Molecular characterization of *gltF* and *gltG* of the *Pseudomonas aeruginosa* glucose ABC transporter

By

Kari L. Kutcher

A Thesis submitted to
the Faculty of Graduate Studies
In Partial Fulfillment of the Requirements for the Degree of

MASTER OF SCIENCE

Department of Microbiology
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THE UNIVERSITY OF MANITOBA

FACULTY OF GRADUATE STUDIES

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ABSTRACT

Pseudomonas aeruginosa is a multidrug-resistant opportunistic human pathogen, capable of causing serious infection in immunocompromised individuals. Previous studies have indicated that high affinity glucose uptake is possible in this organism by means of an ABC transport complex. Due to the ubiquitous nature and medical relevance of ABC transporters, there is much interest in elucidating the ABC complex structures and the mechanism of substrate translocation employed by these complexes.

ABC transporters in Gram-negative bacteria typically consist of two inner membrane proteins with 6 transmembrane domains each, and 2 copies of a peripheral ATPase that associates with the membrane proteins on the cytoplasmic face of the inner membrane. Substrate passes through an outer membrane porin protein, and is received by a periplasmic binding protein that delivers the substrate to the inner membrane ABC complex. The porin, OprB, periplasmic binding protein, GBP (glucose binding protein), and the ATPase, GltK, involved in glucose uptake in *P. aeruginosa* have been identified prior to this study, though the inner membrane ABC proteins have not.

The completion of the *P. aeruginosa* genome sequencing project made it possible to identify an operon, *gltBFGK*, immediately upstream of *oprB* that contains the genes for GBP, GltF, GltG, and GltK, respectively. The predicted amino acid sequences of GltF and GltG indicate that they are likely the transmembrane components of the ABC complex. Each protein is predicted to contain 6 membrane-traversing segments, and contains the EAA motif, an amino acid motif that is

conserved in ABC permeases, and probably interacts with the ATPase component. In addition, GltF and GltG are highly identical (more than 70%) to putative ABC sugar permeases of other *Pseudomonas* species.

Expression studies in *E. coli* and *P. aeruginosa* have confirmed that GltF and GltG are membrane proteins. Transposon insertion mutants in which either *gltF* or *gltG* is disrupted are capable of growing in medium supplemented with glucose as the sole carbon source, likely due to activity of the low affinity glucose uptake system. When the same mutants were tested for whole cell high affinity glucose uptake ability, they were found to be deficient in uptake ability, confirming that they play a role in this system.

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LIST OF ABBREVIATIONS

ABC	ATP binding cassette
Ap	ampicillin
BM2	basal medium 2
Cb	carbenicillin
CD	circular dichroism
CF	cystic fibrosis
CFTR	CF transmembrane conductance regulator
Cm	chloramphenicol
DMSO	dimethylsulfoxide
ED	Entner-Duodoroff
EDTA	ethylene diamine tetraacetic acid
EMP	Embden-Meyerhof pathway
EPI	efflux pump inhibitor
g	gram
G3P	glyceraldehyde-3-phosphate
GBP	glucose binding protein
hr	hour
IM	inner membrane
IPTG	isopropyl-β-D-thiogalactopyranoside
IS	insertion sequence
kDa	kilodalton
Km	kanamycin
LB	Luria-Bertani
LPS	lipopolysaccharide
M	molar
MBP	maltose binding protein
MCS	multiple cloning site
MFP	membrane fusion protein
mg	milligram
min	minute
ml	millilitre
mM	millimolar
NAD	nicotinamide adenine dinucleotide
nm	nanometre
OD	optical density
OEP	outer membrane efflux protein
OM	outer membrane
ORF	open reading frame
PBP	periplasmic binding protein
PCR	polymerase chain reaction
PEP	phosphoenolpyruvate
PIPES	piperazine-N,N'-bis[2-ethanesulfonic acid]
PMF	proton motive force

PMSF	phenylmethylsulfonyl fluoride
psi	pounds per square inch
PTS	phosphotransferase system
QAC	quaternary ammonium compound
RBS	ribosome binding site
RDM	regulatory domain motif
RND	resistance-nodulation cell division
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
TCA	tricarboxylic acid
TMD	transmembrane domain
TYS	tryptone yeast extract salt
µg	microgram
µl	microlitre
µM	micromolar
v/v	volume/volume
w/v	weight/volume
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
XP	5-bromo-4-chloro-3-indolyl phosphate

CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

1.1 General Introduction

The bacterium *Pseudomonas aeruginosa* is a Gram-negative, polarly flagellated rod. Predominantly a soil organism, *P. aeruginosa* is an opportunistic human pathogen capable of causing infection in the immunocompromised, most notably in AIDS and cystic fibrosis patients, and severe burn victims (Bodey *et al*, 1985). *P. aeruginosa* prefers to inhabit aerobic environments, but may also grow anaerobically utilizing nitrate as a terminal electron acceptor. It is due to great metabolic diversity that these chemoorganotrophs are able to survive when supplied with relatively few nutrients, often inhabiting hospital environments.

P. aeruginosa possesses a large number of virulence factors contributing to pathogenicity. There are currently 19 exotoxins known to be secreted by this organism, which are either known or suspected to be involved in virulence (Bitter, 2003). There are several proteases, phospholipases, and ADP-ribosyltransferases produced, as well as an elastase, lipase, and cytotoxin, to name a few (refer to Bitter, 2003 and the references therein). Additionally, *P. aeruginosa* is noted for the production of the blue-green pigment pyocyanin, and the fluorescent pigment pyoverdin (fluoroscein). Pyocyanin aids in host colonization by interfering with the function of human nasal cilia and disrupting the epithelium of the respiratory tract, while pyoverdin serves as an iron-chelator, sequestering iron from competing organisms. Finally, the production of the exopolysaccharide alginate provides mucoid strains of the organism with the potential to grow as a biofilm on surfaces, including medical devices (May *et al*, 1991).

In addition to the production of several virulence factors, *P. aeruginosa* is also noted for a relatively high level of innate antibiotic resistance (Yoshimura & Nikaido, 1982). A common inhabitant of soil, such a high resistance to antibiotics would afford *P. aeruginosa* protection from the large number of antibiotic-producing soil organisms. The inherent mechanisms of antibiotic resistance of *P. aeruginosa* are very effective.

1.2 Innate Antibiotic Resistance of *P. aeruginosa*

P. aeruginosa is resistant to many antimicrobials without the acquisition of specific defenses, due to numerous intrinsic properties preventing antibiotic entry. These include low outer membrane permeability (reviewed in Section 1.2.1), the presence of active efflux pumps (reviewed in Section 1.2.2), and the capability to form biofilms (reviewed in Section 1.2.3).

1.2.1 Outer Membrane Permeability

The first widely-recognized feature of *P. aeruginosa* contributing to innate resistance to antibiotics is the low permeability of the outer membrane. This impermeability is largely due to the characteristics of the outer membrane porins (water-filled channels through which extraneous molecules gain passage into the periplasm). Many of the porins are specific, only granting passage of a specific molecule or class of molecules. The unusual characteristics of the major non-specific porin, and of the phosphorylation patterns of the lipopolysaccharide (LPS) also contribute to outer membrane impermeability.

OprF, a non-specific porin (Woodruff & Hancock, 1989), is the major outer membrane porin of *P. aeruginosa*. OprF functions both in maintaining cell shape, and in

promoting growth at low osmolarity (Gotoh *et al*, 1989; Rawling *et al,* 1998; Woodruff & Hancock, 1989). This porin is unusual in that it is capable of forming two channel sizes (Siehnel *et al*, 1990). The occurance of large pores is estimated at <1%, the predominant OprF pore being quite small. As a result, general diffusion across the outer membrane is slow.

Clinical *P. aeruginosa* isolates displaying multiple antibiotic resistance phenotypes are OprF-deficient (Woodruff & Hancock, 1988). OprF-deficiency is advantageous to the cell, as, not only does this represent the loss of the major non-specific porin, OprF is a significant antigen, the loss of which aids in evasion of the host immune system (Rawling *et al*, 1998). Genetic analysis of such OprF-deficient strains indicate that the phenotype is likely the result of a regulatory mutation, as the gene and the sigma^{70} promoter appear intact (Brinkman *et al*, 1999; Macfarlane *et al*, 1999).

OprH, a porin-like outer membrane protein, is the major outer membrane protein of *P. aeruginosa* when Mg^{2+} is limiting (Bell *et al*, 1991). OprH is a basic (cationic) protein (Bell *et al*, 1991; Young *et al*, 1992) that forms an 8 stranded β-barrel (Rhem & Hancock, 1996). The function of OprH is unknown, but it is believed to occupy Mg^{2+} binding sites in the outer membrane. Negatively-charged LPS molecules of the outer membrane are cross-linked by divalent cations, including Mg^{2+}. When Mg^{2+} levels are low, OprH is overexpressed, presumably occupying the vacant Mg^{2+} binding sites in the membrane, thus contributing to membrane stability (Macfarlane *et al*, 1999). When grown in Mg^{2+}-limiting media, in addition to OprH overexpression, *P. aeruginosa* displays resistance to EDTA, aminoglycosides, and polymyxin B, along with alterations in the LPS (Brown & Melling, 1969).

It is believed that the EDTA-, aminoglycoside-, and polymyxin B-resistant phenotypes are attributed in part to increased levels of OprH, which likely serves to prevent self-promoted uptake of these antibiotics (Bell *et al*, 1991). Self-promoted uptake, a process by which polycationic antibiotics gain entry through the outer membrane, involves the competitive displacement of stabilizing Mg^{2+} by these bulky cations. This displacement leads to distortion of the outer membrane, thereby increasing the permeability.

The isolation of a polymyxin-resistant OprH-deficient mutant is suggestive of another contributing mechanism(s) of *P. aeruginosa* polymyxin resistance (Macfarlane *et al*, 1999). *oprH* is part of a three-component operon, with *phoP-phoQ* residing immediately downstream of *oprH* (Macfarlane *et al*, 1999). PhoP and PhoQ homologues are found in several Gram-negative bacteria, and form a two-component regulatory system that modulates gene expression in response to the environment. PhoQ, the sensor kinase, autophosphorylates at a conserved histidine residue in response to an environmental signal. The DNA-binding response regulator PhoP receives the phosphate from PhoQ via transphosphorylation, and binds upstream to a regulator gene, which then functions to activate or repress the target genes. In *Salmonella typhimurium*, the PhoP-PhoQ regulon controls more than 40 genes, including those involved in Mg^{2+} transport (Soncini *et al*, 1996), LPS structure (Guo *et al*, 1997; Helander *et al*, 1994), and resistance to defensins (Fields *et al*, 1989) and polycationic antibiotics (Groisman *et al*, 1997). Recent studies have determined that both the PhoP-PhoQ system and another newly-identified two-component response system, PmrA-PmrB, are involved in

regulation of resistance to polymyxin B and cationic antimicrobial peptides in this organism (McPhee *et al*, 2003).

Recent studies with quaternary ammonium compound (QAC)-resistant *P. aeruginosa* strains has led to the identification of an additional outer membrane protein that is involved in antibiotic resistance (Tabata *et al*, 2003). QACs are believed to enter the cell by self-promoted uptake. Upon exposure to QACs, it was observed that *oprR* expression was upregulated, and efforts are currently underway to elucidate the role that OprR plays in QAC resistance.

The substrate-specific porin OprD facilitates the diffusion of basic amino acids and small basic amino acid-containing peptides. Due to the small pores of the outer membrane of *P. aeruginosa*, small antibiotics, including imipenem, a small β-lactam structurally resembling a basic amino acid, were designed (Satake *et al*, 1991). Unfortunately, imipenem preferentially traverses the outer membrane via OprD. In fact, imipenem exhibits higher affinity binding to OprD binding sites than do L-amino acids (Trias & Nikaido, 1990). In response to imipenem treatment, *oprD* expression is often downregulated, resulting in resistance to the antibiotic (Martínez-Martínez *et al*, 1999). Transition or deletion mutations in *oprD* leading to premature stop codon generation have been identified in clinical isolates (Pirnay *et al*, 2002). More recently, it was found that insertional activation is another contributing factor to *oprD* downregulation in carbapenem-resistant clinical isolates (Wolter *et al*, 2004).

The unusual LPS phosphorylation patterns of *P. aeruginosa* contribute to membrane stability, and therefore drug resistance (Walsh *et al*, 2000). The LPS of the outer membrane consists of three components: lipid A, core oligosaccharide, and O

antigen. Lipid A serves to anchor the LPS in the outer membrane. Lipid A is followed by the core oligosaccharide region, a short sugar chain on lipid A. The core region is subdivided into the inner core, which tends to be well conserved in Gram-negative organisms, and the outer core, which displays much variability in sugar content amongst bacteria. The O antigen is a polymer of repeating oligosaccharides attached to the core. The lipid A core is that which contributes to outer membrane impermeability. Within this region, there are ionic interactions between divalent cations (displaced in the process of self-promoted uptake) and charged phosphate groups, that likely serves to cross-link the LPS molecules, imparting stability (Nikaido & Hancock, 1986). The influence of the O antigen on impermeability is very little, as mutations in this region do not have any major effect in this respect.

The core region of *P. aeruginosa* is noteworthy in that it is the most phosphorylated of all known Gram-negative cores (Sadovskaya *et al*, 1998), therefore presumably among the most impermeable. Hypersusceptibility of cells to lysis by divalent cation chelators (Eagon & Carson, 1965) reflects this high phosphorylation. Walsh *et al.* (2000) have discovered two novel *P. aeruginosa* LPS core kinase genes encoding putative kinases that phosphorylate inner core heptose, and a third kinase was identified previously (Sadovskaya *et al*, 1998). As no deletion mutants have been successfully isolated, it is likely that inner core heptose-linked phosphate is required for the viability of this organism.

There are limitations associated with current antimicrobials that target LPS phosphorylation. Polymyxin B, when administered at high doses, is toxic to kidney cells (Vinnicombe & Stanley, 1969), and resistance is achieved by attaching aminoarabinose

onto lipid A to reduce the net negative charge of the cell envelope (Cox & Wilkinson, 1991). In the high salt environment of a cystic fibrosis patient's lung, *Pseudomonas aeruginosa*-effective cationic peptides are inactive (Piers & Hancock, 1994).

1.2.2 Efflux Pumps

In the event that an antibiotic successfully penetrates the outer membrane of *P. aeruginosa*, the next mode of defense is active efflux of the antibiotic out of the cell. In fact, data from a large number of recent studies suggests that efflux of antibiotics may be the major intrinsic resistance mechanism of this organism. There are five known families of efflux pumps. *P. aeruginosa* pumps characterized to date all belong to the RND (Resistance-Nodulation-cell Division) family. The completion of the genome sequencing project of *P. aeruginosa* revealed that there are 12 RND pump genes present (Stover *et al*, 2000).

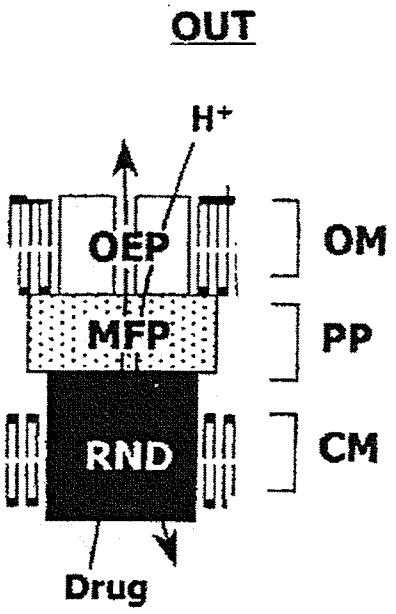
Both the amino acid homology of these pumps to known antiporters (Nies & Silver, 1995; Zgurskaya & Nikaido, 1999), and efflux inhibition by uncouplers (Li *et al*, 1995), suggest that RND pumps are drug/H^+ antiporters, driven by proton motive force (PMF). This family of pumps typically exports antibiotics (Saier *et al*, 1994), and are thus the most interesting from a clinical perspective.

RND efflux pumps serve to pump drugs from the cytoplasm (or periplasm, in the case of β-lactams) directly into the external medium. RND pumps consist of three components: an inner membrane transporter, an outer membrane channel, and a periplasmic accessory protein (Fig. 1.1). Often, the genes encoding the efflux pump components form an operon (Aires *et al*, 1999; Poole *et al*, 1993b).

The inner membrane transporters are responsible for drug recognition (Zgurskaya & Nikaido, 1999) and distinguishing unwanted substances from cell membrane components, the mechanisms by which are not currently understood. RND inner membrane transporters consist of 12 transmembrane domains, with high sequence similarity between the N and C terminal halves (Saier *et al*, 1994). These proteins recognize mostly lipophilic and amphiphilic substrates that have a hydrophobic domain suitable for insertion into the phospholipid bilayer of the membrane (Nikaido, 1996), and may have more than one binding site, facilitating transport of more than one type of drug (Putman *et al*, 1999). Large periplasmic loops have been observed in transporters of *P. aeruginosa* and *E. coli* that are believed to play a role in substrate specificity (Mao *et al*, 2002; Elkins & Nikaido, 2002).

The periplasmic components are referred to as MFPs (Membrane Fusion Proteins), to reflect the homology to paramxyoviral MFP (Dinh *et al*, 1994). These proteins are presumed lipoproteins, anchored to the inner membrane (Yonemaya *et al*, 2000). It is believed that the MFP component serves to connect the inner and outer membranes, such that the substrate is not released by the inner membrane transporter into the periplasm, but rather, extracellularly. Interestingly, there appear to be specific interaction(s) between the MFP and the inner membrane component, but not between the MFP and the outer membrane efflux protein (OEP). Typically, homologous MFPs act only in conjunction with one particular inner membrane transporter, although there has been an exception to this rule observed in an *E. coli* efflux pump MFP (Elkins & Nikaido, 2002). OEPs, on the other hand, can be used with different transporters, and even with different families of pumps (Yoneymaya *et al*, 1998).

Figure 1.1. Schematic diagram representing the organization of RND efflux pumps. Abbreviations: OM, outer membrane; PP, periplasmic space; CM, cell membrane; OEP, outer membrane efflux protein; MFP, membrane fusion protein; RND, resistance-nodulation cell division inner membrane transporter (Adapted from Poole, 2000).


OUT
H+
OEP
OM
MFP
PP
RND
CM
Drug
IN

OEPs of RND efflux pumps are trimers with both transmembrane domains, likely β-strands, and periplasmic domains, likely α-helical regions (Johnson & Church, 1999; Koronakis *et al*, 2000; Wong *et al*, 2001). It is believed that the two halves of the proteins have similar three-dimensional structures, based on the presence of two tandem repeats in the primary sequence. Currently, the mechanism of action of OEPs is unknown, however, it is possible that there is simultaneous interaction between all three components of the system, as tripartite complexes are found to be unstable following substrate translocation (Nikaido & Zgurskaya, 2001; Thanabalu *et al*, 1998). There have been 20 OEPs identified in *P. aeruginosa* (Jo *et al*, 2003).

To date, there are have been seven efflux pumps of *P. aeruginosa* studied, namely: MexAB-OprM (Poole *et al*, 1993b), MexCD-OprJ (Poole *et al*, 1996), MexEF-OprN (Kohler *et al*, 1997), MexXY-OprM (Mine *et al*, 1999), MexJK-OprM (Chuanchuen *et al*, 2001), MexGHI-OpmD (Aendekerk *et al*, 2002), and MexVW-OprM (Li *et al*, 2003). Note that the convention for naming of the pumps is (MFP)(RND)-(OEP).

The most well-characterized *P. aeruginosa* pump, MexAB-OprM, was the first to be discovered in *P. aeruginosa*, and is one of two pumps known to be expressed in wild-type cells (Gotoh *et al*, 1995; Li *et al*, 1995; Poole *et al*, 1993a; Poole *et al*, 1993b). Poole *et al*. (1993a) have demonstrated that a natural role of this pump is the excretion of the iron-sequestering siderophore pyoverdine. MexAB-OprM is also believed to be required for the invasiveness of this organism (Hirakata *et al*, 2002). The MexAB-OprM efflux pump has the most broad substrate specifity of the *P. aeruginosa* pumps studied to

date, and is the only pump of this organism that is known to extrude substrates that reside in the periplasm, namely, β-lactams and β-lactamase inhibitors (Nikaido *et al*, 1998). Additional antimicrobial substrates of this pump include quinolones, chloramphenicol, tetracycline, novobiocin, macrolides, trimethoprim, erythromycin, rifampin, and fusidic acid (Gotoh *et al*, 1995; Köhler *et al*, 1996; Li *et al*, 1995; Poole *et al*, 1993b). When cloned into *E. coli*, cells acquired resistance to crystal violet and SDS, indicating the ability of this pump to efflux dyes and detergents (Srikumar *et al*, 1998).

The expression of MexAB-OprM is growth phase-regulated, with expression in late log phase (Evans & Poole, 1999). In addition, expression of MexAB-OprB has also been observed to affect the expression of the other pumps of *P. aeruginosa*.

The MexGHI-OpmD pump (of which the function of membrane protein MexG is currently unknown) is also active in wild-type cells (Aendekerk *et al*, 2002). Far less is known about this pump, but it is known to contribute to vanadium resistance.

For a discussion of the components, mechanisms, and clinical significance of multidrug efflux pumps in *P. aeruginosa*, see the review by Poole and Srikumar (Poole & Srikumar, 2001), which discusses data obtained up to 2001. For a currrent review on multidrug efflux pumps in Gram-negative bacteria, see the review by Poole (2004) and the references therein.

As efflux of antibiotics is a major resistance mechanism, there is much work being directed towards the production of efflux pump inhibitors (EPIs). Microcide Pharmaceuticals (Mountain View, CA) has identified synthetic EPIs that result in increased accumulation of the antibiotic levofloxacin inside *P. aeruginosa* cells expressing MexAB-OprM, MexCD-OprJ, and MexEF-OprN (Lomovskaya & Watkins,

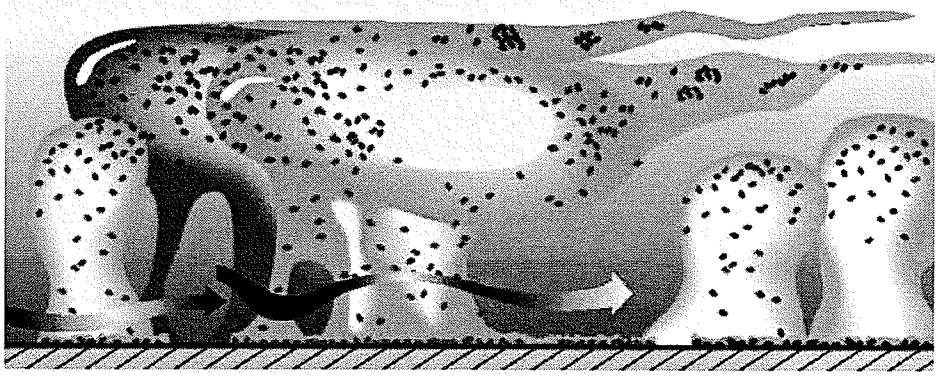
2001; Lomovskaya *et al*, 2001). There is currently much interest in optimization of the pharmacological properties of these compounds.

1.2.3 Biofilm Formation

As a pathogen, *P. aeruginosa* often forms biofilms on tissue or medical implants of the host. Biofilms are communities of bacteria adhered to a solid surface and enveloped within an exopolysaccharide matrix (see the review by Costerton & Lewandowski, 1995, and references therein) (Fig. 1.2). It is now believed that biofilm communities are much more common than planktonic (suspended) bacteria in environments in which nutrients are plentiful (Costerton & Wilson, 2004). The role of biofilms in disease is very significant, and it has been estimated that 65% of all human bacterial infections can be attributed to biofilms (Potera, 1999). Environments in which pathogenic *P. aeruginosa* biofilms occur include contact lenses, lungs of cystic fibrosis patients (where *P. aeruginosa* infection is the leading cause of death), and urinary catheters. *P. aeruginosa* biofilm infection usually necessitates the removal of infected medical devices.

Biofilm cells have been shown to be more resistant to antibiotics than corresponding planktonic cells. This enhanced intrinsic resistance is thought to be attributed to limited penetration of antimicrobials into the biofilm (Stewart, 1996; Chen & Stewart, 1996; Hodges & Gordon, 1991), extrusion of antibiotics via active efflux pumps within biofilms, and subpopulations of superresitant cells within the biofilms (Broun *et al*, 2000). Since many antibiotics are more effective against actively metabolizing cells,

Figure 1.2. Schematic representation of biofilm architecture. Cells are organized into microcolonies (conical mushroom-shaped structures). Arrows represent convective liquid flow in the water channel between the microcolonies (Reproduced from Costerton & Lewandowski, 1995).



the physiological heterogeneity of biofilm cells and the proportion of dormant cells may be proportional to the degree of enhanced resistance. Indeed, it has recently been shown that there is heterogeniety in β-lactamase induction in *P. aeruginosa* biofilms (Bagge *et al*, 2004).

