Characterization of the *Pseudomonas aeruginosa* High Affinity Transport System Periplasmic Glucose Binding Protein Gene, *gltB*

By: Lorelee R. Tschetter
Department of Microbiology
The University of Manitoba
Winnipeg, Manitoba, Canada

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Abstract

Previous research has shown that *Pseudomonas aeruginosa* and *Pseudomonas putida* have similar mechanisms for glucose transport. There is a low-affinity oxidative pathway, and an inducible, high-affinity phosphorylative pathway. The high-affinity pathway consists of several components, which are expressed when grown under glucose-limited conditions. Among these components are: the outer membrane porin, OprB; a periplasmic binding protein, GltB; a two-component response regulator, GltR; and inner membrane-associated proteins.

In order to determine which segment of the *P. aeruginosa* genome encoded for GltB, a mutagenesis vector (pXL118ΩSm) was constructed. This vector contained the *P. putida gltB*, disrupted by a streptomycin resistance cassette from pHP45ΩSm, in the vector pEX100T. Through homologous recombination, *gltB* knock-out mutants were generated in both *P. aeruginosa* and *P. putida* (HX2 and LX8, respectively). Glucose chemotaxis and ^{14}C-D-glucose binding activity assays were performed to confirm gene knock-out. Changes in protein expression between wild type and mutant strains was demonstrated through periplasmic protein expression and SDS-PAGE.

Reconstitution of the mutants HX2 and LX8 was attempted using an intact *gltB* from *P. putida*, and two different DNA segments obtained from the *P. aeruginosa* genome. Glucose chemotaxis, ^{14}C-D-glucose binding activity assays, and periplasmic protein expression were again used to monitor changes in mutant

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The road to completion of this work has been long and taken many turns. As a result, there are a number of people who must be thanked!

❀

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❀

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❀

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List of Abbreviations

w/v	weight per volume
v/v	volume per volume
w/w	weight per weight
LB	Luria-Bertani (broth or agar)
ap	ampicillin
ap^R	ampicillin resistance
km	kanamycin
km^R	kanamycin resistance
sm	streptomycin
sm^R	streptomycin resistance
spc^R	spectinomycin resistance
tc	tetracycline
tc^R	tetracycline resistance
cb	carbenicillin
kDa	kilodaltons
kb	kilobases
bp	base pairs
PMF	proton motive force
SDS	sodium dodecyl sulfate
PAGE	polyacrylamide gel electrophoresis
ATP	adenosine triphosphate

EDTA	ethylene-diaminetetraacetic acid
GBP	periplasmic glucose binding protein
MBP	periplasmic maltose binding protein from *E. coli*

Chapter One

Introduction and Literature Review

1.1 Introduction

Pseudomonas aeruginosa and *Pseudomonas putida* are members of the family *Pseudomonaceae*, whose members are typically Gram-negative, aerobic rods, using a single flagellum for motility. They are extremely adaptable, and growth temperatures are quite variable, ranging from 4°C or lower, to 43°C. These chemoorganotrophs produce energy via respiration, and can utilize a wide variety of carbon sources, including glucose, 2-ketogluconate, geraniol, L-valine, L-alanine, and D- or L-arginine (Doudoroff and Palleroni, 1975).

P. aeruginosa is a well-known opportunistic human pathogen. It preys upon immunocompromised individuals, suffering from another condition, primarily those with cystic fibrosis, diabetes, cancer, and burns. Part of this organism's success in pathogenicity is its ability to resist most antibiotic treatments. This is due, in part, to its unique outer membrane structure (Nikaido and Hancock, 1986), which is 100-500 times less permeable to antibiotics than the *E. coli* outer membrane (Yoshimura and Nikaido, 1982; Angus *et al*, 1982). They also possess the ability to enzymatically deactivate internalized antibiotics (Bryan, 1979) using periplasmic lactamases and penicillin-binding proteins, as well as multidrug efflux pumps to remove potentially harmful drugs from the cell (Nikaido, 1994; Poole *et al*, 1996; Koehler *et al*, 1997; Mine *et al*, 1999).

The inner leaflet of the outer membrane consists mainly of phospholipids such as phosphatidylethanolamine, acidic phospholipids, phosphatidyl glycerol, diphosphatidyl

diglycerol. The predominant fatty acid is cis-vaccenic acid (C18:1) rather than palmitoic acid (C16:1) typically found in Gram negative bacteria, and the lipopolysaccharide composition is similar to that seen in *Enterobacteriaceae* members. Lipopolysaccharide O-chains are unique in their rare sugar content, including aminohexuronic acid and 2,4-diamino-2,4,6-dideoxyhexose. The various O-chain structures between species correspond to the groups identified by serotyping methods (Nikaido and Hancock, 1986).

The uptake of nutrients is accomplished by a variety of periplasmic transport systems, which is commonly seen in most Gram-negative organisms. These transport systems involve the introduction of a substrate through a pore in the outer membrane. These pores may or may not be specific for a given substrate, and may play an important role in effective antibiotic treatment (Trias and Nikaido, 1990). One of these transport systems, the high affinity glucose transport system, is the basis of the study presented in this thesis.

The high affinity glucose transport system is activated when glucose is the sole carbon source available to the cells, and is in limited quantities. Activation of this system is characterized by the production of several specific protein components: OprB, an outer membrane porin specific for glucose (Wylie and Worobec, 1994), GBP (glucose binding protein), a periplasmic glucose binding protein (Stinson *et al*, 1977); GltR, a two-component response regulator, which regulates the expression of GBP (Sage *et al.*, 1996); GltK, an inner membrane ATPase component (Adewoye and Worobec, 2000). The GBP is believed to be an integral component, directing the flow of incoming glucose from the

outer membrane, through the periplasmic space, to the inner membrane where it likely interacts with another transport channel.

The purpose of this study was to create a defined, structural *gltB* mutant strain of *P. aeruginosa*, to allow for accurate interpretations of activity changes in strains upon introduction of DNA harboring *gltB*. To meet this objective, a knock-out mutagenesis vector was created, and introduced into both *P. putida* and *P. aeruginosa*. Protein expression profiles, chemotaxis, and glucose uptake activity were studied to confirm structural gene disruption. Several DNA fragments were then introduced into the mutants in an attempt to determine which fragment harbored *gltB*.

1.2 Pseudomonas Transport Systems

The membrane of Gram-negative bacteria consists of four layers: an impermeable outer membrane, a permeable periplasmic space, a permeable peptidoglycan matrix, and an impermeable cytoplasmic/inner membrane. This structure necessitates directional transport systems for either the internalization of essential nutrients from the surrounding environment, or the export of unwanted materials and byproducts.

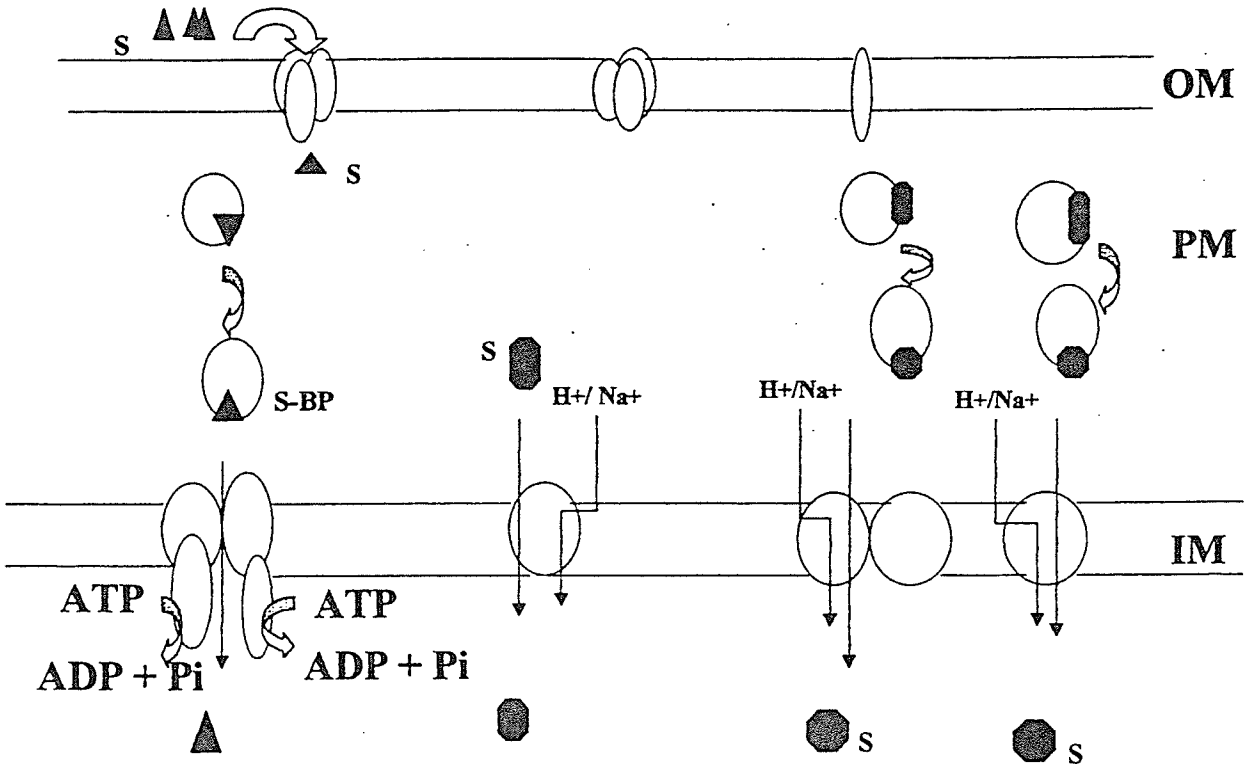
There are two classes of transport systems, identified by their sensitivity to cold osmotic shock; (i) shock-resistant, or (ii) shock-sensitive (often called periplasmic permeases) [Neu and Heppel, 1965]. Shock-sensitive permeases have a periplasmic component which is released upon cold osmotic shock treatment, and require ATP or other compounds with high energy phosphate bonds (primary coupling) (Bishop et al, 1989; Davidson and Nikaido, 1990; Hoshino *et al*, 1992). Periplasmic permeases consist of several components, including an outer membrane porin protein, a periplasmic substrate-

binding protein, hydrophobic integral inner membrane proteins, and peripherally-associated, inner membrane proteins which function as ATPases. The latter components are believed to be involved in energy coupling required for function of the transport system (Neu and Heppel, 1965). Shock-resistant transport systems are membrane-bound, and driven by the electrochemical potential of protons or sodium ions (secondary coupling) (Berger and Heppel, 1974; Hong *et al*, 1979).

The transport systems identified can also be categorized according to their bioenergetics and substrate-binding characteristics, which comprise of three main groups (Figure 1.1). The first is a multicomponent primary transport system which accumulates nutrients against a concentration gradient (Ames, 1986). Its transport function is dependent on the involvement of a periplasmically- localized binding protein (Higgins *et al*, 1982). The second system is commonly referred to as the secondary transport system in which substrate transport is coupled to the co-transport of protons or sodium ions and is driven by the proton motive force (PMF). The third grouping of transporters are those which require both PMF and periplasmic binding proteins (Jacobs *et al*, 1996).

Transport through the outer membrane may occur in one of three different ways: (i) specific and non-specific pores, (ii) specific receptor complexes, or (iii) a hydrophobic pathway (Nikaido and Vaara, 1985). Specific and non-specific pores are those seeming to function in conjunction with periplasmic permeases. These proteins commonly have monomolecular weights ranging from 28 to 48 kilodaltons in size (Hancock, 1987). In the

Figure 1.1. Bacterial substrate transport systems. Class I group represents the multicomponent primary transport system which accumulates nutrients against a concentration gradient. Class 2 group represents the secondary transport system where substrate transport is coupled to the co-transport of proton or sodium ions, and driven by the PMF. Class 3 group represents the transporters which require both PMF and periplasmic binding proteins. Abbreviations: S, substrate; OM, outer membrane. Adapted from Driessen *et al* (1997) as depicted in Adewoye, L., Ph.D. thesis (1999).


S
OM
S
PM
S-BP
S
H+/ Na+
H+/Na+
H+/Na+
IM
ATP
ATP
ADP + Pi
ADP + Pi
S
S
CLASS 1
CLASS 2
CLASS 3

membrane, they usually function as trimers (Nakae *et al*, 1979). These proteins are integral to the outer membrane structure, and are often found closely, but not covalently, linked to the peptidoglycan matrix (Rosenbusch, 1974). These proteins provide the cell with channels to allow for the passage of small, hydrophilic molecules across the outer membrane. Most porins act like a sieve, in that molecules are able to pass through based on their size. If their size exceeds the maximum which the porin can handle, that molecule is excluded from transport. The exclusion limit of a porin depends on the size of the aqueous channel formed by the oligomer (trimer) (Hancock, 1987). Some porins exhibit specificity for a particular substrate via a binding site, as is found for the phosphate-specific OprP protein from *P. aeruginosa* (Poole and Hancock, 1986), and the maltose-specific LamB protein from *E. coli* (Ferenci *et al.*, 1980).

Once the molecule has successfully traveled through the outer membrane, it must now traverse the periplasmic space. For this to happen, a periplasmic binding protein binds its substrate, and carries it from the inner surface of the outer membrane to the outer surface of the inner membrane. The periplasmic binding proteins are monomeric in structure and function, and those identified to date range in size from 25 to 56 kilodaltons (Ames, 1986, Furlong, 1986). X-ray crystallography of the leucine-, isoleucine-, valine-binding protein (LIV-BP) (Saper and Quiocho, 1983) and the galactose-binding (Vyas and Quiocho, 1983) proteins of *E. coli*, and the sulphate-binding proteins of *Salmonella typhimurium* (Mowbray and Petsko, 1983) show that the general shape of these proteins is bilobate. The substrate- binding site is in the cleft between the lobes, and upon binding, a conformational change has been demonstrated for several of these proteins (Ames,

1986). Shen *et al* (1985) demonstrated through NMR spectroscopy, that the protein-substrate interaction for the *E. coli* glutamine-binding protein occurs through hydrogen bonding. Indirect evidence supports the hypothesis for interaction between periplasmic binding proteins and inner-membrane components of the permeases.

Two domains of homology have been identified between binding proteins, suggesting that these areas are important enough to protein function to have been conserved evolutionarily (Higgins and Ames, 1981). One is likely the substrate binding site, and the other is believed to be a recognition site for interaction with the inner membrane components.

There are two types of proteins which comprise the cytoplasmic membrane-associated components of periplasmic permeases. There are two hydrophobic proteins embedded in the inner membrane, involved in the transport of the substrate into the cell, and two peripherally-associated hydrophilic proteins, located on the cytoplasmic face of the inner membrane, involved in the energy coupling to the transport process (Higgins, 1990).

The hydrophobic membrane components have low sequence homology to one another, but are structurally similar, having five or six potential membrane-spanning α-helices (Hiles *et al*, 1987). Helices are separated by short, hydrophilic sequences, one of which appears to be conserved in several of these proteins, and is believed to interact with the peripherally-associated, inner membrane hydrophilic proteins (Dassa and Hofnung, 1985). Although there is variation in size and number of the integral inner membrane proteins, all of them form a transmembrane pore for the passage of substrate (Higgins *et*

al, 1990). There is also indirect evidence, according to Treptow and Shuman (1985), that the cytoplasmic membrane components possess a binding site specific for the substrate.

One or two of the hydrophilic intracellular proteins, peripherally associated in the cytoplasmic membrane, function to provide energy for the transport process (Higgins *et al*, 1986). If only one exists, it functions as a homodimer (Higgins, 1986). It is still unclear as to how exactly their association with the inner membrane proteins occurs. Studies done indicate that they are associated with the integral inner membrane proteins at the cytoplasmic face of the inner membrane as they are only susceptible to proteolytic digestion on that side of the membrane (Gallagher *et al*, 1989). However, the same study also showed that the proteins are tightly associated with the membrane, even in the absence of the integral membrane components. This seems to indicate that there are two recognition sites on these proteins, one to allow for association with the membrane itself, and another to bring it in contact with the integral membrane proteins.

Cytoplasmic membrane-bound permeases, similar to the lactose transport system of *E. coli*, and unlike periplasmic permeases, are driven by the proton motive force (PMF) and transport can be stopped by metabolic inhibitors or uncouplers of the PMF. Periplasmic permeases have been shown to require either ATP hydrolysis, acetyl phosphate, $NADPH_2$, lipoic acid, or succinate for transport (Berger, 1973; Higgins *et al*, 1990). There are three well-characterized systems having consensus ATP-binding sites which bind ATP or ATP analogues through the hydrophilic cytoplasmic membrane proteins (OppD of *S. typhimurium*, and HisP and MalK from *E. coli* (Higgins *et al*, 1985). ATP

hydrolysis has been found to be coupled to the transport process at a rate of one or two molecules of ATP per molecule of substrate entering the cell (Mimmack *et al*, 1989).

Periplasmic permeases are not restricted to Gram-negative bacteria. Systems which are analagous to those seen in Gram-negative cells have been identified in eukaryotes and archaea (Jovell *et al*, 1997). Through studies on *Methanococcus mazei*, a gene cluster encoding proteins with sequence motif, structure, hydrophobicity and hydrophilicity profiles to eubacterial and eukaryotic ABC (ATP Binding Cassette) transporter homologs. In the same year, another group, Koonin *et al* (1997) analyzed the sequence of *Methanococcus jannaschii*, discovering approximately 124 predicted ATPases and GTPases with Walker-type ATP-binding motifs characteristic of ABC transporters.

Eukaryotic ABC transporters function as membrane transport proteins for the ATP-driven expulsion of drugs from the cell. Multidrug resistance to structurally or functionally unrelated chemicals/drugs in *Saccharomyces cerevisiae* has been attributed to over-expression of ABC transporters (Gottesman and Pastan, 1993). Gilson *et al* (1988) reported evidence that *Streptococcus pneumoniae* and a mycoplasma, *Mycoplasma hyorhinis* possess the equivalent to the high affinity periplasmic binding-protein dependent transport systems, termed extra-cytoplasmic binding lipoprotein dependent transport systems. Since these organisms possess no outer membrane, the extra-cytoplasmic protein is maintained in proximity to the cytoplasmic membrane via their N-terminal lipo-amino acid anchor. This proposed mechanism is supported by evidence put forth by Fykes and Bassford (1987). Their research with *E. coli* showed that a mutant MalE protein,

anchored by its uncleaved NH_2-terminal signal peptide to the external face of the cytoplasmic membrane could still operate in the transport of maltose.

The common organization of these high affinity transport systems for the transport of different substrates and their existence in a wide variety of different bacterial species could reveal a common evolutionary origin. How exactly the systems evolved from their common ancestor remains a mystery. Did the ancestral system possess a lipoprotein which evolved into a regular protein, or the opposite?

1.3 The Maltose Transport System of *Escherichia coli*

The best-characterized permease is the maltose transport system of *E. coli*. First described by Monad and Torriani (1948), it is generally used as a model for system function. To date, there have been five proteins discovered which are essential for maltose and maltodextrin uptake by the cell. There are five other components of the maltose regulon, four of which play a role in the transport system, and a fifth which has an unknown function (Schwarz, 1986).

The outer membrane porin (LamB) is a trimer of the *lamB* gene product, with three specific maltose-binding sites per trimer (Gehring *et al*, 1990), and interacts with the periplasmic maltose-binding protein (MBP). MBP transports the maltose through the periplasm where it interacts with the integral inner membrane components, MalF and MalG. The MalF and MalG proteins act conjointly to form a pore through the cytoplasmic membrane. MalG serves a second function in anchoring MalK to the cytoplasmic face of the inner membrane. MalK binds ATP and provides the energy source for the translocation of maltose into the cytoplasm where it can be metabolized (Schwarz, 1986).

The LamB protein is comprised of 421 amino acids and weighs approximately 47.4 kDa, as determined by its gene sequence. It functions as a trimer, with each subunit identical, forming an ionic pore in the outer membrane. Each subunit is capable of producing 13-17 hydrophobic, membrane-spanning helices (Schwarz, 1986). The pore exclusion limit is 600 daltons, allowing the transport of maltodextrins up to the size of maltoheptaose (Neuhaus *et al*, 1983). This pore not only functions in carbohydrate transport, but also serves as a receptor for phage lambda and similar phages (Schwarz, 1986). There are an estimated 10^5 monomers per cell which translates into 1-2 percent of the total cellular protein, significantly more than any other single component of this transport system (Schwarz, 1986).

LamB may act alone, through a binding site, or in conjuction with the periplasmic MBP to convey specificity to the permease (Neuhaus *et al*, 1983). The MBP is the gene product of *malE* and is localized in the periplasmic space of *E. coli* (Boos and Staehelin, 1981). It was believed to be essential for transport of maltose and maltodextrins, as mutants lacking MBP are transport deficient (Schuman, 1986). However, more recent studies have shown that it is possible for the system to function independently of the MBP (Treptow and Shuman, 1985, 1988). Conductance studies performed in the presence of MBP show three distinct steps in the opening and closing of the channel. They are believed to reflect the cooperative opening and closing of three channels (one formed from each subunit of the LamB trimer). The dissociation constant (K_d) of maltoporin from MBP has been determined to be 1.5×10^{-7}M, and is more favored by the closed state of the outer membrane maltoporin (Schwarz, 1986). There is some interaction required between

LamB and MBP, as mutants in the MBP structural gene produce MBP incapable of interacting with LamB, resulting in transport-deficient phenotypes (Bavoil *et al*, 1983). The nature and function of this interaction has yet to be determined, but studies suggest that these mutants are defective in their ability to transport maltose across the outer membrane (Wandersman *et al*, 1979).

Although often purified as a multimer, the functional form of MBP appears to be monomeric, dissociating upon binding maltose (Richarme *et al*, 1982). The DNA sequence reveals a protein of 370 amino acids, with a molecular weight of approximately 40.7 kDa. There are 2-4 x 10^4 monomers per cell, comprising about 0.3 percent of total cellular protein (Manson *et al*, 1985).

The MBP also functions in positive maltose chemotaxis. It interacts with the TAR (taxis to aspartate and some repellents) protein located in the cytoplasmic membrane. This interaction appears to be key in the interaction between maltose-loaded MBP and the cytoplasmic membrane as transport is eliminated in *tar*-defective mutants (Richarme, 1981). Unlike aspartate chemotaxis, the maltose-loaded MBP requires binding to the chemotaxis receptor protein (Risharme *et al*, 1982). Although MBP is required for positive maltose taxis and maltose transport, only 9 percent of the wild type level of MBP is required for full transport activity while 25 percent is required for maltose chemotaxis (Manson *et al*, 1985).

