Mutational Analysis of Genes Involved in Glycogen Degradation in *Escherichia coli*

by

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Declaration

The experimental work in this thesis was supervised by Dr. J.R. Lloyd and was conducted in the Institute for Plant Biotechnology, at Stellenbosch University, South Africa. The results presented are original, and have not been submitted in any form to another university.

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and has not previously in its entirety or in parts been submitted at any other university for a degree

Signed: ……. Date: …….

Abstract

Escherichia coli accumulate or degrade glycogen depending on environmental carbon supply. Glycogen synthesis is carried out when carbon is plentiful but other nutrients such as nitrogen is low. Three enzymes, GlgC (ADP-glucose pyrophosphorylase), GlgA (glycogen synthase) and GlgB (glycogen branching enzyme) directs this process to form a branched homopolysaccharide that consists of linear chains of α-1,4-linked glucose monomers, with α-1,6-linked branches. Glycogen degradation is, in turn, carried out by GlgP (glycogen phosphorylase) and GlgX (glycogen debranching enzyme), under starvation conditions.

It is known that GlgP and GlgX are the two primary enzymes involved in glycogen breakdown, yet in previous work it was demonstrated that a Δ*glgP*/Δ*glgX* double mutant does not eliminate glycogen degradation, indicating that another enzyme must be involved. Given the similarity in structures of glycogen and maltodextrins, MalP was a good candidate to be that enzyme. It shares a similar catalytic activity to that of GlgP, but has a different substrate preference, although there are reports of it acting on glycogen.

This study, therefore, aimed to examine the role of MalP, GlgP and GlgX in *E. coli* by creating a series of knockout mutants lacking combinations of all three enzymes and analysing their ability to degrade glycogen. Additionally, these mutants were studied under scanning electron microscopy to determine whether these mutations had an effect on cell morphology. We demonstrate that in addition to the three genes, there is another gene involve in mobilising glycogen and we observed elongated cell lengths for the strains mutated in *malP*.

Opsomming

Escherichia coli vergader of breek glikogeen af na gelang van koolstof beskikbaarheid in die omgewing. Glikogeen sintese word uitgevoer wanneer koolstof volop is en ander voedingstowwe soos stikstof laag is. Drie ensieme, GlgC (ADP-glukose pirofosforilase), GlgA (glikogeen sintase) en GlgB (glikogeen vertakkingsensiem) regeer hierdie proses om 'n vertakte homopolisakkaried te vorm wat bestaan uit lineêre kettings van α-1,4-gekoppelde glukose monomere met α-1,6-gekoppelde takke. Glikogeen afbraak word, op sy beurt, deur GlgP (glikogeen fosforilase) en GlgX (glikogeen onvertakkings ensiem) uitgevoer, onder hongersnood kondisies.

Dit is bekend dat GlgP en GlgX die twee primêre ensieme is wat by glikogeen afbraak betrokke is, maar in vorige werk is daar getoon dat glikogeen afbraak nie geelimineer word in 'n *ΔglgP/ΔglgX* dubbel mutant nie, wat dui daarop dat nog 'n ensiem betrokke moet wees. Aangesien die struktuur van glikogeen en maltodekstrine baie dieselfde is, was MalP 'n goeie kandidaat. Dit deel 'n soortgelyke katalitiese aktiwiteit aan diè van GlgP, maar dit het 'n voorkeur vir 'n ander substraat, alhoewel daar verslae is wat rapporteer dat dit ook kan inwerk op glikogeen.

Hierdie studie is dus daarop gemik om die rol van MalP, GlgP en GlgX in *E. coli* te ondersoek deur ‘n reeks delesie mutante te skep wat ontbreek in kombinasies van al drie ensieme en sodoende hul vermoë om glikogeen af te breek, te ontleed. Verder is hierdie mutante bestudeer onder skandeerelektronmikroskopie om vas te stel of hierdie mutasies 'n uitwerking op selmorfologie het. Ons demonstreer dat benewens tot die drie gene, is daar nog 'n geen betrokke in die mobilisering van glikogeen, en verlengde sel lengtes is waargeneem vir die stamme gemuteerd in *malP*.

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List of Abbreviations

°C	Degrees Celsius
ADP	Adenosine 5'-diphosphate
ADPG	ADP-glucose
AMP	Adenosine 5'-monophosphate
AmyA	Cytoplasmic α-amylase
ATP	Adenosine 5'-triphosphate
cAMP	Cyclic adenosine 5'-monophosphate
CPR	cAMP receptor protein
CsrA	Carbon storage regulator
Da	Daltons
DP	Degree of polymerization
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
EI	Enzyme I
EII	Enzyme II
EM	Electron microscopy
g	Gram
G1P	Glucose-1-phosphate
G6P	Glucose-6-phosphate
GlgA	Glycogen synthase
GlgB	Glycogen branching enzyme
GlgC	ADP-glucose pyrophosphorylase
GlgP	Glycogen phosphorylase
GlgS	Surface composition regulator
GlgX	Glycogen debranching enzyme
h	Hour(s)
HCl	Hydrogen Chloride
His	Histidine
HPLC	High-performance liquid chromatography

HPr	Heat stable protein
KOH	Potassium hydroxide
LamB	Maltoporin
LB	Lysogeny broth
M	Molar
MalE	Maltose-binding periplasmic protein
MalF	Maltose transport system permease protein
MalG	Maltose transport system permease protein
MalK	Maltose/maltodextrin import ATP-binding protein
MalP	Maltodextrin phosphorylase
MalQ	Amylomaltase
MalS	Periplasmic α-amylase
MalT	HTH-type transcriptional regulator
MalZ	Maltodextrin glucosidase
MBP	Maltose binding protein
mg	Milligram
Mg^{2+}	Magnesium
$MgCl_2$	Magnesium Chloride
min	Minutes
ml	Millilitres
mM	Millimolar
NaCl	Sodium Chloride
NAD^+	Nicotinamide adenine dinucleotide
NaOH	Sodium hydroxide
nm	Nanometres
nt	Nucleotides
Pi	Phosphate ion
PCR	Polymerase chain reaction
PEP	Phosphoenolpyruvate
Pgm	Phosphoglucomutase
pH	Measure of acids and bases
ppGpp	Guanosine 3’-bisphosphate 5’-bisphosphate

PTS	Phosphotransferase system
rpm	Rounds per minute
RpoS	RNA polymerase sigma S
SEM	Scanning electron microscopy
TreT	Trehalose glucosyltransferase
U	Units
V	Volts
$^{v}/_{v}$	Volume per volume
$^{w}/_{v}$	Weight per volume
WT	Wild type
α	Alpha
λ	Lambda
µg	Microgram
µL	Microliters

Chapter 1

1. Literature Overview

1.1 Background

The planet that we exist on contains a myriad of environments where microorganisms can live. Conditions within these environments vary greatly and change over time. To survive within them, microorganisms need to continuously adapt which allows them to withstand many changing environmental conditions and to improve their fitness (Strange 1968). They are able to do this by utilizing a variety of nutrients. One such nutrient that allows many bacterial species to thrive and cope with starvation, is carbon. For many microbes carbon is accumulated and stored in the form of glycogen, especially when environmental nitrogen levels are low, but carbon is plentiful (Wilson 2010).

In 1959, Cedergren and Holme noted a large quantity of 'holes' in the cytoplasm of *Escherichia coli* (*E. coli)* which they attributed to being glycogen granules making this study the first description of bacterial glycogen. Since then much research has been performed to elucidate the biochemical pathway of glycogen metabolism however, this process is still not fully understood. The role of bacterial glycogen has been studied most extensively in *E. coli* due to the ease of mutant production within this species and the availability of fully sequenced genomes. This project studies one aspect of glycogen metabolism in *E. coli* and so both the microorganism and glycogen metabolism will be discussed in greater detail in the remainder of this chapter.