P. aeruginosa biofilm cells produce increased levels of alginate, which is the major component of the exopolysaccharide matrix (Davies *et al*, 1993; Davies & Geesey, 1995). Alginate serves as a physical barrier, preventing access of antibiotics and, due to the highly charged (anionic) nature of the matrix, antibiotics may be trapped by electrostatic interaction within this layer. Secreted β-lactamase may also be trapped in the matrix, hydrolyzing β-lactams at the surface of the biofilm. Such surface degradation of antibiotics has been termed the "reaction-diffusion barrier", as antibiotics are degraded at the surface of the biofilm faster than they can diffuse in (Chen & Stewart, 1996).

The discovery that the cystic fibrosis lung is essentially anaerobic (Worlitzsch *et al*, 2002) has had a large impact on the focus of research directed at antimicrobial treatment of *P. aeruginosa* biofilms. A recent study examined the susceptibility of mature anaerobic *P. aeruginosa* biofilms to a variety of antibiotics, and reported that oxygen limitation is likely a major contributing factor to biofilm resistance against antibiotics in general (Borriello *et al*, 2004).

There are a few chemotherapeutic options available to treat *P. aeruginosa* biofilm infection. Combination therapy with ofloxacin and fosfomycin has been found to be effective. Fosfomycin-induced outer membrane changes appear to lead to increased accumulation of and killing by ofloxacin inside *P. aeruginosa* biofilm cells (Monden *et al*, 2002). In addition, the quinolone levofloxacin has exhibited effectiveness against

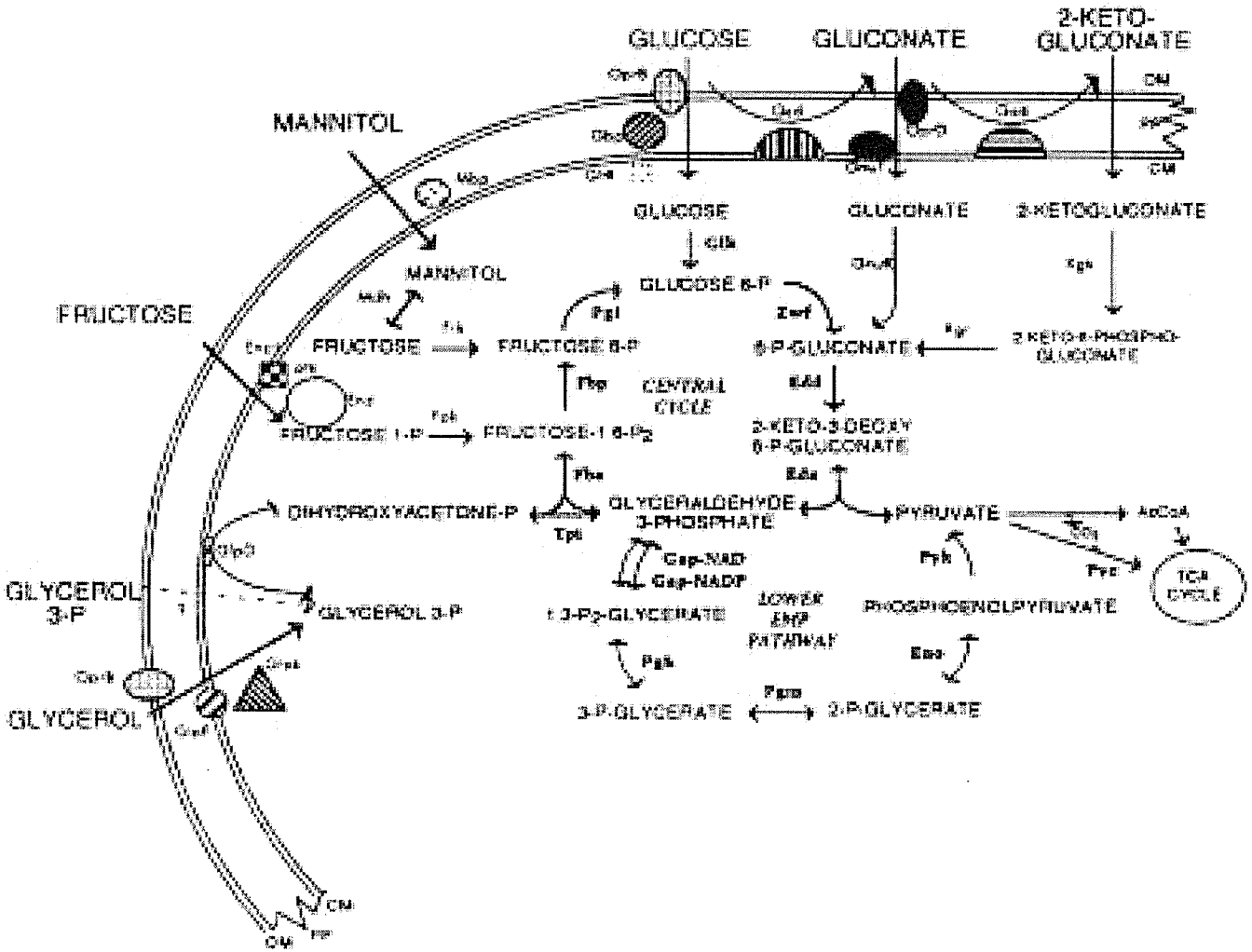
biofilms and non-growing cells of *P. aeruginosa* in phosphate buffer, even at relatively low concentration (Ishida *et al*, 1998).

1.3 Carbohydrate Catabolism by *P. aeruginosa*

P. aeruginosa carbohydrate catabolism is unique in several ways. This organism largely prefers to utilize the enzymes of the Entner-Duodoroff (ED) pathway (Fig. 1.3). Since 6-phosphofructokinase is absent (Tiwari & Campbell, 1969), only the lower Embden Meyerhof pathway (EMP) is operative. However, glyceraldehyde-3-phosphate is rarely converted to pyruvate by the lower EMP, but is usually recycled via the central ED pathway enzymes. *P. aeruginosa* shows a strong preference for TCA cycle intermediates and acids over carbohydrates as carbon sources (Eagon & Phibbs, 1970), with TCA cycle enzymes constitutively produced (Tigerstrom & Campbell, 1966). As such, this organism does not utilize many of the common sugars. Common carbohydrates utilized include fructose, mannitol, glycerol, and glucose, and the corresponding transport and catabolic pathways (as diagrammed in Fig. 1.3) will be discussed in the following subsections.

As illustrated in Fig. 1.3, there are six central cycle enzymes utilized for carbohydrate metabolism. The enzymes Fba (fructose-1,6-bisphosphate aldolase), Fbp (fructose-1,6-bisphosphatase), and Pgi (phosphoglucoisomerase) are constitutively produced. The other three enzymes, Zwf (glucose-6-phosphate dehydrogenase), Edd (6-phosphogluconate dehydratase), and Eda (2-keto-3-deoxy-6 phosphogluconate aldolase) are induced in the presence of glycerol, fructose, mannitol, glucose, or gluconate. The latter enzymes, along with Glk (glucokinase) and NAD-Gap (NAD-dependent

Figure 1.3. 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-Duodoroff) dehydratase; Eda, 2-keto-3-deoxy-6-phosphogluconate aldolase; Pyc, pyruvate decarboxylase; Pyk, pyruvate kinase; Eno, enolase; Pgm, phosphoglucomutase; Pgk, 3-phosphoglycerate kinase; Gap, glyceraldehyde 3-phosphate dehydrogenase; Tpi, triose phosphate isomerase; Pgm, phosphoglucoisomerase; Fba, fructose 1,6-bisphosphate aldolase; Fbp, fructose 1,6-bisphosphatase; Pgi, phosphoglucoisomerase; Frk, fructokinase; Mdh, mannitol dehydrogenase; Fpk, fructose 1-phosphate kinase; GlpD, glycerol 3-phosphate dehydrogenase; GlpK, glycerol kinase. (Reproduced from Temple *et al*, 1998).


GLUCOSE
GLUCONATE
2-KETO-GLUCONATE
MANNITOL
FRUCTOSE
GLUCOSE
GLUCONATE
2-KETOGLUCONATE
MANNITOL
GLUCOSE 6-P
FRUCTOSE
FRUCTOSE 6-P
6-P-GLUCONATE
2-KETO-6-PHOSPHO-GLUCONATE
CENTRAL CYCLE
FRUCTOSE 1-P
FRUCTOSE-1,6-P2
2-KETO-3-DEOXY 6-P-GLUCONATE
DIHYDROXYACETONE-P
GLYCERALDEHYDE 3-PHOSPHATE
PYRUVATE
AcCoA
TCA CYCLE
GLYCEROL 3-P
GLYCEROL
1,3-P2-GLYCERATE
LOWER EMP PATHWAY
PHOSPHOENOLPYRUVATE
3-P-GLYCERATE
2-P-GLYCERATE
CM
OM

glyceraldehyde-3-phosphate dehydrogenase, a lower EMP enzyme), are regulated by the repressor protein HexR, with the genes tightly clustered in the chromosome.

HexR binds to a region of DNA termed the *hexC* locus, which is present in the divergent promoter region between the genes for NAD-Gap and Edd (Proctor *et al*, 1997). An inverted repeat has been identified in this region as the potential binding site for HexR, and a similar sequence has been identified 5' of the transcriptional start site for *zwf*. The current hypothesis is that 2-keto-3-deoxy-6 phosphogluconate (KDPG) inhibits the binding of HexR to the two operator regions, thus allowing transcription of the central cycle enzyme genes. As such, KDPG is an inducer of the *hex*-regulated genes.

The *hex* effect is not fully understood, and is believed to be quite complex. It is unclear if there are HexR-binding sites in the promoter regions of all of the regulated genes, and preliminary data suggests that the expression of each gene is not affected to the same extent by the presence of HexR. Further, the *hex* effect does not explain the mechanism by which catabolite repression control affects central cycle enzyme gene expression.

Presently it is unknown if there is a global regulatory mechanism controlling carbohydrate metabolism in this organism. The identification of the CbrA-CbrB two-component regulatory system suggests that cellular C:N ratios indirectly regulate the catabolism of various natural substrates (Nishijyo *et al*, 2001), but it is unclear at this time if such a global system plays any role in regulation of carbohydrate catabolism. An apparent transregulatory locus, *orfBCD*, has also been identified that, when deleted, results in a significant reduction in [^{14}C]-labeled glucose uptake, and upregulation of [^{14}C]-labeled fructose and [^{14}C]-labeled mannitol uptake, when compared to the wild-

type. Complementation studies are currently underway in an effort to identify which of the open reading frames is responsible for the mutant phenotype. The current knowledge of the regulation of carbohydrate metabolism in *P. aeruginosa* clearly points towards a very complex network of regulatory circuits.

1.3.1 Catabolite Repression

Catabolite repression by succinate has been demonstrated by transferring succinate-grown cells to media containing both succinate and glucose, with the lack of a lag phase indicating that succinate is utilized preferentially (Tiwari & Campbell, 1969). This is further corroborated by the extremely low levels of ED enzymes detected in the presence of such a preferred catabolite. If cells are grown in the presence of both succinate and glucose, diauxic growth is observed (the succinate is used preferentially, and there is a lag phase prior to glucose utilization). The mechanism by which catabolite repression occurs in *P. aeruginosa* is not understood, but it has been determined that cyclic-AMP does not mediate this phenomenon (Siegel *et al*, 1977) (in contrast to the highly-studied catabolite control system of *E. coli*).

A gene has been identified whose product has been shown to be involved in catabolite repression in *P. aeruginosa*. The gene product, Crc (catabolite repression control), is 25-32% identical to prokaryote and eukaryote DNA repair enzymes, but does not seem to bind DNA or exhibit endonuclease activity (Macgregor *et al*, 1996). Crc is required for the expression of most genes that are subject to catabolite repression, but the mechanism by which Crc acts is currently unknown. In the related organism *P. putida*, the nearly identical Crc has been shown to be involved in posttranslational regulation of expression of the branched-chain keto acid dehydrogenase, BkdR (Hester *et al*, 2000),

and it is therefore possible that the protein may function in a similar manner in *P. aeruginosa*. Interestingly, Crc appears to be a sort of global regulator in *P. aeruginosa*, also required for biofilm development and twitching motility (O'Toole *et al*, 2000).

Recently, a gene has been identified that is 91% similar to the catabolite repression protein, CRP, of *E. coli* where it plays a major role in catabolite repression in that organism. The *P. aeruginosa* gene is designated *vfr* (virulence factor regulator), because preliminary studies revealed that the gene product indirectly regulates a large number of virulence factor genes, by regulating the *las* quorum sensing system (a major virulence factor regulator) (Albus *et al*, 1997). Due to the striking resemblance to *E. coli* CRP, studies were undertaken to determine if Vfr plays a similar role in *P. aeruginosa* (Suh *et al*, 2002).

With the dissociation constant, K_D, of 1.6 μM for Vfr binding to cyclic-AMP very similar to that of *E. coli* CRC (0.4 μM), it appears that Vfr is also regulated by cyclic-AMP and may operate by a similar mechanism. Initial complementation experiments with *E. coli crc* null mutants showed that the introduction of *P. aeruginosa vfr* could complement the *E. coli* mutant (West *et al*, 1994), but it was later determined that *vfr* cannot complement a *P. aeruginosa crc* mutant, indicating that, while Vfr may operate via a mechanism similar to that of CRP, it regulates different genes in *P. aeruginosa* and is not involved in catabolite repression in this organism (Suh *et al*, 2002).

1.3.2 Fructose Utilization

Fructose enters the periplasm via OprB, and crosses the cytoplasmic membrane via a PEP-dependent phosphotransferase (PTS) system (Durham & Phibbs, 1982) (Fig. 1.3). This is unusual, as in all other Pseudomonads, carbohydrates are taken up by active

transport systems. PTS systems carry out what is termed group translocation, as substrates are chemically altered (phosphorylated) in the process of translocation. PTS systems typically consist of three components. There is a membrane-bound component called Enzyme II (EII) which is substrate-specific and induced in the presence of the corresponding substrate. The substrate passes through EII into the cytoplasm, where general (non-specific) PTS proteins are involved. In the cytoplasm, PEP is converted to pyruvate, with the phosphate transferred to a soluble general PTS protein Enzyme I (EI). EI-P transfers the phosphate to an Hpr (general PTS) protein, which gives up the phosphate to a subunit of the membrane-bound EII protein. The substrate picks up the phosphate upon entry through EII, with the overall result being PEP + sugar (outside) → pyruvate + sugar-P (inside).

The fructose PTS system of *P. aeruginosa* is unique in that there does not seem to be an Hpr-like protein involved (Durham & Phibbs, 1982). Fructose is trapped in the form of fructose-1-phosphate, and enters the central pathway by conversion to fructose-1,6-bisphosphate via the action of fructose-1-phosphate kinase (Fpk). EI and EII of the PTS system, along with Fpk, are induced when the organism is grown in the presence of fructose. Most of the fructose-1,6-bisphosphate is converted to fructose-6-phosphate, and converted to glyceraldehyde-3-phosphate (G3P) and pyruvate without the use of the lower EMP (Phibbs *et al*, 1978), with only a small portion of the G3P converted to pyruvate via EMP enzymes.

1.3.3 Mannitol Utilization

Mannitol enters the cell by active transport through a putative binding protein-dependent ABC (ATP binding cassette) transport system (ABC transport systems are

described in Section 1.4) (Eisenberg & Phibbs, 1982) (Fig. 1.3). A periplasmic binding protein, MBP (mannitol binding protein), has been identified and shown to bind mannitol specifically. An apparent operon, *mtlEFGK*, appears to encode the binding protein, inner membrane components, and ATPase of the proposed ABC system (Hancock, 2002).

Upon growth on mannitol, production of the outer membrane porin protein OprB is induced (Wylie & Worobec, 1995). Mannitol diffuses through the outer membrane via the carbohydrate-selective porin OprB, is received by MBP, and is presumably transported to and traverses through the inner membrane through the mannitol-induced inner membrane ABC complex, where it enters the cytoplasm unaltered.

The presence of mannitol also induces the enzymes Mdh (mannitol dehydrogenase; converts mannitol to fructose) and Frk (fructokinase; catalyzes the conversion of fructose to fructose-6-phosphate). The resulting fructose-6-phosphate is catabolized as was described in Section 1.3.2 (Fructose Utilization).

1.3.4 Glycerol Utilization

From a medical perspective, information pertaining to *P. aeruginosa* glycerol uptake and metabolism is very important, as this carbohydrate is a good source for biosynthesis of alginate, a major virulence factor. In the lungs, *P. aeruginosa* phospholipase C and extracellular lipases and esterases break down phosphatidylcholine to fatty acids and glycerol. At high concentrations, glycerol passes into the cell by passive diffusion through the carbohydrate selective porin OprB, while at low concentrations, facilitated diffusion is required (Williams *et al*, 1994) (Fig. 1.3). Facilitated diffusion is the least common form of bacterial solute transport (Nikaido & Saier, 1992).

The structural genes for glycerol uptake are a part of the *glp* regulon, which contains at least five operons that map in three regions of the chromosome (Lin, 1976). The *glpFK* operon codes for the membrane-associated glycerol diffusion facilitator, GlpF, and cytoplasmic glycerol kinase, GlpK. Once glycerol passes through GlpF, it is immediately phosphorylated by GlpK to glycerol-3-phosphate. It has also been shown that exogenous glycerol-3-phosphate can enter the cell in unaltered form, but the mechanism by which this occurs is not known. Millimolar concentrations of glycerol have been shown to induce glycerol-3-phosphate uptake, but such uptake cannot be through GlpF, as the diffusion facilitator is not induced at such high concentrations of glycerol.

Glycerol-3-phosphate is converted to dihydroxyacetone phosphate (DHAP) by membrane-bound GlpD (glycerol phosphate dehydrogenase). DHAP is converted to fructose-1,6-bisphosphate by Fba, and is subsequently metabolized by central cycle enzymes. While central cycle enzyme mutants cannot metabolize fructose, mannitol, glucose, or gluconate, such mutants can grow slowly on glycerol. This is because DHAP can be converted to glyceraldehyde-3-phosphate by the action of Tpi (triose phosphate isomerase), and subsequently converted to pyruvate by the lower EMP enzymes.

In *E. coli* glycerol uptake, glycerol-3-phosphate has been found to be the inducer of the system (Cozzarelli *et al*, 1968). It is hypothesized that the same holds true for the *P. aeruginosa* system, as glycerol-3-phosphate induces the *glp* regulon to a greater extent than does the same concentration of glycerol (Schweizer & Po, 1996).

A repressor protein, GlpR, has been identified that negatively regulates the *glp* regulon (Schweizer & Po, 1996). It was previously believed that GlpR was a positive

regulator (Schweizer, 1991a), but more recent data indicates repressor function. *P. aeruginosa* GlpR is 79.4% similar to *E. coli* GlpR (a known repressor). The *E. coli* GlpR operator consensus sequence has been determined and, based on this sequence, putative operator regions have been identified upstream of *P. aeruginosa glpFK* and *glpD*. In addition, GlpR mutants constitutively express GlpF.

1.3.5 Glucose and Gluconate Utilization

P. aeruginosa possesses two systems for glucose uptake: a low affinity oxidative pathway operating with a Km of 2.8 mM (Guymon & Eagon, 1974; Midgley & Dawes, 1973) whereby glucose is oxidized then phosphorylated, and a high affinity phosphorylative pathway operating with a Km of 8 μM (Whiting *et al*, 1976b), whereby glucose is phosphorylated prior to oxidation.

The low affinity oxidative pathway, induced by glucose or gluconate, is functional only under aerobic conditions due to the inactivity of the key enzyme glucose dehydrogenase under anaerobic conditions (Hunt & Phibbs, 1981). In this pathway, periplasmic glucose is oxidized to gluconate by membrane-bound glucose dehydrogenase (Midgley & Dawes, 1973). It has been postulated that periplasmic oxidation of glucose may be advantageous to the cell, as the oxidized form may not be as readily available to other organisms (Whiting *et al*, 1976b).

Extracellular gluconate traverses the outer membrane through OprD (Huang *et al*, 1992), where it is subsequently actively transported across the inner membrane by gluconate permease, the product of the *gnuT* gene (Fig. 1.3). Gluconate is fed into the central cycle as 6-phosphogluconate by the action of GnuK (gluconate kinase), and is

ultimately catabolized to pyruvate. The structural components involved in low affinity glucose uptake require at least 6 mM glucose for induction, with no apparent gluconate threshold (Whiting *et al*, 1976a). When glucose levels exceed 15 mM, an abundance of gluconate represses the system.

By an alternative pathway, gluconate may be even further oxidized extracellularly to 2-ketogluconate by the action of Gad (gluconate dehydrogenase). Recently, the 2-ketogluconate utilization operon, *kgu*, has been identified (Swanson *et al*, 2000). The operon, *kguEKTD*, encodes a potential epimerase (*kguE*), 2-ketogluconate kinase (*kguK*), a 2-ketogluconate transporter (*kguT)*, and 2-ketogluconate-6-phosphoreductase (*kguD*). It is hypothesized that 2-ketogluconate traverses the membrane via KguT, is phosphorylated to 2-ketogluconate-6-phosphate by KguK, and enters the central cycle as 6-phosphogluconate by the action of KguD. The function of the epimerase is not presently known.

The high affinity phosphorylative glucose uptake pathway is induced by growth on minimal media supplemented with glucose (Adewoye & Worobec, 1999). This pathway transports glucose into the cell across a concentration gradient and is therefore subject to catabolite repression. In this pathway, glucose enters the cytoplasm unaltered, where it is subsequently phosphorylated to glucose-6-phosphate by glucokinase, and catabolized by central cycle enzymes (Fig. 1.3).

The high affinity glucose uptake pathway is advantageous to the cell in two respects. Firstly, it allows the cell to grow on glucose in an anaerobic environment. If the oxidative pathway alone existed, this would not be possible, as glucose dehydrogenase (a key enzyme of the low affinity pathway) is active only under aerobic conditions. As it

has been determined that the cystic fibrosis lung is an anaerobic environment (Worlitzsch *et al*, 2002), the high affinity pathway of glucose uptake would be relied upon in cystic fibrosis isolates. Secondly, such a high affinity uptake system may confer a selective advantage. Rather than oxidizing glucose prior to uptake into the cytoplasm, the induction of this system dictates that glucose will be taken up by the cell as rapidly as possible and metabolized afterwards (Whiting *et al*, 1976a).

The components involved in, and the operation of, the low affinity oxidative gluconate uptake pathway of *P. aeruginosa* have been well-characterized, relative to that of the high affinity uptake pathway. Current knowledge has indicated that this latter pathway employs an ABC (ATP-Binding-Cassette) transporter to bring periplasmic glucose into the cytoplasm.

1.4 ABC Transport Systems

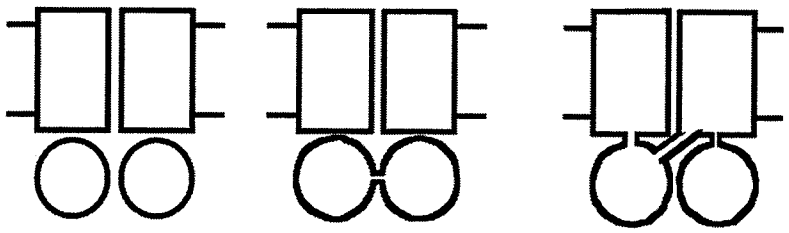
ABC transporters are distributed amongst both prokaryotes (eubacteria and archaebacteria) and eukaryotes, where they function in various processes (Boos & Lucht, 1996) (Wandersman, 1996). In prokaryotes, they function in processes including the uptake of various nutrients and the export of harmful substances including drugs and bacterial toxins (Nikaido, 1994). There are several medically-relevant mammalian ABC transporters. For example, a mutation in the CFTR (Cystic Fibrosis Transmembrane conductance Regulator) transporter leads to the disease cystic fibrosis (Sheppard & Welsh, 1999). Also referred to as Traffic ATPases (Mimura *et al*, 1990), these systems utilize the energy of ATP hydrolysis to transport substrates across concentration gradients.

Fungal and animal ABC transporters are typically a single polypeptide that contains hydrophobic transmembrane spanning domains (TMDs) as well as cytoplasmic ATPase domains (Fig. 1.4). Sometimes the ATPase domains of prokaryotic systems are fused into a single polypeptide that is peripherally-associated with two inner membrane proteins with TMDs on the cytoplasmic face of the membrane (Fig. 1.4). More commonly, prokaryotic ABC transporters consist of four domains encoded by separate polypeptides: two hydrophobic transmembrane domains (TMDs), and two hydrophilic peripheral domains with ATPase activity (associated with the hydrophobic domains on the cytoplasmic face of the membrane) (Fig. 1.4).