After MBP binds maltose, a conformational change occurs, allowing interaction with TAR and the inner membrane components of the maltose periplasmic permease (gene products of *malF* or *malG*, Treptow and Shuman, 1988). While maltose binding provides

specificity to the permease, the conformational change likely provides directionality to the transport system (Manson *et al*, 1985). In addition to the LamB and MBP proteins, there may be other components of the system which convey specificity for maltose. Maltose analogues lacking a reducing end on the glucose unit show wild type interaction with LamB and MBP, but cannot be transported into *E. coli* by the maltose transport system. Neither LamB or MBP require a reducing end on the glucose unit for binding, but it is clearly required for the transport system (Ferenci *et al*, 1986).

The proteins MalF, G and K are the gene products of *malF*, *G* and *K*, respectively. These three proteins are associated with the inner membrane of the cell providing a pore and the energy for transport from the periplasmic space into the cytoplasm. These proteins come together to form a $MalFGK_2$ complex (Davidson and Nikaido, 1991), which allows for translocation of the substrate across the membrane. The presence of substrate in media is thought to be signaled by liganded MalE via interactions with externally exposed loops of MalF and/or MalG (Davidson *et al.*, 1992). Consequently, upon this interaction, conformational changes of MalF and/or MalG are transmitted to the MalK subunits, which then become activated. ATP hydrolysis would then trigger subsequent conformational changes that would lead to the actual translocation of the substrate molecule (Hunke et al., 2000). Experimental data seems to indicate that it is MalK which becomes embedded in the membrane via its helical domain, and surrounded by MalF and MalG (Mourez et al, 1998; Schneider et al., 1995; Hunke, 2000).

The molecular weight of MalF, as determined from its proposed amino acid sequence is approximately 56.9 kDa. Within the protein, there are 6-8 potential

membrane-spanning regions. It functions as a heterodimeric pore through the inner membrane in conjunction with MalG. The molecular weight of MalG is 32.2 kDa and it is also a hydrophobic protein. There is strong sequence homology between the 90 amino acids at the C-terminus of the two proteins (MalF and G). Both these proteins contain a large periplasmic domain of unkown function (Panagiotidis and Shuman, 1998), which could play a role in forming the multisubunit complex at the cytoplasmic membrane.

MalG contacts MalK at a point on the cytoplasmic face of the inner membrane, and serves as a membrane anchor for the MalK (Bavoil et al., 1980; Shuman and Silhavy, 1981; Schwarz, 1986).

Mutagenesis studies of MalF (Tapia et al., 1999) suggest that the first periplasmic loop (P1) is not crucial for the formation/assembly of the MalFGK2 complex or for the constitution of the maltose translocation pathway, but likely contributes to the stability of the protein. P2 appears to be involved in anchoring MalK into the membrane. Partial deletions of loop P4 resulted in high steady-state levels of protein, which were defective in transport and partially mislocated. Data suggests that the mutation likely affects transport by either modifying a substrate binding site or by preventing interaction with MalE. Mutations in the last periplasmic loop of MalG have been proposed to affect substrate recognition (Dassa, 1993), or interaction with MalE (nelson and Traxler, 1998). If these theories are correct, the last periplasmic loops of MalF and MalG might function in the same manner. Cytoplasmic loop C2 likely affects transport-specific functions, while C3 may be involved in the attachment of MalK to the membrane.

Studies of a mutant *E. coli* strain suggest that either the MalF or MalG proteins possess a recognition site for maltose. This mutant is MBP-deficient, yet has maltose binding activity and specificity. These properties are conveyed through another protein component of the system (either MalF or MalG) which is able to interact with contents of the periplasmic space. Maltose binding by one of the cytoplasmic membrane components explains the ability to have transport without the periplasmic MBP. Perhaps the nature of the interaction between MBP and the inner membrane components is via the maltose substrate (Ames *et al*, 1990).

The MalK protein has a molecular weight of approximately 40.7 kDa, as determined by its amino acid composition and does not appear to contain any obvious hydrophobic regions (Morbach et al., 1993). It contains an ATP binding site and has thus been implicated in providing the energy source for the transport process (Ames *et al*, 1990). MalK mutants suggest that there are two additional roles for this protein besides transport. It negatively regulates the expression of the *mal* operons and regulates transport by Enzyme III^{GLC} in the phosphotransferase system (PTS) (Kuhnau *et al*, 1991).

Diederichs et al. (2000) studied the crystal structure of MalK, the ATPase subunit of the trehelose/maltose ABC transporter of *Thermococcus litoralis* and found it to be a dimer formed by the association of the ATPase domains, with the two regulatory domains attached at opposite poles.

There have been a number of substrates hypothesized to be involved as energy sources for maltose transport in *E. coli*. Most recently, Dean *et al*. (1989) showed that ATP hydrolysis was absolutely essential for the transport of maltose into right-side-out

membrane vesicles. The proton motive force has been shown not to be essential for normal levels of transport in the maltose transport system. One or two ATP are believed to be required for the uptake of one maltose unit, although there are other experiments which dispute that ratio (Ames *et al*, 1990).

The maltose regulon encompasses three operons. These are the *malA* region, located at 75 minutes, the *malB* region at 91 minutes, and the *malS* region at 80 minutes on the *E. coli* chromosome. The *malA* region, also known as the *malPQ* operon, is a divergent operon containing *malP* and *malQ* on one operon, and *malT*, the transcriptional activator, on the other. The *malB* region is also divergent, containing *malE*, *malF*, and *malG* genes on one operon, and *malK*, *lamB*, and *malM* genes on the other. Finally, the *malS* gene encoding α-amylase is independent (Schwarz, 1986).

The transcriptional activator, MalT, has a molecular weight of approximately 103.0 kDa, as determined from its amino acid composition (Schwarz, 1986). It is activated by maltose and then activates transcription by binding the DNA at the promotor region of all the operons which are part of the maltose regulon. Mutants of MalT have been isolated which constitutively express the maltose regulon. The two types of mutants isolated show mutations in one of two clustered areas of their DNA (Dardonville and Raibaud, 1990). A second important regulator of the maltose regulon is the catabolic activator protein (CAP). It activates each of the genes involved including the *malT* gene, thus activating the positive regulator of the system (Schwarz, 1986).

Several other components function in, but are not required, for maltose transport. The MalS is a 66 kDa α-amylase which is located in the periplasmic space. As

maltoheptaose is the largest maltodextrin which can be used by *E. coli* through this transport system, α-amylase functions to cut down large maltodextrins which can pass through the outer membrane porin LamB. MalS may allow transport of larger maltodextrins by liberating some glucose or maltose units from the polymer (Schwarz, 1986).

MalQ and MalP are located in the cytoplasm of *E. coli*, and are essential for the metabolism of maltodextrins. MalQ is a 70 kDa amylomaltase which catalyzes the hydrolysis of maltodextrins liberating free glucose which can then be used by the cell for various purposes (Schwarz, 1986). MalP, or maltodextrin phosphorylase, is a homodimer of approximately 90.3 kDa units. It can use the polyglucoside created by the amylomaltase to make glucose-1-phosphate which can then be metabolized by the cell (Schwarz, 1986).

MalM was discovered as part of the maltose regulon. It encodes a 306 amino acid protein which is excreted into the periplasm but whose function remains unkown (Clement and Hofnung, 1981).

This well-characterized system can easily serve as a model with which to compare when studying transport of less well-characterized transport systems for other Gram-negative bacteria, such as the glucose transport system of *P. aeruginosa and P. putida*.

1.4 The High Affinity Glucose Transport System of *Pseudomonas aeruginosa*

Previous studies done on the high affinity glucose transport system of *P. aeruginosa* and *P. putida* suggest that it may be similar to that of the well-characterized maltose transport system of *E. coli*. There are two main mechanisms by which *P. aeruginosa* and *P. putida* may transport glucose. The first is the low affinity system which requires membrane-bound glucose- and gluconate dehydrogenases to oxidize glucose into

gluconate and 2-ketogluconate in the periplasm. Subsequently, these products are converted to 6-phosphogluconate which then enter the Entner-Doudoroff pathway (Figure 1.2) [Cuskey and Phibbs, 1985, Cuskey *et al*, 1985]. The K_m for this low affinity system is 1-2 mM (Hancock and Carey, 1980).

P. aeruginosa also possesses a high affinity system for glucose transport. It appears to be a typical Gram-negative periplasmic permease similar to the maltose transport system of *E. coli*. As previously described, a periplasmic permease transports its substrate (in this case, glucose) into the cytoplasmic space where it is phosphorylated and then enters the Entner-Doudoroff pathway (Cuskey and Phibbs, 1985, Cuskey *et al*, 1985). It is an inducible, high affinity system which has a K_m of 7-8 μM (Guymon and Eagon, 1974, Midgley and Dawes, 1973).

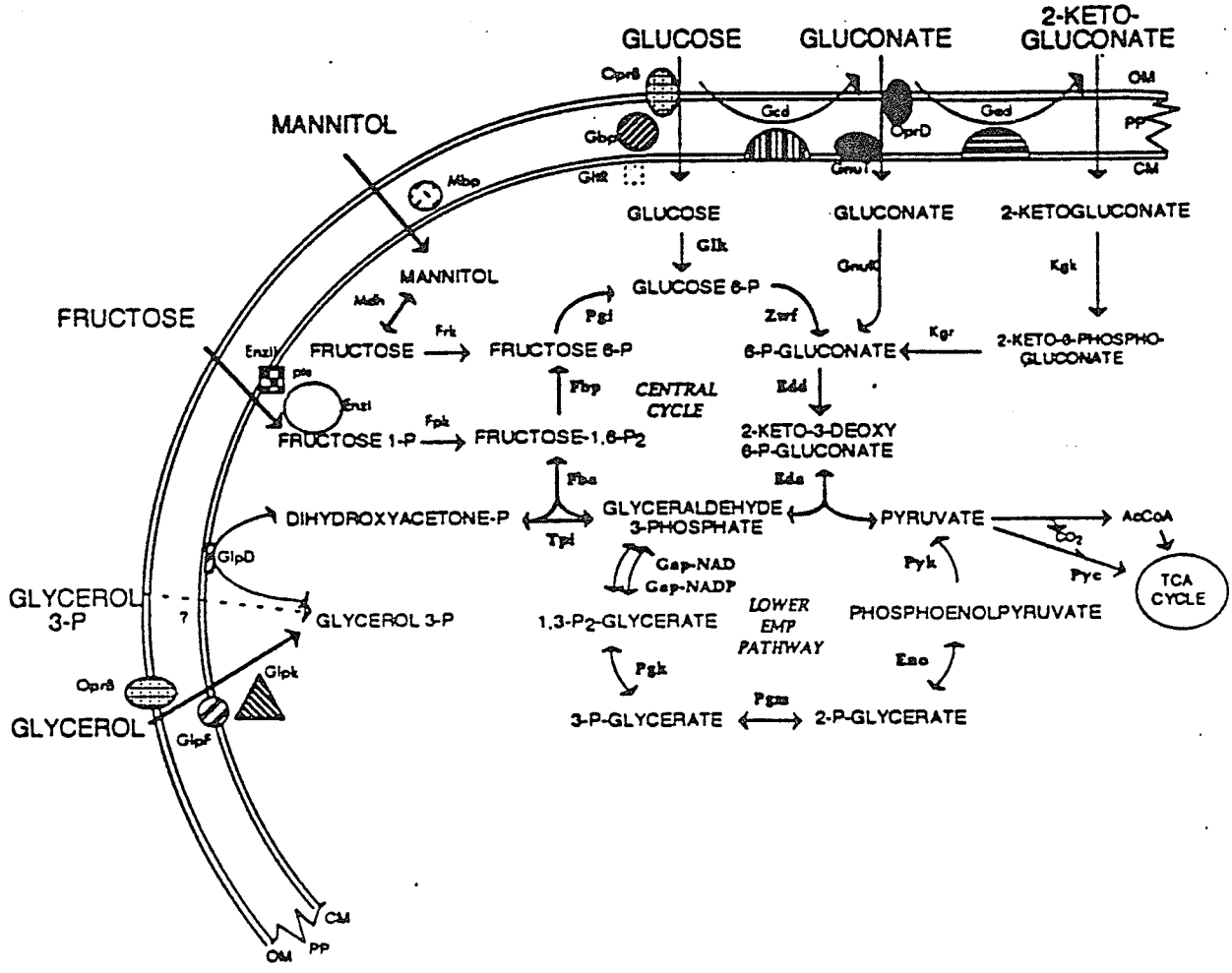
The high affinity glucose transport system is induced by growth on glucose, fructose, mannose, pyruvate plus galactose, 2-deoxy-D-glucoside, and α-methylglucoside but repressed by growth on gluconate, succinate, acetate, and citrate (Cuskey and Phibbs, 1985, Stinson *et al*, 1976, Guymon and Eagon, 1974; Midgley and Dawes, 1973; Mukkada *et al*, 1973; Eagon and Phibbs, 1971). It is both energy and temperature dependent. This system is saturable, suggesting an active transport system involving an enzyme-like carrier (Eagon and Phibbs, 1971). Glucose is internalized as a free sugar and trapped in the cell by phosphorylation (Eagon and Phibbs, 1971). Although the glucose analogue, α-methylglucoside, can also be internalized by this system, its affinity is much lower with a K_m of 7 mM. It can only accumulate in the cell at a concentration of two fold greater than

its external concentration whereas glucose accumulates at 200 fold its external concentration (Guymon and Eagon, 1973).

Upon growth on glucose, specific transport proteins are induced: an outer membrane porin protein (OprB-formally known as D1; Hancock and Carey, 1980), a periplasmic glucose binding protein (GBP) [Cuskey and Phibbs 1985], a two component response regulator protein, GltR (Sage *et al.*, 1996), and several inner membrane ABC transporter proteins, GltF, GltG, and GltK (Adewoye and Worobec, 2000). In addition, induction of glucose catabolizing enzymes also occurs, including glucokinase, glucose-6-phosphate dehydrogenase, dehydratase, and aldolase which function together to convert intracellular glucose to pyruvate and glyceraldehyde-3-phosphate which then enters the Entner-Doudoroff pathway (Hunt and Phibbs, 1983). Figure 1.2 shows the pathways of carbohydrate catabolism in *P. aeruginosa*, including those for glucose. The inducer of these enzymes is believed to be 6-phosphogluconate, an intermediate in the pathway (Hunt and Phibbs, 1983). The induction of these enzymes is prevented by growth on succinate and pyruvate, showing some possible similarities between their regulation and the regulation of the high affinity glucose transport system. However, α-methylglucoside and 2-deoxyglucose, which induce the high affinity glucose transport system, inhibit the production of glucose-catabolizing enzymes (Hylemon and Phibbs, 1972).

OprB, from *P. aeruginosa,* has been shown to be coinduced with the high affinity glucose transport system (Hancock and Carey, 1979, Mizuno and Kageyama, 1978). It was believed to be a glucose-specific channel because of its channel forming properties

Figure 1.2. Pathways of carbohydrate metabolism in *Pseudomonas aeruginosa*. Abbreviations: OM, outer membrane; CM, cytoplasmic membrane; PP, periplasm; Gad, gluconate dehydrogenase; Gcd, glucose dehydrogenase; Gbp, glucose binding protein; Mbp, mannitol binding protein; Kgk, 2-ketogluconate kinase; Gnuk, gluconate kinase; GnuT, gluconate permease; Kgr, 2-keto-6-phosphogluconate reductase; Glk, glucokinase; Zwf, glucose-6-phosphate dehydrogenase; Edd, 6-phosphogluconate (Entner-Doudorof) dehydratase; Eda, 2-keto-3-deoxy-6-phosphogluconate aldolase; Pyc, pyruvate carboxylase; Pyk, pyruvate kinase; Gap, glyceraldehyde 3-phosphate dehydrogenase; Tpi, triose phosphate isomerase; Pgm, phosphoglucoisomerase; Fba, fructose 1,6-bisphosphate alsolase; Fbp, fructose 1,6-bisphosphate; Pgi, phosphoglucoisomerase; Frk, fructokinase; Mdh, mannitol dehydrogenase; Fpk, fructose -phosphate kinase; GlpD, glycerol 3-phosphate dehydrogenase; GlpK, glycerol kinase. Reproduced from Temple *et al*, 1998.


GLUCOSE
GLUCONATE
2-KETO-GLUCONATE
OM
PP
CM
OprB
Gcd
OprD
Gad
Gbp
Mbp
Glt2
Gnu1
MANNITOL
GLUCOSE
GLUCONATE
2-KETOGLUCONATE
Glk
Gnu0
Kgk
MANNITOL
Mdh
GLUCOSE 6-P
Pgi
Zwf
FRUCTOSE
Frk
FRUCTOSE
FRUCTOSE 6-P
6-P-GLUCONATE
Kgr
2-KETO-6-PHOSPHO-GLUCONATE
EnzII
pts
EnzI
Fbp
CENTRAL CYCLE
Edd
FRUCTOSE 1-P
Fpk
FRUCTOSE-1,6-P2
2-KETO-3-DEOXY 6-P-GLUCONATE
Fba
Eda
DIHYDROXYACETONE-P
GLYCERALDEHYDE 3-PHOSPHATE
PYRUVATE
AcCoA
Tpi
CO2
GlpD
Gap-NAD
Gap-NADP
Pyk
Pyc
TCA CYCLE
GLYCEROL 3-P
?
GLYCEROL 3-P
1,3-P2-GLYCERATE
LOWER EMP PATHWAY
PHOSPHOENOLPYRUVATE
OprB
Glpk
Pgk
Eno
GLYCEROL
GlpF
3-P-GLYCERATE
Pgm
2-P-GLYCERATE
CM
OM
PP

and its coinduction with the high affinity glucose transport system. Hancock and Carey (1980) showed that this protein was distinct from the periplasmic GBP as it bound less than 0.01 nmol glucose/mg protein compared to 0.16 nmol glucose/mg for the glucose binding protein. Further, when reconstituted into LPS-phospholipid vesicles, this protein caused a flux of radiolabelled glucose out of the vesicles. Sucrose was also shown to pass through this pore (Hancock and Carey, 1980).

The monomeric molecular weight of OprB is 45-46 kilodaltons (Hancock and Carey, 1979, Hancock and Carey, 1980, Trias *et al*, 1987). Like similar outer membrane porin proteins, it functions in the membrane as an oligomer. Chemical cross-linking studies revealed that its native state in the membrane is trimeric (Angus and Hancock, 1983).

Trias and associates (1987) performed studies using proteoliposomes reconstituted with OprB. Various sugars were assayed for their ability to pass through the OprB pore. Sugars with exceptional ability to enter into proteoliposomes for their size included D-xylose and D-glucose with the b form preferred over the a form. Unlike OprF, a non-specific outer membrane porin protein from *P. aeruginosa*, the permeation rates of sugars through OprB was not directly proportional to the molecular weight of the substrate being transported. This suggests that OprB has specificity for its preferred substrate (glucose), perhaps conveyed by a binding site within the porin channel (Trias *et al*, 1987). This was then confirmed by Wylie *et al.* (1993), where it was demonstrated using black lipid bilayer analysis (Benz *et al.*, 1985, 1987) that OprB possessed a glucose binding site. In the absence of glucose, OprB facilitates the diffusion of other carbohydrates such as mannitol, glycerol, and fructose, which are used by *P. aeruginosa* for growth (Wylie and Worobec,

1995). OprB acts as a central component for carbohydrate uptake though it functions primarily in glucose uptake. Its ability to transport other sugars depends on how similar the stereochemistry of the other substrates is to glucose (Wylie and Worobec, 1993 a, b; Wylie and Worobec, 1994; Wylie and Worobec, 1995, Wylie, J. PhD thesis, 1994).

OprB was previously believed to be involved in the transport of imipenem, an antibiotic used in the treatment of severe *P. aeruginosa* infections. Cells resistant to imipenem were found to be deficient in an outer membrane protein of molecular weight 45-46 kDa (Büscher *et al*, 1987). This protein has since been shown to be OprD, a constitutively expressed outer membrane protein of very similar molecular weight to OprB (Trias and Nikaido, 1990a, Trias and Nikaido, 1990b).

The second component of the high affinity glucose transport system which has been identified is the periplasmic glucose binding protein (GBP). Interestingly, its activity, not its level of synthesis, is inhibited by ten-fold increases in the concentration 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 (K_d=0.35 mM) for the protein-substrate complex (Stinson *et al*, 1977). Like binding proteins described from other periplasmic permeases (Boos, 1974), GBP undergoes a substrate-induced conformational change (Stinson *et al*, 1976) but the substrate is translocated without alteration.

The molecular weight of GBP is 44.5 kDa 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. This

protein was originally believed to be a glycoprotein as it was isolated associated with sugar (Stinson *et al*, 1977). A review of the literature suggests that it is more likely that the protein was isolated with tightly bound substrate.

Stinson *et al* (1977) have isolated a mutant strain, MB723, which lacks both glucose dehydrogenase and GBP activity. This strain is defective in both membrane transport and glucose chemotaxis, thus implicating GBP as an important factor in glucose chemotaxis. Periplasmic binding proteins function as chemoreceptors in systems such as the maltose (Hazelbauer, 1975) and galactose (Hazelbauer and Adler, 1971) transport systems in *E. coli* and the ribose transport system in *S. typhimurium* (Askamit and Koshland, 1972).

The *P. aeruginosa* GBP gene (*gltB*), has been located near several other genes involved in glucose metabolism (Cuskey and Phibbs, 1985). After being isolated on a 6.0 kb *Eco*RI fragment of DNA, the gene was shown to reconstitute GBP expression and activity (Sly and Worobec, 1993) in two mutant strains which lacked GBP activity, PFB360 and PFB362 (Cuskey and Phibbs, 1985, Cuskey *et al*, 1986). 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 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 (Tempel *et al*, 1990).

Another protein which appears to be an integral component of this transport system is GltR, a two-component response regulator. This protein was characterized upon further investigation by Sage *et al.* (1996) after it was shown that a 1.1 kb fragment from *P. aeruginosa* restored glucose transport activity, binding, and chemotaxis (Sly and Worobec, 1993). Upon sequencing, it was discovered that this fragment did not contain *gltB* as originally thought (Sly and Worobec, 1993), but rather a gene researchers designated *gltR* due to its homology to the OmpR family of two-component response regulators (Sage *et al*, 1996). Several residues which are conserved among response regulator proteins were found conserved in this protein, strengthening the view that this was indeed a response regulator protein (Parkinson and Kofoid, 1992; Sage et al., 1996). In an attempt to determine if *gltR* was important to glucose uptake, the gene was insertionally disrupted (Sage *et al*, 1996), and resulting mutants studied for loss of glucose transport activity. GltR-deficient mutants were found to exhibit a loss in glucose transport activity similar to that described by Cuskey *et al.*, 1985) for glucose transport mutants. Glucose-positive revertants displayed glucose transport activity similar to that observed for wild type strains. These results demonstrated that *gltR* encoded a protein (GltR) necessary for expression of the inducible glucose active transport system in *P. aeruginosa*. In combination with the fact that GltR was homologous to the two-component regulator family of proteins, this protein was deemed a response regulator, in control of genes necessary for transport, presumably *gltB* and *oprB*.