1.2 *Escherichia coli* as a model organism

E. coli is a gram negative, rod-shaped bacterium measuring about 1-3µM in length found mostly in the intestines of warm-blooded animals (Schuetz and Strockbine, 2005) and is part of the Enterobacteriaceae family. It can have flagella, a whip-like structure that rotates and enables the bacterium to propel itself forward (thoroughly reviewed in Minamino and Imada 2015), or hair-like pili (Proft and Baker 2008) allowing it to attach to surfaces.

It was first discovered in 1885 during a study examining the gut microbes of infants, which investigated their role in digestion and disease (Escherich 1886). It was originally termed *Bacillus coli communis* but later renamed after its discoverer, Theodor Escherich. *E. coli* quickly became one of the most widely used micro-organisms in research due to its versatility, ease with which it can be propagated, its abundance, rapid growth and non-pathogenicity (Blount 2015). Because of all these advantages it became the model for the study of prokaryotic organisms at the onset of the molecular biological revolution in the 1950's.

Many ground-breaking studies have been conducted using this model organism, for example the elucidation of the genetic code and the mechanisms involved in the central dogma; transcription, translation and replication (Nirenberg et al 1965) and, since then, it has come to play a pivotal role in the field of biotechnology. Recombinant protein production was developed using this bacterium (Cohen et al 1973), due to its rapid replication rate allowing the opportunity for large scale production of protein and today it is the most popular prokaryotic expression system (Huang et al 2012). Examples of these applications include, but are not limited to, the *in vivo* synthesis of therapeutic recombinant proteins such as insulin, human growth hormone and erythropoietin to treat diabetes, pituitary disorders and anaemia, respectively (Huang et al 2012; Kamionka 2011). It has also been used in the metabolic engineering of *E. coli* for production of biofuels (Liu and Khosla 2010). In 2011, its application in biotechnology contributed roughly $500 billion to the global economy (Blount 2015).

One aspect of *E. coli* molecular biology which has greatly aided research is the ability of DNA to be transferred into its genome. Lederberg and Tatum (1946) demonstrated that sexual recombination can occur in *E. coli*, a process whereby cells containing an F^+ fertility factor, present on a plasmid, mate with cells lacking this fertility factor and subsequently transfer DNA from the donor to the recipient (Griffiths et al 1999). This laid the foundation for research into

transfer of DNA between bacteria which allows for manipulation of bacterial genomes. Lenox (1955) later described the ability of P1 bacteriophage to shuttle DNA from one bacterial host to another during a process known as generalized transduction. More recently direct DNA transfer through electroporation has allowed directed genome engineering (Datsenko and Wanner, 2000).

Five primary strains have been developed for laboratory use, *E. coli* K12, B, C, Crooks and W (Archer et al 2011). All of these except for *E. coli* W, can be classified under phylogroup A or Risk Group 1 which is the safest group designated in the biological safety guidelines (Bauer et al 2007). *E. coli* W belongs to phylogroup B1, however A and B1 are considered sister groups as they contain commensal strains while pathogenic strains fall into groups B2, D and E (Archer et al 2011).

The K12 strain was isolated from the stool of a patient recovering from diphtheria in 1922 and was added to the strain collection at Stanford University where it was named (Bachman 1996). It was given to E. L. Tatum in the early 1940's who used it to conduct biochemical and genetic studies. Because it is prototrophic, is very easy to grow and has a short generation time, it quickly became popular for research in recombinant DNA studies and many derivatives were created (Bachman 1996*).* *E. coli* B was originally known as *Bacillus coli* and was first experimented on by Félix d'Herelle who worked on bacteriophages (D'Herelle 1918). This strain was subsequently passed on to a series of scientist until it eventually came into the hands of Delbrück and Luria in 1942 who used it in their study of bacteriophages T1 and T7 (Luria and Delbrück 1943), and today it is the preferred strain to use along with K12 in bacteriophage studies. The *E. coli* C strain is mostly known for its distinct round and oval cell morphology and characteristic globular distribution of its nucleoid at the cell periphery (Lieb et al 1954). In 1988, it was demonstrated to be the preferred *E. coli* host strain for cloning eukaryotic methylated genomic DNA sequences (Gossen and Vijg 1988). *E. coli* W was discovered when Selman A. Waksman isolated the bacterium from a soil sample collected from a cemetery near Rutgers University in 1943. The strain was initially named 'Waksman strain' and is now known as 'W strain' (Archer et al 2011). This strain turned out to have significant properties that make it useful in industrial applications. These include its ability to produce low amounts of acetate to easily grow to high cell densities within a fed-batch system, and to withstand high ethanol concentrations, temperatures, and acidic conditions (Archer et al 2011).

In 1997, the genome of the K12 MG1655 strain was sequenced by Blattner and colleagues, which demonstrated that it consists of approximately 4.6 million base pairs and 4000 genes (Blattner et al 1997). This knowledge allowed research into *E. coli* to progress more rapidly as the precise sequence of all the genes could be annotated and the function of their proteins determined. The closely related, W3110 strain became the second K12-strain to be fully sequenced (Hayashi et al 2006) and today up to 192 fully sequenced genomes for a variety of *E. coli* strains are available (http://www.ncbi.nlm.nih.gov/genome/genomes/167).

E. coli will continue to be one of the most important model organisms used in science, however, despite the large amount of research performed on it there is still much about it to be discovered. One such area is glycogen metabolism and the next section of the chapter will outline what is known about glycogen in terms both of its structure and metabolism in *E. coli* as well as examine areas about it that are poorly researched.

1.3 Glycogen

Glycogen is a branched homopolysaccharide consisting of α-1,4-linked glucose moieties with α-1,6-linked branch points every 6-8 monomers (Fig. 1.1; Preiss and Romeo 1994). The average chain length of bacterial glycogen ranges from 8-12 glucose monomers with a molecular mass of 10^7-10^8 Da (Wilson et al 2010). Its structure is comparable to the amylopectin fraction of starch found in plants, although amylopectin is less highly branched and aggregates in crystalline granules. It is the primary carbohydrate storage compound found in animals, fungi and many species of bacteria but bacterial glycogen have been studied less extensively than that of mammals. In bacteria such as *E. coli*, *Bacillus subtilis* and *Streptomyces coelicolor*, its synthesis has been linked to survival, colonisation, virulence and sporulation (Ballicora et al 2003; Jones et al 2008).

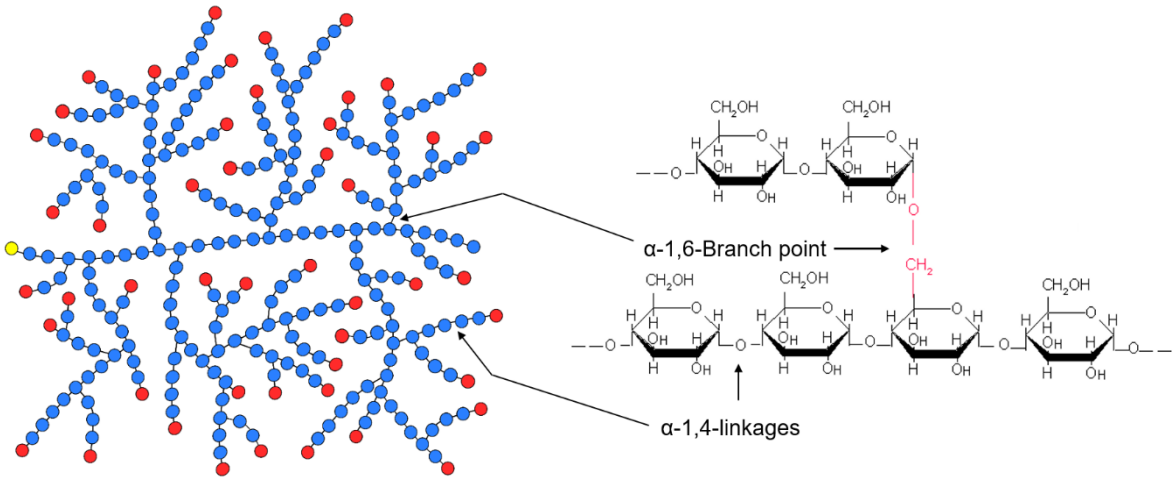

Figure 1.1 The structure of glycogen. Blue circles represent internal glucose residues and the red circles represent non-reducing moieties at the extremity of the molecule. The yellow circle represents the reducing end.