ATP transport systems depend on binding proteins to bind and deliver substrate to the complex. In Gram-positive bacteria, the binding proteins are lipoproteins that extend from the cell membrane. ABC transporters of Gram-negative organisms work in conjunction with periplasmic binding proteins (PBPs) that often function in chemotaxis towards the substrate in addition to substrate binding, as well as outer membrane porin proteins to allow exogenous substrate to enter the periplasm (Fig. 1.5). The remainder of this section will deal solely with Gram-negative ABC transport systems.

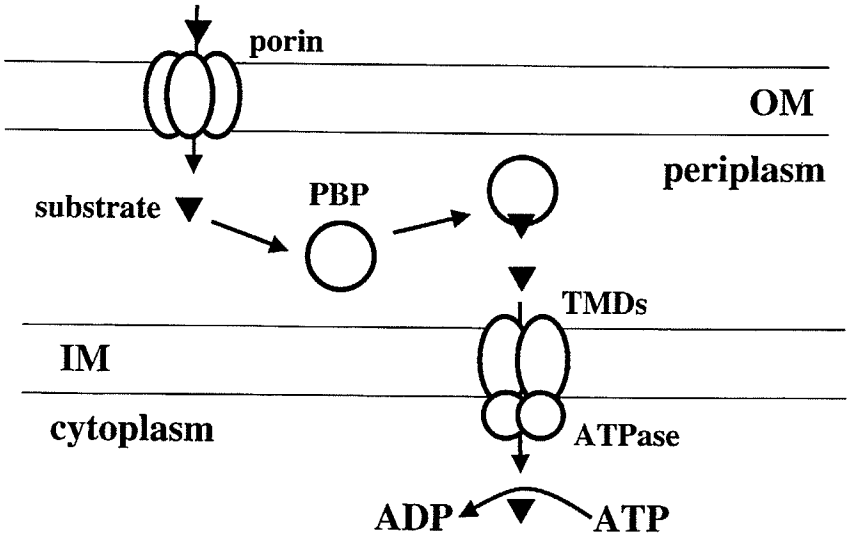
Historically, the PBPs have been the first components of ABC transporters to be identified (Pardee, 1968) (Stinson *et al*, 1977). PBPs of different ABC transport systems are structurally similar to one another. These proteins consist of two nearly

Figure 1.4. Schematic structures of ABC transporters. The most common structure observed in prokaryotes is a four domain structure (A), while fusion between two ATPase components is sometimes observed (B). In fungal and animal systems, the transporter is typically a single polypeptide encoding both transmembrane and ATPase domains (C). Transmembrane domains are represented by rectangles, while ATPase domains are represented by circles. (Reproduced from Nikaido, 2002)



A B C

Figure 1.5. A schematic representation of an ABC transport system present in the inner membrane of a Gram-negative bacterium. The substrate (▼) enters the periplasm through the outer membrane (OM) porin, where it is received by the periplasmic binding protein (PBP). The PBP carries the substrate to the 4-peptide ABC complex at the inner membrane (IM), where passage into the cytoplasm is gained via travel through an inner membrane channel formed through the transmembrane components (TMDs) of the complex. The curved arrow is representative of the conversion of ATP into ADP, upon substrate entry into the cytoplasm, in the direction indicated by the long straight arrow.


porin
OM
periplasm
substrate
PBP
TMDs
IM
cytoplasm
ATPase
ADP
ATP

symmetrical globular domains (lobes) joined by an interior binding cleft (Quiocho & Ledvina, 1996). It is believed that the substrate binds within the binding cleft via hydrogen bonds (Pflugrath & Quiocho, 1988; Quiocho, 1986), with the binding of the substrate by the PBP being the rate-limiting step for substrate ABC transport (Miller *et al*, 1983). Km values for the substrate binding generally range from 0.01-10 μM (Quiocho & Ledvina, 1996).

Upon substrate binding, the PBP undergoes a conformational change, effectively trapping the substrate within the binding cleft. This phenomenon has been referred to as the Venus flytrap model (Mao *et al*, 1982; Sack *et al*, 1989). Once the substrate has been trapped, the PBP is present in a conformation that facilitates interaction with the membrane complex (Prossnitz *et al*, 1988), where it is believed that one lobe of the PBP interacts with one transmembrane subunit, while the second lobe interacts with the other (Hor & Shuman, 1993). As a result, the PBP component is largely responsible for the specificity of the system (Kossman *et al*, 1988; Prossnitz *et al*, 1988; Treptow & Shuman, 1985). In addition, in Gram-negative ABC transport systems, the PBPs are essential for transport activity. This has been demonstrated in studies in which the PBP gene was removed, and transport activity in the resulting knock-out mutants was found to be abolished (Bavoil *et al*, 1983; Wandersman *et al*, 1979).

The TMDs of ABC complexes form a transport pore within the inner membrane, through which the substrate passes into the cytoplasm. These domains are presumed α-helical, with both the N- and the C-termini residing in the cytoplasm. The total number of membrane spanners is usually a multiple of six, with each domain consisting of multiples of 3 extra- and 2 intra-cellular loops (Higgins, 1992). While there is generally little

overall sequence similarity between transmembrane domains of different ABC systems (likely due to the large variety of substrates that are transported), there is, however, a small conserved motif found within each domain. This motif has been termed the EAA motif (Dassa & Hofnung, 1985), with the amino acid sequence EAA-X_3-G-X_9-I-X-L-P (Dassa & Hofnung, 1985). Present in the penultimate cytoplasmic segment and the adjacent TMD (Fig. 1.6), this domain is believed to interact with the hydrophilic ATPase components of the complex (Pearce *et al*, 1992). The EAA motifs appear to be functionally essential, as altering the conserved residues results in decreased or abolished activity of ABC complexes (Mourez *et al*, 1997).

The hydrophilic ATP-binding domains are approximately 200 amino acids long, and typically exhibit 30-50% sequence identity with those of other systems (Higgins *et al*, 1990) (Hyde *et al*, 1990). There are several conserved motifs found within these components: Walker A (or P loop), helical domain, linker peptide (or LSGGQ motif), Walker B, and switch region (Fig. 1.7).

The Walker motifs together form the nucleotide binding pocket, and are involved in ATP binding and hydrolysis. The helical domain, which connects the Walker A and Walker B motifs, contains few conserved residues, but is enriched in hydrophobic amino acids, forming an α-helical structure. It has been proposed that the helical domain interacts with the transmembrane components of the complex (Mourez *et al*, 1997). The linker peptide is a highly conserved region of the proteins sandwiched between the helical domain and Walker B motif. This region is also referred to as the LSGGQ motif, as those are almost invariantly the initial residues of this motif. The linker peptide is believed to interact with the EAA motifs of the transmembrane components of the complex

Figure 1.6. Schematic diagram of MalG, the inner membrane component of the maltose ABC transporter of *E. coli*. Transmembrane-spanning regions are indicated by shaded rectangles, and the location of the EAA motif is shaded and indicated by an arrow. Adapted from (Nelson & Traxler, 1998).

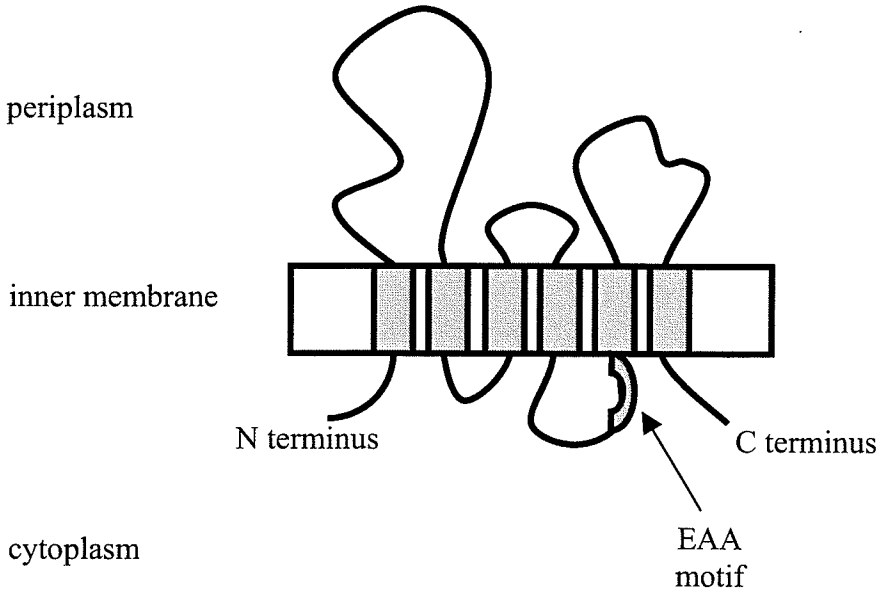
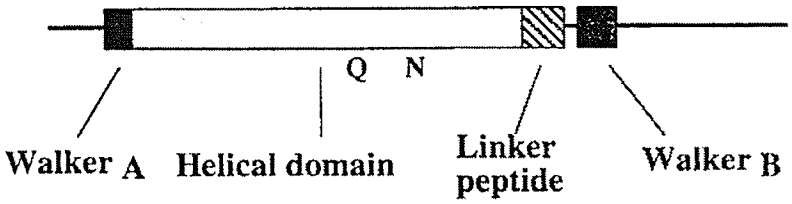

periplasm
inner membrane
N terminus
C terminus
cytoplasm
EAA
motif

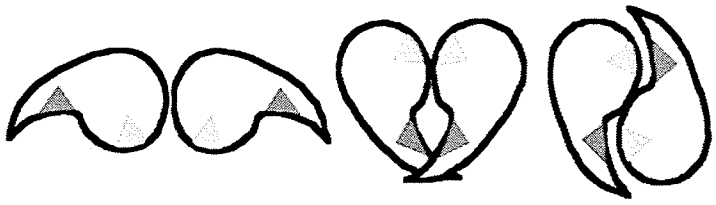
Figure 1.7. Conserved sequence domains of ABC transporter ATPase components. The switch region is not shown. (Adapted from Saurin *et al*, 1999)


Q N
Walker A
Helical domain
Linker peptide
Walker B

(Hunke *et al*, 2000). Following the Walker B motif is the switch region. This region contains a very well conserved histidine residue that appears to be essential for transport function. It has been hypothesized that this histidine residue may sense conformational changes that occur in the transmembrane domains upon substrate binding, and relay such information to the ATPase domains (Speiser & Ames, 1991). The Walker motifs (also referred to as Doolittle motifs or Rossman folds) are characteristic of all ATP hydrolysing proteins (Walker *et al*, 1982), while the linker peptide motif is characteristic of ATPases of the ABC superfamily (Ames *et al*, 1990; Higgins *et al*, 1988).

Due to the relatively large homology of ATPase domains of different ABC transporters to each other, there is much interest in elucidating the structure of these proteins, as there may be widespread applicability. It is possible that the ATPase domains of a wide variety of ABC transporters may exhibit similar structures, and an understanding of such structures may help researchers to gain a better understanding of a common mechanism of substrate transport. There have been several crystal structures reported, however, each structure has thus far served to further confuse rather than to provide additional data for a transport model, as many different types of associations of the ATPase subunits have been reported. For example, the crystal structures obtained for *Salmonella typhimurium* HisP (ATPase component of histidine transporter), *Thermococcus litoralis* MalK (ATPase component of maltose transporter), and *Pyrococcus furiosus* Rad50 (DNA double strand repair enzyme), have all revealed different ATPase:ATPase interfaces (Diederichs *et al*, 2000; Hopfner *et al*, 2000; Hung *et al*, 1998) (Fig 1.8).

Figure 1.8. Proposed arrangement of ATPase dimers of HisP, MalK, and Rad50 based on data obtained from x-ray crystallography. The Walker A motifs are shaded in light grey, and the linker peptides are shaded in dark grey. (Reproduced from Nikaido, 2002)



HisP MalK Rad50

HisP was crystallized bound to ATP with the ATP molecule contacting the Walker A and B motifs, but not the linker peptide region (Hung *et al*, 1998). The monomers are aligned as back-to-back L-shaped domains (Fig. 1.8). Likewise, the MalK crystal (which was formed in the absence of bound nucleotide) revealed an apparent nucleotide binding pocket formed by the Walker A and B motifs, with the linker peptide region located apart from this (Diederichs *et al*, 2000). However, the MalK crystal reveals an interlocking association between the monomers (Fig. 1.8). The DNA double strand repair enzyme Rad50 does not associate with membrane components, but does contain the ABC ATPase signature sequences, and has ATPase activity. The Rad50 dimer was crystallized with two ATP molecules sandwiched between the monomers, with each ATP interacting with the Walker A and B motifs of one ATPase domain, and the linker peptide of the other (Hopfner *et al*, 2000) (Fig. 1.8). Based on the dramatic differences in the structures obtained thus far, it has been suggested that there may be at least two separate classes of ABC transporters (Nikaido, 2002).

The mechanism of energy coupling to substrate translocation in ABC transport systems is unclear, and is currently the topic of much debate in the ABC transporter research community. The traditional view of complex formation and substrate translocation in Gram-negative systems has been described in several papers and reviews (Boos & Shuman, 1998; Locher *et al*, 2002; Schneider, 2001). Briefly, it is generally accepted that the first step in complex formation is the dimerization of the ATPase subunits. The ATPase cassette then associates with the membrane-bound subunits to complete the formation of the ABC complex. Contact with substrate-bound binding protein is required to induce a conformational change in the complex that presumably

increases the affinity of the complex for ATP, possibly by bringing the two ATPase subunits in the correct conformation to complete the ATP binding sites.

Recent data has suggested that it is ATP binding (not hydrolysis) that induces conformational changes in the transmembrane domains that allow for substrate to pass through the membrane and into the cytoplasm (Verdon *et al*, 2003). The binding protein acts as a "plug" throughout the transport process, ensuring unidirectional movement of substrate (Chen *et al*, 2001), then dissociates. Energy obtained from ATP hydrolysis likely facilitates conformational change(s) that bring the complex back to a resting state (Locher & Borths, 2004).

One important question that remains to be answered is, how many ATP molecules are required to transport one molecule of substrate? Attempts at stoichiometric measurements have not been accurate enough to address this question. Does each subunit of the ATPase cassette bind and hydrolyze ATP? There is presently data available to support the idea that either one or two ATP molecules are required.

Studies on the mammalian P glycoprotein transporter have suggested that each site alternates in function (Senior *et al*, 1995), or that interactions between two subunits produce one functional site (Jones & George, 1998). The Davidson lab has been working with the ATP analogue vanadate, and studies with the *E. coli* maltose ABC transporter have shown that only one vanadate molecule binds to the transporter at any one time (Chen *et al*, 2001).

The above data would suggest that only one ATP is required per substrate molecule that is transported. However, it has also been found that nucleotide binding sites are required on both ATPase components in order for transport to occur (Al-Shawi

& Senior, 1993; Davidson & Sharma, 1997). In addition, positive cooperativity in ATP binding (binding of ATP at one site increases the affinity for ATP at the other site) has been demonstrated with the *E. coli* histidine and maltose transporters (Davidson *et al*, 1996; Liu *et al*, 1997).

A study by Moody and colleagues (Moody *et al*, 2002) may help to shed some light on both the assembly of the 4-subunit cassette, and the number of ATP molecules that are bound. This group proposes ATP-dependent ATPase subunit dimerization. This work was prompted by discreptancies between crystal structures obtained for ATPase cassettes that place the linker peptide region distant from the Walker A motif, and biochemical data that suggests otherwise.

Several lines of indirect evidence suggest that the linker peptide region must be in close proximity to the Walker A nucleotide binding site. It has been observed that the linker peptide region of the ATPase domain undergoes a conformational change upon ATP hydrolysis by the Walker motifs (Hunke *et al*, 2000), and mutations in this region have been shown to affect ATP hydrolysis (Schmees *et al*, 1999; Shyamala *et al*, 1991). Vanadate cleavage studies also suggest a close association between these two regions (Fetsch & Davidson, 2002). Vanadate is an ATP analogue that can presumably bind to the transporter in the same manner that ATP would. When exposed to UV, vanadate will catalyze cleavage of the polypeptide backbone near to where it is bound. Cleavage studies with vanadate bound to the *E. coli* MalK ATPase have demonstrated reproducible cleavage at the Walker A and linker peptide regions. Further, in the crystal structure of Rad50, ATP is seen bound to the Walker A and B motifs of one ATPase subunit, and to the linker peptide region of the other (Hopfner *et al*, 2000).

Since the Rad50 structure seems to support the mounting evidence of a close relationship between the linker peptide and nucleotide binding regions, Moody and collaborators set out to determine if the requirement for ATP for dimerization also holds true for other ATPase subunits (Hopfner *et al*, 2000). An elegant set of experiments were carried out to obtain direct evidence to prove that a) the linker peptide and Walker A motifs of the *Methanococcus jannaschii* ATPases MJ0796 and MJ1267 associate in an ATP-dependent manner (Hopfner *et al*, 2000), and b) that the linker peptide and Walker A motifs are in close proximity in the resulting ATP sandwich dimer (Smith *et al*, 2002).

Mutant versions of MJ0796 and MJ1267 were constructed that lack the capacity to hydrolyze ATP (Hopfner *et al*, 2000), with the hypothesis that, if dimerization is indeed ATP-dependent, such mutant proteins should produce stable "ATP sandwich dimers" in the presence of ATP. Wild-type and mutant proteins were mixed with ADP with no formation of wild-type dimers, and a very low proportion of mutant dimers. When mixed with ATP, mutant proteins were seen to form homodimers, thus supporting the hypothesis of ATP-dependent dimerization. Interestingly, when the same experiments were carried out with ATP analogues, very poor dimerization of the mutants was observed. The authors suggest that the subtle differences in chemical properties of the analogues as compared to ATP may account for this, and may explain the difficulties that other groups have experienced with isolating wild-type dimers with non-hydrolyzable ATP analogues.

The crystal structure of the MJ0796 ATP sandwich dimer was solved at 1.9Å resolution (Smith *et al*, 2002). In general, the major structural features of the Rad50 dimer were found to be conserved in the MJ0796 crystal structure. As expected, the linker peptide motifs were found to be in close proximity to the Walker A site, and in fact

appear to "complete" the ATPase active sites. Two ATP molecules were bound between the Walker A motif of one subunit, and the linker peptide of the other, as with the Rad50 structure.

The ATP/substrate stoichiometry of the OpuA ABC transporter of *Lactobacillus lactis* has also been investigated (Patzlaff *et al*, 2003). This system is osmoregulated and requires both the presence of the substrate, glycine betaine, and ionic activation in order for ATP hydrolysis to occur. As such, this system provides highly regulatable and reproducible conditions under which to examine the ATP/substrate stoichiometry. Two independent assays (using proteoliposomes with right-side-out or inside-oriented OpuA) have demonstrated that 2 molecules of ATP are hydrolyzed per substrate molecule transported.

The solving of more structures of ABC transporter components/complexes will continue to provide insight that may be applied to a universal mechanism for the action of these ubiquitous transporters. Due especially to the medical relevance of many of these systems in eukaryotes (ex. CFTR) and the fact that many ABC drug exporters have been identified in prokaryotes, this is currently a hot area of research and is likely to remain so for some time.

1.4.1 *E. coli* Maltose ABC Transporter

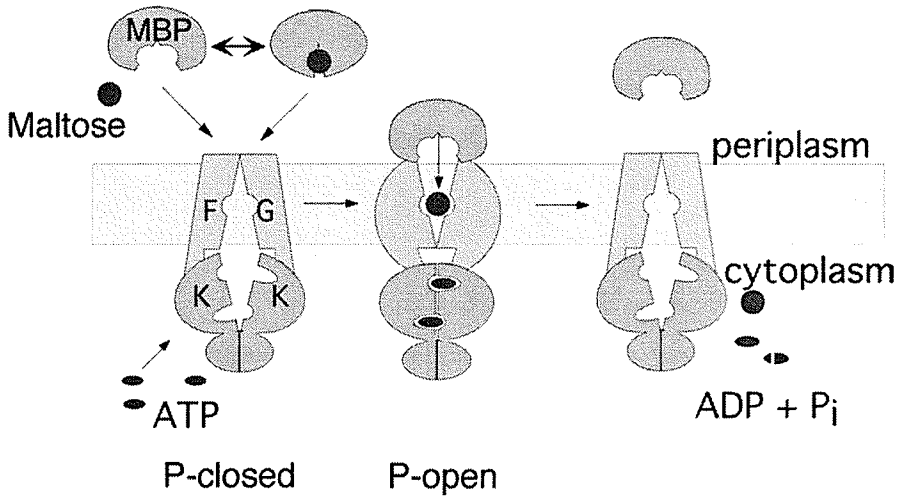
The best-characterized member of the ABC superfamily of transporters is that of the maltose uptake transporter of the Gram-negative organism *E. coli*. This ABC transporter is involved in the uptake of maltose and maltodextrins, $\alpha(1\rightarrow4)$-linked glucose polymers up to 8 glucose units in length. The ABC complex of this transporter is

termed the $MalFGK_2$ complex, with the inner membrane components corresponding to MalF and MalG (Shuman *et al*, 1980; Silhavy *et al*, 1979), and MalK representing the ATPase domain (Bavoil *et al*, 1980). The periplasmic component is referred to as MBP, for maltose binding protein (Kellerman & Szmelcman, 1974), and it has been determined that maltose enters the periplasm via the outer membrane porin protein LamB (Ferenci & Boos, 1980; Szmelcman & Hofnung, 1975).

Structural models proposed for the MBP of *E. coli* based on crystal structures predict a protein with two lobes separated by a substrate binding cleft (Sharff *et al*, 1992), common features of PBPs of Gram-negative ABC transport systems. The current model of maltose transport in *E. coli* includes changes from open to closed conformations of MBP (Fig. 1.9) (Austermuhle *et al*, 2004). Open-conformation MBP binds maltose and changes to closed form, essentially trapping the maltose. At the inner membrane, each lobe of substrate-bound MBP interacts with one of the transmembrane components (Hor & Shuman, 1993), presumably exposing a substrate recognition site of the $MalFGK_2$ complex (Reyes *et al*, 1986). Maltose is released, and MBP reverts back to closed-conformation. A conformational change of the $MalFGK_2$ complex occurs as a result of contact with maltose-bound MBP, and is required to support conformational changes that create a pore for maltose to pass through, and allows for ATP hydrolysis by MalK (Boos & Lucht, 1996; Shilton *et al*, 1996).

The inner membrane components, MalF and MalG, have 8 and 6 transmembrane spanning domains, respectively (Boyd *et al*, 1987; Dassa & Muir, 1993), with the total number of TMDs 14, rather than the usual 12. It is believed that the additional 2 TMDs of MalF contribute to the stability of the protein, and are possibly important for

Figure 1.9. Model for maltose transport in *E. coli*. Open-conformation MBP accepts maltose, and changes to closed-conformation. Upon delivery of maltose to the FGK_2 complex at the membrane, FGK_2 changes from P-closed conformation (periplasmic-closed state) to P-open conformation (periplasmic-open state). Following ATP hydrolysis, maltose is released into the cytoplasm, and open-conformation MBP is released from the FGK_2 complex. (Reproduced from Austermuhle *et al*, 2004)


MBP
Maltose
periplasm
F
G
K
K
cytoplasm
ATP
ADP + Pi
P-closed
P-open

proper folding and membrane insertion (Tapia *et al*, 1999). However, the removal of a portion of the N-terminus of MalF, including the first TMD, does not abolish transport function, indicating that this region is dispensible with respect to transport function (Ehrmann & Beckwith, 1991).

Mutational studies have been carried out in an effort to elucidate the roles of the individual TMDs and hydrophilic regions of MalF (denoted P for periplasmic, and C for cytoplasmic). In enteric organisms, homologues of MalF typically contain a rather large P2 domain (Horlacker *et al*, 1998). Mutational analysis suggests that this domain is essential for function (Tapia *et al*, 1999), and structural studies indicate that the conformation of this region differs in the presence or absence of MalG (Traxler & Beckwith, 1992).

The contribution of TMDs 3 and 4 with respect to the function of the transporter is currently not known, but it is possible that TMD 3 is required for proper assembly of MalF, while mutations in TMD 5 have been implicated in altered substrate specificity (Steinke *et al*, 2001). The C-terminal TMDs, TMDs 6-8, were shown to be important with respect to structure and function of MalF. Mutations targeting TMD 6 and 7 led to reduced maltose uptake, with mutated versions of TMD 6 exhibiting altered substrate specificity, and mutated TMD 7 leading to improper MalF assembly (Ehrle *et al*, 1996). TMD 8, the final TMD, has been linked to transport efficiency.