The most recently characterized component of the high affinity transport system in *P. aeruginosa* is *gltK*. Adewoye and Worobec (2000) identified an open reading frame

(ORF), which, upon sequencing, exhibited homology to the *P. fluorescens mtlK*. Sequence alignment analysis revealed that the *P. aeruginosa gltK* gene product is a member of the MalK subfamily of ABC proteins.

The *gltK* gene product is a membrane-associated protein, and was found to possess a transmembrane helix which is well conserved among ATP-binding proteins from eukaryotes, eubacteria and archaea (Higgins, 1992). As well, there seems to be a putative helical domain which is well conserved among the MalK subfamily of traffic ATPases (Wilken et al, 1996; Schneider and Hunke, 1998). Southern blotting revealed homologues of this gene exist in *P. fluorescens*, *P. chlororaphis*, and *P. putida* (Adewoye and Worobec, 2000), suggesting widespread conservation within this genus.

GltK deficient mutants show decreased glucose transport (Adewoye and Worobec, 2000), indicating the importance of this protein for transport, but without a change in Km as compared to wild type, suggesting that affinity for glucose is not affected by GltK. Unlike the *E. coli* MalK which exhibits an ATPase role in addition to its regulatory role on genes in the maltose regulon, the *P. aeruginosa* GltK does not appear to be involved in regulation of the glucose ABC transporter.

Another interesting discovery was the conservation of two hydrophobic amino acid residues within the helical domain of GltK. They appear to be homologous to two conserved hydrophobic amino acid residues of the *E. coli* MalK that have been shown to "Mediate" the $MalFGK_2$ protein interaction with the cytoplasmic membrane. Two conserved domains, designated Walker motifs A and B, and a putative helical domain located in front of Walker motif B have been identified in ATPase components of several

bacterial solute transport systems mediated by periplasmic binding proteins (Walker *et al.*, 1982).

These findings strongly suggest that the *P. aeruginosa* transport system again functions like that of the *E. coli* maltose transport system and likely forms a complex similar, if not identical to, the MalFGK$_2$ complex. Further studies must be done to confirm this possibility.

1.5 The High Affinity Transport System of Pseudomonas putida

Pseudomonas putida is a Gram negative bacterial species belonging to the fluorescent branch of the *Pseudomonadaceae* family, of which *P. aeruginosa* and *P. fluorescens* are also members (Stanier *et al.*, 1966). The outer membranes of *P. putida* and other related pseudomonads are comprised of a number of proteins which are homologous biochemically, immunologically, and genetically to those described for *P. aeruginosa*. All these bacteria have been shown to possess common pathways for glucose transport (Agbanyo and Taylor, 1985; Lessi and Phibbs, 1983). Vicente *et al.* (1973) first proposed that the initial step in glucose- and gluconate degradation of *P. putida* consisted of glucose being converted to gluconate-6-phosphate via gluconate and 2-ketogluconate. *P. putida* has been shown to utilize glucose primarily via oxidation to 2-ketogluconate (Deley, 1960; Frampton and Wood, 1961; and Vicente and Canovas, 1973), while direct phosphorylation of gluconate was a minor pathway not contributing to significant growth.

This system differs from that of *P. aeruginosa* in several ways. *P. aeruginosa* has an inducible pyridine nucleotide-dependent glucose-6-phosphate dehydrogenase activity

(Lessie and Neidhardt, 1967) while *P. putida* possesses an active system to oxidize glucose to gluconate by means of an oxygen-dependent dehydrogenase. *P. putida* has two forms of glucose dehydrogenase: a thermostable form bound to the membrane and a heat labile form which is soluble (Vicente and Canovas, 1973). In 1974, it was demonstrated by Vicente *et al.* that both glucose and gluconate follow typical saturation kinetics, with the Km for glucose being 6 mM and for gluconate 0.5 mM. Further investigation showed parallel inhibition between glucose and gluconate uptake (Vicente *et al.*, 1975). Strains deficient in glucose transport were also deficient in gluconate transport and vice-versa. Another difference between the two closely related species is that gluconate induces glucose transport in *P. putida* (Vicente *et al*, 1974), but not in *P. aeruginosa* (Guymon *et al.*, 1974), suggesting that a common carrier molecule exists for the two substrates in *P. putida*.

As with *P. aeruginosa*, glucose and gluconate transport was found to be inducible in *P. putida*, followed saturation kinetics, and could be specifically blocked by genetic manipulation. This behavior is consistent with an active transport system mediated by an enzyme-like carrier molecule. Another commonality between the two species' high affinity transport systems is the conservation of the outer membrane protein, OprB. Saravolac *et al.*, (1991) demonstrated that the glucose-inducible, organic acid-repressible OprB of *P. putida* had glucose-specific pore-forming activity, as well as functional homology between the OprB from *P. aeruginosa* and *P. putida*. OprB in *P. putida* was found to act as a glucose-specific porin in the outer membrane (Saravoloc *et al.*, 1991) and co-regulated with the high affinity glucose transport system, as it is with *P. aeruginosa* (Guymon *et al.*,

1974; Hancock and Carey, 1980). OprB from both *P. aeruginosa* and *P. putida* show heat modifiability on SDS-PAGE, which has been attributed to the high β-sheet content on outer membrane proteins (Hancock *et al.*, 1990).

While minimal DNA homology and different monomeric molecular weights between the OprB proteins from *P. aeruginosa* and *P. putida* exists, the two do have similar amino acid composition and similar SDS-PAGE mobilities in the heat-unmodified form (Saravolac *et al*, 1991). Wylie *et al.* (1994) identified several pseudomonad species carrying the OprB gene, but of the representative species examined, only members of the rRNA Group I possessed a protein similar to the *P. aeruginosa* OprB. Immunochemical and genetic analysis suggests that *P. putida* and *P. chlororaphis* OprB proteins are most closely related to the *P. aeruginosa* OprB.

When comparing the binding site of the *P. putida* OprB to its *E. coli* equivalent, the maltodextrin-specific porin, LamB, there appeared to be a difference in the sugar-binding characteristics (Benz *et al.*, 1986; Benz *et al.*, 1987). The binding activity of LamB increases as the pore size of the maltodextrin increases from 2 to 5 units, and then remains constant for maltodextrins with 6 and 7 units. Although the *P. putida* OprB binds glucose and maltose with similar affinities measured for LamB, it binds maltotriose more poorly than maltose, and maltotetrose extremely poorly (Saravolac *et al.*, 1991). This suggested to investigators that the OprB binding site was shorter than that for LamB, with the binding site corresponding to two glucose molecules. There were also differences in their channel specificity. LamB binds galactose 2.5-fold better than glucose and binds sucrose almost as well as maltose (Benz *et al.*, 1987), whereas OprB has a 3-fold

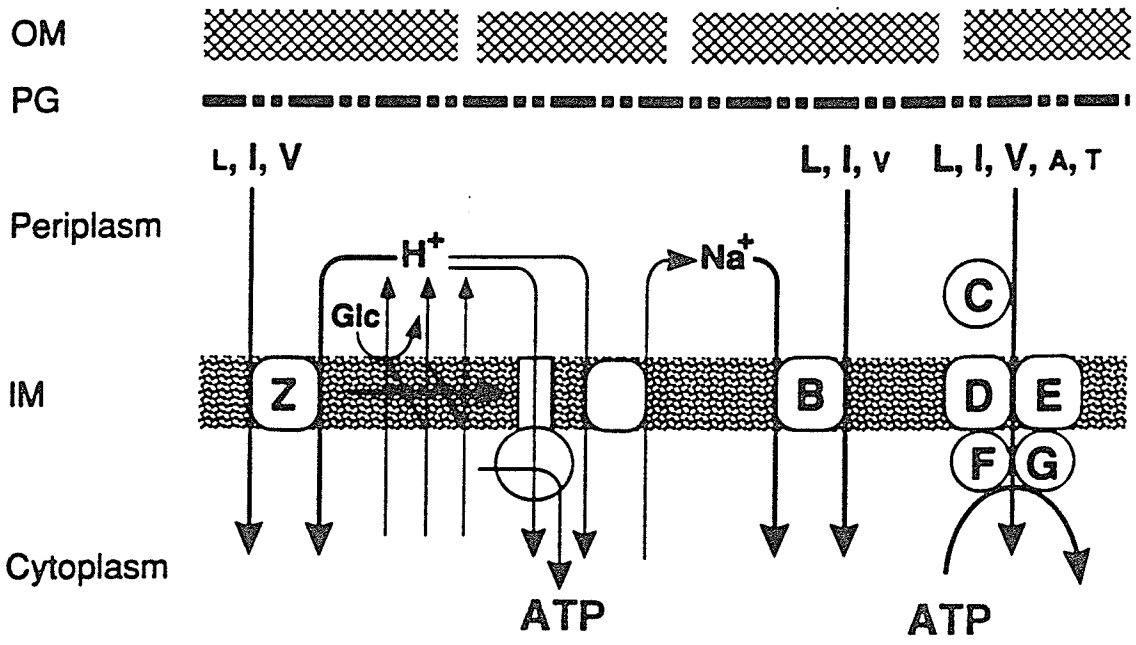
preference for glucose over galactose and a 13-fold preference for maltose over sucrose (Saravolac *et al.*, 1991). OprB induction within the family Pseudomonadaceae showed the *P. aeruginosa* and *P. putida* OprB facilitating the diffusion of xylose and maltose in addition to glucose (Wylie *et al.*, 1994). However, of the several carbohydrates it interacts with, only glucose can support the growth of *P. aeruginosa* and *P. putida*, indicating that OprB function in these species is most likely limited to glucose uptake (Wylie *et al.*, 1994).

1.6 Other Transport Systems of *Pseudomonas aeruginosa*

Pseudomonads have several transport systems which have been identified. These include mechanisms for the transport of fructose (Kundig *et al.*, 1964) and mannitol (Eisenberg and Phibbs, 1982), branched chain amino acids (Hoshino T., 1998), inorganic ions such as phosphate (Lacoste *et al.*, 1981; Hancock *et al.*, 1982; Poole and Hancock, 1983; Poole and Hancock, 1984; Surin *et al.*, 1985), and other compounds which are less well-characterized so will not be discussed. *P. aeruginosa* contains several outer membrane proteins which may form pores (Opr B, C, D, E, F, G, H, I, J, L, M, N, and O). Outer membrane proteins OprP and OprB are induced only under specific growth conditions. Comparatively, outer membrane proteins OprC and OprE have minor porin functions (Siehnel *et al*, 1990).

The importance of OprF, the *oprF* gene product, is controversial. It is constitutively expressed by the cell and functions as a multimer (Siehnel *et al*, 1990). It is present in all serotypes and clinical isolates examined thus far (Mutharia and Hancock, 1983). It shows

Figure 1.3. Schematic representation of the branched chain amino acid transport systems, LIV-I, LIV-II, and LIV-III in *P. aeruginosa*. Abbreviations: OM, outer membrane; PG, peptidoglycan layer; IM, inner (cytoplasmic) membrane; B, *braB*; C, *braC*; D, *braD*;E, braE; F, *braF*; G, *braG*; Z, *braZ*. LIV-I is encoded by the 5 genes *braCDEFG*, and is periplasmic binding protein dependent. LIV-II is encoded by *braB* and driven by the electrochemical gradient of sodium ions. LIV-III is encoded by *braZ*, and probably driven by the proton motive force. Reproduced from Biotechnology Handbooks 10. *Pseudomonas*. 1998. Edited by Thomas C. Montie.


OM
PG
L, I, V
L, I, V
L, I, V, A, T
Periplasm
H+
Na+
Glc
C
IM
Z
B
D
E
F
G
Cytoplasm
ATP
ATP

significant homology with OmpA, a large, non-specific porin forming protein from *E. coli*. OprF is also non-specific and is believed to be involved primarily in the diffusion of nutrients and waste products (Siehnel *et al*, 1990). It has been suggested that OprF forms two distinct pore sizes, with most of the protein being involved in the formation of the smaller pore and allowing only the passage of di- and trisaccharides (Siehnel *et al*, 1990 and Yoshihara and Nakae, 1989).

1.6.1 Phosphate Transport System

Phosphate transport in *P. aeruginosa* is biphasic because it can occur by two different mechanisms, the low and the high affinity transport systems (Lacoste *et al.*, 1981), as is seen in *E. coli* (Rosenberg, 1987). Both obey Michaelis-Menton kinetics. The K_m of the low affinity transport system is 12.0 mM while that of the high affinity system is 0.46 mM (Lacoste *et al*, 1981). The high affinity system is sensitive to osmotic shock and possesses a 37 kDa periplasmic protein (Poole and Hancock, 1984; Hancock *et al*, 1982). The *E. coli* Pst system (the high affinity, periplasmic binding-protein dependent system) for inorganic phosphate transport has three inner membrane components which have been identified, two integral membrane proteins, and one peripheral inner membrane component with an ATP-binding motif (Surin *et al*, 1985). It is possible that the high affinity phosphate transport system of *P. aeruginosa* also possesses similar proteins due to its similarity to *E. coli* transport systems, but no data has been published regarding the cytoplasmic membrane components of this system for Pseudomonads.

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) where 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 K_d for phosphate is 0.3 mM, 100 fold lower than the K_d for chloride ion, which is 40 mM, suggesting that the binding site is specific for inorganic phosphate (Siehnel *et al*, 1990). Although the protein shows no DNA homology to other porins, it contains 50 percent hydrophobic residues and shows an analogous protein folding pattern to PhoE, the phosphate specific outer membrane porin of *E. coli* (Siehnel *et al*, 1990; Worobec *et al*, 1988).

The low affinity phosphate transport system is resistant to osmotic shock (Lacoste *et al*, 1981), and sensitive to inhibitors of the electron transfer chain, suggesting that this system may be mediated by a carrier-type transporter similar to the Pit system (the low affinity, carrier-mediated transport system) of *E. coli* (Rosenberg, 1987).

1.6.2 Mannitol Transport System

Eisenberg and Phibbs (1982) have described an inducible mannitol-binding protein (Man-BP) in *P. aeruginosa*. Intracellularly, mannitol is converted to fructose-6-phosphate which can then be further metabolized and incorporated into the Entner-Doudoroff pathway (Phibbs *et al*, 1978; Allenza *et al*, 1982). Man-BP is the only component characterized thus far in this uptake system. It has a molecular weight of 37 kDa and has been purified and released from a closely associated enzyme, mannitol dehydrogenase (molecular weight of 85 kDa) (Eisenberg and Phibbs, 1982). Brunker *et al* (1997 and 1998) have cloned and characterized several genes encoding components of the *P. fluorescens* mannitol ABC transporter system. Three of these open reading frames

(ORF's), *mtlF*, *mtlG*, and *mtlH* encode putative proteins with strong homology to MalF, G, and K from *E. coli*. Three other ORF's, *mtlD*, *mtlZ*, *mtlY*, have been shown to encode mannitol dehydrogenase, fructokinase, and xylulose kinase, indicating that the mannitol active transport system is energized by the hydrolysis of ATP by the putative MtlK.

1.6.3 Fructose Transport System

Fructose is the only sugar in *Pseudomonas* which is transported by a phosphoenolpyruvate (PEP)-dependent phosphotransferase system (PEP:fructose PTS), and accumulates as fructose-1-phosphate (Kundig et al., 1964; Baumann and Baumann, 1975; Sawyer *et al.*, 1977). Fructose can therefore be metabolized by *Pseudomonas* via the Entner-Doudoroff pathway or the Embden Meyerhof pathway (Baumann and Baumann, 1975). The PEP:fructose PTS is induced by fructose, and mutants have been isolated which cannot grow on fructose, but grow normally on mannitol used after conversion to fructose (Roehl and Phibbs, 1982). These mutants were defective in PEP:fructose PTS activity and exhibited no fructose uptake. This suggests that the PTS is the only route of entry for fructose in most Pseudomonads. It has been suggested by Durham and Phibbs (1982) that the *P. aeruginosa* PTS consists of only two components, a soluble Enzyme I, and a membrane-associated Enzyme II complex. This system appears to differ from most in lacking an Hpr-like low molecular weight phospho-carrier protein.

1.6.4 Branched Chain Amino Acid Transport Systems

There are three mechanisms by which *P. aeruginosa* can transport the branched chain amino acids, LIV-I, LIV-II, and LIV-III (Figure 1.3). LIV-I is specific for branched chain amino acids and alanine and threonine, LIV-II is specific for leucine and isoleucine,

with lower affinity for valine. LIV-III is specific for isoleucine and valine, with lower affinity for leucine .

The high affinity transport system, LIV I, does not require sodium, but rather is a binding protein-dependent transport system (Hoshino, 1979). The binding protein, LIVAT-BP (Hoshino and Kose, 1990), was purified, and previously found to be encoded by *braC* able to restore wild type LIV-I transport activity in a *P. aeruginosa* LIVAT-BP deficient mutant, braC310 (Hoshino and Kose, 1989) .

Since the cloning of the LIVAT-BP gene (*braC*), a 4.0 kb piece of DNA located 3' of *braC* reveals four open reading frames which have been designated *braD*, *E*, *F*, and *G*. The *braF* gene product is a 255 amino acid, 30 kDa protein with a putative ATP-binding site. The *braF* gene product shows 67 percent homology with *livH* gene product, the energy coupling component of the high affinity, branched chain amino acid transport sytem of *E. coli*. The *braG* gene product is 233 amino acids or 27 kDa in size and also contains a putative ATP-binding site. All of these proteins are components of the cell membrane, having been found to localize in the cytoplasmic membrane when overproduced in *E. coli* (Hoshino and Kose, 1990a).

The low affinity system, LIV II, requires sodium to function (Hoshino, 1979), and is resistant to osmotic shock. Properties of the LIV-II system, including its sodium dependence, are similar to those of branched chain amino acid transport systems identified in membrane vesicles (Hoshino, 1979; Hoshino and Kagayama, 1979), suggesting that LIV-II is a membrane-bound, carrier-mediated transport system. Uratani *et al* (1989) showed that sodium and lithium ions are taken up by the *P. aeruginosa* cells

when an inwardly directed concentration gradient of leucine, isoleucine, or valine is imposed on the cells, indicating that LIV-II is a sodium/substrate co-transport system , as suggested by Hoshino and Kagayama (1979). The structural gene for LIV-II carrier protein is *braB*, as it confers LIV-II function to LIV-II-defective mutants (Hoshino *et al*, 1990; Uratani and Hoshino, 1989).

LIV-III was discovered accidentally in *P. aeruginosa* while cloning *braB* for the LIV-II carrier (Hoshino *et al*, 1991). While screening the genomic library on medium containing a high sodium concentration, they failed to isolate *braB*, and found, instead, a gene, *braZ*, which conferred branched-chain amino acid transport activity independent of sodium. There is some evidence which indicates that LIV-III may be coupled with protons (Hoshino *et al*, 1991). BraZ is homologous to BrnQ, the carrier for the LIV-II transport system of *S. typhimurium* (Ohnishi *et al*, 1988), and studies done with the *E. coli* BrnQ/HrbA LIV-II transporter system is homologous to the *S. typhimurium* LIV-II, and is a proton-coupled transport system (Yamoto and Anraku, 1977).

1.7 Research Objectives

With the discovery of the *P. aeruginosa* high affinity glucose transport system, and its induction upon infection of a human host, a deeper understanding and characterization of this transport system could lead to an effective treatment against this organism. The main objective of this study was to create a structural *gltB* knock-out mutant strain, and then introduce DNA fragments in an attempt to identify the location and sequence of the *P. aeruginosa gltB,* as well as elucidate the function of GBP. Previously generated GBP deficient mutants were chemically produced (PFB360 and PFB362), and the actual

location of the mutation was unkown (P.V. Phibbs, 1985). Both mutants were glucose transport deficient, and while PFB362 did not express GBP activity, lack of GBP in PFB360 was not proven.

In order to achieve this objective, a mutagenesis vector was constructed using the structural gene for the *P. putida* GBP, since it has been characterized previously (Tong, H., 1995). This structural gene was disrupted and placed into a replacement vector, then introduced into wild type strains of *P. aeruginosa* and *P. putida*. After the knock-out of *gltB* for both *P. putida* and *P. aeruginosa* was established using periplasmic protein extraction and SDS-PAGE, several *P. aeruginosa* DNA fragments were introduced to determine if a change in protein expression, glucose uptake and chemotaxis was evident.

Chapter Two

Materials and Methods

2.1 Bacterial Strains and Growth Conditions

Bacterial strains and plasmids used in this study are listed in Table 2.1.