When there is an abundance of carbon in the environment alongside low concentrations of nutrients necessary for growth such as nitrogen (Cedergren and Holme 1959), then many bacterial species (both Gram negative and Gram positive) synthesise glycogen as a survival mechanism (Rose and Tempest 1989). Due to the high molecular weight and physical properties of the molecule, it is able to provide a dense store of carbon without affecting osmotic pressure (Ballicora et al 2003) and the accumulation of glycogen within a cell can be

over 50% of its dry weight (Preiss and Romeo 1989). When environmental carbon becomes limiting, the bacteria are able to survive by breaking down the stored glycogen. Two independent studies have demonstrated how bacteria that synthesize glycogen have a competitive advantage over other bacteria. Jones et al (2008) showed that the enteric bacteria in the gut of mice colonised better than other bacterial species due to their ability to synthesise and catabolise glycogen while Goh and Klaenhammer (2014) demonstrated how glycogen synthesis in *Lactobacillus acidophilus* living in the mouse intestinal tract, contributes to the fitness of the host's gut. Interestingly, it was observed that glycogen with short average chain lengths, is degraded very slowly allowing the organism to survive for longer. This occurs because its structure forces bacteria to utilize the compound more slowly and, thus, enhances its durability (Wang and Wise 2011).

Glycogen can be found localised in the cell periphery in the form of granules along with all the enzymes involved in its metabolism (Morán-Zorzano et al 2008). This indicates that the enzymes involved in its metabolism are micro-compartmentalised becoming physically associated in supramolecular clusters (metabolons) in order to aid in the channelling of metabolites (Shepard 2006).

1.4 Glycogen metabolism

The main use of glycogen as a storage carbohydrate, is to build up energy reserves in order to cope with carbon starvation (Ballicora et al 2003). This usually takes place when the cell is under stress during stationary phase due to limited environmental nutrients.

Research indicates that at least five genes (*glgA*, *glgB*, *glgC*, *glgP* and *glgX*) are involved in synthesizing and degrading glycogen in *E. coli*. Originally it was proposed that these are arranged within two tandemly arrayed operons, *GlgBX* and *GlgCAP*, which were thought to operate concomitantly (Preiss and Romeo 1994). Recent evidence suggests, however, that they operate within a single mRNA transcriptional unit under the control of a promotor sequence located upstream of *glgB* (Montero et al 2011). In addition, another promoter was found located within *glgC* that controls the downstream expression of *glgA* and *glgP*. This new model corresponds to the single operon glycogen gene clusters found in other species such as *Agrobacterium tumefaciens* (Ugalde et al 1998).

1.4.1 Enzymatic steps involved in glycogen metabolism

The three main enzymes involved in synthesis of the glycogen polymer are ADP-glucose pyrophosphorylase (GlgC, EC 2.7.7.27), glycogen synthase (GlgA, EC 2.4.1.21) and glycogen branching enzyme (GlgB, EC 2.4.1.18). Glycogen degradation is dependent on the action of GlgX (glycogen debranching enzyme, EC 3.2.1.33) and GlgP (glycogen phosphorylase EC 2.4.1.1). These processes will be examined in more detail below.

1.4.1.1 Glycogen synthesis

The simplest substrate for glycogen synthesis is glucose and this can be taken up directly by the carbohydrate phosphotransferase system (PTS) of *E. coli* before being incorporated into glycogen (Fig. 1.2). Such a system requires a phosphate donor that provides energy in order to facilitate the carbohydrate uptake. In the PTS, phosphoenolpyruvate (PEP) is the source of phosphate (Deutscher et al 2006). The PTS consist out of three integral components, enzyme I (EI), enzyme II (EII) and a low molecular weight heat stable protein, HPr. When glucose is taken up, phosphate is transferred from PEP to EII with the help of EI and HPr. Glucose is then phosphorylated by EII forming glucose-6-phosphate (G6P) which is carried across the membrane, and is converted to glucose-1-phosphate (G1P) by phosphoglucomutase (Pgm;Wilson 2010). Elongation of α-1,4-polyglucan chains is initiated through the action of

GlgC, catalysing the formation of ADP-glucose (ADPG) from ATP and G1P (Ballicora et al 2003). GlgA, transfers the glucose moiety from ADPG to the reducing end of growing α-1,4-chains. Remarkably, Ugalde et al (2003) reported that GlgA not only elongates these glucans but can also catalyse its own glucosylation in order to form the primer required for glycogen chain elongation. Finally, GlgB acts upon these linear chains in a two-step catalytic reaction by which fragments of 6 to 9 glucosyl units in length are cleaved from the non-reducing ends of one chain and are transferred to another chain to form α-1,6-glucosidic branches (Preiss 2014).

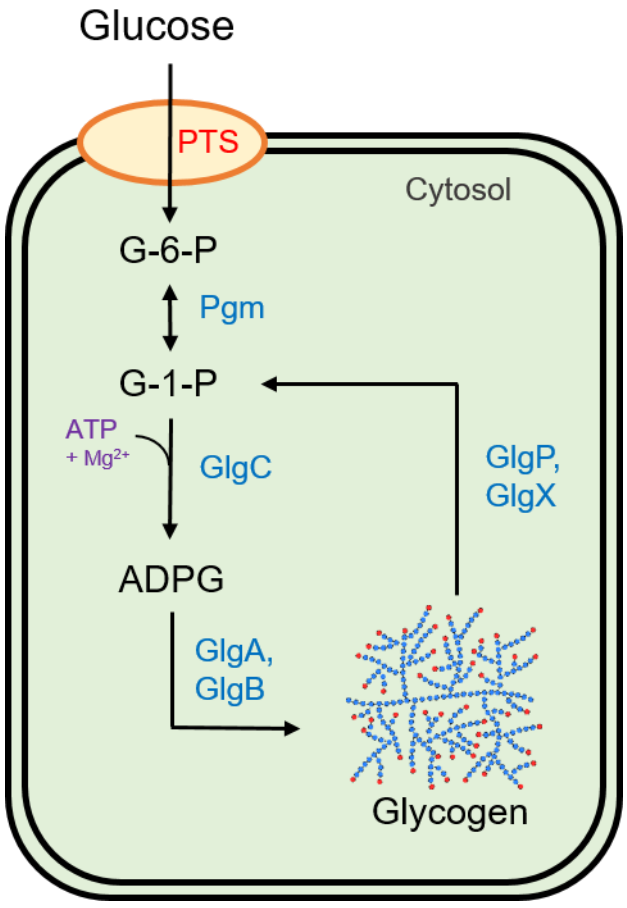

Figure 1.2 Schematic representation of glycogen metabolism in *E. coli*. Glycogen synthesis entails the uptake and conversion of glucose to G6P by the PTS as it enters the cell, hereafter it is catalysed by Pgm, GlgC, GlgA and GlgB to form G1P, ADPG and glycogen, respectively. Glycogen degradation is carried out by GlgP and GlgX.

Evidence for the involvement of PTS and Pgm in the first two steps of this pathway was provided by Eydallin et al (2007a) and Montero et al (2009). In those studies Δ*ptsI*, Δ*ptsH*, Δ*ptsG* and Δ*pgm* deletion mutants were created, all strains carrying these mutations displayed a glycogen-less phenotype.