A collection of MalG mutants (Nelson & Traxler, 1998) has provided insight into the domains of this protein that are responsible for assembly, transport, or both. Surprisingly, insertion mutations within the C terminal cytoplasmic region have been

demonstrated to affect transport activity, by mechanisms as yet unclear. The P3 domain has been found to be important for transport, likely involved in the initial interactions with MBP (prior to ATP hydrolysis and substrate transport) as well as proper assembly of MalG. As expected, the C3 domain (containing the EAA motif) is required for $MalFGK_2$ complex assembly.

MalK, the ATPase component of the *E. coli* maltose uptake system, has been shown to dimerize independently of MalF or MalG, and it has been postulated that this dimerization is the first step towards the formation of the 4-peptide complex (Kennedy & Traxler, 1999). In membrane vesicle experiments, MalK was shown to be accessible to protease digestion from the periplasmic side of the membrane, suggesting that MalK may contain a membrane-traversing segment(s) (Schneider *et al*, 1995a; Schneider *et al*, 1995b). This phenomenon has also been observed in studies of the HisP protein of the prokaryotic histidine ABC transport system (Baichwal *et al*, 1993), and is further supported by the fact that the presence of both MalG and MalK are required to protect MalF from protease digestion (Panagiotidis *et al*, 1993).

The *E. coli* maltose uptake system is regulated in part by MalT, a maltose-induced, positive transcriptional regulator (Richet & Raibaud, 1987). MalT regulates expression of the ABC transporter, binding protein, and LamB. MalT activity is itself regulated in part by MalK (Boos & Shuman, 1998; Kuhnau *et al*, 1991; Reyes & Shuman, 1988). The current hypothesis is that when MalT is bound by the inducer of the maltose uptake system, maltotriose, it is in active form. However, MalT may alternatively be bound by MalK, which holds MalT in an inactive conformation (Joly *et al*, 2004).

Typically, ATPases that also exhibit regulatory function are larger than those that do not, with C-terminal extensions. It has been determined by mutational studies that the regulatory function of MalK resides in a C-terminal domain (Kuhnau *et al*, 1991). A regulatory ABC ATPase is not exclusive to this system, and has been observed in other bacterial and archaeal systems.

Three amino acid motifs were identified in *E. coli* MalK that have been found to be more than 70% identical to sequences found in other systems, when 60 other bacterial and archaeal ABC ATPases were surveyed (Bohm *et al*, 2002). The motifs have been termed RDMs (regulatory domain motifs), and are perhaps a means of communication between the N-terminal ATPase domain, and C-terminal regulatory domain. This proposed function is based on an examination of the structural model for MalK, in which it appears that RDM 1 (present in the ATPase domain) contacts RDMs 2 and 3 of the regulatory domain by a hinge motion. Interactions between the C-terminal RDMs of MalK and MalT have recently been demonstrated (Samanta *et al*, 2003).

A model of substrate travel through the inner membrane complex has been proposed for the maltose uptake system of *E. coli* (Ehrmann *et al*, 1998). This model is based on the hypothesis that there are regions of continuous hydrophobicity as well as regions of continuous hydrophilicity within the MalFG channel. Maltose is predicted to initially interact with a hydrophilic region of the channel, and travel through the channel until a hydrophobic region is encountered that blocks further travel. A subsequent conformational change of the complex is then required in order to transfer maltose to a hydrophilic region. Based on the current models for the channel structure, it is predicted

that two such conformational changes are required in order for the maltose to pass through the entire channel and enter the cytoplasm.

1.5 *P. aeruginosa* Glucose ABC Transporter

The first component of the *P. aeruginosa* glucose ABC transport system to be identified was the periplasmic binding protein, GBP (glucose binding protein) (Stinson et al, 1977). This 44.5 kDa protein is distinct from MBP of *E. coli*, in that it is a glycoprotein, with the carbohydrate moiety accounting for approximately 16% of the total weight. One mole of GBP has been shown to bind one mole of glucose, with a dissociation constant (K_D) of 0.35 μM. Mutant strains of *P. aeruginosa* lacking GBP exhibit a phenotype defective both in high affinity glucose transport and glucose chemotaxis, again supporting the role of this protein as a component of a glucose ABC transport system.

In 1985, *P. aeruginosa* mutants defective in glucose transport were identified (Cuskey & Phibbs, 1985). Upon further characterization in 1993, such mutants were observed to exhibit wild type phenotype with respect to glucose uptake upon addition of purified GBP or periplasmic contents from wild-type *P. aeruginosa* grown on glucose, or by introduction of a plasmid carrying a 1.1 kB open reading frame (ORF) designated *gltB* (Sly *et al*, 1993). While demonstrating that the ORF *gltB* is required for the expression of GBP, this study did not confirm whether *gltB* actually encodes GBP, or a regulatory element of the system.

It was not until 1996 that it was determined that *gltB* encodes a regulatory element, with identity to the OmpR family of two-component response regulators (Sage *et*

al, 1996). As a result, the ORF was renamed *gltR*. The predicted translation product of *gltR* contains conserved residues present in response regulators, and exhibits ~ 47% identity and 70% similarity to OmpR of *E. coli* (Wurtzel *et al*, 1982), a well-characterized response regulator. GltR appears to be a specific regulator of glucose metabolism, as *gltR* mutants cannot utilize glucose, but are unaffected with respect to utilization of fructose, mannitol, or glycerol (Sage *et al*, 1996).

The identification of the *P. aeruginosa* GBP led to an interest in the mechanism by which extracellular glucose enters the periplasm in this system. It has been determined that the outer membrane porin protein OprB (Hancock & Carey, 1980) is the channel facilitating passive diffusion of glucose into the periplasm (Wylie & Worobec, 1995). OprB is a trimeric 47 kDa protein that plays a central role in carbohydrate uptake in *P. aeruginosa*. Initially believed to be glucose-specific (Trias *et al*, 1988), it has been found that OprB is inducible by growth on, and facilitates diffusion of, not only glucose, but fructose, glycerol, mannitol, and gluconate as well (Williams *et al*, 1994; Wylie & Worobec, 1993). As such, OprB is best termed carbohydrate-specific.

Extensive biophysical characterization of *P. aeruginosa* OprB has been carried out (Wylie & Worobec, 1995). Black lipid bilayer experiments were performed in order to determine the magnitude of the pore formed by OprB. The single channel conductance for *P. aeruginosa* OprB was found to be 25 pS (Siemens). This is comparable to that of *P. putida*, which was previously determined to be 35 pS (Saravolac *et al*, 1991), but in contrast to that of *E. coli* LamB, estimated at 160 pS (Benz *et al*, 1986). CD (circular dichroism) spectra analysis has indicated that OprB is approximately 25% α-helical, 40% β-sheet, and 34% random coil (Wylie *et al*, 1993), with topological modeling predicting

that each monomer forms a 16-stranded β-barrel (Wylie, 1994). In addition, ion-selectively experiments suggest that, unlike OprB of *P. putida*, and LamB of *E. coli*, which are highly cation-selective, *P. aeruginosa* OprB is an anion-selective porin.

The peripheral ATPase component of the *P. aeruginosa* glucose ABC transport system has been tentatively identified, and named GltK (Adewoye & Worobec, 2000). Cloning and sequencing of the regions flanking the *oprB* ORF led to the identification of the *gltK* ORF, which exhibits 11-54% amino acid homology to other ATP-binding proteins, and 38-54% homology to carbohydrate ATPases of other ABC transport systems. With 43.5% amino acid homology to MalK of *E. coli*, and possessing conserved helical domain residues implicated in interaction in the cytoplasmic membrane in similar positions to that of *E. coli* MalK, it is likely that GltK is a member of the MalK subfamily of ABC proteins.

In order to assess the involvement of GltK in high affinity glucose uptake, a *gltK* deletion mutant was constructed whereby the 3' end of the gene was removed. The *gltK* deletion mutant exhibited a 2-fold reduction in glucose uptake activity, when compared to wild type *P. aeruginosa* in [^{14}C]-glucose uptake assays. While this result indicates that GltK is physiologically important, further experimentation will be required to interpret the results further. Adewoye and Worobec have suggested two possible explanations for the results of the [^{14}C]-glucose uptake assays. Firstly, it is possible that the truncated version of *gltK* may have recombined with the 3' end of another ATPase-encoding gene, resulting in a hybrid protein with partially functional glucose uptake activity. This hypothesis is supported by *Salmonella typhimurium malK* mutational studies, where a hybrid protein containing the N-terminal end of MalK fused to the C-terminal end of a

histidine transport ATPase was identified and found to be partially functional (Schneider & Walter, 1991). An alternative possibility is that, in the absence of a functional GltK protein, an alternative energy transduction process is utilized in order to transport glucose into the cytoplasm.

With the recent completion of the *P. aeruginosa* genome sequencing project (Stover *et al*, 2000) it became possible to further examine the region of the chromosome containing genes known to be involved in the high affinity glucose uptake system. This has revealed the presence of two ORFs immediately upstream of *gltK* and *oprB* that are possible candidates for the genes of the transmembrane components of this system. These ORFs have been named *gltF* and *gltG* (nomenclature based on the maltose uptake system of *E. coli*), and are described within the *P. aeruginosa* genome database, PseudoCap (Hancock, 2002). These two proteins are the focus of this thesis, with efforts directed towards confirming the inner membrane location and involvement in glucose uptake of GltF and GltG.

In summary, the current knowledge of the high affinity phosphorylative glucose uptake system of *P. aeruginosa* is strongly suggestive of the presence of an inner membrane ABC transporter complex, acting in conjunction with the outer membrane porin OprB and periplasmic GBP. Continued studies focusing on this high affinity glucose uptake system will provide valuable insight into the physiology of this organism.

CHAPTER TWO

MATERIALS AND METHODS

2.1 Bacterial strains, plasmids, and growth conditions

All bacterial strains and plasmids used in this study are described in Table 2.1.

All *P. aeruginosa* and *E. coli* cultures were routinely grown in Luria-Bertani (LB) medium (Difco™) at 37°C. When required, antibiotics were added to the media at the following concentrations: ampicillin (Ap), 100 μg/ml (*E. coli*); tetracycline (Tc), 15 μg/ml (*E. coli*), 60 μg/ml (*P. aeruginosa*); carbenicillin (Cb), 400 μg/ml (*P. aeruginosa*); chloramphenicol (Cm), 30 μg/ml (*E. coli*); and kanamycin (Km), 50 μg/ml (*E. coli*). Blue-white colony screening with *E. coli* NM522 was carried out with the addition of 0.5 mM IPTG (isopropylthio-β-D-galactoside) (Sigma) and 40 mg/ml of X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) to the appropriate antibiotic-containing LB agar plates.

When it was desired to induce the high affinity glucose uptake pathway of *P. aeruginosa*, cells were grown in BM2 medium (Basal Medium 2: 40 mM K_2HPO_4, 22 mM KH_2PO_4, 7 mM $(NH_4)_2SO_4$, 0.5 mM $MgSO_4$, 10 μM $FeSO_4$) (Hancock & Carey, 1979) supplemented with 0.4% D-glucose (w/v). When it was desired to induce protein expression in *E. coli* via induction of the *lac* promoter, IPTG was added to a final concentration of 1 mM to log phase *E. coli* cultures harboring pUCP20::*gltF* or pUCP21::*gltG*. Cultures were incubated for an additional 3 hr to allow for IPTG-inducible gene expression.

Cultures were maintained at –60°C for long term storage. DMSO (dimethyl sulfoxide), cryopreservant, was added to log phase cultures to a concentration of 7% (v/v).

Table 2.1. Bacterial strains and plasmids used in this study.[a,b]

Bacterial strain or plasmid	Relevant Characteristics[a,b]	Source or Reference
P. aeruginosa		
PA01	wild-type prototroph	B. Iglewski (Rochester, NY)
H103	wild-type prototroph derived from PA01	(Hancock & Carey, 1979)
20781	PA01 with IS*lacZ*/hah insertion (in the same direction relative to the ORF) between nucleotides 882 and 883 of gltF	(Jacobs *et al*, 2003)
42725	PA01 with IS*phoA*/hah insertion (in the same direction relative to the ORF) between nucleotides 204 and 205 of gltG	(Jacobs *et al*, 2003)
H18F	H103 (pEX18Tc::ΔF) co-integrate	This study
H18G	H103 (pEX18Tc::ΔG) co-integrate	This study
E. coli		
NM522	supE thi Δ(lac-proAB) hsd5 F'[proAB$^+$lacI^q lacZΔM15]	Promega
MT616	MT607 (pro-82 thi-l hsdR17 supE44)::pRK600	(Finan *et al*, 1986)
Plasmids		
pKS$^+$	Ap^R, high copy number cloning vector (ColE1 replicon)	Stratagene
pKS:: ΔG2	pKS$^+$ with 1.72 kb ΔG2 PCR product	This study

pUC18	Ap^R, high copy number cloning vector (ColE1 replicon)	(Yannisch-Perron *et al*, 1985)
pUC18::ΔF1	pUC18 with 0.36 kb ΔF1 insert	This study
pUC18::ΔF2	pUC18 with 0.23 kb ΔF2 insert	This study
pUC18::ΔF	pUC18 with 0.59 kb (ΔF1+ΔF2) insert	This study
pUC18:: ΔG1	pUC18 with 1.15 kb ΔG1 insert	This study
pUC18:: ΔG	pUC18 with 2.87 kb (ΔG1+ΔG2) insert	This study
pUCP20	Ap^R/Cb^R, high copy number, broad host range cloning vector derived from pUC18	(Schweizer, 1991b)
pUCP20::*gltF*	pUCP20 with 1.03 kb gltF insert (FD3/F3 PCR product from H103 genome)	This study
pUCP21	Ap^R/Cb^R, high copy number, broad host range cloning vector derived from pUC19	(Schweizer, 1991b)
pUCP21::*gltG*	pUCP21 with 1.06 kb gltG insert (G3/G2 PCR product from H103 genome)	This study
pEX18Tc	Tet^R, sacB-containing replacement vector with pUC18 MCS (ColE1 replicon)	(Hoang *et al*, 1998)
pEX18Tc:: ΔF	pEX18Tc with 0.59 kb (ΔF1+ΔF2) insert	This study
pEX18Tc:: ΔG	pEX18Tc with 2.87 kb (ΔG1+ΔG2) insert	This study
pRK600	Cm^R, tra^+ helper plasmid (ColE1 replicon)	(Finan *et al*, 1986)

[a] Refer to Table 2.2 for a description of the plasmid inserts.

[b] The following abbreviations have been used: Ap^R = ampicillin resistance; Cb^R = carbenicillin resistance; Tc^R = tetracycline resistance; Km^R = kanamycin resistance; Cm^R = chloramphenicol resistance; MCS = multiple cloning site.

2.2 *P. aeruginosa* Chromosomal DNA Extraction

P. aeruginosa chromosomal DNA extraction was carried out as described by Hancock (Hancock, 2001) with some modifications. Approximately 500-700 µl of an overnight culture was pelleted in a microfuge and resuspended in 400 µl of TES buffer (50 mM Tris-HCl, 10 mM NaCl, 10 mM EDTA, pH 7.5). Seventeen µl of 30% (w/v) sarkosyl was added to achieve a final concentration of 1%, and 2.5 µl of 20 mg/ml Proteinase K was added to obtain a final concentration of 100 µg/ml. The microfuge tube was gently mixed and lysis was allowed to proceed at 37°C for 30-60 minutes, or until the solution was clear.

Following lysis, 400 µl of 4 M ammonium acetate was added, the resulting solution was mixed gently and extracted once with an equal volume of 25:24:1 phenol:chloroform:isoamyl alcohol. The aqueous layer was washed with 1 ml of 70% ethanol, and a small DNA sample was then hooked out of the solution onto the end of a flame-sealed capillary tube, and placed into a fresh microfuge tube. The DNA was dried at 37°C and resuspended in 50 µl of TE buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0) with the addition of 1 µl of 1 mg/ml RNaseA. One µl of DNA prepared by this method was routinely used per 25 µl PCR (Polymerase Chain Reaction).

2.3 Polymerase Chain Reaction

All PCR reactions were carried out in a PTC-150 Minicycler, with either *P. aeruginosa* genomic DNA or plasmid DNA as the template. The template was denatured at 95°C, annealing temperatures used ranged from 48-52°C, and elongation was carried out at 72°C for 30 seconds per every 1 kb of product expected. Glycerol and DMSO

(dimethyl sulfoxide) were used as enhancing agents at concentrations of 10% (v/v) and 5% (v/v), respectively. PCR primers used in this study are described in Table 2.2.

Table 2.2 PCR primers used in this study. Relevant restriction endonuclease recognition sites are underlined.

Primer Pairs (Restriction Site)	Primer Sequence (5'→ 3')	Product (kb)
FD3 (*Eco*RI) F3 (*Hind*III)	CGC TGA ATT CTC CCG ACT G CTG AAG CTT GGG TTC AGG	*gltF* (1.03)
FD3 (*Eco*RI) HAH-166	CGC TGA ATT CTC CCG ACT G TCA CCC GTT AAA CGG CGA	5' end of *gltF* + 5' end of IS*lacZ*/hah (1.16)
G2 (*Eco*RI) G3 (*Hind*III)	AGG AAT TCG CCG GAG TCG CTG AAG CTT GGC GCT GTA CT	*gltG* (1.06)
G3 (*Hind*III) LACZ-211	CTG AAG CTT GGC GCT GTA CT CGG GCC TCT TCG CTA TTA	5' end of *gltG* + 5' end of IS*phoA*/hah (0.44)
F1 F3 (*Hind*III)	CGA CGT GGT GAC CAA CT CTG AAG CTT GGG TTC AGG	*gltF* (1.2)
FD1 (*Bam*HI) FD3 (*Eco*RI)	CGA TGA AGG ATC CGC CGA CGC TGA ATT CTC CCG ACT G	ΔF1 (0.36) [containing the 5' end of *gltF*]
FD2 (*Bam*HI) F3 (*Hind*III)	TCA AGG GAT CCG ACC TG CTG AAG CTT GGG TTC AGG	ΔF2 (0.23) [containing the 3' end of *gltF*]
GDO1 (*Kpn*I) GD1 (*Bam*HI)	CGC TGG TAC CTC CCG ACT G CAG CAG GAT CCC GGT GCG	ΔG1 (1.15) [containing the 5' end of *gltG*]
GD2 (*Bam*HI) K2 (*Hind*III)	TCC TGG ATC CGA TGT CCA CTC AAG CTT GAA GTC GTA GC	ΔG2 (1.72) [containing the 3' end of *gltG*]

2.4 DNA Sequencing

PCR products to be sequenced were purified with the UltraClean 15 kit (Mo Bio), and sequencing-grade plasmid DNA was prepared by the alkaline lysis method using the Qiaprep Spin Miniprep Kit (Qiagen) according to the manufacturer's instructions. DNA sequencing was carried out at the automated sequencing facility of the National Research Council/Plant Biotechnology Institute, Saskatoon, Saskatchewan, Canada.

2.5 Computer Analysis of DNA and Protein Sequences

P. aeruginosa PA01 gene sequences were obtained from the PseudoCAP genome sequence database (Hancock, 2002) and the gene sequences and the corresponding protein sequences were analyzed with the Omiga 2.0 software package (Oxford Molecular Ltd.). Further topological analysis was carried out with the PSIPRED protein structure prediction server (McGuffin *et al*, 2000), and amino acid sequences were examined for N-terminal signalling sequences via PSORT (Nakai & Kanehisa, 1992).

2.6 Plasmid DNA Isolation

Plasmids were routinely isolated from *E. coli* and *P. aeruginosa* strains by the alkaline lysis method (Ausubel *et al*, 1989). Cultures were grown overnight in media supplemented with the appropriate antibiotic. Approximately 1.5 ml of the overnight culture was pelleted in a microfuge at room temperature, resuspended in 100 µl of GTE buffer (10 mM EDTA, 50 mM glucose, 25 mM Tris-HCl, pH 8.8), and incubated at room temperature for at least 5 minutes. Two hundred µl of freshly-prepared lysis solution (0.2 M NaOH, 1 % SDS) was added dropwise, and the tube was mixed gently and incubated on ice for no longer than 5 minutes. The lysed solution was neutralized by the addition of 150 µl of 3 M sodium

acetate pH 5.2, followed by a 10 minute incubation period on ice. The neutralized solution was microfuged for 10 min at room temperature to remove the SDS-cell debris, and the clear supernatant was transferred to a clean tube. If necessary, this step was repeated with an additional 5 min spin if the supernatant was not clear.

The supernatant was extracted with an equal volume of 25:24:1 phenol:chloroform:isoamyl alcohol. To the resulting aqueous phase, 0.7X volume of room temperature isopropanol was added, and the mixture was immediately microfuged for 15 minutes at 4°C. The pellet was washed with 70% ethanol, dried under vacuum, and then resuspended in an appropriate volume of sterile water. Plasmids were stored at 4°C for short term storage, or –20°C for long term storage.

2.7 Recombinant DNA Techniques

All DNA manipulations and cloning procedures were carried out by standard recombinant DNA techniques, as described by Ausubel *et al* (Ausubel *et al*, 1989).

2.8 Cloning of *P. aeruginosa gltF* and *gltG*

P. aeruginosa H103 genomic DNA was isolated and utilized as the template for the PCR amplification of *gltF* and *gltG*. PCR primers were designed based on the gene sequences of *gltF* and *gltG,* as described on the *P. aeruginosa* genome database, PseudoCAP (Hancock, 2002), where the genes appear as PA3189 and PA3188, respectively. Amplification of *gltF* was carried out with the primers FD3 (forward) and F3 (reverse), while *gltG* was amplified with primers G3 (forward) and G2 (reverse). The genes were ligated into the high copy number, broad host range plasmid vectors pUCP20 (*gltF*) and pUCP21 (*gltG*), by ligating *Eco*RI/*Hind*III-digested inserts into the plasmids

treated with the same enzymes. The multiple cloning sites of pUCP20 and pUCP21 are in the opposite orientation relative to each other, and a different plasmid was used to clone each gene in order to ensure that the gene was ligated in the correct orientation to allow for expression from the *lac* promoter on the plasmid. The pUCP constructs were transformed into competent *E. coli* NM522. All DNA manipulations and cloning procedures were carried out by standard recombinant DNA techniques, as described by Ausubel *et al* (1989).

2.9 *E. coli* Competent Cell Preparation and Transformation

E. coli competent cells were prepared by the $CaCl_2$ method (Ausubel *et al*, 1989). Cells were grown overnight in LB broth at 37°C. Fresh LB broth was inoculated with a 1% inoculum of the overnight culture, and incubated with shaking to an $OD_{600} = 0.3$ at 37°C. Cells were harvested by centrifugation in a Sorvall centrifuge at 1000 x g for 5 minutes at 4°C, and were resuspended in 1/10 of the original culture volume of 0.1 M ice cold $CaCl_2$. Following 30 min incubation on ice, cells were harvested by centrifugation at 1000 x g for 5 min at 4°C, and resuspended in 1/25 volume of ice cold 0.1 M $CaCl_2$. Glycerol was added to a final concentration of 23%, and 200 µl aliquots were stored at -60°C.

For transformation, approximately 10 ng to 1µg DNA in a volume of 10 µl was added to 100 µl of competent cells. After incubation on ice for 30 min, the mixture was heat-shocked at 42°C for 3 min, and cold-shocked on ice for 5 min. Seven hundred µl of room temperature LB broth was added, and the cells were incubated without shaking for 50 min at 37°C. One hundred µl aliquots were plated in duplicate on selective media, and

the remainder of the cells were pelleted and resuspended in 100 µl of fresh room temperature LB broth and plated.

2.10 *P. aeruginosa* Competent Cell Preparation and Transformation

Competent *P. aeruginosa* cells were prepared by the method of Olsen *et al* (1982) with some modifications. Cells were grown overnight in TN broth (5% w/v tryptone, 1% w/v glucose, 2.5% w/v yeast extract) at 37°C. Fresh TN broth was inoculated with a 1% inoculum of the overnight culture, and incubated with shaking to an OD_{600} = 0.3 at 30°C. Cells were harvested by centrifugation in a Sorvall centrifuge at 1000 x g for 5 min at 4°C, and were resuspended in ½ of the original culture volume of cold transformation buffer (10 mM Tris, pH 8.0, 50 mM $MgCl_2$, 10 mM $CaCl_2$). Following a 10 min incubation period on ice, cells were again pelleted and resuspended in ½ of the original culture volume of transformation buffer. Cells were incubated for 20 min on ice, harvested, and resuspended in 1/10 of the original culture volume of 0.15 M $MgCl_2$ with 15 % (v/v) glycerol and 10 mM PIPES (piperazine-N,N'-bis[2-ethanesulfonic acid]), pH 7.0. Cells were stored at –60°C in 200 µl aliquots.