Table 2.1 Bacterial Strains and Plasmids

Bacterium/Plasmid	Characteristics	Source/Reference
Pseudomonas		
Pseudomonas aeruginosa H103	wt PAO1 prototroph reference strain	REW Hancock/Hancock & Carey, 1979
HX2	*gltB* knock-out mutant	This study
HX2/pGR7	HX2 reconstituted with *P. putida gltB*	This study
HX2/pCL306	HX2 reconstituted with a 3.0 kb *P. aeruginosa* fragment containing the putative *P. aeruginosa gltB*	This study
HXK5	HX2 reconstituted with a 1.65 kb *P. aeruginosa* PCR fragment containing the putative *P. aeruginosa gltB*	This study
AK5	wt PAO1 reference strain with a 1.65 kb *P. aeruginosa* PCR fragment containing the putative *P. aeruginosa gltB*	This study
Pseudomonas putida	wt prototroph reference strain	ATCC 12633
LX8	*gltB* knock-out mutant	This study
LX8/pGR7	LX8 reconstituted with *P. putida gltB*	This study
LX8/pCL306	LX8 reconstituted with a 3.0 kb *P. aeruginosa* fragment containing the putative *P. aeruginosa gltB*	This study
LXK5	LX8 reconstituted with a 1.65 kb *P. aeruginosa* PCR fragment containing the putative *P. aeruginosa gltB*	This study

Bacterium/Plasmid	Characteristics	Source/Reference
PK5	wt prototroph reference strain with a 1.65 kb *P. aeruginosa* PCR fragment containing the putative *P. aeruginosa gltB*	This study
Escherichia coli		
NM522	*supE thi*Δ*(lac-proAB) hsd*5F'[*proAB*±*lacI*^{q}*lacZ* Δ*M15*]	UMCC
JM109	*F'traD36proA+proB+laclq delta(lacZ)M15 delta(lac-proAB)supE44 hsdR17 recA1 gyrA96 thi-1 endA1 relA1 e14-* lambda-	Yanisch-Perron C. *et al.* 1985 ATCC 53323
CC118	*AraD*139 (*ara, leu*)7697Δ*lacX74phoA*Δ *20galEgalK thi rpsE rpoB arg Eam recA1*	C. Manoil, Seattle, USA.
MM294	*pro thi endA hsrW km*^{R} *tra*$^+$ contains plasmid pK2013	Goldberg and Ohman, 1984
Plasmids		
pCL306	Plasmid comprised of a 3.0 kb *P. aeruginosa* genome fragment containing the putative *gltB* ligated to pPZ375.	This study
pEX100T	5.8 kb pUC19-based gene replacement vector, *oriT, lacZα, sacB, Cb*^{R}	Schweizer & Hoang, 1995
pGR7	*pPZ129* containing *P. putida gltB*	H. Tong, 1995
pPZ129	A 1.1 kb *Sal*I fragment from pR01839 containing *gltB* ligated to the broad host range plasmid pR01614	Sly and Worobec, 1993
pR01839	*gltB*$^+$ *glk*$^+$ in pR01614	Temple *et al.*, 1990
pR01614	*Ap*^{R} *Tc*^{R} OriV in pBR322	Olsen *et al.*, 1982
pHP45ΩSm/Spc	*Ap*^{R}*Sm*^{R}*Spc*^{R}	Felley *et al*, 1987
pM13	*Ap*^{R}*ColE1*	
pPZ375	*Ap*^{R}; Broad host range derivative of pGEM-3Z(+)	PV Phibbs
pRK415	*Tc*^{R} broad-host-range plasmid vector	Keen *et al*, 1988
PCR1	pRK415 with the 1.65 kb	This study

Bacterium/Plasmid	Characteristics	Source/Reference
	fragment from *P. aeruginosa* believed to harbor *gltB*	
pRK2013	$Km^R tra^+$ helper plasmid carried by *E. coli* MM294 (*pro, thi, endA, hsdR*)	Godberg & Ohman, 1984
pXL100	Plasmid consisting of the *P. putida gltB* ligated to pEX100T replacement vector	This study
pXL118ΩSm	Sm^R, *gltB* mutagenesis vector	This study
pXL122ΩSm	Sm^R, *gltB* mutagenesis vector	This study
pM13 SK^+/KS^-	Ap^R, cloning vector, 3.0 kb	Stratagene
Phage		
R408	Helper phage	Promega

Abbreviations: Ap^R, ampicillin resistance; Cb^R, carbenicillin resistance; Km^R, kanamycin resistance; Sm^R, streptomycin resistance; Spc^R, spectinomycin resistance; Tc^R, tetracycline resistance; *sacB*, levansucrase gene; *gltB*, periplasmic glucose binding protein structural gene

Induction of the high affinity glucose transport system was achieved by growing cultures on Basal Minimal Salts (BM2) media [0.04 M K_2HPO_4, 0.022 M KH_2PO_4, 0.007 M $(NH_4)_2SO_4$, 0.005 M $MgSO_4$, 0.0001 M $FeSO_4$] (Hancock and Carey, 1979) supplemented with 20 mM glucose. *P. aeruginosa* and *P. putida* strains were grown overnight at 37°C and 28°C, respectively. *E. coli* strains were incubated overnight at 37°C. When required, antibiotics were added to the media in the following concentrations: ampicillin (Ap), 100 μg/ml; carbenicillin (Cb), 400 μg/ml; streptomycin (Sm), 500 μg/ml; kanamycin (Km), 15 μg/ml; tetracycline (Tc), 25 μg/ml; sucrose, 5% (v/v).

2.2 Genomic DNA Isolation

Genomic DNA was prepared via the miniprep method according to Murray and Thompson (1980). A 5 ml aliquot of liquid medium was inoculated with the appropriate strain, and incubated overnight at the appropriate temperature in the presence of the appropriate antibiotics. The following day, 1.5 ml of the overnight culture was centrifuged for 2 minutes at maximum speed (14 000 x g). The pellet was resuspended in 567 µl TE (1 M Tris[hydroxymethyl]aminomethane, 0.1 M Ethylenediaminetetraacetic acid) buffer by repeated pipetting. Thirty µl of 10% SDS and 3 µl of 20 mg/ml proteinase K were added, mixed, and incubated 1 hour at 37°C. One hundred µl of 5 M NaCl was then added, and mixed thoroughly, followed by the addition of 80 µl CTAB/NaCl (10 % hexadecyltrimethyl ammonium bromide, 0.7 M sodium chloride) solution. The mixture was inverted gently several times, and incubated at 65°C for 10 minutes to remove polysaccharides and other contaminating macromolecules. An equal volume of chloroform/isoamylalcohol was added, the tube gently inverted several times to mix, and microcentrifuged at maximum speed (18 000 rpm) for 4-5 minutes. The supernatant was transferred to a fresh Eppendorf tube, an equal volume of phenol/chloroform/isoamylalcohol added, mixed and microcentrifuged 5 minutes as described previously. The supernatant was again transferred to a fresh Eppendorf tube. A 0.6 volume of isopropanol was added and mixed gently until the DNA precipitated. The precipitate was then transferred with a sealed Pasteur pipet (ends heated over a flame until melted closed so as to not remove any liquid via capillary action) to 1 ml of 70% ethanol and washed. After microcentrifuging 5 minutes, the supernatant

was discarded, the pellet dried in a lyophilizer, and resuspended in 100µl TE buffer. Genomic DNA was then frozen until needed.

2.3 Plasmid DNA Isolation

Plasmid DNA was prepared on a large scale according to the alkaline lysis protocol of Ausubel *et al.* (1989).

For small-scale DNA preparation, 3 ml of an overnight culture was centrifuged 2 minutes, and resuspended in 100 µl GTE (50 mM glucose, 25 mM Tris pH 8.0, 10 mM EDTA) containing lysozyme (4 mg/ml). Freshly prepared lysis solution (0.2 M NaOH and 1% SDS) and 3 M sodium acetate, pH 5.2 were added (200 and 150 µl, respectively), and placed on ice for 10 minutes. Following centrifugation for 10 minutes, a phenol/chloroform/isoamylalcohol (25:24:1) extraction was performed, 0.8 volumes of isopropanol added, and the mixture centrifuged 15 minutes at room temperature.

2.4 DNA Digestion and Electrophoresis

Restriction enzyme digests and electrophoresis were carried out by standard procedures (Ausubel *et al.*, 1989) or manufacturer's instructions.

Restriction enzymes used were from GibcoBRL Life Technologies, and digestions performed according to manufacturers instructions. Electrophoresis was carried out in TAE (40mM Tris HCl, 0.1% acetate, 1mM EDTA) using 0.8% (w/v) agarose gels containing 0.5 µg/ml ethidium bromide. The 1kb Plus ladder (GibcoBRL Life Technologies) was used as a standard for determining fragment sizes. DNA bands were visualized under UV (ultraviolet) light using the Gel-Doc system (Bio-Rad Laboratories Life Sciences Group, Hercules, CA.).

2.5 Preparation of Competent *E. coli* Cells

Preparation of competent cells was according to standard procedures (Ausubel *et al.*, 1989). An overnight broth culture of *E. coli* (grown in the presence of the appropriate antibiotics) was diluted 1/100 in fresh medium (without antibiotics) in a volume of 50 ml, and grown to an OD_{600} of 0.2-0.5. The culture was kept on ice 15 minutes, followed by centrifugation at 4000 x g for 5 minutes. The pellet was resuspended in 5 ml of ice-cold 0.1M calcium chloride, and placed on ice for 30 minutes. Following a 5 minute cenrifugation at 4000 x g, 2 ml of ice-cold 0.1M calcium chloride containing 600 µl of 50% glycerol was used to resuspend the pellet. Aliquots of 200 µl were dispensed into sterile Eppendorf tubes, and either used immediately, or stored at –60°C until needed.

2.6 Transformation of Plasmid DNA into *E. coli*

Transformation of cells was done according to Ausubel *et al.* (1989). Up to 40 ng of DNA was added to 200 µl of competent *E. coli* cells, and left on ice 1 hour. DNA concentration was determined by taking a spectrophotometric reading at 260 nm and converting to nanograms by dividing the reading by the dilution factor used, multiplying by 2 to compensate for double stranded DNA, dividing by 1000 to determine the concentration per microliter. Using the concentration, a volume of up to 10 µl was added to the competent cells, whichever gave 40 ng or less of DNA. The transformation mixture was transferred to a 42°C water bath for 2-3 minutes, and placed on ice for 5 minutes. A volume of 1 ml of LB broth was added, and the

tube incubated at 37°C for 2 hours. Cells were centrifuged 2 minutes and resuspended in a smaller volume of LB, and then 0.1 ml spread-plated onto LB plates containing the appropriate antibiotic. Plates were left at room temperature until dry, then inverted and incubated overnight at the appropriate temperature.

2.7 Preparation of Competent *Pseudomonas* cells

Competent *Pseudomonas* cells were prepared according to Olsen *et al* (1982). A 3 ml overnight culture (grown in the presence of the appropriate antibiotics) was diluted 1/100 into 50 ml of fresh medium without antibiotics, and grown 2.5 hours (~$1x10^8$ cells/ml). Cells were harvested by centrifugation at 1000 x g for 10 minutes, at 4°C, and resuspended in 25 ml of ice-cold magnesium chloride solution (0.15 M MgCl2, 15% v/v glycerol, and 10 mM PIPES [pH 7.0]). Cells were placed on ice for 5 minutes. Cells were centrifuged again, as before, and placed on ice for 20 minutes. Another centrifugation at 1000 x g was performed, and the cells resuspended in 5 ml ice-cold magnesium chloride solution. Aliquots of 200 µl were dispersed into sterile Eppendorf tubes, and the cells used immediately, or stored at –60°C until needed.

2.8 Transformation of Plasmid DNA into *Pseudomonas*

Transformations were carried out as described in Ausubel *et al.* (1989). DNA (0.2 µg) in a volume of 25 µl was added to 200 µl of competent *Pseudomonas* cells, and placed on ice 1 hour. Cells were transferred to a 37°C water bath for 3 minutes, and then placed on ice 5 minutes. A volume of 500 µl of LB broth was added, and incubated at 37°C for 2.5 hours. Cells were concentrated by centrifugation for 2-3 minutes, and resuspension in a lesser volume of LB broth.

An appropriate volume was then plated onto LB plates containing the appropriate antibiotics.

2.9 Introduction of Plasmid DNA via Conjugation

Conjugation was performed using pRK2013 (Keen *et al*, 1988). A 3 ml culture of the donor (pXL118::Sm or pXL122::Sm or pGR7 or pCL306), recipient (wild type *P. aeruginosa* H103 or *P. putida*), and helper (pRK2013) strains were grown overnight in the presence of the appropriate antibiotics. Each culture was diluted to 5% in the same media the following morning, and grown to an OD_{600} of 0.5-0.6. Cultures were centrifuged at 4000 x g for 5 minutes, and washed with 10-20 ml LB twice. Cells were resuspended in 200 µl LB. The helper and donor cells were incubated at room temperature, and the acceptor cells at 37°C, for 1-1.5 hours. An LB plate was partitioned into 4 sections, and 10 µl of the donor, helper, and acceptor were plated onto separate sections. On the fourth section, 50 µl of each were mixed together and plated. The plate was left at room temperature to dry, then inverted and incubated at 28°C overnight. The following morning, each of the 4 samples was restreaked onto selective media, and incubated overnight.

2.10 PCR and Sequencing/Sequencing Analysis of *P. aeruginosa gltB*

PCR (polymerase chain reaction) was performed using *P. aeruginosa* H103 genomic DNA. The reaction mix was made up of 2.5 µl DMSO (Dimethyl Sulfoxide), 10 µl 50% glycerol, 5.0 µl of 10X Taq (PCR) buffer, 2.5 µl of 25 mM $MgCl_2$, 4.0 µl of 2.5 mM dNTP's, 2.5 µl of 40 µM forward and reverse primers (B1 and B2, respectively; GIBCO BRL/Invitrogen, Burlington, ON.), 0.5 µl Taq, 2.0 µl template, and 18.5 µl water to make final volume 50 µl.

B1 5' GGG AAT TCG TCG CCA TGC 3'

B2 5' AGA AGC TTC TCG GGC CGT 3'

PCR conditions were as follows: initial denaturation of DNA was at 95°C for 1 minute, followed by 30 cycles of; 95°C for 45 seconds, 44°C for 45 seconds, 72°C for 1.5 minutes, followed by a final extension at 72°C for 5 minutes, followed by 4°C until removed from the thermalcycler (PE Biosystems).

The band of interest, approximately 1.7 kilobases (kb) in size was cut from the gel following electrophoresis (see Section 2.4) and purified using a QIAquick Gel Extraction kit (QIAGEN Inc., Mississauga, ON). After quantifying the purified DNA, 50 ng DNA was used for sequencing on an ABI Prism 310 Genetic Analyzer automated capillary sequencer (PE Biosystems), using the Big Dye Terminator Sequencing Reaction kit according to manufacturer's instructions.

Following sequencing, the data was saved as a text file and used in a standard nucleotide - nucleotide BLAST (blastn) search on the NCBI (National Center for Biotechnology Information) website at http://www.ncbi.nlm.nih.gov/ and also the Pseudomonas Genome Project website at http://www.pseudomonas.com/.

2.11 Mutagenesis Vector Construction

Construction of the *gltB* mutagenesis vector was accomplished by using the vector pEX100T (Schweizer, 1995). First, the plasmid pGR7 (Tong, 1995) was digested with *Bam*HI and *Xho*I to generate a 1.65 kb fragment containing the *P. putida gltB*. According to restriction endonuclease mapping (Tong, 1985),

the *P. putida gltB* contains a unique *Pst*I site. Simultaneously, pEX100T was digested with *Sma*I to linearize the plasmid. Digestion with *Sma*I results in the disruption of the *lacZ* gene, which contains a unique *Sma*I site. Both the linearized plasmid and the excised *gltB* fragment were treated with Klenow, and a blunt-ended ligation performed to generate a new 7.5 kb plasmid, named pXL100. The pXL100 was digested with *Pst*I to disrupt the *gltB*, treated with Klenow to make it blunt-ended, and *Sma*I linkers ligated to the ends. Simultaneously, the plasmid pHP45ΩSm (Fellay *et al*, 1987) was digested with *Sma*I, and a 1.7 kb fragment containing a streptomycin resistance cassette isolated. This fragment was then ligated to the altered pXL100 vector to yield a 9.2 kb mutagenesis vector named pXL118ΩSm. Recombinants were screened by growing on plates containing streptomycin, 5% sucrose (v/v), X-gal , and IPTG (isopropyl-1-thio- β -D-galactoside) The *sacB* gene of pEX100T, and subsequently pXL100, confers sucrose intolerance to an *E. coli* host. In the presence of sucrose, the *E. coli* cells are destroyed, causing the suicide vector to relocate to a *Pseudomonas* host. As a result, any colonies forming on the sucrose-containing LB plates will be pXL100-containing Pseudomonads. The IPTG and X-gal in the media allowed for identification of those colonies having their *lacZ* gene disrupted by the insertion of the streptomycin cassette. See Figure 2.1.

2.12 Generation of *P. aeruginosa* and *P. putida gltB* mutants

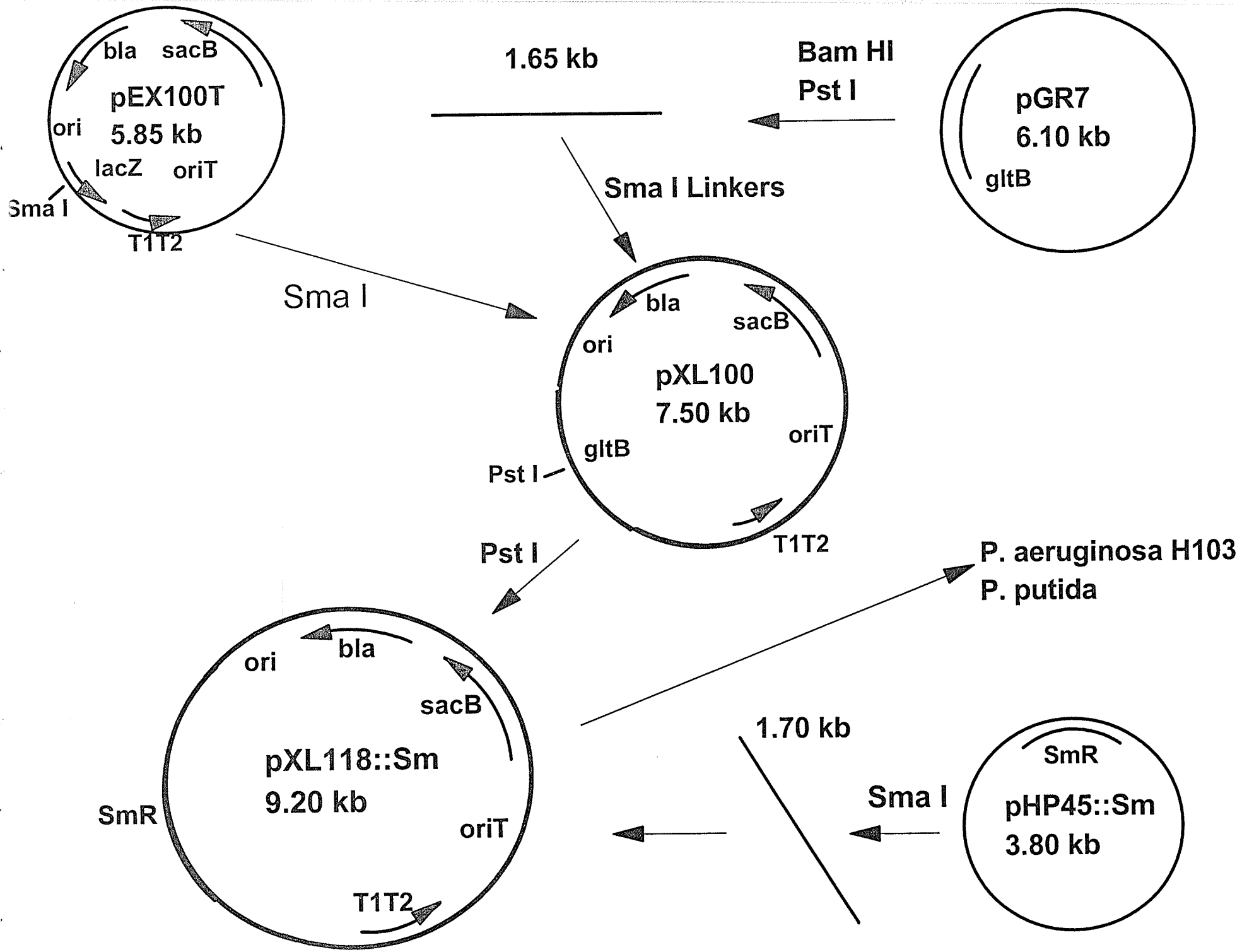
P. aeruginosa and *P. putida gltB* knock-out mutants were generated using the mutagenesis vector described in Section 2.11. The pXL118ΩSm was

introduced into wild type *P. aeruginosa* and *P. putida* strains via conjugation (see Section 2.10). Mutants were selected by growth on LB plates containing carbenicillin, streptomycin, and 5% sucrose. The presence of carbenicillin allows for selection of *Pseudomonas* colonies as they are inherently resistant to carbenicillin, the streptomycin allows for the selection of cells containing the pXL118ΩSm, and the sucrose allows for selection of those colonies possessing pXL100, as *sacB* allows only the *Pseudomonas spp.* colonies to survive (Schweizer, 1995).

2.13 Periplasmic Protein Extraction

Wild type and mutant periplasmic proteins were isolated using the heat shock method of Hoshino and Kageyama (1980), as modified by Poole and Hancock (1984). Cultures were grown up overnight in Luria Bertani (LB) liquid media, on a shaker to provide appropriate aeration, transferred into BM2 liquid media supplemented with 20 mM glucose (Hancock and Carey, 1979), and incubated overnight again with shaking. Cells were harvested by centrifugation at 10 000 x g for 10 minutes. Cells were washed in TM buffer (50 mM Tris HCl, pH 7.3 and 0.2 M $MgCl_2$), and resuspended in 80 ml TM buffer/litre of culture grown. This suspension was then subjected to two rounds of heat (30°C)/cold (0°C) treatment. The suspension was then centrifuged 10 minutes at 10 000 x g, and the supernatant concentrated with a 50 ml Amicon concentrator (Model 52, Diaflo, Amicon Div, WR Grace & Co., Danvers, Massachussets).

Figure 2.1. GBP knock-out mutagenesis vector construction. A 1.65 kb fragment harboring the *P. putida gltB* was isolated from pGR7, and ligated to pEX100T. The resulting 7.5 kb plasmid was designated pXL100. Digestion with PstI and Klenow treatment disrupted *gltB*, leaving the *bla* and *sacB* genes intact to allow for selection. The final step was to ligate a 1.7 kb fragment from pHP45::Sm to pXL100 containing streptomycin resistance gene, thus making the final vector selectable by growth in the presence of antibiotic. The final vector was 9.2 kb in size and named pXL100::Sm. The vector was then ready to introduce to both *P. aeruginosa* and *P. putida* wild type strains to create GBP-deficient mutants.


bla
sacB
pEX100T
ori
5.85 kb
lacZ
oriT
Sma I
T1T2
1.65 kb
Bam HI
Pst I
pGR7
6.10 kb
gltB
Sma I Linkers
Sma I
bla
sacB
ori
pXL100
7.50 kb
oriT
gltB
Pst I
T1T2
Pst I
P. aeruginosa H103
P. putida
ori
bla
sacB
pXL118::Sm
9.20 kb
SmR
oriT
T1T2
1.70 kb
SmR
Sma I
pHP45::Sm
3.80 kb

2.14 Whole Cell ^{14}C-D-Glucose Binding Activity Assays

Wild type and mutant cultures were grown in 5 ml BM2 supplemented with 20 mM glucose to mid log phase (OD_{600} of 0.50-0.60), transferred to 50 ml of the same medium, and grown to mid log phase again. Cells were harvested by centrifugation at 5000 x g for 10 minutes, and washed twice in BM2 without glucose before resuspending in BM2 to give an OD_{600} of 0.2. A 30 ml aliquot of this suspension was prewarmed at 30°C for 15 minutes prior to initiation of the assay. The uptake of ^{14}C-D-glucose (3.7 MBq/ml, DuPont Canada Inc., Markham, Ontario, Canada) was monitored every 10 minutes over a 60 minute period with a 500 µl aliquot taken and filtered through a 0.45 µm Millipore nitrocellulose filter. Filters were washed twice with 5 ml of BM2 without glucose. Nonspecific binding was determined under the same conditions by incubating the cells with formaldehyde, at a final concentration of 18%, for 15 minutes prior to assay initiation. Filters were placed into scintillation vials and counted in 10 ml of Ecolume (ICN Biochemicals Inc., Irvine, California) in a Beckman LS 6500 Multi Purpose Scintillation Counter. User program number 5 was used with a preset time of 2 minutes per sample, and quench limits of 3.619 to 327.17.

2.15 Glucose Binding Assays of Shock Fluids

Periplasmic protein extracts were assayed for glucose binding activity using a modified Stinson *et al* (1976) procedure. Shock fluids were diluted to an absorbance of 1.70 at 280 nm, incubated at 30°C for 5 minutes with ^{14}C-D-glucose (3.7 MBq/ml, DuPont Canada Inc.) and filtered through 0.45 µm Millipore nitrocellulose filters as described above, only 50mM Tris HCl (pH 7.3) was used in

place of BM2 without glucose. Filters were then counted by liquid scintillation spectrometry as described in Section 2.14.