GlgC plays a pivotal role in glycogen synthesis as it catalyses the first committed step in the synthetic pathway. It is allosterically regulated by the metabolic intermediates, fructose 1,6-bisphosphate and AMP (discussed in more detail in section 1.4.2.3). Mutants containing an inactive form of *glgC* were reported to be unable to synthesize glycogen (Leung et al 1986, Ballicora et al 2003), however, one mutant allele (*glgC16*) leads to the accumulation of increased amounts of glycogen as it lacks the allosteric inhibition caused by metabolic intermediates (Damotte et al 1968; Creuzat-Sigal et al 1972), indicating that GlgC has a profound effect on glycogen accumulation. More recently the influence of GlgC has been questioned. Edyallin et al (2007b) used a semi quantitative glycogen stain to demonstrate that a *glgC* deletion strain displayed a negative glycogen stain phenotype. However, when glycogen was quantified in the mutant it was found that it accumulated measurable amounts. This indicates that another, as yet unidentified source of ADPG exists (Wilson 2010). One possibility is that this comes from trehalose metabolism as it has been demonstrated that, in the archeon *Thermococcus litoralis*, trehalose glucosyltransferase (TreT) can catalyse the reversible reaction of trehalose and ADP into ADPG and glucose (Qu et al 2004). Further studies are required to confirm if this pathway plays a role in glycogen metabolism in *E. coli*.

It has been demonstrated that GlgA is essential for glycogen synthesis as Δ*glgA* knockout mutants are unable to accumulate it (Damotte et al 1968) when grown on glucose. Park et al (2011) demonstrated, however, that when grown on maltodextrins Δ*glgA* mutants can form glycogen, demonstrating interaction between the glycogen and maltodextrin pathways. This will be discussed in more detail in the context of maltodextrin metabolism in section 1.5.

GlgB is not essential for glycogen synthesis as Δ*glgB* knockout mutants are still able to form linear glucans, but they are unable to introduce branch points and accumulate linear α-1,4-linked polyglucan chains. This leads to colonies of Δ*glgB* mutants staining blue when exposed to iodine, rather than brown which would be indicative of the presence of branched polyglucans (Damotte et al 1968).

1.4.1.2 Glycogen degradation

Glycogen degradation occurs when cells are in stationary phase and have been deprived of nutrients for an extended period of time. In order to survive the cell has to catabolize the glycogen it has accumulated. There are two main enzymes known to be involved in this process, GlgX (glycogen debranching enzyme) and GlgP. Although glycogen degradation is not as well characterised as glycogen synthesis, I will go into this process in detail with this section as it is the focal point of the project.

Debranching enzymes can be categorized into two distinct classes, isoamylases (EC 3.2.1.68) or pullulanases (EC 3.2.1.41). Isoamylase type enzymes catalyse the hydrolysis of α-1,6-glucosidic branches within glycogen, amylopectin and related β-limit dextrins but not those within pullulan (Cheng et al 2011). The first isoamylase was isolated from *Pseudomonas amyloderamosa* by Harada et al (1968) and by 1972, the enzyme had been extensively characterized (Yokobayashi et al 1970; Harada et al 1972). This revealed that it could hydrolyze linkages of amylopectin occurring on both inner and outer branches whereas pullulanase was more effective on outer linkages and barely cleaved inner linkages (Sebald 2012). Pullulanase, first discovered in *Klebsiella aerogenes* (originally known as *Aerobacter aerogenes*; Katsuragi et al 1987), can catalyse the hydrolysis of α-1,6-glucosidic linkages in pullulan but does not hydrolyze the α-1,4-linkages in glucans or amylose or α-1,6-glucosidic linkages present in glycogen (Kashiwabara et al 1999).

In 1973 Palmer et al identified a debranching activity from *E. coli* which hydrolysed limit dextrins and proposed the first model for intracellular carbohydrate polysaccharide catabolism in bacteria. They hypothesized that the debranching enzyme, when degrading glycogen, produces maltodextrins which enters the maltodextrin pathway (described in detail below) and are subsequently hydrolysed. Evidence for this was later provided by Jeanningros et al (1976) who purified the enzyme and studied its kinetic properties. They observed a strong specificity towards cleavage of the branch points of chains within glycogen that are, at maximum, three or four glucosyl residues in length. They concluded that it could be classified as a debranching enzyme but it was unclear to which class (isoamylases or pullulanase), it belonged as it displayed properties of both. In a similar manner to isoamylases it is unable to hydrolyse pullulan, while similar to pullulanase it has a low affinity towards glycogen.

To provide further insight into its role as a glycogen debranching enzyme, Dauvillée et al (2004) demonstrated that it not only plays a role in glycogen metabolism but also contributes to the overall structure of the carbohydrate. They found that GlgX acts specifically on α-1,6 bonds of chains that are only three or four glucosyl residues in length, releasing maltotriose and maltotetraose as products. As the majority of chains within glycogen normally contain between 5-20 glucose moieties, another enzyme would have to remove external glucose residues prior to GlgX being able to act (Dauvillée et al 2004). It has been proposed that the maltotetraose released by GlgX would be metabolised by a number of proteins involved in maltodextrin metabolism, such as amylomaltase (MalQ) and maltodextrin phosphorylase (MalP) (Song et al 2010). *glgX* has a high degree of sequence similarity to *glgB* and due to its location in the genome of *E. coli* next to *glgB*, it has been suggested by Romeo et al (1988) that it may play a role in determining glycogen structure. Evidence to support this comes from the observation that it is expressed during glycogen synthesis, albeit to a lesser extent than *glgC*, *glgA* or *glgB*. It has been proposed that GlgX could shorten the external chains of glycogen during synthesis to ensure a balance between glycogen synthesis and degradation, leading to the production of glycogen containing optimal chain lengths (Dauvillée et al 2004).

As was said above, the external chains of glycogen need to be degraded before GlgX can act on them and recent evidence indicates that this is accomplished by glycogen phosphorylase (GlgP). The first description of GlgP was reported in a study led by Chen and Segel (1968a) as a type of α-glucan phosphorylase present in *E. coli*, which they characterised as a glycogen phosphorylase due to its greater affinity for glycogen over maltodextrin. They demonstrated that GlgP degrades glycogen at a relatively slow rate during the course of extended stationary periods (Chen and Segel 1986b) but it wasn't until recently that formal evidence was provided on the function of GlgP and its influence on the structure of glycogen (Alonso-Casjús et al 2006). It was found that a Δ*glgP* deletion mutant exhibited no glycogen phosphorylase activity whilst a *glgP*-overexpressing strain displayed a nearly 20-fold increase in enzyme activity. Analysis of the glycogen chain lengths within Δ*glgP* cells demonstrated a clear difference in outer chain lengths compared to the parent strain, showing an increase in chains with a DP of 13-30 at the cost of chains with a DP of 6-8. This is due to the action of GlgA in the absence of GlgP whereby the outer chains are elongated providing evidence that GlgP plays a role in shaping the structure of glycogen during synthesis (Alonso-Casjús et al 2006).

1.4.2 Other enzymes potentially involved in glycogen metabolism

Although the core enzymes involved in glycogen metabolism are present in the *glg* operon, a number of other proteins have been demonstrated to affect this process, or encode activities that could act on glycogen. One of these, *glgS*, is not part of the *glg* operon being located approximately 300 000bp distant in the *E. coli* genome, and rather makes up a separate monocistronic operon (Hengge-Aronis et al 1992). GlgS positively effects glycogen accumulation and is itself positively regulated by a number of controlling factors involved in controlling glycogen metabolism (See section 1.4.3 for more details). No significant sequence homology, outside of the Enterobacteriaceae family has yet been found for GlgS making the precise function of this enzyme still poorly understood, however a recent study by Rahimpour et al (2013) has shown that GlgS plays a significant role in *E. coli* motility and biofilm formation by negatively regulating propulsion, adhesion and synthesis of biofilm EPSs (exopolysaccharides). They proposed that GlgS plays a major role in *E. coli* surface composition and only effects glycogen metabolism indirectly and therefore they suggested renaming it to ScoR (surface composition regulator).

AmyA is a cytoplasmic α-amylase found in *E. coli*. Raha et al (1992) were the first to not only biochemically characterize AmyA but also demonstrated that it is encoded by the *amyA* gene. This gene showed sequence similarity to other α-amylases that are secreted by other bacterial species, although the AmyA peptide sequence contains no obvious secretion signal. Raha et al (1992) found that AmyA can effectively digest maltodextrins consisting of six glucose units or larger. However, *amyA* lies far outside the *mal*-regulon, the maltose/maltodextrin system of *E. coli*, and no MalT box characteristics (discussed in more detail in section 1.5) are located upstream of it. This led them to consider a potential role for it in glycogen metabolism and, although they found that glycogen was a seemingly poor substrate, they hypothesised that it was still the most likely natural substrate present for it within the cytoplasm of *E. coli*.

