gene to produce novel site directly

altered proteins which will be examined for changes in structure, glucose binding and OprB-GBP interaction; and identification of the glucose binding domain to understand the structure/function relationship of GBP which includes circular dichroism, fluorescence quenching, molecular model building, and crystallographic methods. By comparing *P. putida* GBP to other systems such as the maltose transport systems of *E. coli* and *S. typhimirium* and the glucose transport systems of *P. aeruginosa* and *Agrobacterium radiobacter* (Greenwood *et al.*, 1990) should also provide evidence for the evolution of binding protein dependent sugar transport systems in gram negative bacteria.

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