2.16 Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as outlined by Lugtenberg *et al* (1975) using 11% (w/v) acrylamide in the running gel. Gels were stained using Coomassie Brilliant Blue R-250 as described by Maizel (1966), omitting the fixing step. Samples were solubilized at 90-100°C for 10 minutes prior to loading, in the presence of 0.12% (w/v) SDS and 5% (v/v) 2-β- mercaptoethanol. A prestained protein molecular weight standard (GibcoBRL, Life Technologies) was used (14 300-200 000 molecular weight range) to determine protein sizes.

2.17 Reconstitution of Mutants

Wild type and mutant cultures of *P. aeruginosa* and *P. putida* were grown to mid log phase in 3 ml of Luria Bertani (LB) medium, and then transferred to 100 ml of BM2 supplemented with 20 mM glucose and grown to mid log phase (OD_{600} 0.5-0.6). Cells were harvested by centrifugation at 10 000 x g for 10 minutes and were resuspended in BM2 containing 20 mM glucose to an absorbance of 1.00 at 578nm.

Reconstitution was performed by using one of three different DNA fragments: the 1.65 kb *Bam*HI/*Xho*I fragment from pGR7 harboring the *P. putida gltB* (pGR7); a 3.0 kb *Pst*I fragment from *P. aeruginosa* (pCL306), believed to harbor the *P. aeruginosa gltB*; or a 1.7 kb PCR fragment obtained from *P. aeruginosa*, also believed to harbor the *P. aeruginosa gltB* (PCR1). Each of the three fragments

(from pGR7, pCL306, or PCR1) were introduced to each of the *gltB* knock-out mutants via transformation (Section 2.8) or conjugation (Section 2.9).

2.18 ^{14}C-D-glucose Binding Activity Assays of Reconstituted Mutants

Reconstituted wild type and mutant cultures were prepared at an absorbance of 0.2 at 600 nm and prewarmed at 30°C for 10 minutes prior to the initiation of the binding assays. The uptake of the ^{14}C-D-glucose (3.7 MBq/ml, DuPont Canada Inc.) was monitored for 500 µl aliquots taken at 10 minute intervals over 1 hour. Samples were filtered through a 0.45 µm Millipore nitrocellulose filter and filters were washed with 5 ml of BM2 without glucose. Filters were placed into scintillation vials and liquid scintillation spectroscopy performed as described in Section 2.14.

2.19 Chemotaxis Assays of Wild Type and Mutant Strains

After mutation and reconstitution, wild type and mutant cultures were harvested by centrifugation at 10 000 x g for 10 minutes and resuspended in BM2 to an absorbance of 0.2-0.4 at 590 nm. These assays were also performed on wild type and mutant cells in the presence of periplasmic protein shock fluid. All assays were done in duplicate.

Standard capillary assays were used as described by Adler (1973). The attractant (1 µl of 20 mM glucose) was drawn up into a 5 µl disposable borosilicate glass micropipet (Fisher Scientific, Nepean, Ontario, Canada). This was placed inside a U-shaped glass disposable borosilicate micropipet (formed by heat-bending), horizontally, onto a glass microscope slide (Canlab, Toronto, Ontario, Canada). A 250 µl aliquot of each culture was added such that the capillary with

attractant was in constant contact with the culture, and a glass cover slip (Corning Science Products, Richmond Hill, Ontario, Canada) placed over it. The assay was left to proceed for 60 minutes at 30°C. The number of cells that entered the capillary was determined by serially diluting the capillary contents in saline (0.9% NaCl), and spread-plating 0.1ml of several dilutions, in duplicate, on LB agar plates. Plates were incubated overnight at either 28°C or 37°C, depending on the bacterial strain, and colonies counted the following morning.

2.20 N-Terminal Amino Acid Sequencing

Protein sequencing was performed by S. Kielland (University of Victoria, Victoria, B.C., Canada). The protein was isolated by first performing heat shock to isolate the periplasmic proteins, as described in Section 2.13, and then running the protein samples on SDS-PAGE, as described in Section 2.16. The proteins were then transferred onto a PVDF (polyvinylidene difluoride)-Immobilon-P membrane (Millipore Corp., Bedford, Massachussets) as outlined by Matsudaira (1987) and Yuen *et al* (1988). The PVDF membrane was first wet in methanol briefly (2-3 seconds) and then placed in a Petri dish containing 1x CAPS buffer (10 mM 3'-[cyclohexylamino]-1-propanesulfonic acid, pH 11; 10% (v/v) methanol). The gel was also soaked in buffer for 5 minutes. Electroblotting took place at room temperature for 10-30 minutes at 90 volts (300 mA) in a Transblot cell (BioRad) in CAPS buffer.

The membrane was then rinsed with distilled water for 5 minutes, and then placed in Ponceau S stain (0.2% w/v Ponceau S in 100 ml of 3% w/v trichloroacetic acid) for 5 minutes. The membrane was rinsed briefly in distilled

water, washed twice in destain (5% v/v acetic acid) for 3-5 minutes, washed twice with distilled water for 1-2 minutes, and dried between two sheets of Whatmann filter paper and left to dry overnight. The protein band was cut from the dried Immobilon membrane and sent away to be sequenced.

Chapter Three

Results and Discussion

After searching the *Pseudomonas* genome and locating a region predicted to contain the structural glucose binding protein gene, *gltB*, primers were designed to isolate the fragment of interest to be used in further investigations. This region was selected as a target for PCR because it was within a cluster of genes involved in carbohydrate transport, and was designated by the Pseudomonas Genome Project as a possible binding protein involved in carbohydrate transport, and possibly *gltB*. In addition, a previously isolated DNA fragment believed to harbour the *P. aeruginosa gltB* was cloned and used along with the previously characterized *P. putida gltB* (Tong, 1995) to determine if these fragments were, indeed, *gltB*. In order to determine the effect of these DNA fragments on glucose transport and chemotaxis, a genetically designed *gltB* knock-out mutant was required. Since previous glucose transport mutants were chemically generated and the precise location of the mutation not determined, the previously cloned and sequenced *P. putida gltB* (Tong, 1995) was disrupted and used to create a *P. aeruginosa gltB*-deficient mutant via homologous recombination. Once the mutagenesis vector was constructed, experiments were performed to verify the mutation and study the gene fragments of interest. The mutagenesis vector was then used to create mutants in both *P. aeruginosa* wild type in order that a fragment encoding *gltB* could be found, and *P. putida* wild type in order to determine that the mutagenesis vector was effective in both species. In keeping with effective mutagenesis in both species, the effect of the DNA fragments could then be studied in both species to further

emphasize the homology between the two species should the fragment(s) demonstrate changes in mutants from both species.

3.1 Mutagenesis Vector Construction

Construction of a *gltB* mutagenesis vector was performed as described in section 2.11 (Figure 2.1). pEX100T (a 5.8 kb pUC19-based gene replacement vector) was first cleaved with restriction enzyme *Sma*I, and the linearized plasmid was treated with Klenow to remove the restriction site. Simultaneously, pGR7 (pPZ129 containing *P. putida gltB*; Tong, 1995) was *Bam* HI and *Xho*I digested to remove a 1.65 kb fragment containing the *P. putida gltB*. This fragment was then also treated with Klenow to give blunt ends. A blunt-ended ligation of the 1.65 kb BamHI/XhoI fragment to pEX100T produced the vector pXL100 (7.5 kb in size). Successful recombinants were selected by growing on LB agar supplemented with X-gal and IPTG. Recombinants were white, and non-recombinants blue, due to the disruption of the *lacZ* gene of pEX100T (achieved with first step of *Sma*I digestion). To confirm successful formation of this vector, plasmid DNA was extracted, and run on an agarose gel to confirm its approximate size.

The resulting vector, pXL100, was then digested with *Pst*I to cleave a unique *Pst*I site within the cloned *gltB*. The linearized plasmid was then treated with Klenow to remove this *Pst*I site, and *Sma*I linkers ligated to its ends. A 1.70 kb streptomycin resistance cassette was extracted from a plasmid pHP45::Sm by digestion with *Sma*I. After ligation of the streptomycin resistance cassette to pXL100, successful recombinants were selected for by growth on LB media containing 500 µg/ml streptomycin.

The final mutagenesis vector construct was confirmed by plasmid DNA extraction and digestion with *Sma*I to yield a 1.75 kb fragment (Sm cassette) and a 7.5 kb fragment (pXL100). The final construct was named pXL100::Sm. After screening recombinants, 2 were found to yield the correct banding pattern (clones 18, and 22), and subsequently named pXL118::Sm and pXL122::Sm (Figure 3.1).

3.2 Generation of *P. aeruginosa* and *P. putida* mutants

The mutagenesis vectors pXL118::Sm and pXL122::Sm were introduced to wild type strains of both *P. aeruginosa* and *P. putida* via conjugation. Selection of knock-out mutants was achieved by growth on LB medium containing 5% (w/v) sucrose and 500 µg/ml streptomycin. The *sacB* gene of pEX100T confers sucrose intolerance to an *E. coli* host, allowing only *Pseudomonas spp.* cells to grow.

The successfully isolated *P. aeruginosa* and *P. putida* knock-out mutants were designated as HX2 and LX8, respectively, and plasmid DNA extracted to confirm the presence of the 9.20 kb mutagenesis vector (Figure 3.2). Wild type *P. putida* does not yield any plasmid DNA until either of the isolated mutagenesis vectors (pXL118::Sm or pXL122::Sm) are introduced. In this case, plasmid DNA is present, and runs with the same pattern as that for pXL118::Sm and pXL122::Sm alone. Similar results for *P. aeruginosa* were seen (gel image not included).

3.3 Periplasmic Protein Expression/Extraction

Since GBP is a periplasmic protein, it is released in response to heat shock treatment. If the *gltB* knock-out was successful, *gltb* should not be produced. To confirm this, periplasmic proteins of wild type and mutant strains for both species were extracted using the Hoshino and Kagayama heat shock method. Proteins

Figure 3.1. Agarose gel confirming successful mutagenesis vector construction. Lane 1, 1 kb ladder; Lane 2, pXL118::Sm digested with *Sma*I; Lane 3, pXL122::Sm digested with *Sma*I; Lane 4, 1 kb ladder.

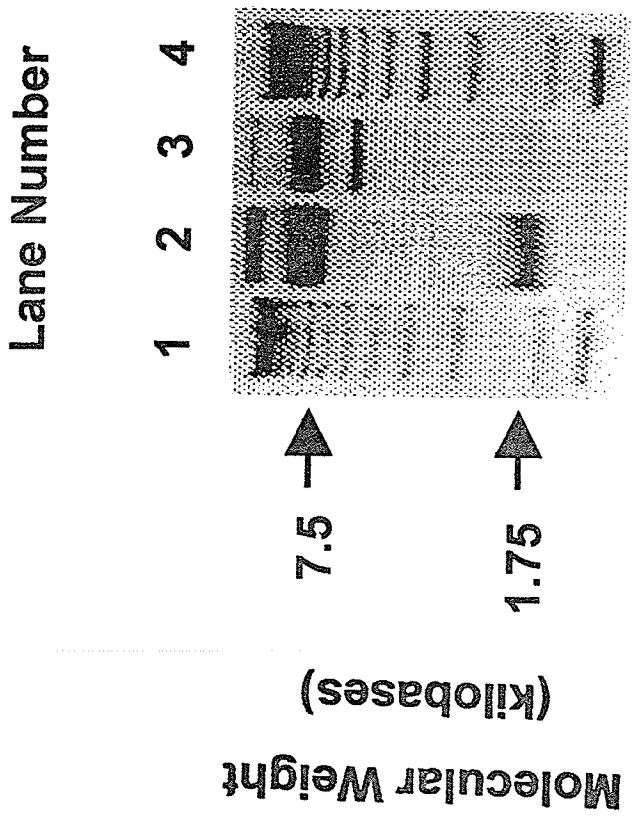
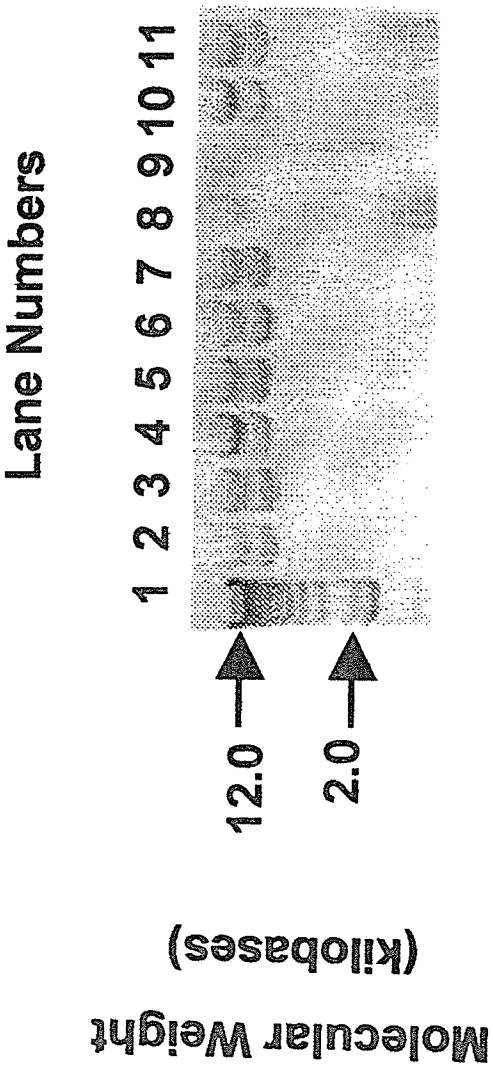

Lane Number
1 2 3 4
7.5
1.75
Molecular Weight
(kilobases)

Figure 3.2. Agarose gel confirming the presence of mutagenesis vector plasmid DNA introduced into *P. putida* via conjugation. Lane1, 1 kb DNA ladder; Lanes 2 and 3, pXL118::Sm plasmid DNA; Lanes 4 and 5, *P. put*ida plus pXL118::Sm conjugant plasmid DNA; Lanes 6 and 7, pXL122::Sm plasmid DNA; Lanes 8 and 9, *P. putida* plasmid DNA; Lanes 10 and 11, *P. putida* plus pXL122::Sm conjugant plasmid DNA. Two molecular weights are indicated in the figure (2.0 and 12.0 kb). These points are provided to indicate the range of the DNA marker used. These points also indicate the size of the plasmid isolated from each strain, which is expected to be 9.2 kb in size.


Lane Numbers
1 2 3 4 5 6 7 8 9 10 11
12.0
2.0
Molecular Weight
(kilobases)

were run on SDS-PAGE to compare protein banding patterns, and are seen in Figures 3.3 and 3.4.

Figure 3.3 depicts the periplasmic protein expression profile for wild type *P. aeruginosa* and the knock-out mutant strains. As can be seen by comparing lanes 2 and 3 (wild type and knock-out mutant strains, respectively), there is again a difference in protein profiles. The predicted size for the *P. aeruginosa gltB* is 44.5 kDa. There is an approximately 44.5 kDa protein expression in the wild type strain, which is not present in HX2. There are again, a series of proteins which differ in expression between the wild type and mutant strains. There are 2 proteins (approximately 30.0 and 47.0 kDa in size) which are expressed in HX2 that are not evident in the wild type. As there were two proteins of similar size expressed in LX8, this would lead further credence to the possibility of alternate proteins or an alternate transport system being expressed to compensate for the damaged high affinity transport system.

Figure 3.4 depicts the periplasmic expression profiles for *P. putida*. As can be seen, there are several noticeable differences in protein expression between the wild type strain (lane 2) and the knock-out mutant strain, LX8 (lane 3). Most noticeable is the lack of expression of an approximately 43.0 kDa protein, which is the molecular weight of the *P. putida gltB*, in the mutant, as compared to the wild type strain. The mutant strain also shows expression of four proteins not evident in the wild type strain. These proteins are approximately 29.0, 31.0, 37.0 and 46.0 kDa in size. This could be an indication that when the high affinity transport system is damaged, there are alternate proteins expressed in an attempt to compensate for

Figure 3.3. SDS-PAGE gel of periplasmic proteins extracted from *P. aeruginosa.* Ten µg of each sample was loaded into each well to accurately compare band intensity/protein expression. Lane 1, wild type *E. coli* CC118; Lane 2, CC118/PCR1 (*E. coli* CC118 with the 1.65 PCR fragment from *P. aeruginosa*); Lane 3, HXK5 (HX2 reconstituted with the 1.65 kb PCR fragment from *P. aeruginosa*); Lane 4, AK5 (wild type *P. aeruginosa* H103 with the 1.65 kb PCR fragment from *P. aeruginosa*); Lane 5, *gltB* knock-out mutant HX2; Lane 6, wild type *P. aeruginosa* H103; Lane 7, BioRad prestained high molecular weight marker.

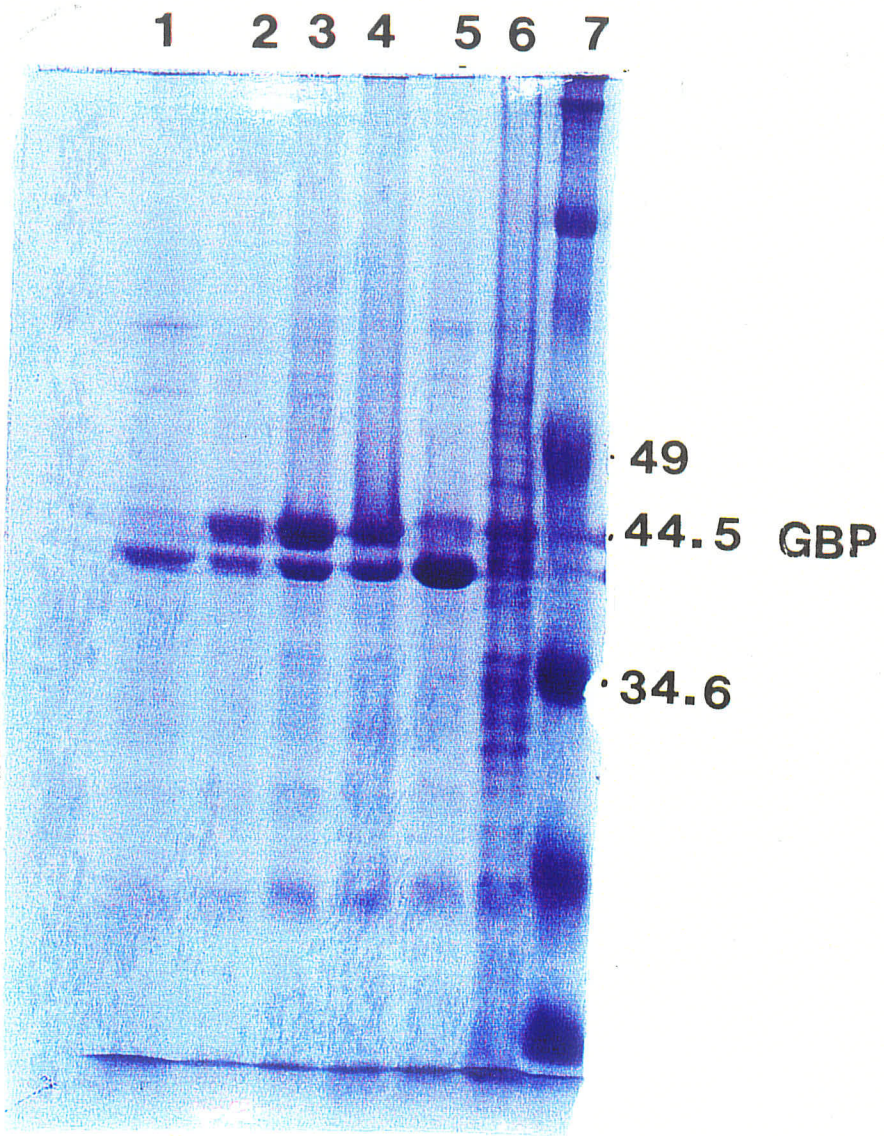
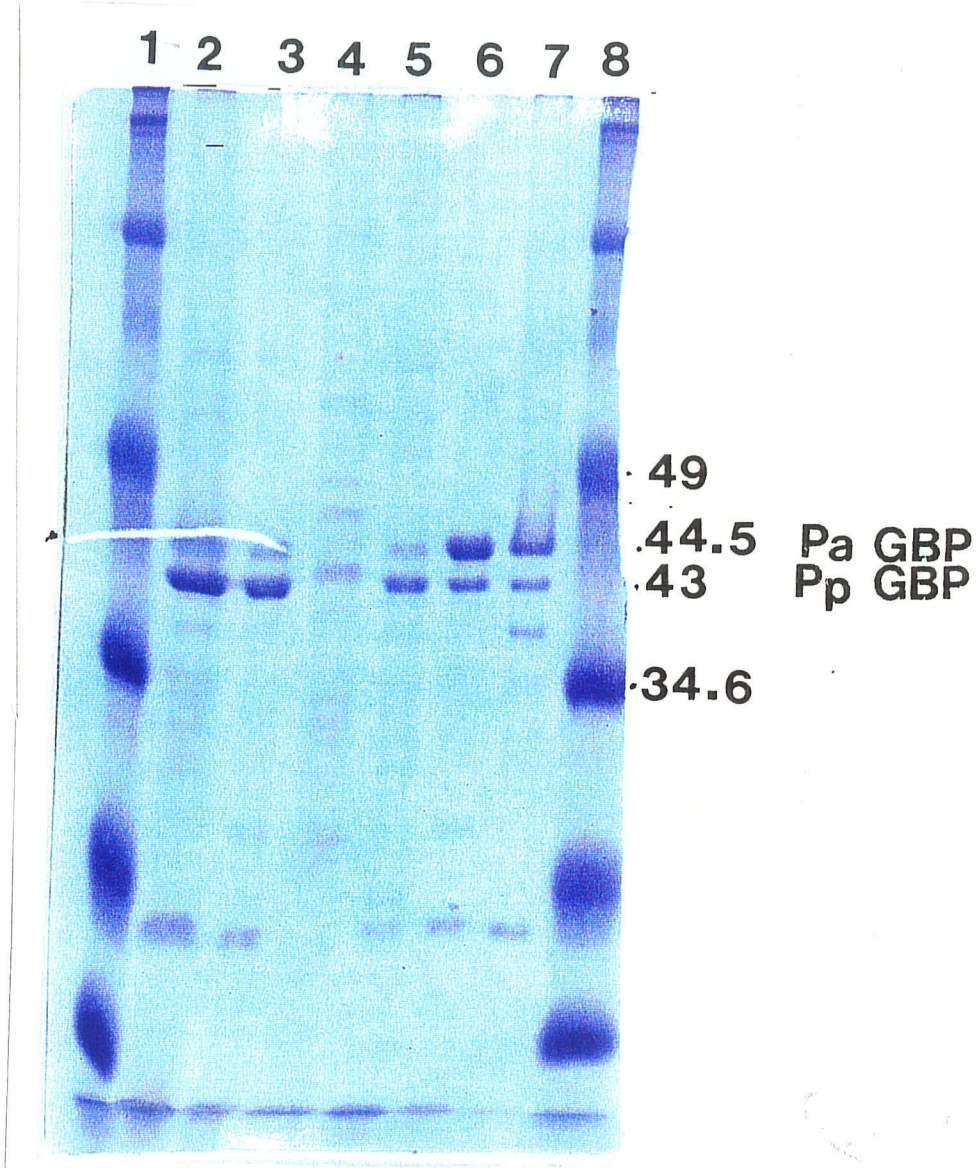

1 2 3 4 5 6 7
49
44.5
GBP
34.6

Figure 3.4. SDS-PAGE gel of periplasmic proteins extracted from *P.aeruginosa* and *P. putida* strains. Ten µg of each sample was loaded into each well to accurately compare band intensity/protein expression. Lane 1, BioRad prestained high molecular weight protein marker; Lane 2, HX2/pGR7 (HX2 reconstituted with the *P. putida gltB*); Lane 3, HX2/pCL306 (HX2 reconstituted with the *P. aeruginosa gltB*); Lane 4, wild type *P. putida*; Lane 5, LX8 (*P. putida gltB* knock-out mutant); Lane 6, PK5 (wild type *P. putida* with the 1.65 kb PCR fragment from *P. aeruginosa*); Lane 7, LXK5 (LX8 reconstituted with the 1.65 kb fragment from *P. aeruginosa*).