A second α-amylase, encoded by *malS*, is found in *E. coli* (Freundlieb and Boos 1986) but differs from AmyA in several ways. The amino acid sequences of the two enzymes demonstrate little similarity and MalS localises to the periplasm. It is thought, therefore, that MalS digests periplasmic maltodextrins (Freundlieb and Boos 1986; Raha et al 1992) and is likely, therefore to have no effect on cytosolic glycogen (See section 1.5 for a more in depth description of the role of this enzyme in maltodextrin metabolism).

1.4.3 Regulation of glycogen metabolism

The regulation of glycogen metabolism in bacteria usually involves an intricate assemblage of factors (Fig. 1.3) that are controlled by the metabolic status of the cell and its immediate environment (Preiss and Romeo 1994). It occurs at both the transcriptional and enzymatic level through the alternation of positive and negative regulation and crosstalk between the flux of glycogen synthesis and degradation (Eydallin et al 2007).

1.4.3.1 Negative regulation

When cells within an *E.coli* culture are still growing, negative regulation occurs at the post-transcriptional level by a gene known as the carbon storage regulator, *csrA*, which effects the expression of structural genes involved in both glycogen synthesis and gluconeogenesis (Romeo et al 1993, Montero et al 2009). Within genes involved in glycogen metabolism, CsrA prevents *glgC* translation by enhancing the rate of decay of *glgC* mRNA (Yang, Lui and Romeo 1996) which can lead to the cessation of glycogen synthesis (Fig. 1.3a).

1.4.3.2 Positive regulation

There are three levels of control that positively mediates glycogen synthesis at the level of gene expression; (i) the stringent response involving the cAMP/CPR complex and the alarmone ppGpp, (ii) the general stress response with the RpoS factor and (iii) the PhoP/PhoQ regulatory system. The roles of these are outlined in more detail below:

(i) When *E. coli* cells enter stationary phase, they undergo physiological changes which elicit a specific 'stringent response' that prepare the cell for survival and puts a halt on further growth. This process requires the cAMP/cAMP receptor protein (CRP) complex and guanosine 3'-bisphosphate 5'-bisphosphate (ppGpp) for normal glycogen accumulation and regulation of *glgCAP* expression, respectively (Romeo and Preiss 1989; Romeo et al 1993). GlgC and GlgA are thus induced at this point, but GlgB is regulated independently (Romeo et al 1993). cAMP is produced by adenylate cyclase, encoded by *cya*, a membrane bound protein which responds to the presence of extracellular glucose leading to the initiation of glycogen synthesis (Fig. 1.3b). It is also required for the expression of *glgS* and PTS-related genes (Fig. 1.3c) all of which plays an important role in glycogen production (Hengge-Aronis and Fischer 1992; Montero et al 2009). ppGpp is an alarmone that is synthesized by the product of *relA* (GTP

pyrophosphokinase) and Δ*relA* cells are glycogen deficient (Eydallin et al 2007a; Montero et al 2009). When ppGpp accumulates within the cell, it contributes directly to the upregulation of *glgCAP* transcription (Fig. 1.3b). At the same time intracellular ATP levels are known to rise due to the temporary cessation of nucleic acid and protein synthesis, which also stimulates glycogen synthesis (Montero et al 2009).

(ii) RpoS is an alternative sigma factor of the RNA polymerase that is induced when the cell undergoes stress and positively regulates the expression of *glgS* (Fig. 1.3d), a gene that plays an undefined role in glycogen accumulation (Montero et al 2009, Eydallin et al 2007a). *rpoS* is not required for the transcription of the *glgCAP* operon, however *rpoS* mutants are glycogen free, so the protein is required for glycogen synthesis (Hengge-Aronis et al 1992).

(iii) PhoP-PhoQ is a regulatory system in *E. coli* and *Salmonella spp*. that reacts to changes in extracellular Mg^{2+} and in doing so, controls the expression of many genes involved in the energy and metabolic status of the cell (Fig. 1.3e). Cells lacking *phoP* and *phoQ* in conditions of low Mg^{2+}, demonstrate very low *glgC* expression and this effect is restored when the Mg^{2+} is added (Montero et al 2009).

1.4.3.3 Allosteric regulation

Allosteric enzymatic regulation of GlgC leading to alterations in the production of ADPG, occurs by small effector molecules within the cell (Preiss 1984, Ballicora et al 2003). When carbon is abundant, glycolysis is activated causing an increase in metabolic intermediates such as fructose 1,6-bisphosphate, which activates GlgC (Fig. 1.3f). These, in turn, increase the affinity of the enzyme for the substrates G1P and ATP, and glycogen synthesis is initiated. When energy levels within cells drop there is an increase of AMP, an allosteric inhibitor of GlgC (Fig. 1.3g), which causes glycogen synthesis to be inhibited (Govons et al 1973).

1.4.3.4 Crosstalk between glycogen synthesis and degradation

Regulatory crosstalk linking glycogen synthesis and degradation occurs throughout the *E. coli* growth phase (Seok et al 2001). This is mediated by the strong specificity GlgP has towards the HPr component of the PTS, shown by surface plasmon resonance-based ligand fishing (Seok et al 1997). Its affinity for phosphorylated HPr (P-His-HPr) is up to four times higher than for the unphosphorylated form, however only binding of unphosphorylated HPr leads to an increase in GlgP activity (Seok et al 1997). Overall, the majority of cellular GlgP should be

complexed with either HPr or P-His-HPr due to the much higher concentration of these proteins compared to GlgP (Deutscher et al 2006).

Consequently GlgP activity is regulated by the phosphorylation status of HPr in the following way: At the beginning of the stationary phase when glycogen synthesis is upregulated due to an increase in cAMP and the expression of *glgCAP*, the concentration of P-His-HPr increases meaning the activity of GlgP will be low allowing the cells to accumulate glycogen (Seok et al 2001). However, in late stationary phase when the growth of the cells has advanced and nutrients are nearly depleted, cAMP levels drop, the expression of *glgCAP* decreases and the concentration of PEP also declines leading to dephosphorylation of P-His-HPr proteins. This causes GlgP to bind to the now increasing amount of unphosphorylated HPr proteins and become active leading to the commencement of glycogen break down (Deutscher et al 2006).