For transformation, approximately 0.2 µg of plasmid in a volume of 25 µl was added to 200 µl of competent cells. After incubation on ice for 1 hr, the mixture was heat-shocked at 42°C for 3 min, and cold-shocked on ice for 5 min. Five hundred µl of room temperature LB broth was added, and the cells were incubated without shaking for 2 hours at 37°C. One hundred µl aliquots were plated in duplicate on selective media, and the remainder of the cells were pelleted and resuspended in 100 µl of fresh room temperature LB broth and plated.

2.11 Triparental Mating

Triparental mating was carried out as described by Goldberg and Ohman (Goldberg & Ohman, 1984). *P. aeruginosa* recipient cells were grown overnight in L broth (a modification of LB broth with an NaCl concentration of only 0.5%) at 42°C, while donor and helper cells (carrying either the mobilizing plasmid pRK2013 or pRK600) were grown overnight in LB broth supplemented with the appropriate antibiotic at 28°C or 30°C. One hundred µl of the recipient culture, followed by 100 µl of the donor and helper cultures were added to 2 ml of L broth. This mixture was then filtered through a 0.45 µM Nalgene filter unit, and the filter was removed and placed cell-side up on a fresh L agar plate. The plate was incubated at 28°C overnight, after which the cells were soaked off of the filter into 3 ml of sterile saline (0.8 % NaCl). The cell suspension was diluted ten-fold to 1/1 000, and 100 µl of each dilution, along with the undiluted cell suspension, were plated onto the appropriate selective plates and incubated overnight at 37°C, or until colonies developed.

2.12 Cell Envelope Preparation

Cell envelopes were prepared by the method described by Lugtenberg *et. al* (1975). Cells were grown overnight in 50 ml of culture medium, and harvested by centrifugation at 5000 x g for 5 min. Cells were resuspended in 10 ml of 50 mM Tris, 2.5 mM EDTA, pH 8.5, and passed through a French Press at 18 000 psi twice. Unlysed cells and cell debris was removed by centrifugation at 1200 x g for 10 min, and cell envelopes were collected by centrifuging the supernatant at 100 000 x g for 1 hr. Membrane

samples were resuspended in 50 mM Tris, pH 8.0. A few crystals of PMSF (phenylmethylsulfonyl fluoride) were added to inhibit protease activity. Samples were stored at –20°C.

2.13 SDS-Polyacrylamide Gel Electrophoresis

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Laemmli (Laemmli, 1970). Protein samples were boiled in the presence of loading dye (0.4% w/v SDS, 12 mM Tris-HCl, pH 8.0, 2% w/v glycerol, 1% v/v β-mercaptoethanol) for 10 min prior to loading on a 12% polyacrylamide gel. A pre-stained molecular weight marker (New England Biolabs) was utilized, and electrophoresis was carried out at 100 – 130 V. Gels were stained in Coomassie Brilliant Blue R-250 (0.05 % w/v Coomassie Brilliant Blue R-250, 10 % v/v acetic acid, 30 % v/v ethanol) for 1 hr and destained for 45 min and then overnight in fresh destain solution (7.5 % acetic acid, 20 % methanol).

Typically, 50 – 100 μg of cell envelope protein samples were analyzed on mini-gels.

2.14 [^{14}C] Glucose Uptake Assays

Whole cell [U-^{14}C]-glucose uptake assays were performed by the membrane filtration assay of Eagon and Phibbs (Eagon & Phibbs, 1970), modified by Wylie and Worobec (Wylie & Worobec, 1993). *P. aeruginosa* strains were grown overnight in BM2 medium supplemented with 0.4% glucose at 37°C. Fresh medium was inoculated with a ~5% inoculum of the overnight culture (to achieve an OD_{600} = 0.1), and cells were grown

at 37°C to mid-log phase (OD_{600} = 0.5). Cells were harvested by centrifugation at 10 000 x g for 10 min, and washed in 10 ml of fresh BM2 (with no glucose supplementation). This was repeated, and cells were resuspended in an appropriate volume of BM2 (with no glucose supplementation) to achieve an OD_{600} = 0.04.

Cells were divided into 10 ml aliquots and placed at 37°C for 10 minutes to prewarm the cells. At time zero, [U-^{14}C]-glucose (Amersham Biosciences) was added to a concentration of 33 μM. Five hundred μl samples were removed at 30 second intervals, and placed onto 0.45 μm Durapure® membrane filters (Millipore) on a manifold (Millipore). Immediately following sample application to the filter, 5 ml of BM2 (with no glucose supplementation) was applied to wash unadsorbed glucose from the filter. Formaldehyde-killed cells (exposed to 18% formaldehyde for at least 10 min prior to the start of the assay) were used as a control.

Filters were placed into scintillation vials, 10 ml of ScintiSafe Plus 50% scintillation fluid (Fisher Scientific) was added, and cpm for each samples was read by an LS 6500 scintillation counter (Beckman). To obtain the final reported cpm for each time point, the backround cpm obtained from the formaldehyde-killed cells (attributed to non-specific filter binding) was subtracted. Each strain of interest was analyzed at least three times by this method, and the reported cpm values are an average of all trials.

2.15 *gltF* and *gltG* Replacement Systems

The construction of *gltF* and *gltG* replacement vectors was approached by a PCR-based method, and constructs were introduced into *E. coli* NM522 via transformation. The PCR was utilized to amplify the 5' and 3' ends of the gene of interest in separate

reactions, omitting amplification of the interior region of the gene (this region being of a size divisible by 3, so as not to disrupt the reading frame of downstream genes). Primers for these reactions were designed such that the two resulting gene fragments could be ligated together upon digestion with a common restriction endonuclease, *Bam*HI, the recognition site for which being engineered into the appropriate primer (see Table 2.2 for a description of the primer pairs used, and Figure 2.1 for an illustration of the experimental design). Preliminary cloning steps to capture the PCR products were carried out in the ColEI replicon pUC18 or pKS^+, due to the relative ease of cloning into these plasmid vectors. The resulting truncated version of the gene was then ligated into the ColEI replicon pEX18Tc to complete the construction of the replacement vector.

The replacement vectors were introduced into *P. aeruginosa* by triparental mating, with initial selection of co-integrates on LB plates supplemented with 60 μg/ml tetracycline. Since pEX18Tc cannot replicate in *P. aeruginosa*, and 60 μg/ml of tetracycline is too high a concentration for the donor *E. coli* strain to withstand, colonies growing on the LB/tetracycline plates represent co-integrates in which the plasmid has been integrated into the chromosome by a cross-over event at a homologous region.

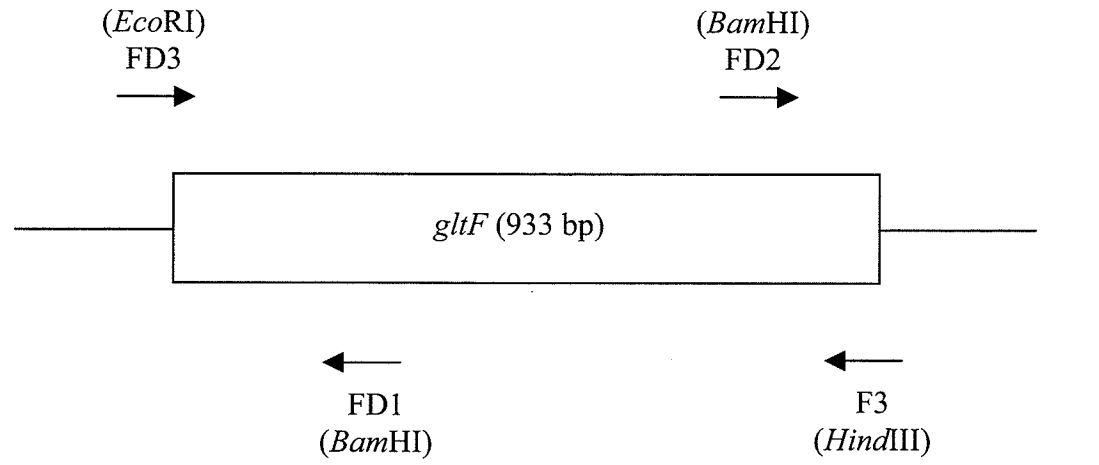
Co-integrates were streaked onto LB plates supplemented with 5% sucrose to promote plasmid excision and curing. pEX18Tc contains the *sacB* gene which encodes levansucrase, an enzyme that synthesizes the production of levans (sucrose polymers) which accumulate in the periplasm. Therefore, growth on LB/sucrose agar plates is expected to be lethal for *P. aeruginosa* harboring pEX18Tc, and is a means to promote excision of the plasmid from the chromosome by a second cross-over event, followed by curing of the plasmid. Several modifications to this step were carried out separately and

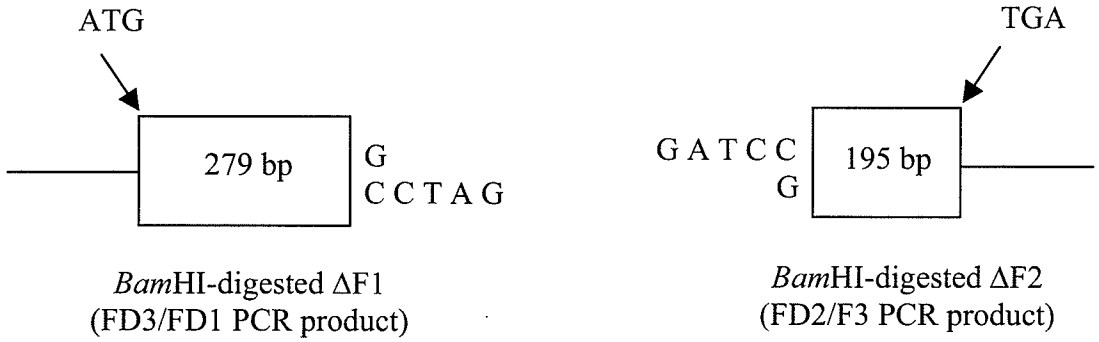
in combination, and include alternate growth temperatures (28°C vs 37°C), alternate growth medium (TYS agar plates vs LB agar plates), and initial growth in TYS broth (Tryptone-Yeast extract Salt medium: 10 g trypticase peptone, 5 g yeast extract, 5 g NaCl per litre) supplemented with 5% sucrose (w/v) prior to plating on solid medium. It has been observed that the rate of spontaneous mutation of *sacB* is increased at temperatures above 30°C (Blomfield *et al,* 1991), and that temperature and salt concentration also affect the level of expression of *sacB* (likely by altering the degree of DNA supercoiling) (Goldstein & Drlica, 1984).

Screening of colonies for the desired homologous recombination events (replacement of the chromosomal copy of the gene of interest with the truncated version carried on the replacement vector) was by the PCR. Genomic DNA was isolated and PCR was carried out with the primer pairs FD3/F3 (for *gltF*) or G3/G2 (for *gltG*) utilized to amplify the genes. The size of the PCR amplicons from potential mutants were compared to the amplicon obtained from known wild-type genomic DNA and that from the replacement vector.

Figure 2.1. Schematic diagram representing PCR-based approach taken to construct a) ΔF and b) ΔG from *gltF* and *gltG* found within the genome of *P. aeruginosa* H103. Arrows indicate primer binding sites and direction of extension (dashed lines up- and downstream of *gltG* indicate that primer binding sites for GDO1 and K2 are farther away from the gene than depicted). Relevant restriction sites engineered within primers are indicated in brackets next to the primer names.

a)



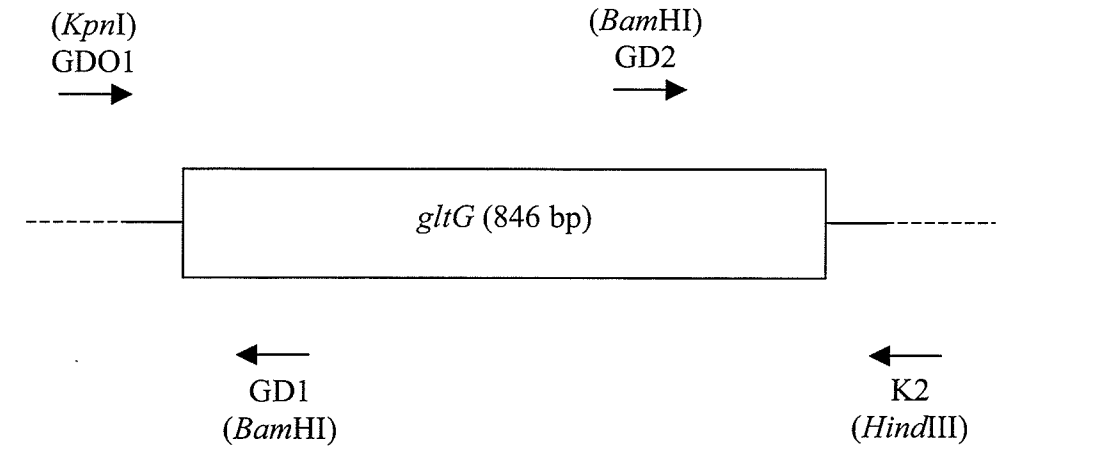


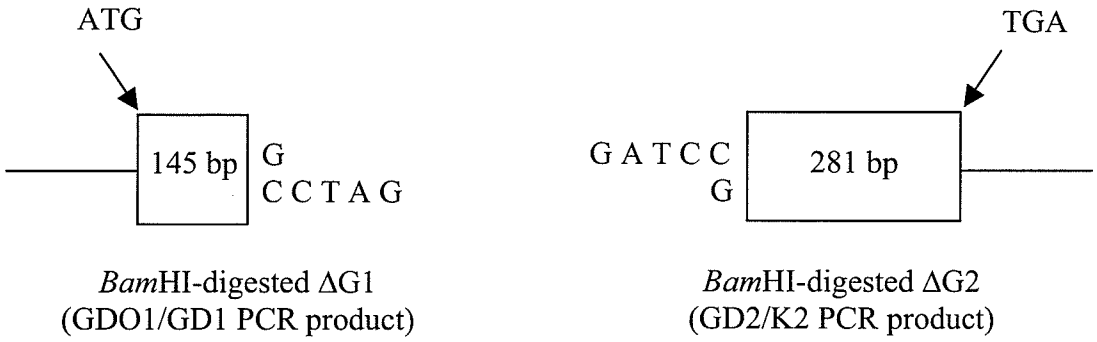
*Bam*HI-digested ΔF1
(FD3/FD1 PCR product)

*Bam*HI-digested ΔF2
(FD2/F3 PCR product)

ΔF: ΔF1 ligated to ΔF2, interior 453 bp of original 933 bp ORF absent

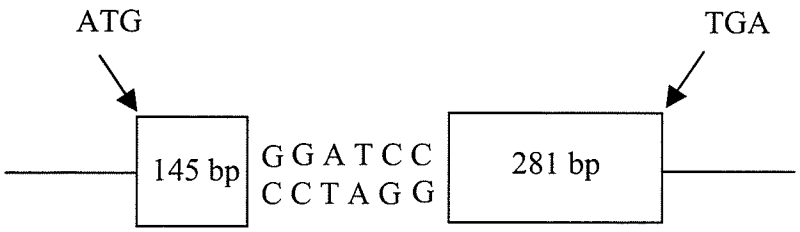
b)





*Bam*HI-digested ΔG1
(GDO1/GD1 PCR product)

*Bam*HI-digested ΔG2
(GD2/K2 PCR product)



ΔG: ΔG1 ligated to ΔG2, interior 414 bp of original 846 bp ORF absent

CHAPTER THREE

RESULTS AND DISCUSSION

3.1 Identification, cloning, and molecular characterization of *gltF* and *gltG*

3.1.1 Results

In order to identify the genes for the inner membrane components of the *P. aeruginosa* glucose ABC transporter and to analyze the predicted amino acid sequences of the corresponding proteins, computer-aided analysis was carried out. This was followed by cloning and sequencing of the genes from wild-type *P. aeruginosa* strain H103.

The complete genome sequence of *P. aeruginosa* PA01 has been reported (Stover *et al*, 2000), and a genome database maintained by the Pseudomonas Community Annotation Project collaborators (PseudoCAP; www. pseudomonas.com) has been made available. The PseudoCAP database was scanned to identify the ORFs surrounding *oprB*, the gene known to encode the outer membrane porin through which glucose gains entry into the periplasm of *P. aeruginosa*. This search revealed the presence of what appears to be an operon downstream of genes for a putative two component response system (*gltR* and its probable sensor kinase) and immediately upstream of *oprB*. This region of the genome also contains other genes known to be involved in carbohydrate metabolism (Fig. 3.1). The genes are described by PseudoCAP as *gltBFGK* (to reflect the nomenclature used to describe the well-characterized *E. coli* maltose ABC transporter), and are annotated as PA 3190-3187, respectively. The predicted amino acid sequence of GltB is 63% identical to a periplasmic sugar-binding protein of *P. putida*, while GltK is 89% identical to a sugar ATP-binding subunit of *P. putida,* and contains Walker A and

Figure 3.1. The arrangement of a cluster of *P. aeruginosa* carbohydrate utilization genes, as pictured on the *P. aeruginosa* genome database, PseudoCAP. From left to right, the genes correspond to PA3181 - PA3195 on the database. The *hexR* gene and those known to be under the control of HexR are shaded as▩, the genes believed to be involved in high affinity glucose uptake are shaded as⊠, and the putative two-component response system genes are shaded as ⊡. The genes correspond to the following proteins: *gapA*, NAD-dependent glyceraldehyde 3-phosphate dehydrogenase; *edd*, 6-phosphogluconate (Entner-Duodoroff) dehydratase; *glk*, glucokinase; *gltR*, GltR; *gltB*, GBP (Glucose Binding Protein); *gltF*, GltF; *gltG*, GltG; *gltK*, GltK; *oprB*, OprB; PA3185, predicted protein of unknown function; *hexR*, HexR; *zwf*, glucose 6-phosphate dehydrogenase; *pgl*, 6-phosphogluconolactonase; *eda*, 2-keto-3-deoxy-6-phosphogluconate aldolase. The direction of transcription of genes pictured below the line is leftward, while the genes pictured above the line are transcribed in a rightward direction (as indicated by arrows).

edd
glk
glxR
sensor
glxB
glxF
glxG
glxK
oprB
PA3185
hexR
zwf
pgl
eda
gapA
1 kbp

Walker B ATP-binding motifs. Involvement of GltK in high affinity glucose uptake has previously been confirmed (Adewoye & Worobec, 2000).

There is a putative promoter upstream of *gltB* closely resembling the experimentally-determined promoter of *oprB* (Adewoye & Worobec, 1992) (Fig. 3.2), while there are no obvious -35 or -10 promoter-like elements observed in the intergenic regions between *gltB* and *gltF* or between *gltG* and *gltK*. There are no terminator sequences found downstream of *gltB*, *gltF*, or *gltG*, although there is potential for secondary structure formation between *gltK* and *oprB* (Fig. 3.2) that may function to halt transcription in a Rho-independent manner.

There are two potential reading frames for *gltF*, with the potential start codon for the larger of the two potential ORFs a distance of 129 bp upstream of the other. PSORT analysis (http://psort.ims.u-tokyo.ac.jp), which predicts protein localization to the inner membrane based on the presence of hydrophobic stretches or N-terminal signal sequences (Nakai & Kanehisa, 1992), did not predict the presence of N-terminal signal sequences in either GltF or GltG. The *gltF* and *gltG* genes overlap by 7 bp with putative ribsome binding sites upstream of both genes (Fig. 3.2), allowing for production of both GltF and GltG from a single transcript.

Primer pairs FD3/F3 and G3/G2 were designed based on the sequences of the flanking DNA of *gltF* (FD3 was designed to bind upstream of the larger ORF in the event that the larger reading frame is transcribed) and *gltG*, respectively, and were utilized to amplify both genes by the PCR from the *P. aeruginosa* H103 genome. Subsequent sequence analysis of the PCR products revealed that the H103 gene

Figure 3.2. Nucleotide sequence of *gltBFGK* and flanking DNA. Start and stop codons are shaded, putative ribosome binding sites (RBS) are underlined and labelled, and putative –35 and –10 promoter elements are boxed. Palindromes upstream of *gltF* and downstream of *gltK* are underlined.