1 2 3 4 5 6 7 8
49
44.5
43
34.6
Pa GBP
Pp GBP

the lack of certain integral proteins, or that these proteins are from an alternate transport system.

3.4 Reconstitution of Wild Type Phenotype

Reconstitution of each *gltB* deletion mutant strain was attempted using either pGR7 (carrying the *P. putida gltB*), pCL306 (carrying the putative *P. aeruginosa gltB*), or a 1.2 kb fragment obtained via PCR from *P. aeruginosa* genomic DNA using primers B1 and B2, designed from a putative *gltB* sequence obtained from the *P. aeruginosa* genome project website (www.pseudomonas.com) (Figure 3.5). The 1.2 kb fragment was introduced into the plasmid pRK415, and the successful construct designated PCR1 confirmed by plasmid DNA extraction and restriction endonuclease digestion using *Eco*RI and *Hind*III. Then either pGR7 or PCR1 were introduced into *P. aeruginosa*, *P. putida*, and *E. coli* CC118 via conjugation. Successful *P. aeruginosa* conjugants were designated as AK5 (*P. aeruginosa* H103 plus pRK) HXK5 (*P. aeruginosa gltB* deficient mutant plus PCR1), HX2/R7 (*P. aeruginosa gltB* deficient mutant plus pGR7), and HX2/306 (*P. aeruginosa gltB* deficient mutant plus pCL306). Successful *P. putida* conjugants were designated as PK5 (*P. putida* wild type plus pRK), LXK5 (*P. putida gltB* deficient mutant plus pRK415), LX8/R7 (*P. putida gltB* deficient mutant plus pGR7), and LX8/306 (*P. putida gltB* deficient mutant plus pCL306). The successful *E. coli* conjugant was designated CC118/PCR1 (*E. coli* CC118 plus PCR1). Only the fragment housed in PCR1 was used with *E. coli* in order to determine if the *P. aeruginosa* fragment would actually be expressed in *E. coli*. In this way, N-terminal amino acid sequencing results for both *P. aeruginosa* and *E. coli* proteins could be

Figure 3.5. Putative *gltB* sequence for *P. aeruginosa*. Obtained from the *Pseudomonas* genome project website (www.pseudomonas.com)

→B1

GGCTGAAGTTTCGTCGGC**AGAAGGTTCTCGGGCCGTA**CATCGTCGATTTCGTCTGTCATGAGCGGAT
GCTGGTGATCGAACTGGATGGTGGGCAGCATGTGGGTTCGCCGGCCGATGCCTGGCTGGAGAGCCG
GGGTCCAGGGTACTGCGTTTCTGGAACGACAAGGTGCTGCCGCGCAGGGAGGTGCTGCTGGAGGTC
ATTCTCGTGTTGTAGGAGGTTTCTGTCGCGAAACCGCCTTGCCGAGTTCAAGTCCAGATTTTCCTGCTC
TGCCTCAATCCCAATGTAACCGCTCCGTGACAAACCCGGATCGCTTCGTTACCCGCGACGCGGAACG
CTTGAATAGACTCGGAGCATTGCAAGCGCAGACTTGCTCCGAATAACAAGAAGAAGGTTTTCCTCACN
TGAACCCGGATCGCTTCGTTACCCGCGACGCGGAACGCTTGAATAGACTCGGAGCATTGCAAGCGCA
GACTTGCTCCGAATAACAAGAAGAAGGTTTTCCTCATGAATGCGATCCGTCGCCTCTCTGCCGTCATCT
GTCTTTCCTCCCTCTGCCTCTCCCCGCTGCTGGCCCAGGCCGGCGAAGTCGAAGTGCTGCACTGGTG
GACCTCCGCCGGCGAGAAGCGCGCCGCCGAAACCCTGAAGAAGCTGGTCGAAGCCAAGGGCCACAC
ATGGAAGGACTTCGCCGTGGCCGGCGGTGGCGGCGAGGCGGCGATGACCGTGCTGAAGACCCGCG
CGGTGTCCGGCAATCCGCCGGCCGCGGCGCAGATCAAGGGGCCGGATATCCAGGAGTGGGGCGAA
CTGGGCCTGCTCGCCGACCTCAACGCGGTGGCCGCCGAAGGCAAGTGGGACAGCCTGCTGCCGAAG
CAGGTGGCGCAGATCATGAAGTACGACGGCGACTACGTAGCAGTGCCGATCAACGTACACCGGGTGA
ACTGGCTCTACATCAACCCGGAAGTGTTCAAGAAGGCCGGCGCCACCCCGCCGACCACCCTCGACGA
ACTGTTCGTCGCCGCCGACAAGCTCAAGGCCGCCGGTTTCACGCCGCTGGCCCATGGCAGCCAACC
GTGGCAGGACGGCACCGTGTTCGAGAACCTGGTGCTGAGCAAGATGGGTCCGGAAGGCTATCGCAA
GGCCTTCGTCGAACAGGACAAGGCGACCCTCACCGGAGCGCAGATGGTCGAGGTGTTCGCCGCGCT
GAAGAAGCTCCGCGGCTACGTCGATGCCGACGCCGCCGGTCGCGAATGGAGCGCCGCCACGGCGA
TGGTGATCAACGGCAAGGCCGGGATGCAGATCATGGGCGACTGGGCGAAGAGCGAGTTCACCGCCG
CCGGCAAGGTGCCGGGCAAGGACTACCAGTGCCTGCCGTTCCCCGGCACGCAGAAAGCCTTCGACT
ACAACATCGACTCGCTGGTGATGTTCAAGCTGAGCAACGCGGAGAACCGCAAGGCCCAGGAAGACCT
GGCGCGCAGCGTGCTCGACCCGTCCTTCCAGAAGGACTTCAACCTCAACAAGGGCTCCATCCCGGTT
CGCCTGGACGCCGACATGGCGCCGTTCGACAGTTGCGCCCAGCAGTCGATGAAGGACTTCAAGCAG
GCTTCCCAGGACGGCAACCTGGTGCCGAGCATGGCCCACAGCATGGCCGCTTCCAGCTACGTGCAG
GGGGCGATCTTCGACGTGGTGACCAACTTCTTCAACGACCCCGCCGCGGATCCGCAGAAGGCCGCC
CAGCAACTGGCGGCCGCCATCGAGGCGGCGGCGCAGTAAGTCCCTGGCGCGGATCCCGGCCAGGG
CTCCGCTCGATCCTCCCGACTGGCTCCGTGCCGCCGAGCGGCACGGGTGGGTTCCTTCGCACGCGA
GAAAA**CGCATGGCGACCAATTCCCCT**GTCTATCCCACGGCT

B2 ←

Start codon ATG;(indicated in red) Stop codon TAA (indicated in red)

Forward primer (B1) ^{5}AGAAGGTTCTCGGGCCGT $^{3'}$ G→C to create a HindIII site

Reverse Primer (B2) $^{5'}$ GGGGAATTGGTCGCCATGCG $^{3'}$ G→C to create an EcoRI site

(Primers are indicated in bold print, with solid arrows indicating their orientation)

gltB sequence is underlined.

compared (see Section 3.8).

As can be seen in Figures 3.3 and 3.4, altered periplasmic protein expression was observed. When the knock-out mutant was introduced, there was a lack of expression of the expected band at 44.5 kDa. However, upon introduction of pGR7, pCL306, and PCR1, both *P. aeruginosa* and *P. putida* showed reconstitution of the expected 44.5 kDa GBP band, indicating that these fragments did, indeed potentially harbor *gltB*.

3.5 Whole Cell ^{14}C-D-Glucose Binding Activity Assays

Whole cell glucose uptake assays were performed on wild type and mutant strains at similar cell and glucose concentrations to allow for the comparison of the different strains' abilities to incorporate radiolabelled glucose (Figures 3.6 and 3.7).

The curve for the wild type *P. aeruginosa* H103 strain demonstrates a maximum of glucose binding activity at 1238 pmol (Figure 3.6). The *P. aeruginosa* knock-out mutant strain HX2 was found to have a glucose binding capacity slightly higher than that of the wild type strain at 1309 pmol. This is an increase of 5%. HX2/pGR7 had binding activity somewhat higher than HX2 (1371 pmol), and HX2/pCL306 exhibited a maximum of glucose transport higher than that for HX2 at 1432 pmol. Both results are slightly above wild type levels. Introduction of pRK415 containing the PCR fragment from *P. aeruginosa* genome into wild type *P. aeruginosa* resulted in a slight decrease in transport activity at 1147 pmol, and an even more noticeable decrease for the knock-out mutant HX2 (964.7 pmol).

These results appear to indicate that the knock-out mutagenesis was actually unsuccessful. One would expect to see a marked decrease in glucose

Figure 3.6. ^{14}C-D-Glucose uptake assay results for *P. aeruginosa* wild type and mutant strains. Different strains are designated on the graph itself and include *P. aeruginosa* H103 (wt strain), *gltB* knock-out mutant HX2, HX2/pGR7 (HX2 reconstituted with pGR7-containing *P. putida gltB*), HX2/pCL306 (HX2 reconstituted with pCL306-containing putative *P. aeruginosa gltB*), AK5 (*P. aeruginosa* H103 wt strain transformed with pRK415 harboring *P. aeruginosa gltB* within the 1.7 kb PCR fragment), and HXK5 (HX2 reconstituted with pRK415 harboring *P. aeruginosa gltB* within the 1.7 kb PCR fragment). The H103 control was a kill control used to monitor background levels of radioactivity. The control had 30 % formaldehyde added to the flask and left for at least 10 minutes to allow the formaldehyde to destroy the cells. Each strain was tested 3 times, and the data graphed represents the average of those 3 trials.

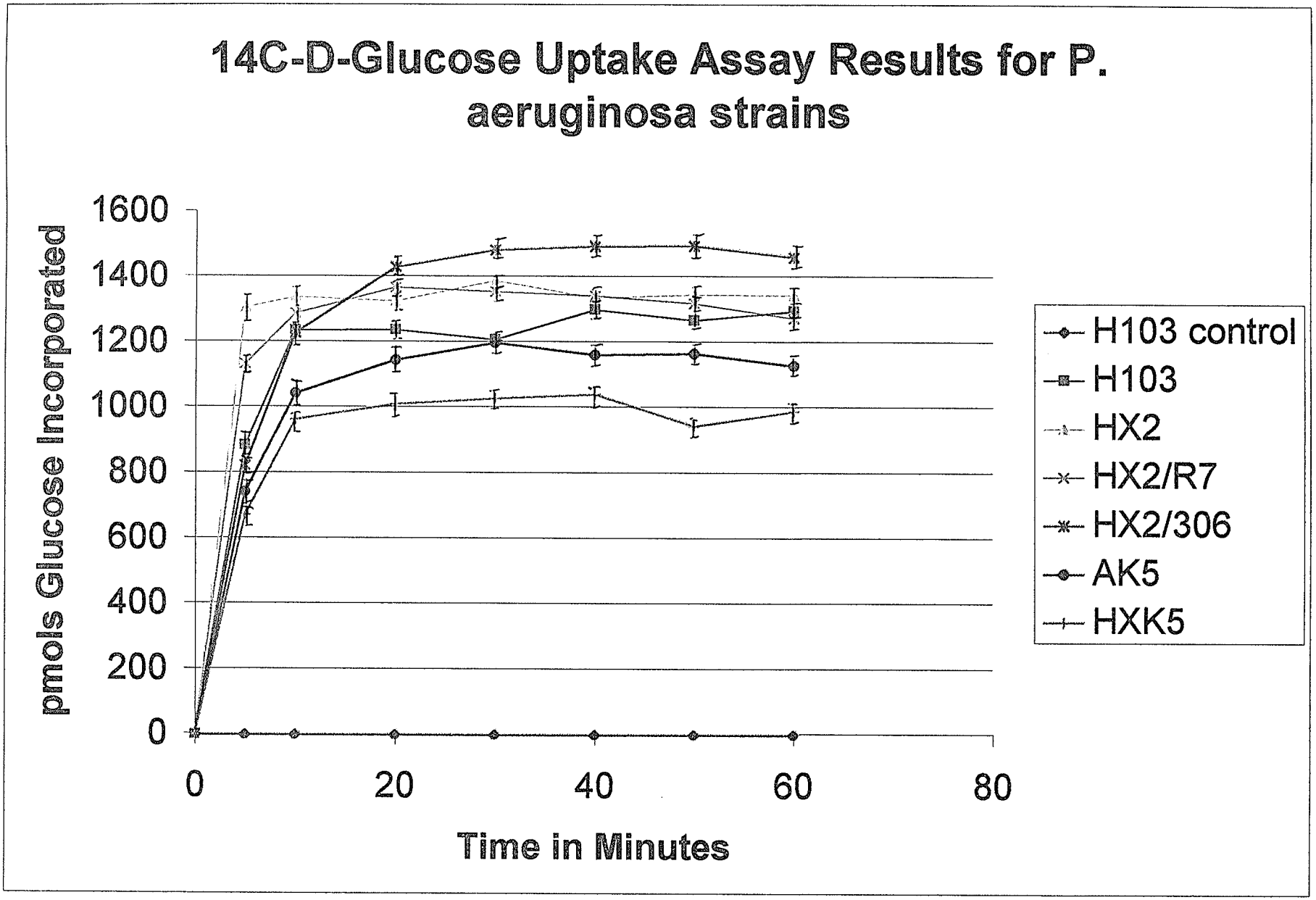
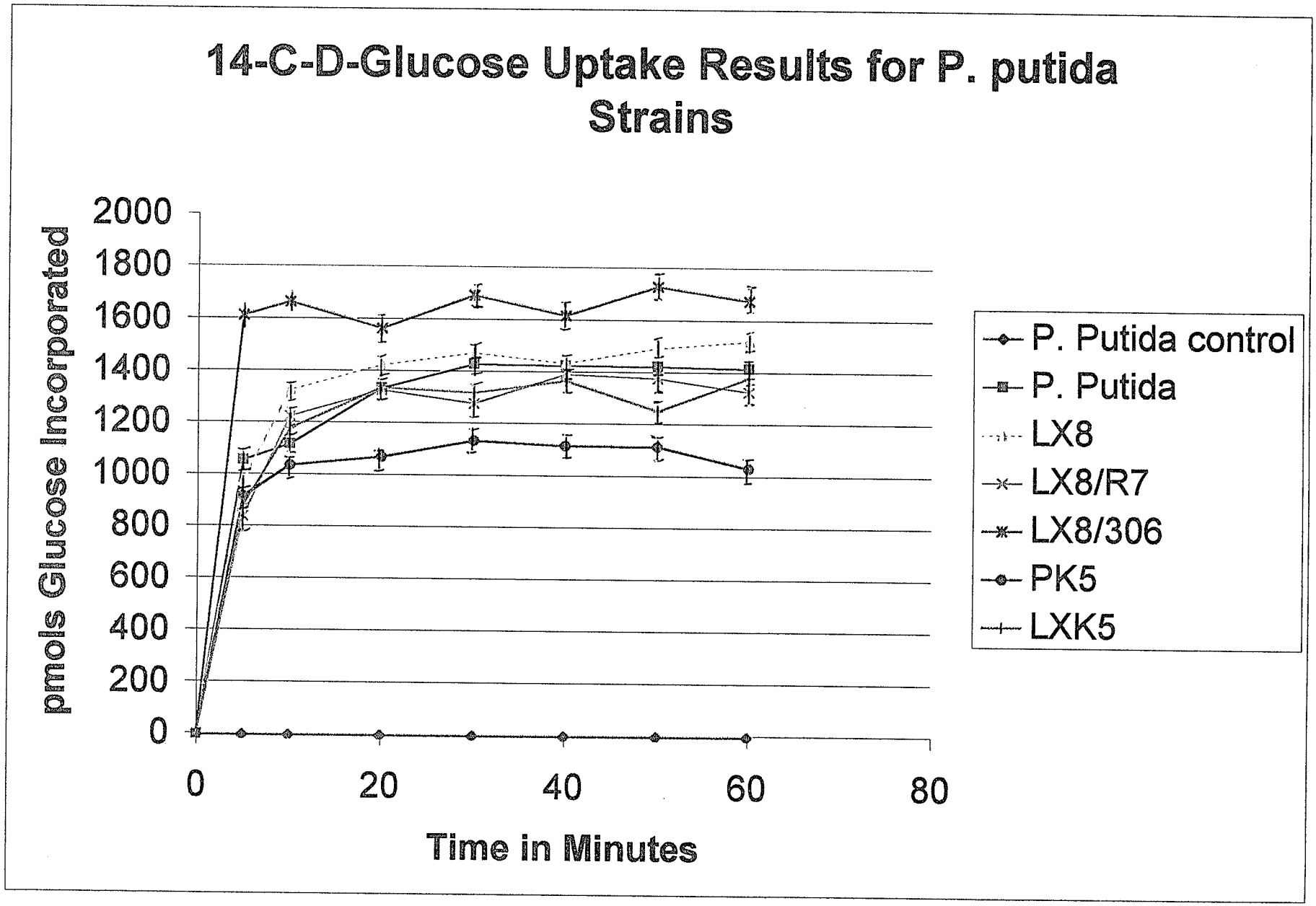

14C-D-Glucose Uptake Assay Results for P. aeruginosa strains
pmols Glucose Incorporated
1600
1400
1200
1000
800
600
400
200
0
0
20
40
60
80
Time in Minutes
H103 control
H103
HX2
HX2/R7
HX2/306
AK5
HXK5

Figure 3.7. ^{14}C-D-Glucose uptake assay results for *P. putida* wild type and mutant strains. Different strains are designated on the graph itself and include: *P. putida* (wt strain), *gltB* knock-out mutant LX8, LX8 reconstituted with pGR7 (containing *P. putida gltB*), LX8 reconstituted with pCL306 (containing putative *P. aeruginosa gltB*), *P. putida* (wt strain) transformed with pRK415 harboring *P. aeruginosa gltB* within the 1.7 kb PCR fragment (PK5), and LX8 reconstituted with pRK415 harboring *P. aeruginosa gltB* within the 1.7 kb PCR fragment (LXK5). The *putida* control was a kill control used to monitor background levels of radioactivity. The control had 30 % formaldehyde added to the flask and left for at least 10 minutes to allow the formaldehyde to destroy the cells. Each strain was tested 3 times, and the data graphed represents the average of those 3 trials.


14-C-D-Glucose Uptake Results for P. putida Strains
pmols Glucose Incorporated
2000
1800
1600
1400
1200
1000
800
600
400
200
0
0
20
40
60
80
Time in Minutes
P. Putida control
P. Putida
LX8
LX8/R7
LX8/306
PK5
LXK5

uptake/incorporation if *gltB* was indeed disrupted and rendered inactive, whereas we see the opposite, with the *gltB* knock-out mutant having a slightly higher glucose uptake rate than that of the wild type. This would seem to indicate the removal of a regulatory region rather than a structural gene for GBP. However, this may not be the case.

Treptow and Shuman (1985, 1988) have found *E. coli* mutants capable of transporting maltose via the high affinity maltose transport system independently of the MalE (the periplasmic maltose binding protein). Through experimental observations, they determined that these mutant strains are capable of transporting maltose at, or near, wild type levels without the involvement of a binding protein.

They have speculated that the maltose, once transported through the outer membrane, migrates on its own to the inner membrane, whereupon it interacts with one, or perhaps both, of the inner membrane proteins (MalF and MalG). Upon introducing a fully functional, intact MBP, one of two different phenotype classes were observed. The first (henceforth referred to as Class I mutant phenotype) showed inhibition of maltose transport, which the investigators have speculated that the MBP interacts nonproductively with altered plasma membrane components (MalF and/or MalG), inhibiting transport. In the second (referred to as Class II mutant phenotype), an increase of maltose transport was observed. This was presumably the result of the MBP still able to interact with altered MalF and/or MalG.