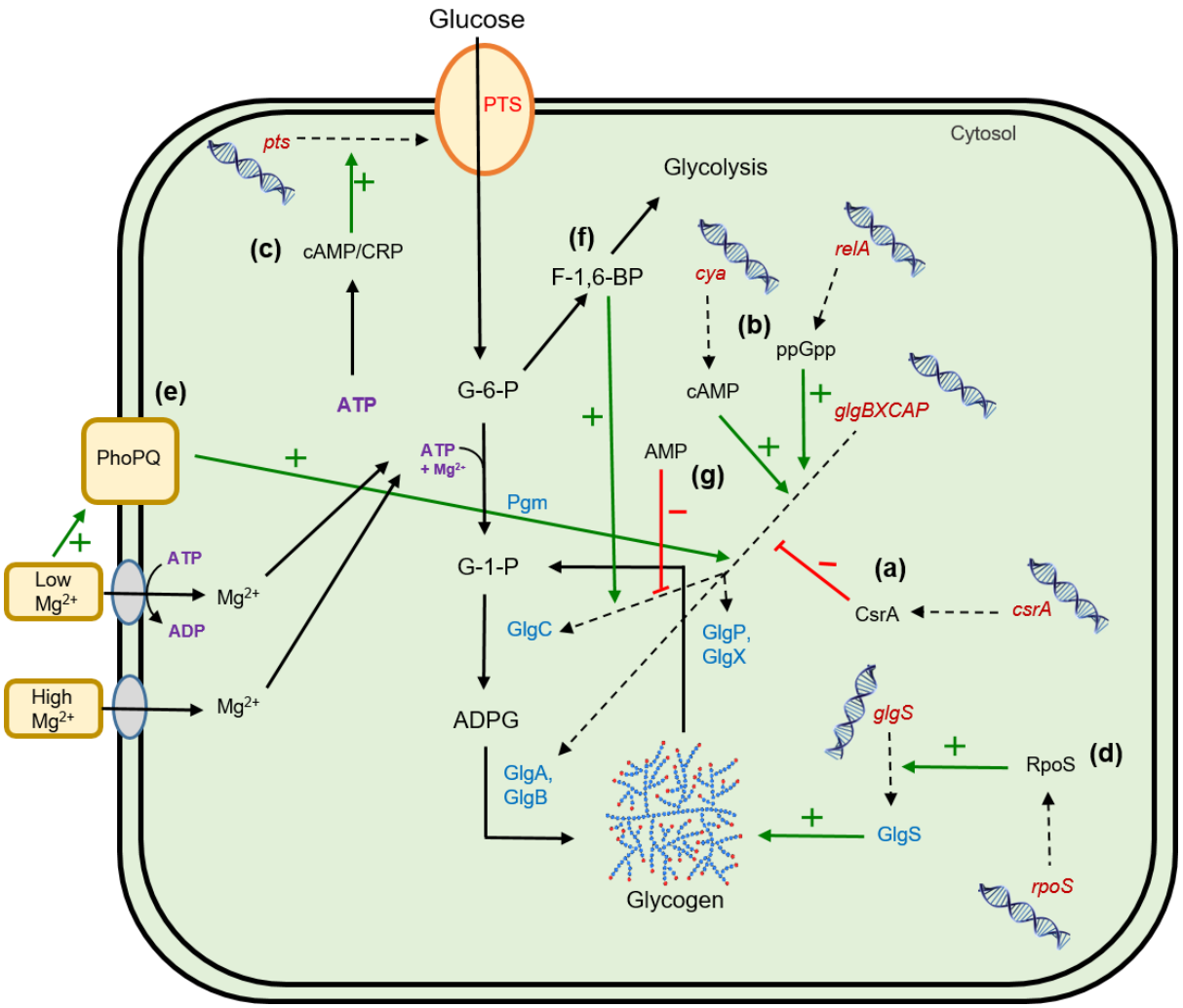

Figure 1.3 Proposed diagram for the intricate assemblage of factors involved in the regulation of glycogen metabolism in *E. coli* (Adapted from Wilson 2010). (a) Negative regulation by *csrA* encoding a protein, CsrA, that prevents *glgC* translation. (b) Positive regulation by both cAMP and ppGpp, encoded by *cya* and *relA*, respectively, is required for the upregulation of *glgCAP* so that glycogen synthesis can commence. (c) cAMP/CRP positively regulates PTS-related gene expression necessary for normal glycogen production. (d) Rpos positively regulates *glgS* transcription when the cell undergoes stress promoting glycogen accumulation. (e) PhoP-PhoQ system reacts to extracellular Mg^{2+} levels and positively regulates *glgC* transcription. (f) GlgC is allosterically regulated in a nutrient dependent way, when carbon is abundant, fructose 1,6-bisphoshate (F-1,6-BP) enhances GlgC activity and when carbon is low, GlgC is inhibited by increasing levels of AMP.

1.5 Maltodextrin metabolism

In *E. coli*, the metabolism of maltose/maltodextrin can be closely related to that of glycogen. Both maltose and maltodextrins can act as substrates that, in turn, lead to the production of glycogen and G1P which enters glycolysis (Fig. 1.4; Boos and Schuman 1998). Maltose is a disaccharide sugar consisting of two glucose monomers containing an α-1,4-glycosidic linkage whereas maltodextrin is a chain of glucose monomers connected by the same linkage but consists of three to eight glucose residues (Boos and Schuman 1998).

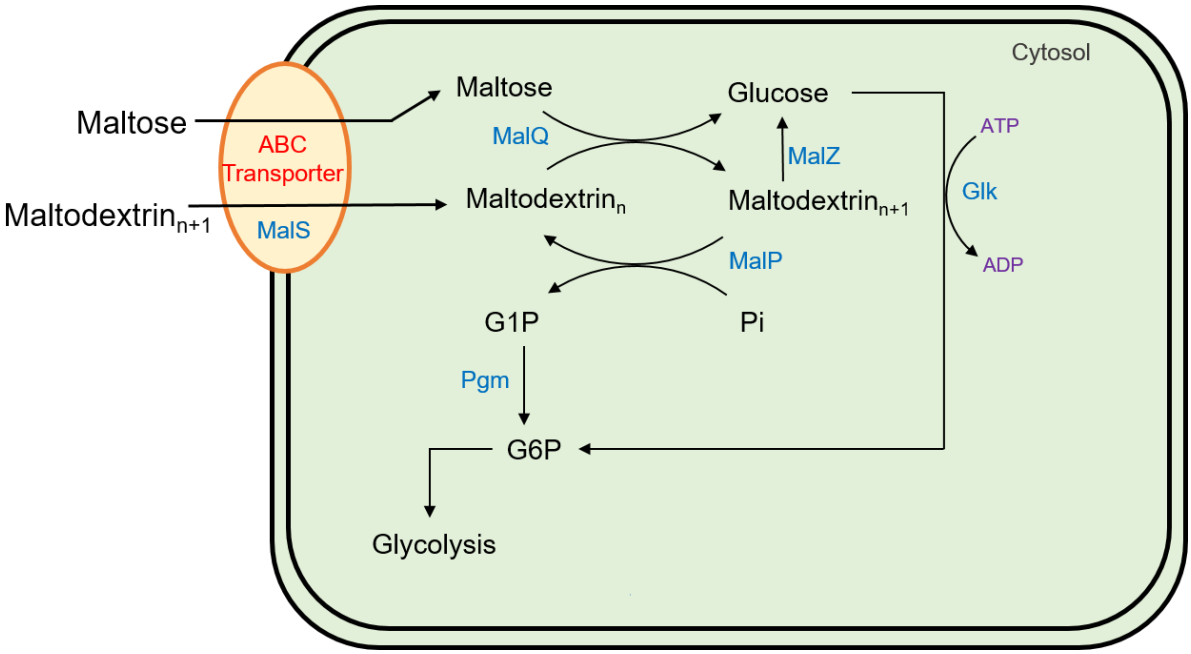

Figure 1.4 Schematic representation of the maltose/maltodextrin system in *E. coli*. When cells are grown on maltose, it is taken up directly into the cytosol where it is converted to glucose and longer chain maltodextrins by MalQ. When cells are grown on maltodextrins they are taken up, shortened to the appropriate length by MalS and enters the cytosol where they are catalysed by MalQ to form longer chains. These are, in turn, cleaved by MalP and MalZ to release G1P and glucose, respectively.

The maltose/maltodextrin regulon consists of ten genes present within five operons, all under the control of MalT, the central transcriptional activator. These genes function to regulate the uptake of maltose and maltodextrins through a periplasmic binding protein dependant ABC high-affinity transporter. This consists of five integral components, LamB, a diffusion porin

located on the outer membrane of the cell which also acts as the λ phage receptor (Freundlieb, Ehmann and Boos 1988); MalE, a binding protein with high affinity for maltose and maltodextrin located in the periplasm, often referred to as the maltose binding protein (MBP) (Kellerman and Szmelcman 1974); and a multimeric membrane spanning complex consisting of three subunits, (i) MalF, (Froshauer et al 1988) (ii) MalG, both inner membrane components (Dassa and Muir 1993), and (iii) MalK, an ATP-hydrolyzing subunit of the ABC transporter located within the cytoplasm (Shuman and Silhavy 1981, Morbach, Tebbe and Schneider 1993). This system however, is subject to strong catabolite repression by glucose because both the ABC transporter and the expression of *malT* is highly dependent on the cAMP/CAP complex (Chapon 1982).