-35 -10 RBS

1 gaacgcttga atagactcgg agcattgcaa gcgcagactt gctccgaata acaagaagaa

***gltB* start**

61 ggttttcctc atgaatgcga tccgtcgcct ctctgccgtc atctgtcttt cctccctctg

121 cctctccccg ctgctggccc aggccggcga agtcgaagtg ctgcactggt ggacctccgc

181 cggcgagaag cgcgccgccg aaaccctgaa gaagctggtc gaagccaagg gccacacatg

241 gaaggacttc gccgtggccg gcggtggcgg cgaggcggcg atgaccgtgc tgaagacccg

301 cgcggtgtcc ggcaatccgc cggccgcggc gcagatcaag gggccggata tccaggagtg

361 gggcgaactg ggcctgctcg ccgacctcaa cgcggtggcc gccgaaggca agtgggacag

421 cctgctgccg aagcaggtgg cgcagatcat gaagtacgac ggcgactacg tagcagtgcc

481 gatcaacgta caccgggtga actggctcta catcaacccg gaagtgttca agaaggccgg

541 cgccaccccg ccgaccaccc tcgacgaact gttcgtcgcc gccgacaagc tcaaggccgc

601 cggtttcacg ccgctggccc atggcagcca accgtggcag gacggcaccg tgttcgagaa

661 cctggtgctg agcaagatgg gtccggaagg ctatcgcaag gccttcgtcg aacaggacaa

721 ggcgaccctc accggagcgc agatggtcga ggtgttcgcc gcgctgaaga agctccgcgg

781 ctacgtcgat gccgacgccg ccggtcgcga atggagcgcc gccacggcga tggtgatcaa

841 cggcaaggcc gggatgcaga tcatgggcga ctgggcgaag agcgagttca ccgccgccgg

901 caaggtgccg ggcaaggact accagtgcct gccgttcccc ggcacgcaga aagccttcga

961 ctacaacatc gactcgctgg tgatgttcaa gctgagcaac gcggagaacc gcaaggccca

1021 ggaagacctg gcgcgcagcg tgctcgaccc gtccttccag aaggacttca acctcaacaa

1081 gggctccatc ccggttcgcc tggacgccga catggcgccg ttcgacagtt gcgcccagca

1141 gtcgatgaag gacttcaagc aggcttccca ggacggcaac ctggtgccga gcatggccca

1201 cagcatggcc gcttccagct acgtgcaggg ggcgatcttc gacgtggtga ccaacttctt

1261 caacgacccc gccgcggatc cgcagaaggc cgcccagcaa ctggcggccg ccatcgaggc

***gltB* stop**

1321 ggcggcgcag taagtccctg gcgcggatcc cggccagggc tccgctcgat cctcccgact

RBS ***gltF* start**

1381 ggctccgtgc cgccgagcgg cacgggtggg ttccttcgca cgcgagaaaa cgcatggcga

1441 ccaattcccc tgtctatccc acggctccgg tagcgcctcg tcgcgtctcg ccactcgatc

1501 gcctgcaagg ctggctgccg aagctggtgc tggcgccaag cgtggtcgtg gtgctgttct

alternate *gltF* start

1561 gcatgtatgg ctacattggc tggacgctgc tgctgtcctt caccaactcg cgcttcatgc

1621 ccagctacaa gtgggccggg ctgagccagt accagcgcct gtgggacaac gaccgctggt

1681 gggtggcgag caagaacctg ctgctcttcg gcggctcgtt catcgccatc tgcctggtgc

1741 tcggagtgtt cctcgcagtg ctgctggacc agcgcatccg ccgcgaaggc ttcatccgca

1801 ccctctacct gtatcccatg gcgttgtcga tgatcgtcac cggcaccgcc tggaagtggc

1861 tgctcaatcc gggcctgggc ctggacaagc tgctccgcga ctggggctgg gaaggtttcc

1921 gcttcgactg gctggtggac ccggagcggg tcgtctactg cctggtgatc gccgccgtgt

1981 ggcaggcctc cggcttcgtc atggcgctgt tcctcgccgg cctgcgcggg gtcgacccgg

2041 ccatcgtgcg cgccgcccag gtcgacggtg cgagcctgcc gaccatctac ctgcgcatcg

2101 tcctgccgag cctgcgcccg gtgttcttca gcgcgctgat gatcctcgcc catatcgcga

2161 tcaagagctt cgacctggtg gcggcgatga ccgccggcgg cccgggttat tcctccgacc

2221 tgccggcgat gttcatgtat gcgcatacct tcacccgtgg ccagatgggg ctcggcgcgg

2281 cgagcgcgat gctgatgctc ggcgctgtac tggcgatcgt ggtgccttac ctgtattccg

RBS **_gltG_ start** **_gltF_ stop**

2341 aactgcgagg caagcgccat gcgtgacgcg tccctgaacc cgagcttcag cgtcagccgc

2401 ctggccgtgc acctgaccct ctggggcgcc tgcgcgcttt acctggtgcc gctgctggtg

2461 atgctgctga ccagcttcaa gacgccggac gacatccgca ccggcaacct gctgtcgctg

2521 cccgacacct tcaccgtcat cggctggctc aaggcctggg acagcgtcgg cgcgttcttc

2581 tggaactcgg tgctgatcac cgtgcccggc gtgctgatct ccaccttcat cggcgcgttg

2641 aacggctacg tgctgtcgat gtggcgcttc cgcggttccc agctgttctt cggcgcgctg

2701 ttgttcggct gcttcctgcc gttccaggtg atcctcctgc cgatgtcctt caccctcggc

2761 aagctcggcc tggccaatac caccagtggc ctggtgctgg tccacgtgat ctacggcctg

2821 gccttcacca cgctgttctt ccgcaactat ttcgtggcga tcccggatgc cctggtcaag

2881 gccgcgcgcc tggacggcgc cggcttcttc accatcttcc tgcgcatcct gctgccgatg

2941 tccacgccca tcgtgatggt ctgcctgatc tggcagttca cccagatctg gaacgacttc

3001 ctgttcggcg tggtgttcgc cagcggcgat tcgcaaccga tcaccgtggc cctgaacaac

3061 ctggtgaaca tcaccaccgg ggtcaaggaa tacaacgtcg acatggccgc ggcgatgatc

3121 gccgcgctgc ccacgctggt ggtctacgta ctggccggca agtacttcgt gcgcggcctc

gltG stop **RBS** **_gltK_ start**

3181 accgccggcg ccgtgaaagg ctgagacggg tcgctgaccc cggagacgaa cgaaacatgg

3241 caaccctcga actgcgcaac ctgcacaaga gctacggcag cggcctggcg gataccctca

3301 gggacatccg gctgaagatc gactccggcg aattcctgat cctcgtcggg ccctcgggtt

3361 gcggcaagtc gaccctgatg aactgcatcg ccgggctgga gagcatcacc ggcggtgcga

3421 tcctggtgga cggccaggac atcagcggca tgagccccaa ggaccgcgac atcgccatgg

3481 tcttccagtc ctacgccctg tacccgacca tgagcgtgcg cgagaacatc gccttcggcc

3541 tgaagatccg caagatgccc caggccgcca tcgacgagga ggtggcgcgg gtggccaggc

3601 tgttgcagat cgagcacctg ctcgaacgca agccctcgca gctctccggc ggccagcagc

3661 agcgggtggc catgggccgg gcgctggcgc ggcggcccaa ggtctacctg ttcgacgagc

3721 cgctgtccaa cctcgatgcc aagctgcggg tggaaatgcg caccgagctg aagctgatgc

3781 accagcggct gaagaccacg accgtgtatg tcacccacga ccagatcgag gccatgaccc

3841 tcggcgacaa ggtggcggtg atgaaggacg gcgtcatcca gcagttcggc acgccgcagc

3901 agatctacaa cgacccggcc aacctgttcg tcgccagctt catcggttcg ccgccgatga

3961 atttcattcc gttgcgcctg cagcgcggga cggacgctgg accggcctgc tcgatagcgg

***gltK* stop**

4021 ccaggaccgt atcgaactgc cgttgagcct ggagcccggc ctggaggagg ggcgcgagct

4081 gatcctcggc gtacgaccgg agcagatcgc cctgggcgat ggcagcgctg cccatgacct

4141 gcgtaccgag gtcgaggtgc tcgaaccgac cgggccggac accctggtct tcgtcaccgt

4201 caaccaggtc aaggtctgtt gccgcctggc gccggacgtc gcgccggccg cgggcagcgg

4261 catgagcctg cgcttcgatc ccgcgcgggt cctgctgttc gacgcgcaga gcggcgagcg

-35

4321 cgtggccgtg cgcacggcgg gcgtggcggc ggagaaggtc accgccttga acggcaatcg

-10 **RBS** ***oprB* start**

4381 ccagaagaac tcctgaatcc tttcgtactg cctagaccac aaccacgagg acgctggaaa

4441 tg

sequences were 100% identical to the PA01 sequences reported by PseudoCAP, thus there are no apparent strain-specific sequences.

A BLASTX search (Altschul *et al*, 1990) with the predicted amino acid sequence of GltF (based on the larger potential reading frame) revealed 74% identity to that of putative ABC sugar transporters of *P. putida* and *P. syringae*. When the shorter potential amino acid sequence was submitted for analysis, the identities to the above-mentioned *P. putida* and *P. syringae* proteins increased to 75%. Upon viewing the amino acid alignment of GltF and the *P. putida* putative permease (GenBank accession no. AAC98811), it is evident that the decrease in identity of the larger GltF sequence is due to the fact that the *P. putida* protein has a smaller N-terminal cytoplasmic region (Fig. 3.3). A BLASTX search with the predicted amino acid sequence of GltG revealed 73% identity to an ABC permease of *P. putida*, and 72% identity to an ABC permease of *P. syringae*.

Further analysis of the predicted amino sequences was carried out using Omiga 2.0 software (Oxford Moleculars). The molecular weight of GltF is predicted to be 29.9 or 34.5 kDa, depending on the size of the reading frame, and the molecular weight of GltG is predicted to be 30.7 kDa. The EAA amino acid motif that is characteristic of ABC transporter permeases was found to be present in both GltF and GltG (Fig. 3.4).

von Heijne transmembrane helix prediction plots were prepared by Omiga 2.0 software. The von Heijne method of topological analysis generates a hydrophobicity profile of the primary sequence, based on the von Heijne algorithm (von Heijne, 1992). Hydrophobicity is plotted against residue number, and positive hydrophobicity peaks indicate probable membrane-spanning regions of the protein. von Heijne analysis of the predicted amino acid sequences of GltF and GltG indicate that both proteins contain 6

Figure 3.3. Amino acid alignment of *P. aeruginosa* GltF and *P. putida* probable ABC-type permease (GenBank accession no. AAC98811). The position of the first amino acid of the smaller potential GltF protein is indicated with a *. Non-identical, non-similar amino acid residues are given as "X" in the consensus sequence, while non-identical, similar amino acid residues are given as "X" in the consensus sequence.

```
          1                                                   50

P. putida          MS SIAVFSKASP FDALQRWLPK LVLAPSMFIV LVGFYGYILW
GltF       MATNSPVYPT APVAPRRVSP LDRLQGWLPK LVLAPSVVVV LFCMYGYIGW
Consensus  XXXXXXXXXX XXXXXXXXSP XDXLQXWLPK LVLAPSXXXV LXXXXGYIXW
                                                              *

          51                                                  100
P. putida  TFVLSFTTST FLPSYNWAGL AQYQRLFDND RWWVASKNLA VFGGMFIGIS
GltF       TLLLSFTNSR FMPSYKWAGL SQYQRLWDND RWWVASKNLL LFGGSFIAIC
Consensus  TXXLSFTXSX FXPSYXWAGL XQYQELXDND RWWVASKNLX XFGGXFIXIX

          101                                                 150
P. putida  LVVGVILAVF LDQRIRREGF IRTIYLYPMA LSMIVTGTAW KWLLNPGMGL
GltF       LVLGVFLAVL LDQRIRREGF IRTLYLYPMA LSMIVTGTAW KWLLNPGLGL
Consensus  LVXGVXLAVX LDQRIRREGF IRTXYLYPMA LSMIVTGTAW KWLLNPGXGL

          151                                                 200
P. putida  DKLLRDWGWE GFRLDWLIDP DRVVYCLVIA AVWQASGFIM AMFLAGLRGV
GltF       DKLLRDWGWE GFRFDWLVDP ERVVYCLVIA AVWQASGFVM ALFLAGLRGV
Consensus  DKLLRDWGWE GFRFDWLXDP XRVVYCLVIA AVWQASGFXM AXFLAGLRGV

          201                                                 250
P. putida  DQSIIRAAQI DGASLPTIYL KVVLPSLRPV FFSAVMILAH IAIKSFDLVA
GltF       DPAIVRAAQV DGASLPTIYL RIVLPSLRPV FFSALMILAH IAIKSFDLVA
Consensus  DXXIXRAAQX DGASLPTIYL XXVLPSLRPV FFSAXMILAH IAIKSFDLVA

          251                                                 300
P._putida  AMTAGGPGYS SDLPAMFMYS FTFSRGQMGM GSASAILMLG AIRAIVVPYL
GltF       AMTAGGPGYS SDLPAMFMYA HTFTRGQMGL GAASAMLMLG AVLAIVVPYL
Consensus  AMTAGGPGYS SDLPAMFMYX XTFXRGQMGX GXASAXLMLG AXXAIVVPYL

          301    310
P. putida  YSELRNKRHE
GltF       YSELRGKRHA
Consensus  YSELRXKRHX
```

Figure 3.4. EAA amino acid motifs of GltF and GltG. Residues that are typically conserved are boxed, while residues that tend to be variable are represented by an X.

gltF (residues 206-226)	RAAQVDGASLPTIYLRIVLP
gltG (residues 174-193)	KAARLDGAGFFTIFLRILLP
consensus	EAAXXXGXXXXXXXXXIXLP

transmembrane spanning regions (Fig. 3.5). The von Heijne plot of a putative 34.5 kDa GltF indicates that the initial 43 amino acids (that would be lacking in a 29.9 kDa GltF) contribute to transmembrane structure.

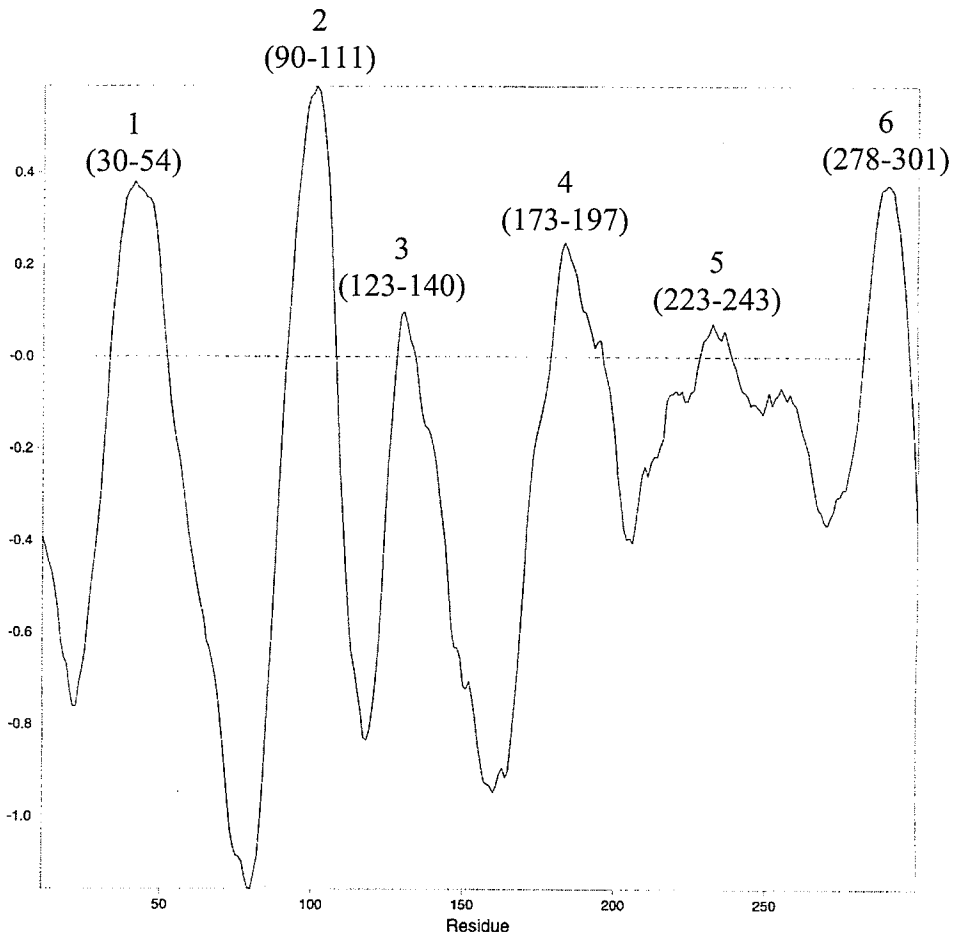
The MEMSAT 2 transmembrane topology prediction method available from the PSIPRED protein structure prediction server (McGuffin *et al*, 2000) was utilized to provide a detailed analysis of the location of individual amino acid residues of GltF and GltG (Fig. 3.6). This method predicts protein secondary structure and topology by statistical analysis of submitted sequences to those of well-characterized membrane protein sequences. This data correlates with the von Heijne plots, also predicting the presence of 6 transmembrane-spanning regions in each protein, and indicates that the EAA motifs of both proteins reside in the penultimate cytoplasmic segment and transmembrane spanning region.

3.1.2 Discussion

Examination of the genomic context of *oprB* revealed that the gene for the carbohydrate-selective porin resides in a region of the chromosome that contains a cluster of sugar-utilization genes that encode regulatory proteins, enzymes involved in central pathway sugar catalysis, and ABC transport. Sequence analysis of the genes sandwiched between components of a probable two-component response system (*gltR* and its sensor kinase) and *oprB* has indicated that *gltBFGK* is likely under the control of a single promoter upstream of *oprB*. As the gene products of *gltK* and *gltR* have been previously been identified as playing a role in glucose transport in this organism, the other components of the operon likely also encode glucose transport proteins.

Figure 3.5. von Heijne hydrophobicity plots of a) GltF and b) GltG, as calculated with Omiga 2.0 software (Oxford Moleculars). Peaks with positive hydrophobicity (Y axis) represent amino acid residues likely to traverse the inner membrane, and predicted transmembrane domains are numbered 1 – 6, with the corresponding amino acid numbers in brackets.

a)


2
(90-111)
1
(30-54)
6
(278-301)
4
(173-197)
3
(123-140)
5
(223-243)
0.4
0.2
-0.0
-0.2
-0.4
-0.6
-0.8
-1.0
50
100
150
200
250
Residue

b)

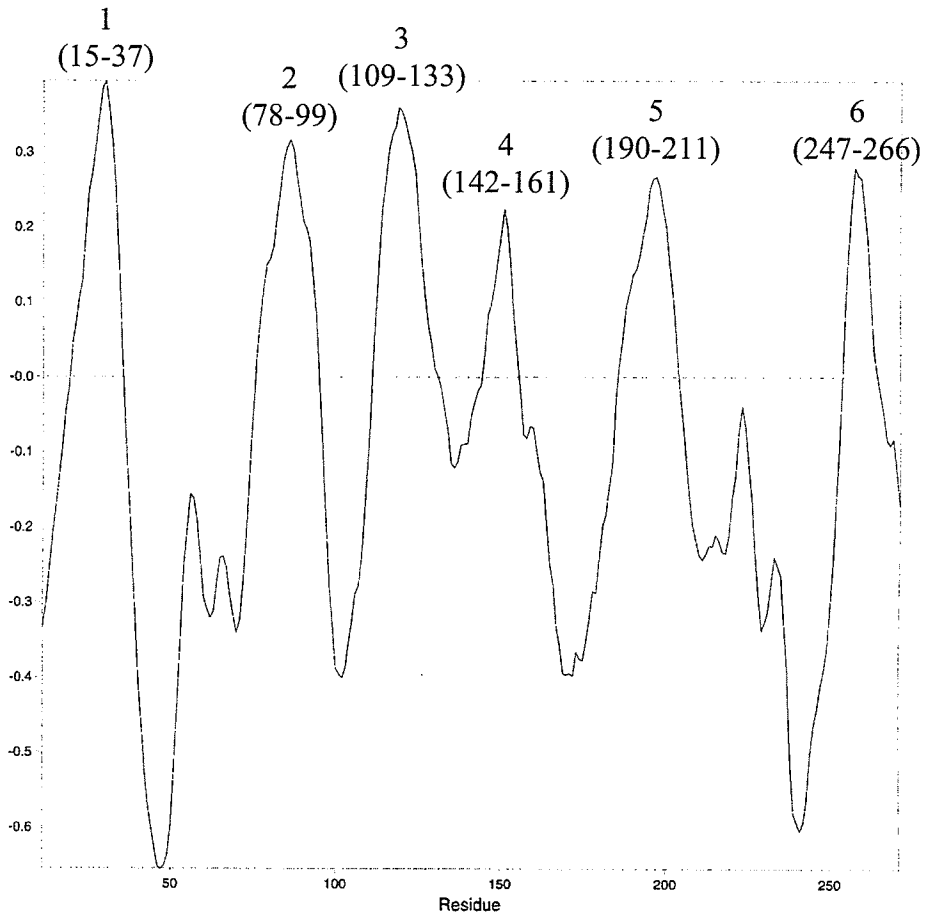

1
(15-37)
2
(78-99)
3
(109-133)
4
(142-161)
5
(190-211)
6
(247-266)
0.3
0.2
0.1
-0.0
-0.1
-0.2
-0.3
-0.4
-0.5
-0.6
50
100
150
200
250
Residue

Figure 3.6. MEMSAT 2 topological analysis of a) GltF and b) GltG (McGuffin *et al*, 2000). The first amino acid residue predicted by the smaller *gltF* ORF is labelled with a *. Cytoplasmic residues are indicated by "+," transmembrane residues are indicated by "X," and periplasmic residues are indicated by "-." EAA motifs, located in the penultimate cytoplasmic loop and transmembrane-spanning region, are shaded.

a)

```
        10        20        30        40        50        60
++++++++++++++++++++++++++++XXXXXXXXXXXXXXXXXXXXXXXXX------
MATNSPVYPTAPVAPRRVSPLDRLQGWLPKLVLAPSVVVVLFCMYGYIGWTLLLSFTNSR
                                          *

        70        80        90       100       110       120
----------------------------XXXXXXXXXXXXXXXXXXXXXXX+++++++++
FMPSYKWAGLSQYQRLWDNDRWWVASKNLLLFGGSFIAICLVLGVFLAVLLDQRIRREGF

       130       140       150       160       170       180
++XXXXXXXXXXXXXXXXXX------------------------------XXXXXXXX
IRTLYLYPMALSMIVTGTAWKWLLNPGLGLDKLLRDWGWEGFRFDWLVDPERVVYCLVIA

       190       200       210       220       230       240
XXXXXXXXXXXXXXXXXX++++++++++++++++++++++XXXXXXXXXXXXXXXXXXXX
AVWQASGFVMALFLAGLRGVDPAIVRAAQVDGASLPTIYLRIVLPSLRPVFFSALMILAH

       250       260       270       280       290       300
XXX---------------------------------XXXXXXXXXXXXXXXXXXXXXXX
IAIKSFDLVAAMTAGGPGYSSDLPAMFMYAHTFTRGQMGLGAASAMLMLGAVLAIVVPYL

       310
X++++++++++
YSELRGKRHAAA
```

b)


10 20 30 40 50 60
++++++++++++++XXXXXXXXXXXXXXXXXXXXXX----------
MRDASLNPSFSVSRLAVHLTLWGACALYLVPLLVMLLTSFKTPDDIRTGNLLSLPDTFTV
70 80 90 100 110 120
----------XXXXXXXXXXXXXXXXXXXXX++++++++XXXXXXXXXXXX
IGWLKAWDSVGAFFWNSVLITVPGVLISTFIGALNGYVLSMWRFRGSQLFFGALLFGCFL
130 140 150 160 170 180
XXXXXXXXXXXXX--------XXXXXXXXXXXXXXXXXXXX++++++++++++++++++
PFQVILLPMSFTLGKLGLANTTSGLVLVHVIYGLAFTTLFFRNYFVAIPDALVKAARLDG
190 200 210 220 230 240
+++++++++XXXXXXXXXXXXXXXXXXXXX----------
AGFFTIFLRILLPMSTPIVMVCLIWQFTQIWNDFLFGVVFASGDSQPITVALNNLVNITT
250 260 270 280
------XXXXXXXXXXXXXXXXXXXX++++++++++++++++
GVKEYNVDMAAAMIAALPTLVVYVLAGKYFVRGLTAGAVKGGG

While there are no obvious promoter-like elements between the genes of the *gltBFGK* operon, there are three small palindromic sequences between the stop codon of *gltB* and the start codon of *gltF* (one of which being the *Bam*HI restriction site). The presence of the palindromes may result in enhanced transcription of *gltB* which codes for the periplasmic glucose binding protein GBP, relative to the other members of the operon. This is a feature that may be explained by observations made concerning the maltose ABC transporter of *E. coli*, where it was found that the binding protein, MBP, is present in 20- to 40-fold higher concentration than the other ABC transport components (Manson & Boos, 1985). It has been determined that increased levels of MBP are attributed to the fact that more MBP is involved in chemotaxis than in maltose transport. GBP of *P. aeruginosa* is also known to be involved in chemotaxis towards glucose, so it seems plausible that enhanced levels of expression from *gltB* would be required.

There are several lines of evidence that suggest that *gltF* is transcribed from the first potential start codon, for the larger ORF. Firstly, there is a string of 7 consecutive purines upstream of this start codon which may act as a ribosome binding site, while this is a feature that is not observed the appropriate distance upstream of the second potential start codon. Secondly, it is generally accepted that the most common number of transmembrane spanning regions of ABC inner membrane complexes is 12 (Higgins, 1992). There have been exceptions to the 6 + 6 rule identified, although, in bacterial systems, this is typically towards one inner membrane component consisting of more than 6 transmembrane spanning regions, not less. For example, MalF of the *E. coli* maltose ABC transporter is known to contain 8

transmembrane domains, while MalG, the inner membrane partner, traverses the membrane only 6 times (Boyd *et al*, 1987) (Dassa & Muir, 1993).

When the larger predicted GltF sequence was compared to the *P. putida* transport protein that was identified as the closest match via BLASTX, the % identity decreased slightly. Upon viewing an alignment of the predicted amino acid sequences of both proteins, it is evident that the decrease in identity is due solely to the fact that the first cytoplasmic loop of the *P. putida* protein is shorter (Fig. 3.3). This alignment provides further evidence to support the significance of the larger *gltF* ORF, as there is significant identity between the *P. aeruginosa* and *P. putida* gene sequences in the region of GltF that is present only from the larger transcript. There are also a number of amino acids in this region that are similar, though not identical, between the two sequences.

The presence of the EAA motif within the penultimate cytoplasmic segment and transmembrane domain of GltF and GltG provides strong evidence of involvement of the proteins in ABC transport.

3.2 Acquisition of *gltF* and *gltG* knock-out mutants

3.2.1 Results

It was necessary to acquire *gltF* and *gltG* knock-out mutants so that subsequent experiments directed towards confirmation of the membrane location of GltF and GltG, and the roles of GltF and GltG in glucose uptake could be carried out.

Initial efforts were directed towards construction of in-frame *gltF* and *gltG* deletion mutants. Replacement vectors pEX18Tc::ΔF and pEX18Tc::ΔG were

successfully constructed, and *P. aeruginosa* H103 (pEX18Tc::ΔF) and H103 (pEX18Tc::ΔG) co-integrates were isolated from LB agar plates supplemented with 60 μg/ml tetracycline, following triparental mating with *E. coli* donor cells. Refer to Section 2.15 for a description of the experimental approach taken in the replacement vector construction.

The presence of the replacement vector in the genome of the recipient H103 cells was confirmed by PCR-based analysis (data obtained with the *gltF* replacement vector is shown in Fig. 3.7) and Southern blotting (data not shown). PCR with gltF- or *gltG*-specific primer pairs FD3/F3 or G3/G2, respectively, from genomic DNA of co-integrate cells should yield two products (one representing ΔF/ΔG, and the other representing *gltF/G*). The same PCR from either the H103 genome or the replacement vector should yield only one product (either the complete gene or the truncated version of the gene).

Additional PCR-based analysis of a pEX18Tc::ΔF co-integrate genomic DNA sample further confirmed replacement vector integration, while also indicating that the cross-over event leading to plasmic integration occurred at the 5' end of the gene. This was determined by utilizing the F1/F3 primer pair, in which the forward primer, F1, binds to a region of H103 DNA not cloned into pEX18Tc (upstream of the FD3 binding site). As a result, F1/F3 PCR amplification from co-integrate DNA should occur only from ΔF or *gltF* (Fig. 3.8). PCR amplification from co-integrate DNA resulted in a PCR product of ~ 0.6 kb (in contrast to a product of ~ 1.2 kbp from wild-type DNA, not shown), indicating that the cross-over event occurred at the 5' end of *gltF* (Fig. 3.9).

Figure 3.7. FD3/F3 PCR from H103 genomic DNA, pEX18Tc::ΔF, and H103 (pEX18Tc::ΔF) co-integrate genomic DNA. Lane 1: H103 and pEX18Tc::ΔF templates (separate reactions were carried out, and reaction mixtures were mixed prior to loading); lane 2: co-integrate genomic DNA template; lane 3: 1 kb Plus DNA Ladder (Invitrogen). Samples were resolved on a 1.5% agarose gel. The white arrow points to the presence of ΔF in the co-integrate PCR reaction lane. Wild-type H103 and pEX18Tc::ΔF PCR products were analyzed separately prior to this experiment.