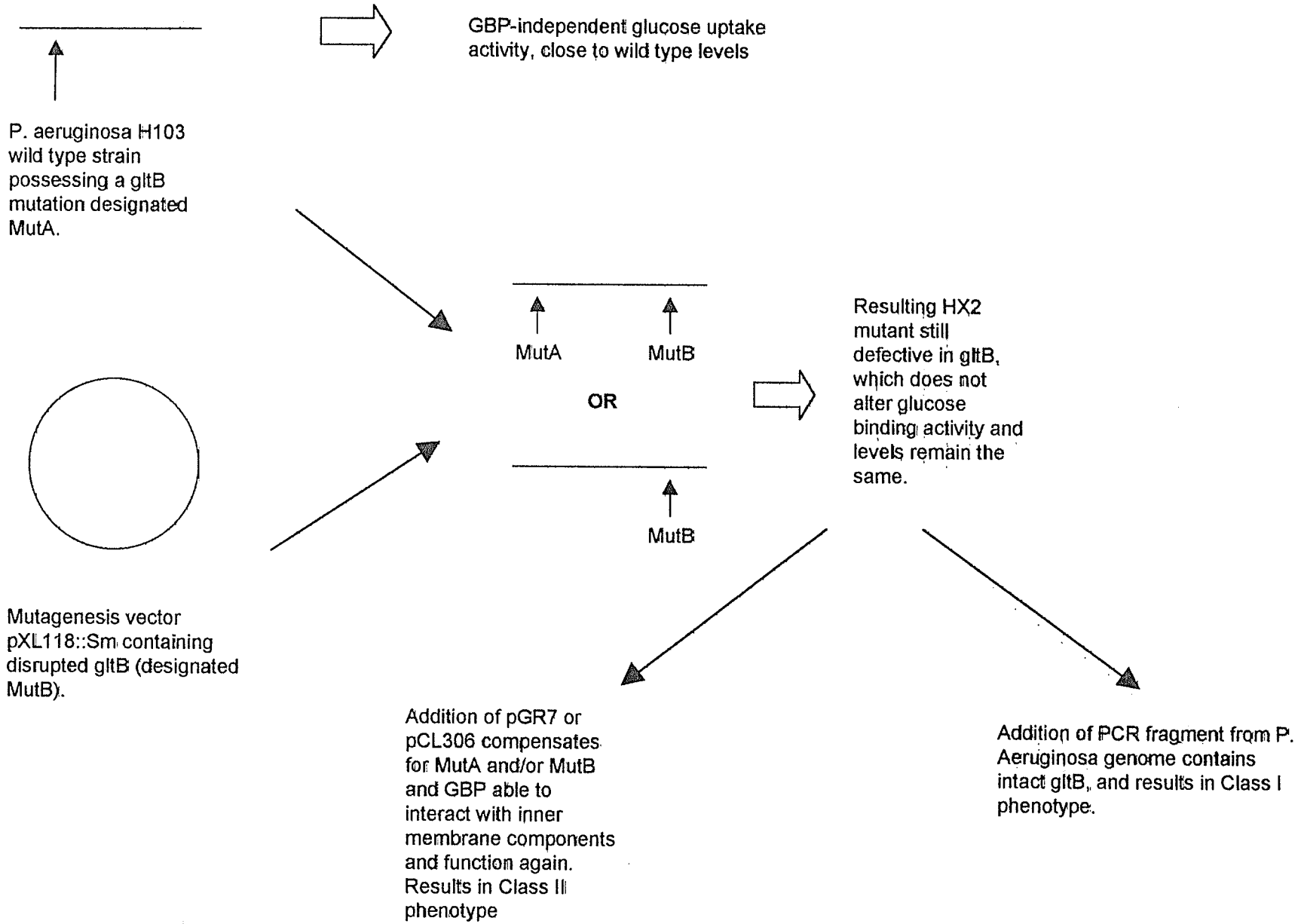
As can be seen from the *P. aeruginosa* data in Figure 3.6, the behavior of the Class II mutant phenotype is observed. When *gltB* was disrupted via the

mutagenesis vector, no change in glucose uptake/incorporation was observed. The data from the reconstituted mutants using pGR7 and pCL306 supports the possibility of a Class II mutant phenotype. When the functional *gltB* from *P. putida* is introduced (pGR7), there is an increase of glucose transport compared to both mutant and wild type strain levels. A similar effect is seen when a DNA fragment believed to carry the *P. aeruginosa gltB* (pCL306) is introduced, resulting in an even greater increase in transport activity. However, the inhibitory response observed by Treptow and Shuman (1985,1988) with *E. coli* Class I mutants was evident when pRK415 containing the PCR fragment of *P. aeruginosa* DNA was introduced to both wild type and knock-out mutant strains. There was a notable decrease in binding activity as compared to the wild type strain. When Treptow and Shuman (1985, 1988) introduced an intact MBP, maltose transport showed a similar inhibition.

This data would seem to suggest that both pGR7 and pCL306 have the capability to restore/improve glucose binding/transport activity to the knock-out mutant created, while the PCR fragment is inhibitory.

Why does there seem to be two mutant phenotypes being displayed? One possible explanation is that the *P. aeruginosa* H103 wild type colony selected from solid media and used throughout this study was actually a GBP-deficient mutant to begin with (See Figure 3.8). If this was the case, recombination with the disrupted *gltB* in the mutagenesis vector would have no effect on uptake activity, as seen in the uptake assay results. Addition of either pGR7 or pCL306 compensated for whichever mutation was present in HX2, allowing for increased uptake levels, but by introducing an intact gene obtained by PCR from *P. aeruginosa* genomic DNA

Figure 3.8 Diagramatic Representation of *P. aeruginosa* Glucose Binding Uptake Activity Assay Phenotypes.


GBP-independent glucose uptake activity, close to wild type levels
P. aeruginosa H103 wild type strain possessing a gltB mutation designated MutA.
MutA
MutB
OR
MutB
Resulting HX2 mutant still defective in gltB, which does not alter glucose binding activity and levels remain the same.
Mutagenesis vector pXL118::Sm containing disrupted gltB (designated MutB).
Addition of pGR7 or pCL306 compensates for MutA and/or MutB and GBP able to interact with inner membrane components and function again. Results in Class II phenotype
Addition of PCR fragment from P. Aeruginosa genome contains intact gltB, and results in Class I phenotype.

prepared previously and stored in the freezer until needed for PCR, inhibition of transport activity was the result.

A second scenario is that the mutant strain high affinity transport system is able to function independently of the GBP, and that introduction of the DNA fragments housed within pGR7 and pCL306 were able to compensate for the initial mutation, thus causing an increase in uptake activity upon reconstitution of the mutant. And perhaps the PCR fragment actually encodes for a repressor protein rather than the suspected GBP, thus resulting in a decrease in glucose transport activity when it is introduced into the wild type and the GBP-independent mutant.

The final explanation is that perhaps the mutagenesis vector did not result in the disruption of the *gltB*, but rather some other protein involved in glucose transport, which would explain the similar activity rates for both wild type and mutant strains. Once pGR7 or pCL306 were introduced, the mutation was neutralized and there was an increase in uptake activity seen. However, if the intact *gltB* was located within the PCR fragment, and was introduced to both the wild type and mutant strains, it could still exhibit the Class I phenotype which has transport activity inhibited upon introduction of an intact GBP (Treptow and Shuman, 1985, 1988).

The curve for the wild type *P. putida* strain demonstrates a plateau at 1431 pmol (Figure 3.7). The knock-out mutant strain LX8 showed a slight increase in its uptake activity, with a maximum at 1476 pmol. Reconstitution with pGR7 resulted in decreased activity, with a plateau around 1330 pmol, lower than wild type levels. Reconstitution with pCL306 exhibited a plateau around 1667 pmol, which was

slightly above wild type levels. Introduction of the PCR fragment into wild type *P. putida*, however, resulted in a decrease of glucose equilibrium to 1137 pmol. Introduction of the PCR fragment into LX8 resulted in binding activity of approximately 1340 pmol, also lower than wild type and mutant levels.

These results differ somewhat to those seen with *P. aeruginosa*. Uptake data is consistent with Treptow and Shuman's Class I phenotype (1985, 1988). There was no appreciable difference seen with respect to wild type and knock-out mutant strains other than a slight increase in maximum uptake for HX2. However, when DNA from either *P. putida* or *P. aeruginosa* were introduced, there was an inhibitory effect, with transport activity decreasing to below wild type and mutant levels. The only exception was with reconstitution with pCL306, which resulted in an increase above wild type levels.

3.6 Chemotaxis Assays

The optimal concentration of glucose to be used as a chemotactic attractant was determined previously (Sly, L. 1991). By using the 10 mM concentration of glucose in 1X BM2, chemotaxis was observed for all wild type and mutants strains of *P. aeruginosa* and *P. putida* (Table 3.1).

The *P. aeruginosa* knock-out mutant strain (HX2) shows a decrease in chemotaxis compared to the wild type strain (52% versus 100%, respectively). Reconstitution with either pGR7 or pCL306 results in no appreciable increase in chemotaxis, while the introduction of PCR1 into wild type *P. aeruginosa*

Table 3.1. Chemotaxis Assay Results For All *Pseudomonas* Strains

Strain	Chemotaxis Assay Results ($x 10^6$)	Percent of Wild Type Chemotaxis [a]
P. aeruginosa H103	2.25	100.00
HX2	1.17	52.00
HX2/pGR7	0.69	30.67
HX2/pCL306	1.25	55.56
AK5	1.54	68.44
HXK5	7.02	312.00
P. putida	22.86	100.00
LX8	4.61	20.17
LX8/ PGR7	2.03	6.60
LX8/pCL306	2.03	8.87
PK5	0.96	4.20
LXK5	0.76	3.32

ND, Not determined

[a]Percent chemotaxis determined by calculating the average percent chemotaxis, and designating the wild type strain for each species as 100%

NOTE: Standard deviations for results not included. Please see Appendix.

contributes a moderate increase in chemotaxis compared to that for the knock-out mutant HX2. These results coincide with those of the glucose uptake/incorporation assays, and the explanation is presumably the same. Since the results are mimicking those of a mutant strain capable of operating independently of the periplasmic binding protein, it is possible that there is an alternate method of chemotactic response as well. Reconstitution of the knock-out mutant with the PCR fragment results in an increase of chemotactic activity to twice that of the wild type strain, which is curious since the glucose uptake/incorporation results seem to indicate that the protein encoded by this fragment is likely not the GBP.

The *P. putida* knock-out mutant strain shows a marked decrease in activity compared to that of the wild type strain (20.17% versus 100%), exhibiting the same behavior as observed for the glucose uptake/incorporation assays. Reconstitution with pGR7 resulted in lowered activity, as did reconstitution with pCL306, indicating inhibition consistent with class I mutant behavior. Introduction of the PCR fragment into wild type *P. putida* and the knock-out mutant resulted in marked decreases in activity as well, again exhibiting class I mutant behavior.

3.7 PCR and Sequencing/Sequencing Analysis of *P. aeruginosa gltB*

Primers were designed based on a study of the *Pseudomonas aeruginosa* genome from the *Pseudomonas* genome project website at http://www.pseudomonas.com by GIBCO BRL/Invitrogen (Burlington. ON). The forward primer was termed B1, with the composition of: 5' GGG AAT TCG TCG CCA TGC 3', having a *Hind* III cleavage recognition site. The reverse primer, B2, had the sequence 5' AGA AGC TTC TCG GGC CGT 3', which contains an *Eco* RI

cleavage recognition site. The cleavage sites were essential to have present so that the PCR product (expected size of 1.7 kb) could be subjected to restriction enzyme digestion and used for directional cloning.

After the PCR was performed, there were three main products visible on the agarose gel (1.7, 0.74, and 0.42 kb) (Figure 3.9). Since attempts to optimize the PCR reaction described in Section 2.10 resulted in no product at all, the three products were each cut out of the agarose and the DNA purified according to manufacturer's instructions in the QIAGEN Gel Extraction Mini-Kit (QIAGEN Inc., Mississauga, ON.).

Once each PCR product was obtained, the DNA was quantified and 50 ng of DNA used for preparing sequencing reactions. Sequencing reaction products were then loaded onto the ABI 310 Prism Genetic Analyzer automated capillary sequencer (PE Biosystems), and allowed to run for 42 minutes each. The sequencing results are shown in Figure 3.10.

Each of the sequencing results was then subjected to a BLAST search (See Section 2.10). The largest band (1.7 Kb) showed homology to *P. aeruginosa* PA01, sections 304 of 529 (accession numbers AE004743, AE004742), and section 398 of the complete genome (accession number AE004837). Sequence AE004743 is a region of 10 146 bp, and consists of a probable binding protein component of ABC sugar transporter, a probable two-component sensor, two-component response regulator GltR, glucokinase, phosphogluconate dehydratase, glyceraldehyde-3-phosphate-dehydrogenase, and two hypothetical proteins of unlisted function. Sequence AE004742 is a region of 12 902 bp, consisting of 4

Figure 3.9 Agarose gel of PCR performed on *P. aeruginosa* and *P. putida* genomic DNA using primers B1 and B2. Loading order is: Lane 1, 1 kb ladder; Lane 2, *P. aeruginosa* (2.0 µl genomic DNA); Lane 3, *P. aeruginosa* (1.0 µl); Lane 4, *P. putida* (2.0 µl genomic DNA); Lane 5, negative control (water used as template); Lane 6, *P. aeruginosa* (2.0 µl genomic DNA); Lane 7, *P. aeruginosa* (1.0 µl genomic DNA); Lane 8, *P. putida* (2.0 µl genomic DNA); Lane 9, negative control (water used as a template). Reactions were done in duplicate to ensure reproducibility.

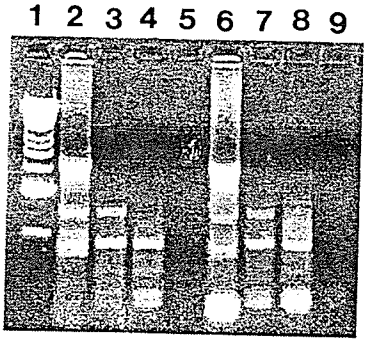

1 2 3 4 5 6 7 8 9

Figure 3.10. Sequence Alignment of the *P. aeruginosa gltB* sequence from the *Pseudomonas* genome project website (http//www.pseudomonas.com) and the 1.7 kb fragment obtained by PCR using primers B1 and B2.

B1 CTCCGTGCGAAGGAACCCACCCGTGCCGCTCGGCGGCACGGAGCCAGTC

B1 GGGAGGATCGAGCGGAGCCCTGGCCGGGATCCGCGCCAGGGACTTACTG
gltB TTACTG

B1 CGCCGCCGCCTCGATGGCGGCCGCCAGTTGCTGGGCGGCCTTCTGCGAT
gltB CGCCGCCGCCTCGATGGCGGCCGCCAGTTGCTGGGCGGCCTTCTGCGAT

B1 CCGCGGCGGGGTCGTTGAAGAAGTTGGTCACCACGTCGAAGATCGCCCC
gltB CCGCGGCGGGGTCGTTGAAGAAGTTGGTCACCACGTCGAAGATCGCCCC

B1 TGCACGTAGCTGGAAGCGGCCATGCTGTGGGCCATGCTCGGCACCAGTT
gltB TGCACGTAGCTGGAAGCGGCCATGCTGTGGGCCATGCTCGGCACCAGTT

B1 GCCGTCCTGGGAAGCCTGCTTGAAGTCCTTCATCGACTGCTGGGCGCAAC
gltB GCCGTCCTGGGAAGCCTGCTTGAAGTCCTTCATCGACTGCTGGGCGCAAC

B1 TGTCGAACGGCGCCATGTCGGCGTCCAGGCGAACCGGGATGGAGCCCTG
gltB TGTCGAACGGCGCCATGTCGGCGTCCAGGCGAACCGGGATGGAGCCTTG

B1 TTGAGGTTGAAGTCCTTCTGGAAGGACGGGTCGAGCACGCTGCGCGCAG
gltB TTGAGGTTGAAGTCCTTCTGGAAGGACGGGTCGAGCACGCTGCGCGCAG

B1 GTCTTCCTGGGCCTTGCGGTTCTCC
gltB GTCTTCCTGGGCCTTGCGGTTCTCCGCGTTGCTCAGCTTGAACATCACCAG

gltB CGAGTCGATGTTGTAGTCGAAGGCTTTCTGCGTGCCGGGGAACGGCAGC

gltB ACTGGTAGTCCTTGCCCGGCACCTTGCCGGCGGCGGTGAACTCGCTCTTC

gltB GCCCAGTCGCCCATGATCTGCATCCCGGCCTTGCCGTTGATCACCATGCC

gltB GTGGCGGCGCTCCATTCGCGACCGGCGGCGTCGGCATCGACGTAGCCG

gltB CGGAGCTTCTTCAGCGCGGCGAACACCTCGACCATCTGCGCTCCGGTGA

gltB GGGTCGCCTTGTCCTGTTCGACGAAGGCCTTGCGATAGCCTTCCGGA

gltB CATCTTGCTCAGCACCAGGTTCTCGAACACGGTGCCGTCCTGCCACGGT

gltB TGGCTGCCATGGGCCAGCGGCGTGAAACCGGCGGCCTTGAGCTTGTCGG

gltB CGGCGACGAACAGTTCGTCGAGGGTGGTCGGCGGGGTGGCGCCGGCCT

gltB TCTTGAACACTTCCGGGTTGATGTAGAGCCAGTTCACCCGGTGTACGTTG

gltB ATCGGCACTGCTACGTAGTCGCCGTCGTACTTCATGATCTGCGCCACCTG

gltB CTTCGGCAGCAGGCTGTCCCACTTGCCTTCGGCGGCCACCGCGTTGAGGT

gltB CGGCGAGCAGGCCCAGTTCGCCCCACTCCTGGATATCCGGCCCCTTGATC

gltB TGCGCCGCGGCCGGCGGATTGCCGGACACCGCGCGGGTCTTCAGCAGG

gltB TCATCGCCGCCTCGCCGCCACCGCCGGCCACGGCGAAGTCCTTCCATTG

gltB TGGCCCTTGGCTTCGACCAGCTTCTTCAGGGTTTCGGCGGCGCGCTTTCG

gltB CCGGCGGAGGTCCACCAGTGCAGCACTTCGACTTCGCCGGCCTGGGCAG
B2 CCGGGGAGGTCCACCAGTGCAGCACTTCGACTTCGCCGGCCTGGGCCAG

gltB CAGCGGGGAGAGGCAGAGGGAGGAAAGACAGATGACGGCAGAGAGCGA
B2 CAGCGGGGAGAGGCAGAGGGAGGAAAGACAGATGACGGCAGAGAGCGA

gltB CGGATCGCATTCAT
B2 CGGATCGCATTCATGAGGAAAACCTTCTTCTTGTTATTCGGAGCAAGCTGC

B2 GCTTGCAATGCTCCGAGTCTATTCAAGCGTTCCGCGTCGCGGGTAACAAG

B2 CGTCCGGGTTTGTCACGGAGCGGTTACATTGGGATTGAGGCAGAGCAGG

B2 AAAATCTGGACTTGAACTCGGCAAGGCGGTTTCGCGACAGAAACCTCCAC

B2 AACACGAGAATGACCTCCAGCAGCACCTCCCTGCGCGGCAGCACCTTTCG

B2 TTCCAGAAACGCAGTACCCTGGACCCCGGCTCTCCAGCCAGGCATCGGCC

B2 GGCGAACCCACATGCTGCCCACCATCCAGTTCGATCACCAGCATCCGTCA

B2 TACAGAGAAATCGACG

hypothetical proteins, 2 conserved hypothetical proteins, 2-keto-3-deoxy-6-phosphogluconate aldolase, glucose-6-phosphate 1-dehydrogenase, a probable transcriptional regulator, the outer membrane porin OprB precursor, a probable ATP-binding component of ABC transporter, and 2 probable permeases of ABC sugar transporter. Sequence AE004837 is a region of 10 357 bp which encodes for a probable permease of ABC sugar transporter, a conserved hypothetical protein, a hypothetical protein, a probable RND efflux membrane fusion protein precursor, a probable RND efflux transporter, a probable outer membrane efflux protein precursor, and a probable O-methyltransferase.

The 0.73 kb band also showed homology to the *P. aeruginosa* PA01 complete genome, with sections 223 (AE004662) and 8 (AE004447) of 529. Sequence AE004662 consists of 6 hypothetical proteins, a probable transcriptional regulator, and 2 conserved hypothetical proteins. Sequence AE004447 encodes for 9 hypothetical proteins, 4 conserved hypothetical proteins, and a probable ClpA/B-type chaperone.

The smallest product (0.42 kb) showed homology to *P. aeruginosa* as well, specifically section 82 of 529 of the complete genome (AE004521). This region of the genome encodes 3 hypothetical proteins, a conserved hypothetical protein, a probable oxidoreductase, a probable transcriptional regulator, 4-hydroxyphenylpyruvate dioxygenase, an aromatic amino acid transport protein AroP2, and D-alanyl-D-alanine-endopeptidase.

A general search of the *Pseudomonas* genome using the term *gltB* resulted in two matches: PA3190 and PA5036. PA3190 is described as being a "probable

binding protein component of ABC sugar transporter", and ranges from 3581645 to 3580383 of the complete *P. aeruginosa* genome. PA5036 is described as a glutamate synthase large chain precursor, with the alternate protein name of NADPH-GOGAT, GltS alpha chain, and an alternate gene name of *aspB*, falling within the range of 5672140 to 5667695.

Using the range for PA3190, as it was deemed to be the most likely candidate for being *gltB*, the sequence was translated in both the forward and reverse orientation to obtain six possible amino acid sequences (depicted in Figure 3.11), which were then compared to the N-terminal amino acid sequencing results obtained and described in the following section.

3.8 N-Terminal Amino Acid Sequencing

Upon introducing the PCR fragment into the wild type and mutant strains for *P. aeruginosa*, *P. putida*, and *E. coli*, over-expression of 2 main proteins were observed (Figures 3.3 and 3.4). Several protein bands of approximately 44.5 kDa were sent away for N-terminal amino acid sequencing. The sequences obtained were AEVYNKDGNKLDLYGKVDGLHYFSDNKDVDSDQTXMRLG. The results obtained were unexpected in that they indicated a strong sequence homology to an *E. coli* outer membrane protein, OmpC. There is also no homology seen between this N-terminal sequence and those obtained in Section 3.7. The most likely explanation for this result is that there was overexpression of this particular protein, which obscured the actual band of interest. Consequently, when the band for sequencing was isolated, the incorrect band was sent, resulting in a protein sequence which does not match what was expected from the nucleotide sequence

obtained. Should a further attempt be made to obtain the correct N-terminal amino acid sequence, two-dimensional gel electrophoresis should be performed in order to obtain better resolution of bands around the expected size than can be seen by either isoelectric focusing or SDS-PAGE alone.