There are four main enzymes involved in maltose/maltodextrin metabolism that will be discussed in more detail below. These enzymes are MalS (periplasmic α-amylase), MalQ (amylomaltase also known as a glucosyltransferase, EC 2.4.1.25), MalP (maltodextrin phosphorylase, EC 2.4.1.1) and MalZ (maltodextrin glucosidase, EC 3.2.1.20).

When *E. coli* is grown on maltose, this disaccharide diffuses first into the periplasm of the cell and is then directly transported into the cytosol where it is degraded to glucose and G1P (Boos and Schuman 1998). However, if *E. coli* is grown on longer maltodextrins, these enter the periplasm and are degraded by MalS, a periplasmic α-amylase, releasing shorter dextrins, with a strong preference for maltohexaose, however the maximum chain length that is then transported by the ABC transporter into the cytosol is seven glucosyl units (Schneider et al 1992).

MalQ was originally thought to transfer only single glucosyl units utilising maltose as its primary substrate. A study led by Palmer, Ryman and Whelan (1976) demonstrated, however, that maltose acts as an acceptor rather than a donor molecule, and that MalQ can release not only glucose but also longer dextrins from the reducing end of its maltodextrin substrates. MalQ thus acts on maltodextrins that enter the cell via the ABC transporter, by cleaving off glucose or longer dextrins from the reducing end and transferring the remaining maltodextrinyl chain to the non-reducing end of either glucose or maltodextrin acceptors to produce longer chains (Boos and Schuman 1998). Maltotriose is the shortest donor substrate that MalQ can utilize. The longer chains can then function as substrate for GlgB (Park et al 2011). *ΔmalQ* deletion mutants cannot grow when maltose is the sole carbon source but can when maltotetraose and longer dextrins are supplied. The ABC transporter is unable to

transport maltodextrins longer than seven glucosyl units in length, and so they are too short to act as a substrate for GlgB. Therefore, in the absence of MalQ, these dextrins are catabolized by MalP and MalZ to produce glucose and G1P that provides energy for the cell (Park et al 2011).

MalP cleaves glucose moieties from the non-reducing ends of maltodextrins, through a phosphorylase reaction with the resulting G1P molecules entering glycolysis (Park et al 2011, Boos and Schuman 1998). Maltotetraose is the shortest maltodextrin that MalP can act on. When Δ*malP* deletion mutants are grown on glucose the amount of glycogen accumulated is similar to that of the wild type however when grown on maltose or other short maltodextrins they accumulate a significant amount of longer dextrins due to the action of MalQ ultimately leading to enlarged and elongated cell shape (Park et al 2011).

MalZ has a similar function to that of MalP, however it releases glucose as opposed to G1P and targets maltodextrins of three glucose units and longer from the reducing ends (Boos and Schuman 1998). When Δ*malZ* deletion mutants are grown on maltose or maltodextrins, an increase in glycogen is also observed, but to a lesser extent than observed for $MalP^-$ strains and it has been found that this enzyme is not essential for maltose and maltodextrin utilization (Park et al 2011).

1.6 *E. coli* cell size and division

Bacterial cell size control is an essential part of how cells grows and divides, however, there is clearly still much to be learned about how this process is governed (Robert et al 2014). In *E. coli*, there are several main proteins involved in cell division, the best studied ones, applicable to rod-shaped bacteria, are FtsZ and MreB (Wiley et al 2011).

FtsZ is a highly conserved tubulin-like GTPase protein, which forms both a Z-ring, in the centre of a cell, and a septum which will eventually divide the two daughter cells (Lutkenhaus and Addinall 1997). The Z-ring formation is influenced by three *min* genes encoding the proteins MinC, MinD and MinE and which operate within a MinCDE system. These proteins oscillate from one end of the cell to the other leading to accumulation of MinC at the poles and in doing so, prevents FtsZ from forming a Z-ring anywhere else but at the centre of the cell when the cell reaches a specific size (Lutkenhaus 2007). Once the Z-ring is formed, the rest of the division machinery can be assembled.

MreB (murein cluster B) is a bacterial homologue of eukaryotic actin. It is only found in rod-shaped bacteria as it assists with cell shape. Inhibition of MreB in rod-shaped cells turn them into a coccoid shape (Jones et al 2001). This protein was studied in *Caulobacter crescentus* and was shown to form heliacal spirals all around the inside periphery of the cell where it plays a role in peptidoglycan synthesis (Figge et al 2004).

Recently an additional protein, RodZ, a membrane spanning protein, was discovered to interact with MreB and is found to be essential in the proper assembly of MreB spirals (Shiomi et al 2008; Shiomi et al 2009). They observed that a deletion mutant causes the cell to be effected lengthwise and not along its width forming a similar coccoid shape as a Δ*mreB* mutant.

It is known that cell size control is necessary especially during the growth phase in order to maintain cell size homeostasis (Robert et al 2014). A number of models have been suggested to explain this size control. In 1968, Donachie proposed the idea that DNA replication is initiated when an *E. coli* cell reaches a specific size and that the mass of the cells are constant at the time of replication, irrespective of the growth rate. This view was based on the work done by Schaechter et al (1958) and Cooper and Helmstetter (1968), and is supported by several other studies (see Donachie and Blakely 2003 and references therein). It became known as the ‘sloppy size model’, later termed the ‘sizer’ model. This assumes that cell division is dependent

on cell size, and that all cells divide when they are the same size, irrespective of their size at birth. This concept was generally accepted for many years, however, direct experimental evidence for it was lacking. Other models were proposed on the basis that there is a stronger connection between the initiation of replication and the time elapsed since birth (cell age) than to cell mass (Bates et al 2005; Bates and Kleckner 2005; Boye and Nordström 2003), leading to the introduction of the 'timer' model. This assumes that cell division is dependent on cell age rather than size and is supported by studies that showed a constant time between the initiation of DNA replication and cell division across a variety of different growth rates (Cooper and Helmstetter 1968).

An updated version of the sizer-based model, originally called the 'incremental size' model, was presented by Voorn and Koppes (1998) and later supported by Amir (2014), where they argued that a growing cell adds a constant volume at each generation. However, those studies (similar to the sizer and timer models) focussed only on population-averaged data instead of either single-cell data or a combination of the two. This lead Taheri-Aragahi and colleagues (2015) to build a device called the 'mother machine', where individual cells could be monitored from birth to cell division in order to get a clearer picture on cell size homeostasis. They discovered that instead of cells doubling in size before dividing, each cell rather added a constant volume (Δ) under a steady-state growth condition, where the constant volume is a product of the difference in volume between a new born cell and a cell prior to division (Taheri-Aragahi 2015). This model was renamed the 'adder' model and, because of the new single-cell data, has overtaken both the sizer and timer models (Sauls et al 2016).

A challenge to the 'adder' model was recently published (Tanouchi et al 2015). In that study they demonstrated that a cell's initial size does in fact determine the amount of growth the cell will undergo before it divides. They, too, carried out their experimentation on the 'mother machine' and found a direct linear relationship within the results and concluded that each cell add a different amount of biomass before beginning division and that amount is determined by its initial cell size (Tanouchi et al 2015).

In addition to all the proposed models, nutrient availability (Fig. 1.5a, b) has been shown to play a major role in cell size, as both *E. coli* (Grover and Woldringh 2001) and *B. subtillis* (Schaechter et al 1958) cells grown in nutrient rich media are larger than when they are grown in nutrient poor media (Weart et al 2007). This begs the question how nutrient availability and growth rate are regulated in the control of cell size? Several studies point towards a signalling

pathway that transmits information between the metabolic status of the cell and its division machinery (Hill et al 2013).