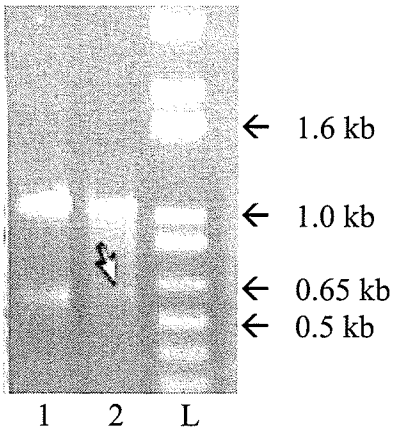
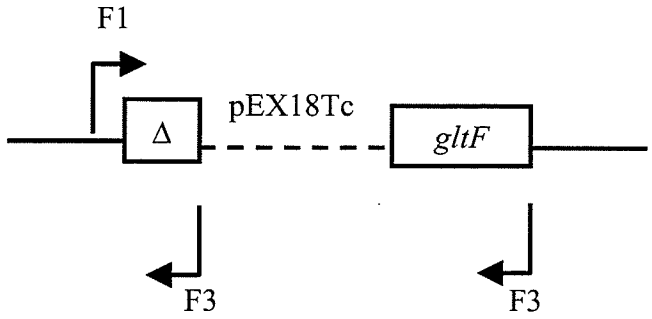

1.6 kb
1.0 kb
0.65 kb
0.5 kb
1
2
L

Figure 3.8. Schematic diagram of a region of the genome of H103 (pEX18Tc::ΔF) co-integrate DNA. a) Product of cross-over between pEX18Tc::ΔF and genome at the 5' end of *gltF*. b) Product of cross-over between pEX18Tc::ΔF and genome at the 3' end of *gltF*. Approximate primer binding sites are marked with an arrow, also depicting the direction of PCR amplification. The pEX18Tc DNA is depicted with a dashed line. ΔF is labelled as "Δ." The drawing is not to scale.

a)



b)

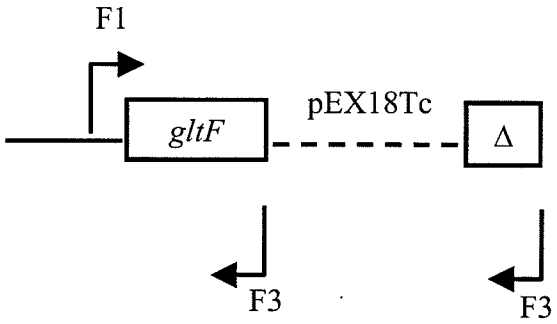
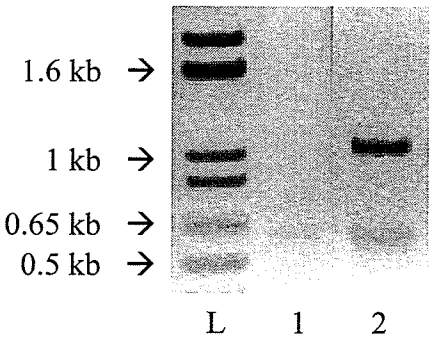


Figure 3.9. PCR analysis of H103 (pEX18Tc::ΔF) co-integrate genomic DNA.

Lane 1: F1/F3 PCR; lane 2: FD3/F3 PCR; L: 1 kb Plus DNA Ladder (Invitrogen).


1.6 kb
1 kb
0.65 kb
0.5 kb
L
1
2

Following growth of co-integrates on medium supplemented with 5% (w/v) sucrose, subsequent PCR-based analysis (as described above, and with *sacB*-specific primers to target pEX18Tc) of sucrose-resistant, tetracycline-sensitive cells indicated that plasmid excision was not successful (data not shown). Refer to Section 2.15 for a description of experimental modifications taken in an effort to optimize excision.

As an alternate approach, *P. aeruginosa* PA01 *gltF* and *gltG* transposon insertion mutants, strains 20781 and 42725, respectively, were obtained from M. Jacobs at the University of Washington Genome Center, Seattle. The mutants were isolated following random insertion mutagenesis of PA01 with IS*lacZ*/hah (20781) or IS*phoA*/hah (42725) (Jacobs *et al*, 2003). Both transposons utilized by Jacobs *et al.* were derived from the IS*50*L element of Tn5, and contain an outward-facing neomycin phosphotransferase promoter at the 3' end of each transposon (see the paper by Jacobs *et al,* 2003 for more details).

The location of IS*lacZ*/hah within strain 20781 was mapped by Jacobs *et al.*, and found to be between nucleotides 882 and 883 of the 933 nucleotide *gltF* ORF, inserted in the same direction as the gene. As such, the neomycin phosphatase promoter at the end of the transposon would allow for expression of *gltG* and *gltK,* preventing polar effects of the insertion. 20781 colonies are white in color on 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal)-containing medium, indicating inactivity of *lacZ.*

The location of IS*phoA*/hah within strain 42725 was mapped by Jacobs *et al.*, and found to be between nucleotides 204 and 205 of the 846 nucleotide *gltG* ORF, inserted in the same direction as the gene. As such, the neomycin phosphatase

promoter at the end of the transposon would allow for expression of *gltK,* preventing polar effects of the insertion. 42725 colonies are white in color on 5-bromo-4-chloro-3-indolyl phosphate (XP)-containing medium, indicating that *phoA* is not active.

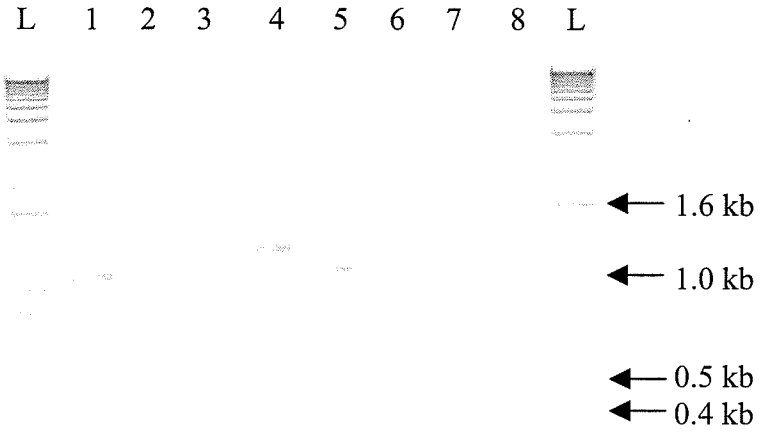
Prior to experimentation with these strains, the presence and location of the insertions were confirmed by a PCR-based method. The PCR was carried out from genomic DNA with *gltF*- or *gltG*-specific primers (FD3/F3 or G3/G2, respectively). Due to the size of the transposons used, insertion into *gltF* or *gltG* would result in a failed reaction, as the primer binding sites would be too far apart for amplification to be achieved under the conditions used. Amplification from 20781 with FD3/F3 failed, as did amplification from 42725 with G3/G2 failed (Fig. 3.10). Wild-type PA01 DNA was used as a positive control.

In order to confirm the location of the transposons within the genes, FD3 and G3 primers were paired with transposon-specific reverse primers LACZ-211 (binding site 211 bases within IS*lacZ*/hah) or HAH-166 (binding site 166 bases within IS*phoA*/hah), respectively (see Jacobs *et al*, 2003 for transposon-specific primer sequences). As expected, PCR from the 20781 genome with FD3/ LACZ-211 resulted in a ~1.2 kb product, while PCR from the 42725 genome with G3/ HAH-166 resulted in a ~0.44 kb product (Fig. 3.10). Wild-type PA01 DNA was used as a negative control.

3.2.2 Discussion

Attempts to create *P. aeruginosa* in-frame *gltF* and *gltG* deletion mutants were unsuccessful. While co-integrates in which the replacement vector had integrated into

Figure 3.10. PCR-based confirmation of transposon insertion location within *gltF* or *gltG* of *P. aeruginosa* PA01 (strains 20781 and 42725, respectively). Genomic DNA was utilized as PCR templates, and the resulting PCR reaction mixtures were resolved on a 1.5% agarose gel.



Legend
Lane 1: FD3/F3 PCR (PA01 template)
Lane 2: FD3/F3 PCR (20781 template)
Lane 3: FD3/LACZ-211 PCR (PA01 template)
Lane 4: FD3/LACZ-211 PCR (20781 template)
Lane 5: G3/G2 PCR (PA01 template)
Lane 6: G3/G2 PCR (42725 template)
Lane 7: G3/HAH-166 PCR (PA01 template)
Lane 8: G3/HAH-166 PCR (42725 template)
L: 1 kb Plus DNA ladder (Invitrogen)

the chromosome were isolated, attempts to promote excision of the replacement vector failed.

The presence of *sacB* in medium containing at least 3% sucrose should be lethal for Gram-negative organisms, as the product of *sacB*, levansucrase, is responsible for producing sucrose polymers that build up in the periplasm. Unexpectedly, PCR-based analysis of co-integrates indicated that *sacB* was present in the cells following growth in the presence of sucrose. Survival must be due to either inactivity of levansucrase or to extrusion of levans from the periplasm. Perhaps survival of co-integrates was possible due to mutation of *sacB* (which has been observed to occur spontaneously at frequencies from 2 - 94%) (Geissler & Drummond, 1993; Kamoun *et al,* 1992; Quandt & Hynes, 1993; Ried & Collmer, 1987; Schafer *et al,* 1994; Wu & Kaiser, 1996) or due to active extrusion of levans.

The use of insertion mutants in this study was initially deemed inappropriate, as this would result in polar effects on downstream genes in the operon. However, the genome-wide library of insertion mutants constructed at the University of Washington Genome Center (Jacobs *et al*, 2003) were prepared with transposons carrying a *Pseudomonas*-recognized promoter at the 3' end. A transposon insertion mutant received for *gltF* (strain 20781) contains the transposon IS*lacZ*/hah inserted in the appropriate orientation such that continued expression of *gltGK* from the promoter on the transposon is possible. Similarly, the *gltG* transposon insertion mutant (strain 42725) contains the transposon IS*phoA*/hah insertion in the appropriate orientation such that continued expression of *gltK* is possible. As a result, the *P. aeruginosa*

PA01 *gltF* and *gltG* transposon insertion mutants, strains 20781 and 42725, respectively, are suitable for this study.

3.3 Confirmation of the membrane location of GltF and GltG

3.3.1 Results

In order to confirm that GltF and GltG are membrane proteins, cell envelope preparations of several *E. coli* and *P. aeruginosa* strains were examined by SDS-PAGE.

Initial expression studies were carried out in *E. coli* NM522 transformed with either pUCP20::*gltF*, pUCP21::*gltG*, or pUCP21 (control). SDS-PAGE revealed the overexpression of membrane proteins of approximately 30 kDa in size in both the NM522 (pUCP20::*gltF*) and NM522 (pUCP21::*gltG*) protein profiles, that was not apparent in the NM522 (pUCP21) cell envelope protein profile (Fig. 3.11). The presence of an approximately 30 kDa protein in the NM522 (pUCP20::*gltF*) profile indicates that it is likely the smaller ORF that is transcribed in *E. coli*.

Cell envelope fractions were isolated from *P. aeruginosa* H103 grown in BM2 medium supplemented with 0.4% glucose (growth conditions known to induce the high affinity glucose uptake system). The protein profiles of H103 (pUCP21), H103 (pUCP20::*gltF*), and H103 (pUCP21::*gltG*) were compared via 12% SDS-PAGE, although overexpression was not observed in the strains carrying an extra copy of *gltF* or *gltG* (Fig. 3.12).

In a final effort to examine for expression of GltF and GltG in *P. aeruginosa*, *gltF* and *gltG* insertion mutants 20781 and 42725 were utilized (Jacobs *et al*, 2003),

Figure 3.11. SDS-PAGE of *E. coli* cell envelope fractions to examine for IPTG-induced GltF and GltG expression. One hundred μg of each protein sample was resolved via 12% SDS-PAGE. Expression of GltF and GltG is observed in lanes 2 and 3 at the arrow. Lane 1: *E. coli* NM522 (pUCP21); Lane 2: *E. coli* NM522 (pUCP21::*gltG*); Lane 3: *E. coli* NM522 (pUCP20::*gltF*).

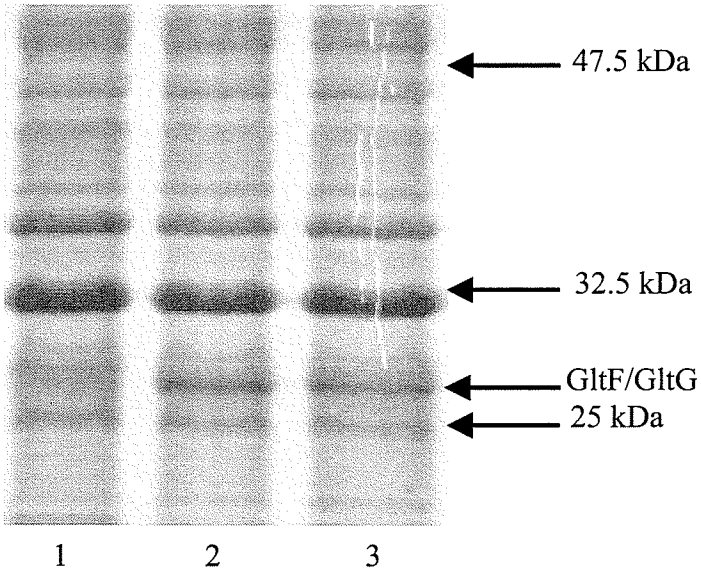
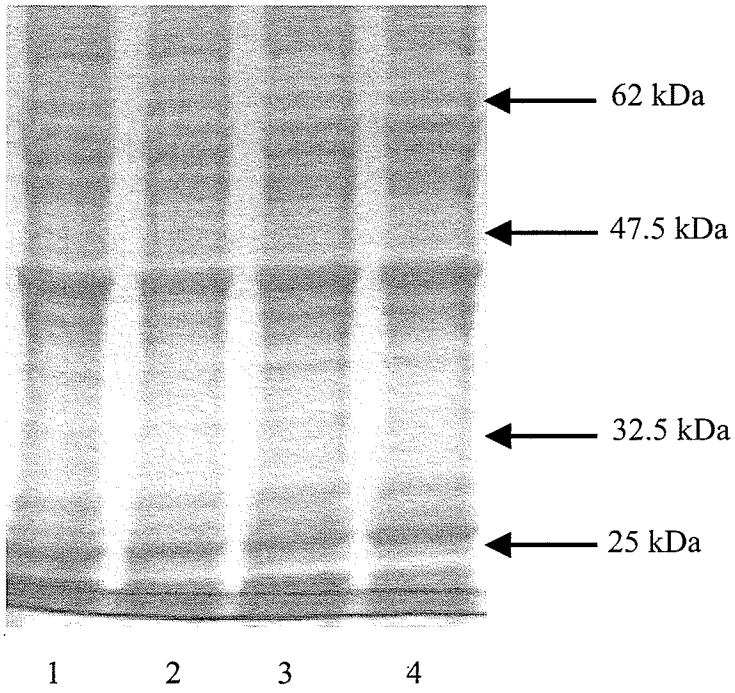

47.5 kDa
32.5 kDa
GltF/GltG
25 kDa
1
2
3

Figure 3.12. SDS-PAGE of *P. aeruginosa* H103 cell envelope fractions from BM2 + 0.4% glucose-grown cells to examine for overexpression of GltF and GltG. Fifty μg of each protein sample was resolved via 12% SDS-PAGE. Lane 1: *P. aeruginosa* H103; Lane 2: *P. aeruginosa* H103 (pUCP21); Lane 3: *P. aeruginosa* H103 (pUCP20::*gltF*); Lane 4: *P. aeruginosa* H103 (pUCP21::*gltG*).


62 kDa
47.5 kDa
32.5 kDa
25 kDa
1
2
3
4

and membrane protein profiles of the insertion mutants were compared to mutants which were complemented with either pUCP20::*gltF* or pUCP21::*gltG* (Fig. 3.13). While it does not appear as though the introduction of either pUCP construct altered protein profile of the mutants, it does appear as though the mutant profiles are different from that of the wild-type profile.

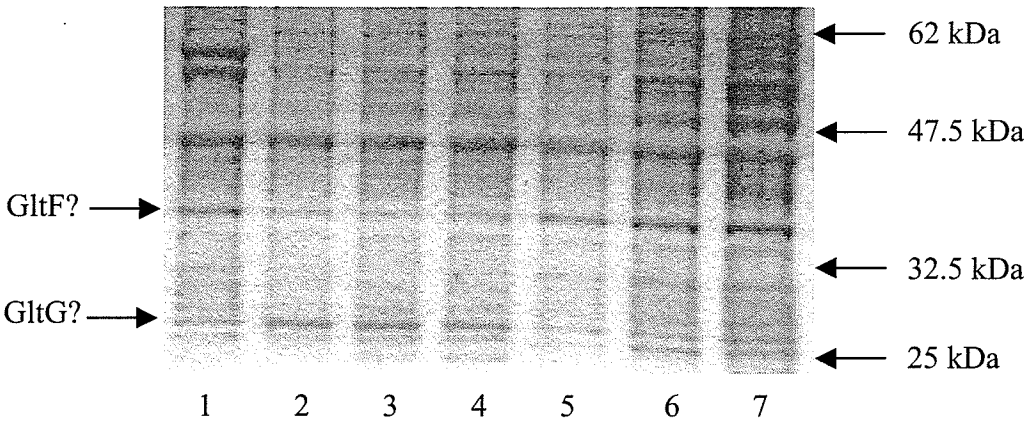
There is one band in the protein profiles of strain 20781 that appears to be less intense compared to the wild-type profile and the 42725 profiles (labelled with an arrow as "GltF?"on Fig. 3.13). This band is of an appropriate size to represent GltF transcribed from the larger reading frame, as it falls between the 47.5 and 32.5 kDa ladder markers. Similarly, there is one band in the protein profiles of strain 42725 that appears to be less intense compared to the wild-type profile and the 20781 profiles (labelled with an arrow as "GltG?" on Fig. 3.13). This band is of approximately 30 kDa, and may represent GltG. This band appears to be overexpressed in the 20781 protein profiles, relative to the PA01 profile.

3.3.2 Discussion

Expression studies in *E. coli* NM522 have established that GltF and GltG are membrane proteins. Cell envelope preparations of *E. coli* NM522 carrying either pUCP20::*gltF* or pUCP21::*gltG* revealed overexpression of protein bands of approximately 30 kDa in size (Fig. 3.11).

While overexpression of a 30 kDa protein from pUCP21::*gltG* was expected, overexpression of a 30 kDa protein from pUCP20::*gltF* was not. This would indicate that *gltF* was transcribed from the smaller ORF, rather from the larger ORF

Figure 3.13. SDS-PAGE of *P. aeruginosa* PA01 transposon insertion mutant cell envelope fractions from BM2 + 0.4% glucose-grown cells to examine for expression of GltF and GltG. Fifty μg of each protein sample was resolved via 12% SDS-PAGE. Arrows indicate possible locations of GltF and GltG. Lane 1: *P. aeruginosa* PA01; Lane 2: *P. aeruginosa* 20781; Lane 3: *P. aeruginosa* 20781 (pUCP21); Lane 4: *P. aeruginosa* 20781 (pUCP20::*gltF*); Lane 5: *P. aeruginosa* 42725; Lane 6: *P. aeruginosa* 42725 (pUCP21); Lane 7: *P. aeruginosa* 42725 (pUCP21::*gltG*).


62 kDa
47.5 kDa
GltF?
32.5 kDa
GltG?
25 kDa
1
2
3
4
5
6
7

from which a protein of approximately 34.5 kDa is expected. As computer-aided analysis did not indicate the presence of an N-terminal signalling sequence in the predicted amino acid sequence of either protein (refer to Section 3.1.1), it is unlikely that the larger *gltF* ORF was transcribed and the 34.5 kDa protein processed into a mature 30 kDa protein. It is possible that there may be a ribosome binding site recognized by *E. coli* translation machinery upstream of the start codon of the smaller ORF.

Overexpression of GltF and GltG was not observed in *P. aeruginosa* H103 carrying either pUCP20::*gltF* or pUCP21::*gltG* (Fig. 3.12). This was not entirely unexpected, as there was not a *Pseudomonas*-specific promoter cloned along with the genes into the plasmids. Typically, when the pUCP cloning vectors are utilized in experiments when expression in *Pseudomonas* is desired, the promoter region of the gene(s) of interest is included in the insert. This is done as expression from the *lac* promoter contained on the plasmids cannot be guaranteed in *Pseudomonas* species. In this study, the forward primers utilized to clone *gltF* and *gltG* were designed such that a region upstream of the start codon of a sufficient in size to contain a promoter was contained within the PCR product. However, sequence analysis has indicated that the presence of a promoter sequence within these regions is doubtful.

In the event that *gltF* and/or *gltG* were transcribed from either the pUCP *lac* promoter or an unidentified piece of regulatory DNA upstream of the start codons, it is possible that overexpression would not be observed by this method. The copy number of the pUCP vectors in *Pseudomonas* tends to be quite low, ranging from 1 to 10 copies per cell (Schweizer, 1991b).

Expression of GltF and GltG could not be clearly identified in 20781 (pUCP20::*gltF*) or 42725 (pUCP21::*gltG*) when compared to uncomplemented mutants and PA01. Again, this is likely due to the absence of a *Pseudomonas*-specific promoter upstream of the genes in the pUCP constructs. However, it does appear as though there are protein bands that are of the appropriate size to contain GltF (as a 34.5 kDa protein) or GltG within the mutant protein profiles that may be less intense when compared to the profiles of the strains that are wild-type for the relevant gene (Fig. 3.13).

The protein band likely to contain GltG in the 20781 protein profiles is more intense than the same band in the PA01 protein profile. This is likely due to the fact that *gltG* is under the control of different promoters in these two strains. *gltG* of PA01 is controlled by the *gltBFGK* promoter, while *gltG* of 20781 is under the control of the neomycin phosphatase promoter contained within IS*lacZ*/hah. As expected, the band likely to contain GltF in the 42725 protein profiles is of the same intensity as the same band in the PA01 profile, as *gltF* is under the control of the same promoter in both strains.

3.4 Confirmation of a role of GltF and GltG in high affinity glucose uptake in *P. aeruginosa*

3.4.1 Results

In order to assess the effect of the absence of GltF or GltG on glucose uptake by *P. aeruginosa*, two sets of experiments were carried out with the *gltF* and *gltG* insertion mutants and PA01. Growth rate analysis in minimal medium supplemented with glucose as the sole carbon source was carried out in order to compare the

abilities of the strains to use glucose as a carbon source. Whole cell glucose uptake assays were carried out in order to compare abilities of the strains to take up glucose via the high affinity uptake pathway.

The relative rates of growth in BM2 + 0.4% glucose of the *gltF* and *gltG* insertion mutants (20781 and 42725, respectively), were compared to the growth of the parental strain, PA01. The rates of growth of the mutants carrying a complementing pUCP construct (pUCP20::*gltF* or pUCP21::*gltG*) were compared to the mutants carrying pUCP21 (with no insert). From Fig. 3.14, it is evident that the rate of growth of 20781, with or without pUCP20::*gltF* is comparable to that of PA01. Similarly, 42725 was also found to grow at a rate comparable to PA01, with or without pUCP21::*gltG* (Fig. 3.15).

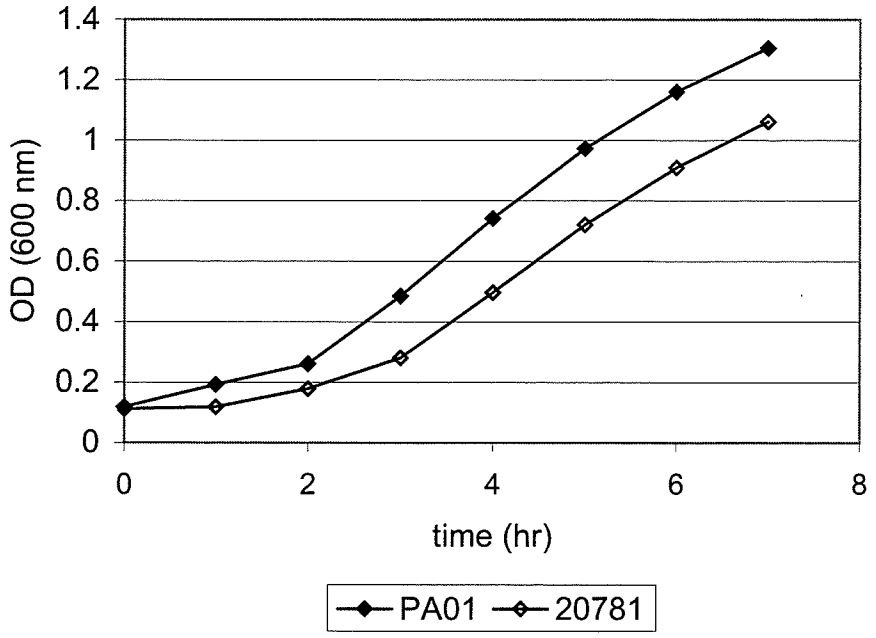
The ability of the various *P. aeruginosa* strains to take up [U-^{14}C]-glucose by the high affinity glucose uptake pathway was examined. It was discovered that both mutants were deficient in high affinity glucose-uptake ability (Figs. 3.16 and 3.17). Introduction of either pUCP20::*gltF* or pUCP21::*gltG* to 20781 or 42725, respectively, did not have an appreciable effect on the glucose uptake ability of the mutant strains (Figs. 3.16 and 3.17).