Figure 3.11. Sequence translation of the *Pseudomonas* genome project *P. aeruginosa gltB* sequence. (A) is the forward sequence translation in all 3 possible frames. (B) is the reverse complement sequence translation in all 3 possible frames. (C) is the N-terminal amino acid sequence obtained for the 44.5 kDa protein over-expressed in *E. coli* CC118, *P. aeruginosa* H103 and *P. putida*

Ranges: 3580383 - 3581645
>P_aeruginosa 3580383 - 3581645 Frame 1
LLRRRLDGGRQLLGGLLRIRGGVVEEVGHHVEDRPLHVAGSGHAVGHARH
QVAVLGSLLEVLHRLLGATVERRHVGVQANRDGALVEVEVLLEGRVEHAA
RQVFLGLAVLRVAQLEHHQRVDVVVEGFLRAGERQALVVLARHLAGGGEL
ALRPVAHDLHPGLAVDHHRRGGAPFATGGVGIDVAAELLQRGEHLDHLRS
GEGRLVLFDEGLAIAFRTHLAQHQVLEHGAVLPRLAAMGQRRETGGLELV
GGDEQFVEGGRRGGAGLLEHFRVDVEPVHPVYVDRHCYVVAVVLHDLRHL
LRQQAVPLAFGGHRVEVGEQAQFAPLLDIRPLDLRRGRRIAGHRAGLQHG
HRRLAATAGHGEVLPCVALGFDQLLQGFGGALLAGGGPPVQHFDFAGLGQ
QRGEAEGGKTDDGREATDRIH

>P_aeruginosa 3580383 - 3581645 Frame 2
YCAAASMAAASCWAAFCGSAAGSLKKLVTTSKIAPCT*LEAAMLWAMLGT
RLPSWEACLKSFIDCWAQLSNGAMSASRRTGMEPLLRLKSFWKDGSSTLR
ARSSWALRFSALLSLNITSESML*SKAFCVPGNGRHW*SLPGTLPAAVNS
LFAQSPMICIPALPLITIAVAALHSRPAASAST*PRSFFSAANTSTICAP
VRVALSCSTKALR*PSGPILLSTRFSNTVPSCHGWLPWASGVKPAALSLS
AATNSSSRVVGGVAPAFLNTSGLM*SQFTRCTLIGTAT*SPSYFMICATC
FGSRLSHLPSAATALRSASRPSSPHSWISGPLICAAAGGLPDTARVFSTV
IAASPPPPATAKSFHVWPLASTSFFRVSAARFSPAEVHQCSTSTSPAWAS
SGERQREERQMTAERRRIAF

>P_aeruginosa 3580383 - 3581645 Frame 3
TAPPPRWRPPVAGRPSADPRRGR*RSWSPRRRSPPARSWKRPCCGPCSAP
GCRPGKPA*SPSSTAGRNCRTAPCRRPGEPGWSPC*G*SPSGRTGRARCA
PGLPGPCGSPRCSA*TSPASRCCSRRLSACRGTAGTGSPCPAPCRRR*TR
SSPSRP*SASRPCR*SPSPWRRSIRDRRRRHRRSRGASSARRTPRPSALR
*GSPCPVRRRPCDSLPDPSCSAPGSRTRCRPATVGCHGPAA*NRRP*ACR
RRRTVRRGWSAGWRRPS*TLPG*CRASSPGVR*SALLRSRRRTS*SAPPA
SAAGCPTCLRRPPR*GRRAGPVRPTPGYPAP*SAPRPADCRTPRGSSARS
SPPRRHRRPRRSPSMCGPWLRPASSGFRRRASRRRRSTSAALRLRRPGPA
AGRGRGRKDR*RQRGDGSHS

A

Ranges: 3580383 - 3581645
>P_aeruginosa 3580383 - 3581645 Reverse Frame 1
MNAIRRLSAVICLSSLCLSPLLAQAGEVEVLHWWTSAGEKRAAETLKKLV
EAKGHTWKDFAVAGGGGEAAMTVLKTRAVSGNPPAAAQIKGPDIQEWGEL
GLLADLNAVAAEGKWDSLLPKQVAQIMKYDGDYVAVPINVHRVNWLYINP
EVFKKAGATPPTTLDELFVAADKLKAAGFTPLAHGSQPWQDGTVFENLVL
SKMGPEGYRKAFVEQDKATLTGAQMVEVFAALKKLRGYVDADAAGREWSA
ATAMVINGKAGMQIMGDWAKSEFTAAGKVPGKDYQCLPFPGTQKAFDYNI
DSLVMFKLSNAENRKAQEDLARSVLDPSFQKDFNLNKGSIPVRLDADMAP
FDSCAQQSMKDFKQASQDGNLVPSMAHSMAASSYVQGAIFDVVTNFFNDP
AADPQKAAQQLAAAIEAAAQ*

>P_aeruginosa 3580383 - 3581645 Reverse Frame 2
*MRSVASLPSSVFPPSASPRCWPRPAKSKCCTGGPPPARSAPPKP*RSWS
KPRATHGRTSPWPAVAARRR*PC*RPARCPAIRRPRRRSRGRISRSGANW
ACSPTSTRWPPKASGTACCRSRWRRS*STTATT*QCRSTYTG*TGSTSTR
KCSRRPAPPRRPPSTNCSSPPTSSRPPVSRRWPMAANRGRTAPCSRTWC*
ARWVRKAIARPSSNRTRRPSPERRWSRCSPR*RSSAATSMPTPPVANGAP
PRRW*STARPGCRSWATGRRASSPPPARCRARTTSACRSPARRKPSTTTS
TRW*CSS*ATRRTARPRKTWRAACSTRPSRRTSTSTRAPSRFAWTPTWRR
STVAPSSR*RTSSRLPRTATWCRAWPTAWPLPATCRGRSSTW*PTSSTTP
PRIRRRPPSNWRPPSRRRRS

>P_aeruginosa 3580383 - 3581645 Reverse Frame 3
ECDPSPLCRHLSFLPLPLPAAGPGRRSRSAALVDLRRREARRRNPEEAGR
SQGPHMEGLRRGRRWRRGGDDRAEDPRGVRQSAGRGADQGAGYPGVGRTG
PARRPQRGGRRRQVGQPAAEAGGADHEVRRRLRSSADQRTPGELALHQPG
SVQEGRRHPADHPRRTVRRRRQAQGRRFHAAGPWQPTVAGRHRVREPGAE
QDGSGRLSQGLRRTGQGDPHRSADGRGVRRAEEAPRLRRCRRRRSRMERR
HGDGDQRQGRDADHGRLGEERVHRRRQGAGQGLPVPAVPRHAESLRLQHR
LAGDVQAEQRGEPQGPGRPGAQRARPVLPEGLQPQQGLHPGSPGRRHGAV
RQLRPAVDEGLQAGFPGRQPGAEHGPQHGRFQLRAGGDLRRGDQLLQRPR
RGSAEGRPATGGRHRGGGAV

B

AEVYNKDGNKLDLYGKVDGLHYFSDNKDVDSDQTXMRLG

C

Chapter Four

Conclusions and Future Work

In an attempt to further understand the function/role of *gltB* in *P. aeruginosa* within the high affinity glucose transport system, a *gltB* knock-out mutagenesis vector was constructed using the *P. putida gltB*. It was thought that due to the high degree of homology between these two species that homologous recombination between their genes would be successful, allowing for the creation of a defined GBP-deficient mutant. Chemotaxis, glucose uptake assays, and sequencing were performed in order to confirm mutagenesis and reconstitution of mutant strains, in an attempt to compare wild type and mutant behavior. While some of the results obtained were unexpected, there is evidence to warrant further investigation.

4.1 Mutagenesis Vector Construction

A *gltB* mutagenesis vector was successfully constructed by disrupting the *P. putida gltB*. This was confirmed by a series of plasmid DNA extractions and restriction enzyme digestions at various stages of cloning to ensure the plasmid exhibited the correct expected size and restriction digest banding patterns (Figures 3.1 and 3.2). Two mutagenesis vectors, pXL118::Sm and pXL122::Sm were isolated and used for the remainder of this study.

4.2 Generation of *P. aeruginosa* and *P. putida* Knock-Out Mutants

Knock-out mutant strains were created by introducing the inactivated *P. putida gltB* on either pXL118::Sm or 122::Sm into the genome of wild type *P.*

aeruginosa and *P. putida*. Creation of knock-out mutants was confirmed by SDS-PAGE analysis of periplasmic protein expression profiles, which showed a lack of expression of a 44.5 and 43.0 kDa protein in *P. aeruginosa* and *P. putida*, respectively, suggesting that they were now GBP-deficient (Figures 3.3 and 3.4).

4.3 Reconstitution of Mutants

Reconstitution of the knock-out mutant strains was attempted and achieved by one of three methods. The first was to introduce the intact *P. putida gltB* from pGR7 into the created mutant strains. The second was to introduce the putative, intact *P. aeruginosa gltB* from pCL306 to the mutant strains, and the third, to introduce the putative, intact *P. aeruginosa gltB* from a PCR fragment obtained from the *P. aeruginosa* genome.

Upon introducing these fragments into *P. aeruginosa*, and analyzing the data obtained (See Figures 3.6), it can be concluded that the *P. aeruginosa* strain used for this study possesses the ability to transport glucose independently of the periplasmic GBP, and introduction of fragments harboring intact *gltB* causes inhibition of glucose uptake/incorporation (Treptow and Shuman (1985, 1988), and chemotaxis (Figure 3.8).

A similar trend is observed in *P. putida* (Figure 3.7), although the mutant phenotype appears to be slightly different from that observed in *P. aeruginosa*.

4.4 Future Work

Based on the preliminary work done in this study, there are a number of

interesting follow-up studies which can be done to further characterize the *P. aeruginosa gltB*.

Since it has now been established that the 1.7 kb PCR fragment obtained in this study likely possesses the *P. aeruginosa gltB* (based on the sequence match for sequences obtained by PCR as compared to the *Pseudomonas* Genome Project and its assessment of that region's likely function), primer extension analysis (Ausubel et al, 1989) can be done to determine the transcriptional start site for the gene, and the promotor and other surrounding regions of *gltB* sequenced in an effort to locate repressor/activator-binding domains in the upstream region, and by any other glucose transport genes which may be present in that region.

Since expected results were not obtained for uptake assays or chemotaxis, and data seems to suggest that the systems are functioning independently of the binding protein, a study of the sequences for GltF, GltG and GltK need to be done in order to confirm that there is a mutation within one or more of these genes to support the evidence presented by Treptow and Shuman (1985, 1988) on *E. coli* mutants functioning independently of the maltose binding protein.

Another area of interest would be to determine the actual glucose binding domain. Site-directed mutagenesis (Ausubel et al, 1989) can be used to observe changes due to specifically-designed mutations introduced via an oligomeric primer. Restriction fragment deletions (Kunkel et al, 1987) could also be used to delete small sections of the gene and observe any changes which occur. The final method which could be applied is linker insertions (Kunkel et al, 1987), which allows the

gradual removal of small sections of the gene and/or flanking regions to observe the effects various areas have on transport activity.

The conservation of GBP among other Pseudomonads is another area of interest, and can easily be accomplished by comparing the sequenced fragment against various completed genomes posted on the internet. Southern blotting can also be done using the *P. aeruginosa gltB* sequence as a probe.

Determining the interactions (Bavoil and Nikaido, 1981) between the various outer and inner membrane protein components of the high affinity transport system can also be examined. It would be possible to determine exactly how GBP interacts with its substrate and membrane components. Once this has been established, a comprehensive model of glucose/carbohydrate transport in Pseudomonads can be constructed.

Previous studies have shown that the glucose-inducible porin, OprB (Hancock and Carey, 1980) is expressed during *P. aeruginosa* growth in human tissues. Patients with *P. aeruginosa* sepsis have an extremely high mortality rate. It is essential that we establish how this system functions so that effective treatments can be developed against this organism. Due to its resiliant nature, *P. aeruginosa* is extremely hard to treat, and rapidly develops resistance against antibiotics. However, if a treatment targeting the disruption of the high affinity glucose transport system can be developed, there may be a more optimistic outcome for those patients suffering from *P. aeruginosa* sepsis.

Appendix

Pursuant to the results and conclusions reached throughout the course of this study, and published in this thesis, it is necessary to discuss several concerns which may, or may not, affect the final conclusions and future work.

The first area which should be addressed is with respect to the procedure followed for the construction of the mutagenesis vector. The use of pEX100T was prefaced with a cautionary note that it was imperative to monitor the Suc^S phenotype at each step during plasmid constructions and transformations through streaking on Sucrose-containing medium. If the phenotype is not checked, mutations leading to a spontaneous Suc^R phenotype may accumulate in the process. Since pEX100T, is a pUC19-based replacement vector (Schweizer and Hoang, 1995) it is expected that, after successful introduction to the host, there should be complete integration of the plasmid into the host genome. This was not the case in this study. Plasmid DNA isolation proved that after introduction of the vector into the host, there was plasmid DNA isolated, indicating a lack of integration. This could be indicative of the plasmid being able to survive within the host without integration into the genome, or that the actual conjugation was unsuccessful. If we consider that the conjugation was actually unsuccessful, that presents the possibility that the mutants studied were not actually *Pseudomonas*, but rather *E. coli*. However, as an added control to monitor the effects of different DNA fragments, periplasmic protein isolation and uptake studies were also done on wild type *E. coli* CC118 as well as PCR1 (*E. coli* CC118 with the PCR fragment introduced). The *E. coli* protein profiles were not the same as those for the mutants used in the study, nor were the glucose uptake profiles. This, then, would indicate that the mutants used were indeed different than the *E. coli*

strains used, and are most likely *Pseudomonas*. In order to confirm this, the final concern with the mutagenesis vector construction needs to be discussed. Schweizer and Hoang (1995) used *Pseudomonas* selection agar to confirm that the colonies used were indeed Pseudomonas and not *E. coli*. This step was omitted in my procedure as it was felt that the amount, and type, of antibiotics used, in combination with the 5% sucrose would be sufficient to ensure only *Pseudomonas* would grow. What would be recommended is to streak the stocks of HX2, HX2/pCL306, HX2/pGR7, HX2/PCR1, LX8, LX8/pCL306, LX8/pGR7, and LX8/PCR1 prepared in this study onto *Pseudomonas* selection agar and redo the periplasmic protein extraction and uptake assay studies. Once the studies have been repeated, the results can be compared to those obtained in this study.

There was some concern with respect to Figures 3.3 and 3.4. As can be seen in both Figures, all periplasmic protein expression profiles appear the same between the *E. coli* and *Pseudomonas* strains. It should be noted here, that only in the last protein extraction was this similarity in overexpression seen among all strains. Prior to that time, all strains showed different expression profiles, but as one, or several, of the strains being studied were not clearly visible, those gels were not used. However, I have included a gel here to verify that this profile was not normally seen. Indeed, the profiles exhibited in Figures 3.3 and 3.4 would seem to support the theory that the mutants used were not *Pseudomonas*, but rather *E. coli*. However, as I have already noted, different profiles were normally seen, and obtaining such similar expression is likely indicitive of a mixed *Pseudomonas-E. coli* population. This would also explain the N-terminal amino acid sequencing results which most closely matched that of the *E. coli* OmpC protein. The use

of *Pseudomonas* selection agar should eliminate these irregularities and provide a clearer picture.

It should also be noted that the chemotaxis assays done had extensive variability, and are not statistically significant, and as such, should only be regarded as an example.

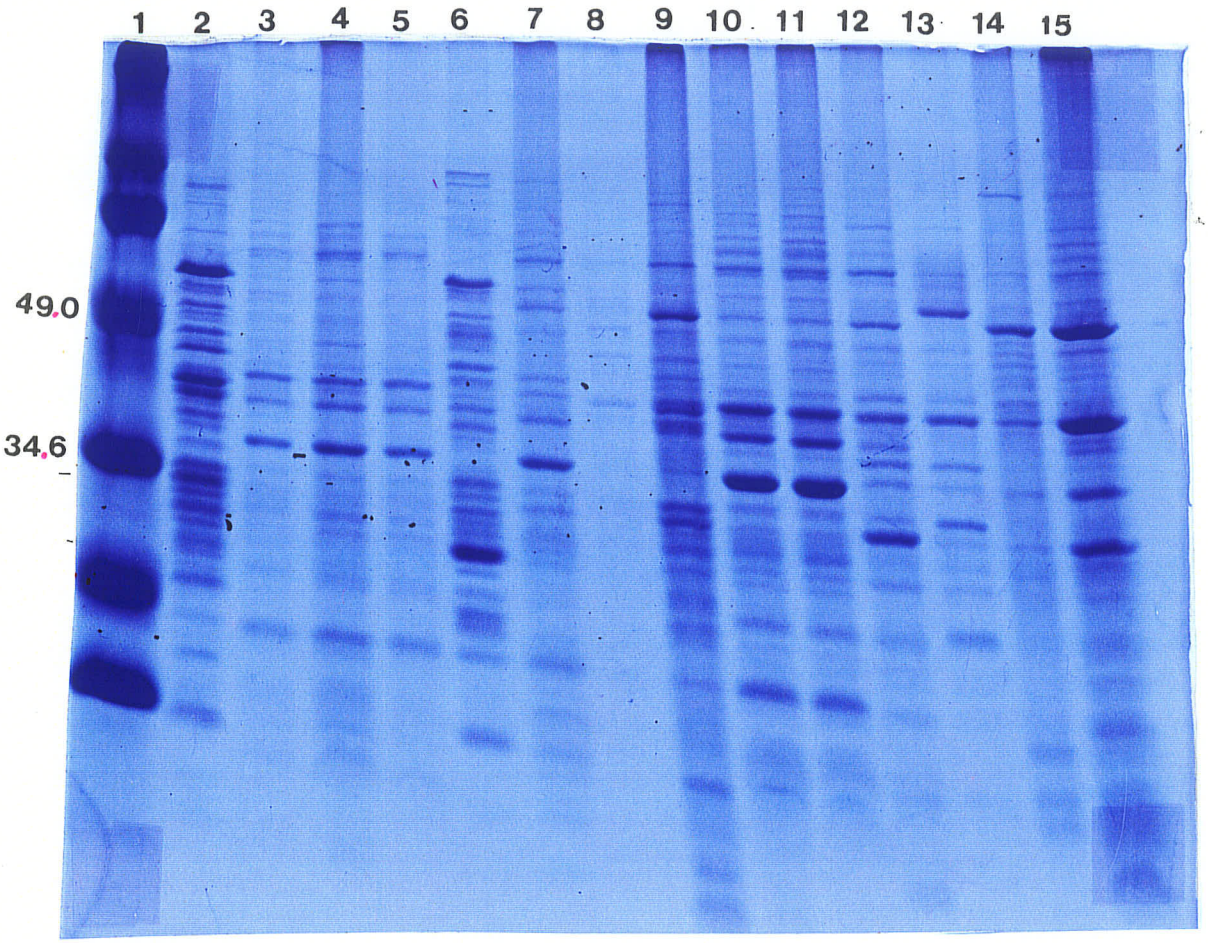
Table A1. Chemotaxis Assay Results For All *Pseudomonas* and *E. coli* Strains

Strain	Chemotaxis Assay Results				Percent Chemotaxis [a]
	Trial 1	Trial 2	Trial 3	Trial 4	
P. aeruginosa H103	5.11	1.24	0.41	ND	100.00
HX2	1.31	0.28	1.93	ND	52.00
HX2/pGR7	1.29	0.32	0.47	ND	30.67
HX2/pCL306	2.24	0.85	0.65	ND	55.56
AK5	2.40	0.68	ND	ND	68.44
HXK5	10.59	3.44	ND	ND	312.00
P. putida	38.82	4.64	45.91	2.1	100.00
LX8	3.30	10.12	0.42	ND	20.17
LX8/ PGR7	1.51	ND	ND	ND	6.60
LX8/pCL306	5.64	1.20	1.06	0.21	8.87
PK5	0.96	ND	ND	ND	4.20
LXK5	0.76	ND	ND	ND	3.32
E. coli CC118	0.81	ND	ND	ND	100.00
CC118/PCR1	0.91	ND	ND	ND	112.35

ND, Not determined

[a]Percent chemotaxis determined by calculating the average percent chemotaxis, and designating the wild type strain for each species as 100%

Figure A1. SDS-PAGE gel of periplasmic protein expression profiles for *Pseudomonas* and *E. coli* strains used in this study.



Lane 1, HMW BioRad Prestained Protein Marker; Lane 2, *P. aeruginosa* H103 wild type; Lane 3, HX2 (*P. aeruginosa gltB* mutant); Lane 4, HX2/pGR7 (HX2 with *P. putida gltB* introduced via plasmid pGR7); Lane 5, HX2/pCL306 (HX2 with *P. aeruginosa gltB* introduced via plasmid pCL306); Lane 6, AK5 (*P. aeruginosa* H103 wild type with PCR fragment of *P. aeruginosa gltB*); Lane 7, HXK5 (HX2 with PCR fragment of *P. aeruginosa gltB*); Lane 8, *P. putida* wild type; Lane 9, LX8 (*P. putida gltB* mutant); Lane 10, LX8/pGR7 (LX8 with *P. putida gltB* introduced via plasmid pGR7); Lane 11, LX8/pCL306 (LX8 with *P. aeruginosa gltB* introduced via plasmid pCL306); Lane 12, PK5 (*P. putida* wild type with PCR fragment of *P. aeruginosa gltB*); Lane 13, LXK5 (LX8 with PCR fragment of *P. aeruginosa gltB*); Lane 14, *E. coli* CC118 wild type; Lane 15, CC118/PCR1 (*E. coli* CC118 wild type with PCR fragment of *P. aeruginosa gltB*).

Below are included the uptake results over the initial 1 minute interval for most strains. These are included here as the conventional method for comparing uptake is over the first several minutes as opposed to the 1 hour time period used in the main body of this thesis. This is to prevent any carbohydrate usage/conversion by the cells from skewing the results. As can be seen by these uptake results, the *Psudomonas aeruginosa* results show a slight increase in uptake activity upon *gltB* mutagenesis, and inhibition upon introduction of both pGR7 and pCL306. There is inhibition upon introduction of the PCR1 fragment to wild type *P. aeruginosa* H103, and only a slight increase with its introduction to the mutant HX2. This is synonymous with the results obtained for the 1 hour uptake period.

P. putida results show a somewhat similar trend in that there is only a slight decrease upon gltB mutagenesis, with a marked decrease in activity upon introduction of pGR7. Introduction of pCL306 results in an increase in activity, while introduction of the PCR fragment to wild type and mutant LX8 strains results in slightly lower uptake levels to that of the wild type strain.

Uptake activity for that of *E. coli* CC118, hoever, shows a marked increase in uptake activity upon introduction of PCR1 to the wild type strain. The exact nature of this behavior is not clear, as glucose uptake in *E. coli* is not governed by a high affinity transport sytem, but rather a PTS system.

Table A2. ^{14}C-D-Glucose Uptake Assay Results for All Strains Over a 1 Minute Interval

Strain	Average cpm			Average pmol		
	20 sec	40 sec	60 sec	20 sec	40 sec	60 sec
P. aeruginosa H103 control	0	0	0	0	0	0
P. aeruginosa H103	380.0	614.1	627.5	54.72	88.43	90.36
HX2	631.3	824.7	794.5	90.90	118.7	114.4
HX2/pGR7	171.3	204.2	259.8	24.67	29.41	37.41
HX2/pCL306	361.3	441.6	557.2	52.02	63.58	80.21
AK5	496.2	549.7	685.7	71.45	79.15	98.75
HXK5	637.3	993.8	817.6	91.77	143.1	117.7
P. putida control	0	0	0	0	0	0
P. putida	555.6	828.9	973.0	80.01	119.4	140.1
LX8	472.2	727.5	719.3	67.99	104.8	103.6
LX8/pGR7	240.5	342.6	455.2	34.63	49.34	65.54
LX8/pCL306	929.8	1472	1886	133.9	211.9	271.6
PK5	638.0	695.1	848.5	91.88	100.1	122.2
LXK5	670.1	730.6	772.0	96.50	105.2	111.2
E. coli CC118 control	0	0	0	0	0	0
E. coli CC118	283.3	302.6	605.2	40.79	43.57	87.14
E. coi CC118/PCR1	685.5	1031	1312	98.71	148.4	188.9

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