3.4.2 Discussion

The relative rates of growth of wild-type and mutant *P. aeruginosa* strains in BM2 medium supplemented with 0.4% (w/v) glucose (equivalent to 22 mM) was examined. It has been previously determined that the high affinity glucose uptake pathway is operative under these conditions (Stinson *et al*, 1977). The low affinity

Figure 3.14. Growth of a) *P. aeruginosa* strain PA01 (wild-type), 20781 (*gltF* insertion mutant), and b) 20781 transformants carrying pUCP vectors in BM2 minimal medium supplemented with 0.4% glucose. BM2 medium supplemented with 0.4% glucose was inoculated with overnight cultures to an OD_{600nm} of approximately 0.1, and cultures were grown with shaking at 37°C for 7 hrs. Data is representative of at least 3 independent trials.

a)


1.4
1.2
1
0.8
0.6
0.4
0.2
0
OD (600 nm)
0
2
4
6
8
time (hr)
PA01
20781

b)

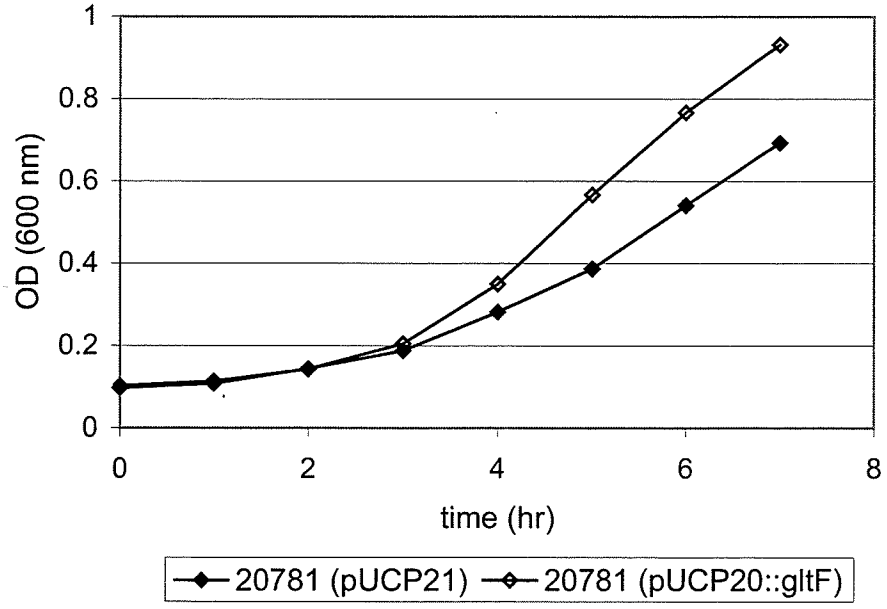
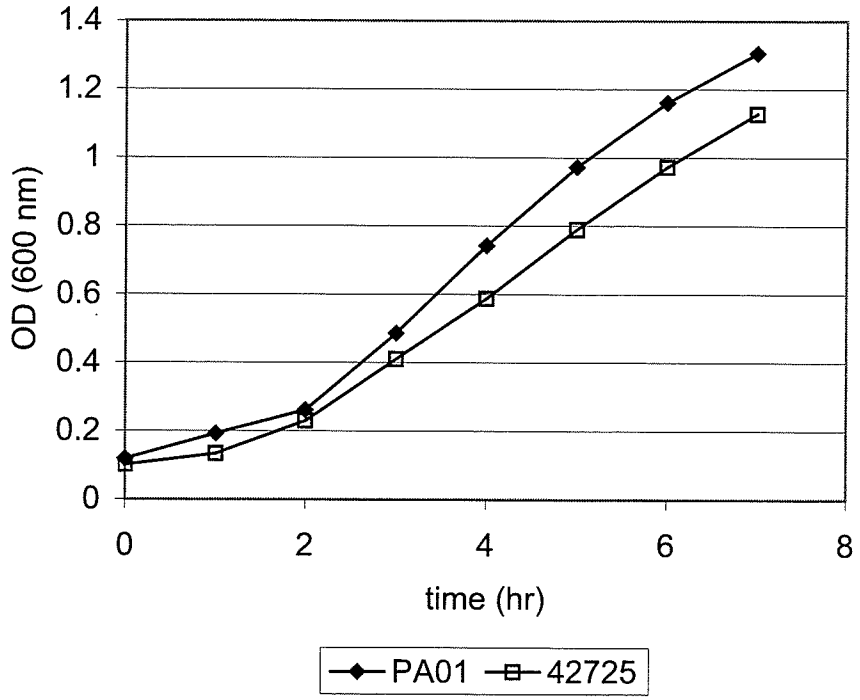

1
0.8
0.6
0.4
0.2
0
OD (600 nm)
0
2
4
6
8
time (hr)
20781 (pUCP21)
20781 (pUCP20::gltF)

Figure 3.15. Growth of a) *P. aeruginosa* strain PA01 (wild-type), 42725 (*gltG* insertion mutant), and b) 42725 transformants carrying pUCP vectors in BM2 minimal medium supplemented with 0.4% glucose. BM2 medium supplemented with 0.4% glucose was inoculated with overnight cultures to an OD_{600nm} of approximately 0.1, and cultures were grown with shaking at 37°C for 7 hrs. Data is representative of at least 3 independent trials.

a)


1.4
1.2
1
0.8
0.6
0.4
0.2
0
OD (600 nm)
0
2
4
6
8
time (hr)
PA01
42725

b)

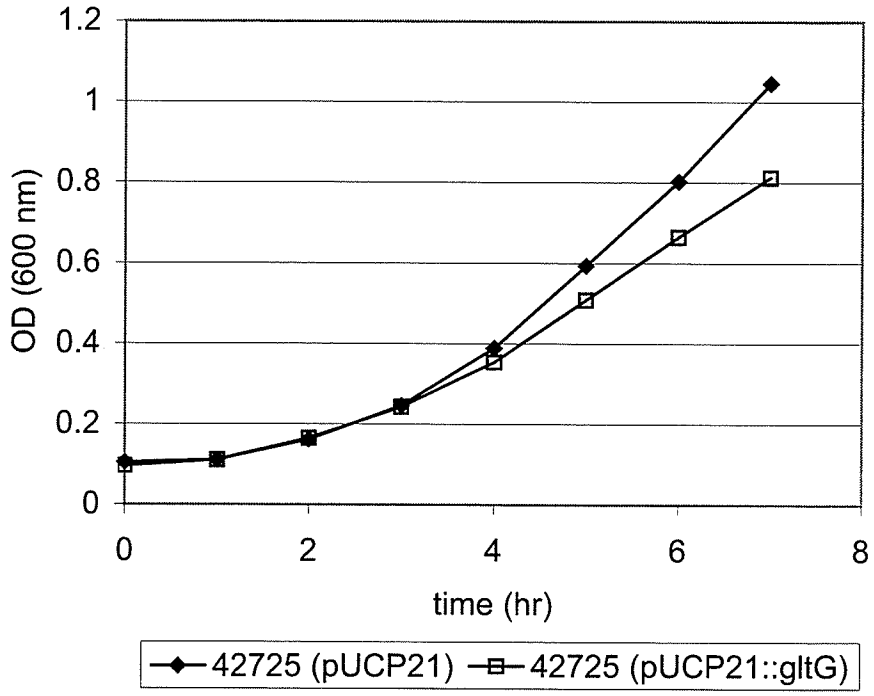

1.2
1
0.8
0.6
0.4
0.2
0
OD (600 nm)
0
2
4
6
8
time (hr)
42725 (pUCP21)
42725 (pUCP21::gltG)

Figure 3.16. Whole cell uptake of [U-^{14}C]-glucose by glucose-starved *P. aeruginosa* PA01, 20781, and 20781 (pUCP20::*gltF*). Log phase cell samples (diluted to an OD_{600}= 0.04) were applied to 0.45 μm membrane filters on a manifold 30, 60, 90, and 120 s after addition of [U-^{14}C]-glucose to a concentration of 33 μM. Filters were immediately washed with glucose-free BM2 medium. Cpm represents the amount of radioactivity detected from the filters, with background counts obtained from formaldehyde-killed cells subtracted. The data presented is representative of at least 4 independent assays.

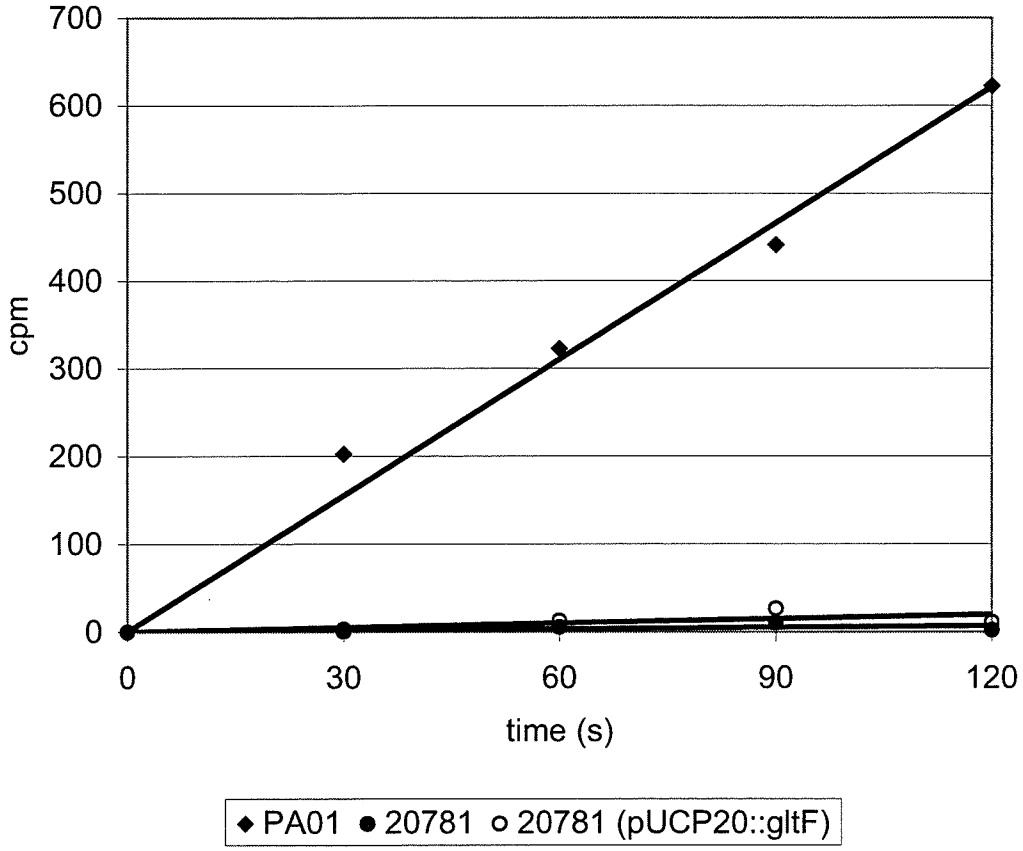
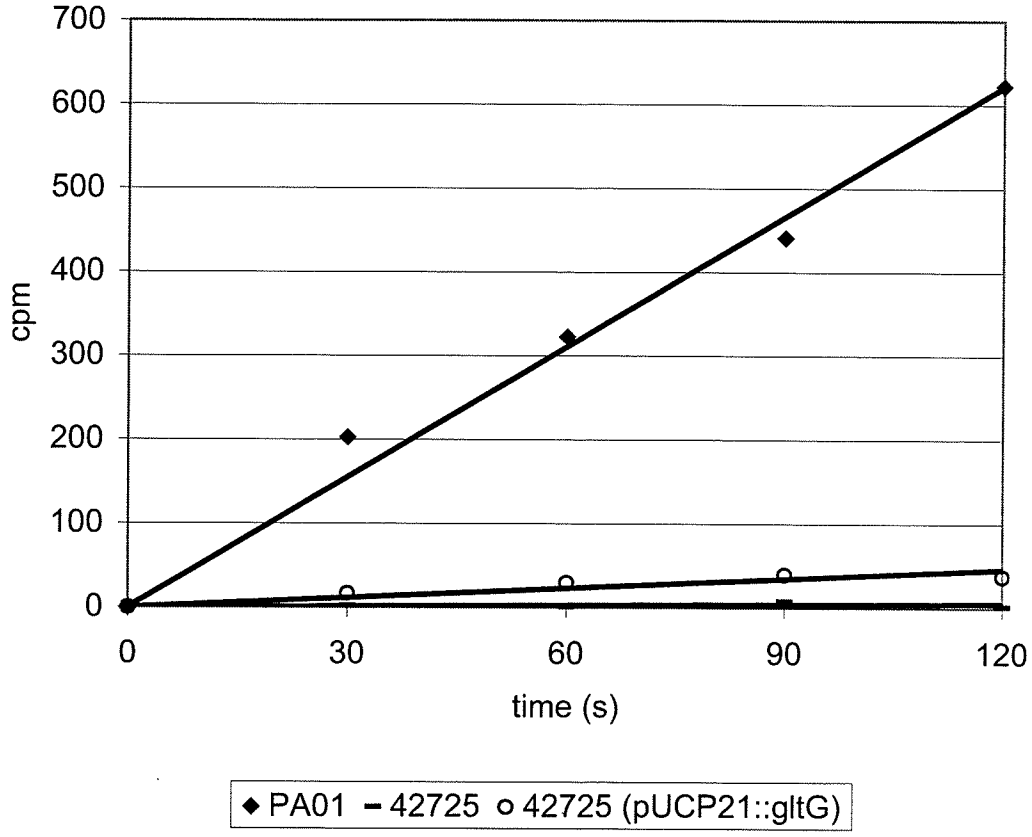

700
600
500
400
300
200
100
0
cpm
0
30
60
90
120
time (s)
◆ PA01 ● 20781 ○ 20781 (pUCP20::gltF)

Figure 3.17. Whole cell uptake of [U-^{14}C]-glucose by glucose-starved *P. aeruginosa* PA01, 42725, and 42725 (pUCP21::*gltG*). Log phase cell samples (diluted to an OD_{600}= 0.04) were applied to 0.45 μm membrane filters on a manifold 30, 60, 90, and 120 s after addition of [U-^{14}C]-glucose to a concentration of 33 μM. Filters were immediately washed with glucose-free BM2 medium. Cpm represents the amount of radioactivity detected from the filters, with background counts obtained from formaldehyde-killed cells subtracted. The data presented is representative of at least 4 independent assays.


700
600
500
400
300
200
100
0
cpm
0
30
60
90
120
time (s)
◆ PA01 – 42725 o 42725 (pUCP21::gltG)

oxidative pathway of glucose utilization has been found to be partially repressed when levels of gluconate exceed 15 mM (Whiting *et al*, 1976b).

Figures 3.14 and 3.15 indicate that the ability of the *gltF* and *gltG* transposon insertion mutants to grow under these conditions was not compromised relative to the wild-type strain. These results suggest that either transposon insertion into *gltF* or *gltG* does not impact the functioning of the high affinity glucose uptake pathway, or that incomplete repression of the low affinity glucose uptake pathway can compensate for problems in high affinity uptake. The latter possibility is plausible, as early work on glucose uptake pathways of *P. aeruginosa* has indicated that repression of the low affinity glucose uptake pathway is not complete (Eagon & Phibbs, 1970) (Midgley & Dawes, 1973) (Whiting *et al*, 1976a). Further, it has been observed that cells unable to produce glucose dehydrogenase, the key enzyme of the low affinity glucose uptake pathway, grow at wild-type rates in medium with glucose as the sole carbon source (Midgley & Dawes, 1973). It has been suggested that regulatory mechanisms compensate for the loss of one uptake system by stimulating upregulation of the other.

Figures 3.16 and 3.17 demonstrate that *P. aeruginosa* strains 20781 and 42725 are unable to take up appreciable amounts of radiolabelled glucose via the high affinity glucose uptake pathway. Introduction of the appropriate complementing pUCP construct did not restore uptake activity to wild-type levels. This is likely due to low or complete lack of expression from the pUCP constructs (see Section 3.3.2 for details).

CHAPTER FOUR

CONCLUSIONS AND FUTURE STUDIES

4.1 Concluding remarks and summary

This study has completed the identification of the components involved in ABC transporter-mediated uptake of glucose in *P. aeruginosa*. Although the complete ABC transporter complex has not been isolated, there is an abundance of evidence that suggests that GltF and GltG form the inner membrane components in association with a GltK dimer.

The completion of the *P. aeruginosa* genome sequencing project and online availability of the sequence data has made PCR-based cloning of genes as well as analysis of flanking DNA of genes of interest relatively straightforward. The major findings of computer-aided analysis can be summarized as follows:

1. The structural genes for GltF and GltG are found in an apparent operon with the genes for the binding protein, GBP, and the ATPase, GltK;
2. Structural features of ABC permeases were identified (the presence of 6 transmembrane spanners from each inner membrane partner);
3. Amino acid sequences characteristic of ABC permeases were identified (EAA motifs were found within the penultimate cytoplasmic segment and transmembrane spanner); and
4. The predicted amino acid sequences of GltF and GltG show very high identity (> 70%) to permeases of ABC systems of other *Pseudomonas* species.

Expression studies in *E. coli* proved that GltF and GltG are membrane proteins, and involvement of GltF and GltF in high affinity glucose uptake was demonstrated experimentally, by monitoring uptake of radiolabelled glucose by glucose-starved cells. Transposon insertion *gltF* and *gltG* mutants were found to be unable to take up glucose by the high affinity pathway. While mutant complementation was unsuccessful, the dramatic difference in glucose uptake abilities of the mutants compared to the wild-type clearly indicate that the lack of a functional GltF or GltG shuts down this uptake pathway.

With the identification of *gltF* and *gltG*, each of the structural components involved in transporting extracellular glucose into the cytoplasm in the high affinity pathway have now been identified and characterized, to some degree.

4.2 Future Studies

To date, the only protein involved in high affinity glucose uptake to be extensively characterized is OprB, the outer membrane porin. The proposed future work described in the proceeding sections outlines several avenues that may be explored in order to further characterize the components of this system.

4.2.1 Purification and sequencing GltF and GltG

Expression studies described in Section 3.2 have tentatively identified GltF and GltG from *E. coli* and *P.aeruginosa* cell envelope preparations. In order to definitively determine the size and sequence of the proteins, it is necessary to purify the proteins and have N-terminal amino acid sequencing carried out. Because expression studies in *E. coli* and *P. aeruginosa* have suggested that *gltF* may be

processed differently in the different organisms, it is desirable to isolate GltF from *P. aeruginosa*. One approach would be to clone the putative promoter element found upstream of *gltB* into a pUCP vector, such that the genes may be cloned into the plasmid downstream of the promoter. Cloning of *gltF* and *gltG* should be PCR-based, such that a His-tagged construct can be generated by adding a His tag sequence to the reverse primer to achieve C-terminal tagging. The presence of a His-tagged protein will allow for positive identification of GltF- and GltG-containing bands from cell envelope preparations, via Western blotting with a His antibody. A nickel affinity column can be used to purify the tagged proteins for N-terminal amino acid sequencing.

4.2.2 Assessing the role of the EAA motifs present in GltF and GltG

Within the EAA motif of ABC permeases, there are 4 residues that are particularly well-conserved, namely E1, A3, G7, and P20 (Saurin & Dassa, 1994). E1 (glutamate) is conserved in approximately 50% of ABC transporters examined, and appears as D (aspartate) in approximately 12% of ABC transporters (Saurin & Dassa, 1994). Interestingly, in *P. aeruginosa* GltF and GltG, E1 (negatively-charged glutamate) is not conserved, and is present as either R (positively-charged arginine) or K (positively-charged lysine) (see Fig. 3.4).

The conserved EAA motif residues have been the target of site-directed mutagenesis experiments in studies of the maltose ABC transporter of *E. coli* (Mourez *et al*, 1997). When E1 was substituted in MalF or MalG, maltose transport was found to occur at a rate 2- to 3-fold less that of wild-type. This same residue may be targeted in GltF and GltG by site-directed mutagenesis, and converted to the

consensus E, as well as to A and L to examine the effect of a negatively-charged, and large non-polar residue.

The EAA motif is believed to be involved in interaction with the ATPase component of ABC transporters. An understanding of the importance of residues in this region will provide clues as to the mechanism of assembly of the complete complex, which is currently a topic of much debate in the ABC transporter research community.

4.2.3 Isolation of the glucose ABC transporter and assay of ATP hydrolysis

An *in vitro* method for reconstitution of the maltose ABC transporter of *E. coli* into proteoliposome vesicles has been devised (Hall *et al*, 1998). The same method may be used for isolation of vesicles containing the *P. aeruginosa* glucose ABC transporter. This would allow for kinetic studies of ATP hydrolysis, by methods described previously (Hall *et al*, 1998), and would confirm that GltK is a functional ATPase.

4.2.4 Assessing the importance of the C-terminal extension of GltK

Examination of the predicted amino acid sequence of GltK revealed the presence of the RDM1, RDM2, and RDM3 regulatory motifs that have been identified in *E. coli* MalK (Bohm *et al*, 2002), and shown to interact with the regulatory protein MalT (Bohm *et al*, 2002) (Samanta *et al*, 2003) (Joly *et al*, 2004). As such, it is quite likely that GltK also plays a role in regulating expression of *gltBFGK*, possibly by binding to GltR or an as-yet unidentified regulatory protein.

Bohm *et al* (2002) have identified 3 amino acid residues within the RDM subdomain of *E. coli* MalK (also conserved in the RDM subdomain of *Thermococcus litoralis* MalK) that, when targeted by site-directed mutagenesis, resulted in strains defective for maltose utilization. Glutamate^{308}, glycine^{340}, and phenylalanine^{355} were targeted, and were altered to similar amino acids (glutamine, alanine, and tyrosine, respectively). These amino acids are far away from the ATPase domain of MalK (and therefore effects observed should not be due to altered ATPase activity), and the mutations did not affect the amount of MalK protein produced (indicating that the mutations did not have a large effect on proper folding of the protein). Upon viewing the alignment of the MalK/GltK sequences (data not shown), it was found that the same amino acids are conserved in *P. aeruginosa* GltK, at positions 310, 340, and 355.

These 3 amino acid residues should be targeted by site-directed mutagenesis in *P. aeruginosa* in a similar fashion, and whole cell glucose uptake assays can be carried out to assess the effects. Should high affinity glucose uptake be severely affected, this would indicate that the C-terminal extension of GltK is an important regulatory feature.

4.2.5 Assessing the role of *oprB2* in carbohydrate uptake

A homologue of *oprB* has been identified that is >90% identical to the gene for OprB. The homologous gene has been named *oprB2* and has been annotated as PA2291 (Hancock, 2002). The gene product of *oprB2* has not yet been characterized, but the predicted amino acid sequence has been analyzed. PSORT analysis predicts

an N-terminal cleavage site that would result in a mature OprB2 protein identical to OprB, but for a single amino acid substitution. o*prB2* is found distant from *oprB*, and is actually immediately adjacent to glucose dehydrogenase, a key enzyme of the low affinity uptake system. The involvement of the gene product of *oprB2*, if any, in carbohydrate uptake should be investigated.

Northern blot analysis of total cellular RNA of wild-type and *oprB*-deficient *P. aeruginosa* strain WW100 (Wylie & Worobec, 1993) grown under conditions in which OprB is required, would be a preliminary experiment to determine if *oprB2* is transcribed or is merely a silent gene. The probe used should be based on the 5' sequence of *oprB2* (the region in which the *oprB* and *oprB2* gene sequences differ), so that it is specific for *oprB2* mRNA. If expression of *oprB2* can be demonstrated in *P. aeruginosa* from a plasmid construct, it would also be interesting to determine if overexpression of *oprB2* can complement the carbohydrate uptake activity of WW100 and restore the wild-type phenotype. This could be determined by whole cell [^{14}C]-labelled carbohydrate uptake assays and growth curve analysis.

CHAPTER FIVE
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