In 2007, Weart and colleagues reported that *B. subtilis* (Fig. 1.5d) responds to the intracellular concentration of uridine diphosphate glucose (UDP-glucose) which coordinates cell size by promoting an interaction between a membrane-associated protein, glucosyltransferase (UgtP), and FtsZ. They observed that in nutrient rich conditions (when UDP-glucose concentrations are high), this interaction caused a delay in cell division, while in nutrient poor conditions (when UDP-glucose levels are low) UgtP is sequestered and has little effect on FtsZ. This leads to the activation of the cell division machinery and commencement of cell division (Weart et al 2007). Interestingly, they reported that cells only divide once they have reached a certain mass for a given growth rate, called the 'critical mass'. This requires the cell to possess a sensor for cell mass which alleviates the UDP-glucose dependant inhibition of UgtP once critical mass has been reached. However, the identification of such a sensor is currently unknown.

In 2013, Hill et al reported on a parallel pathway to the one described above within *E. coli* (Fig. 1.5c) that links growth rate with cell size homeostasis in a nutrient-dependant manner. However instead of UgtP, an unrelated functionally analogous glucosyltransferase, OpgH, acts as the sensor. That polypeptide localises to the inner membrane of *E. coli* and it situates at the cell division plate (Hill et al. 2013). They proposed that in *E. coli*, OpgH binds UDP-glucose in nutrient rich conditions, causing a conformational change to occur at the N-terminus of the protein. UDP-glucose, in this regard, again acts as a metabolic signal as it is synthesised from G1P when the cell takes up glucose. This conformational change exposes a binding site which has a strong affinity for FtsZ, and once FtsZ is bound, the cell's division machinery is obstructed and cell growth is promoted. In nutrient poor conditions, UDP-glucose levels are low and FtsZ cannot bind the N-terminal of OpgH, allowing FtsZ assembly to proceed unhindered and cell division to occur (Hill et al 2013).

In order to confirm that it is OpgH that is regulated by UDP-glucose in this process, as opposed to Pgm or GalU (UDP-glucose pyrophosphorylase that catalyses the conversion of G1P to UDP-glucose), *opgH*-, *pgm*- and *galU*-overexpressing cells were produced and cultured in nutrient rich media, and their average cell sizes measured (Hill et al 2013). They found that only the *opgH*-overexpressing cells were affected by displaying a significant increase in cell

length of up to five fold that of the wild-type, whereas the other two strains demonstrated no significant alterations in their cell size.

A second pathway that informs the cell of its metabolic status and transmits the information to its cell division machinery, is glycolysis. A recent study (Monahan et al 2014) demonstrated that glycolysis is closely linked to cell division in *B. subtilis*. This is governed by the presence of pyruvate, the final metabolite in the glycolytic pathway produced by pyruvate kinase, the presence of which is dependent on nutrient availability. Pyruvate effects cell division *via* a large multi-enzyme complex, pyruvate dehydrogenase (PDH), which is made up of four subunits. One of these subunits, E1α, exercises a pyruvate-dependent control on Z-ring assembly as it localises over the nucleoid. This was confirmed by demonstrating that cells lacking their PDH E1α subunit displayed significant defects in Z-ring formation. Similarly, PDH E1α cannot interact with the nucleoid in *pyk* mutants (cells mutated in the gene, *pyk*, which encodes pyruvate kinase), due to the lack of pyruvate, and aberrant cell division thus occurs in these cells as PDH E1α accumulates at the poles.

A recent study has identified a mutant *E. coli* strain that produces extremely long (750µ M) cells (El Hajja and Newman 2015), when it was grown in nutrient rich media (LB and LB devoid of salt). They determined the amount of FtsZ within the cells by Western blotting and found that these cells were nearly depleted in FtsZ however the reason for this is still unclear as there were no mutation present in the *ftsZ* gene and no other discovered mutation in the genome could be demonstrated to lead to the observed phenotype. This indicated that there must be other, as yet undiscovered, factors involved in determining *E. coli* cell size.

All of these studies point to an intricate assemblage of factors involved in growth related cell division. Precisely how they all come together and regulate cell size homeostasis is not well understood, however many breakthroughs are being made that adds to the ever growing elucidation of cell cycle dynamics.

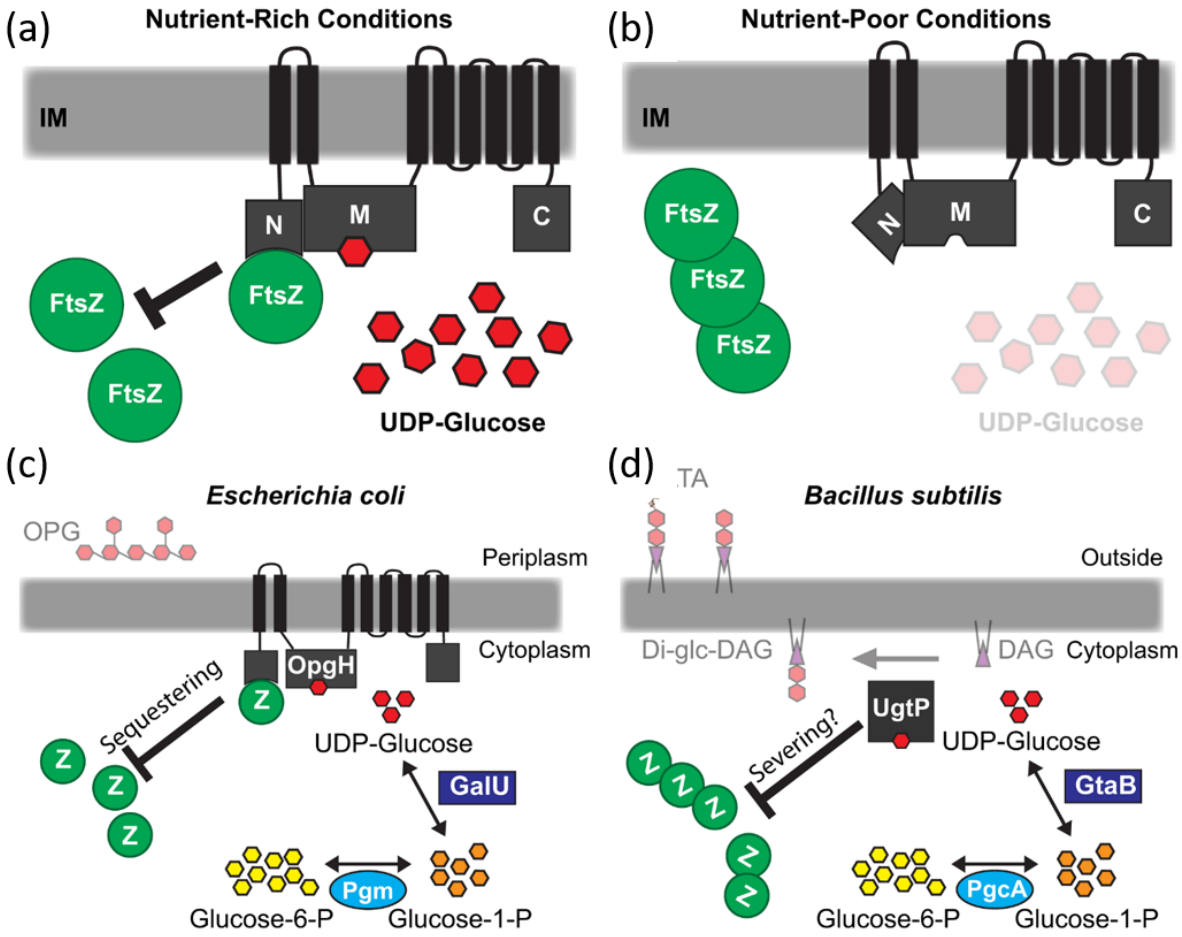

Figure 1.5 UDP-glucose and OpgH regulates *E.coli* cell size in a nutrient dependent manner (Hill et al 2013). (a) In nutrient rich conditions, there is an abundance of UDP-glucose which causes OpgH to undergo a conformational change on its N-terminal. OpgH now has a high affinity for FtsZ, which puts a halt on FtsZ-mediated Z-ring formation and cell growth seizes. (b) In nutrient poor conditions, the level of UDP-glucose are low, allowing FtsZ to continue unhindered as the N-terminal of OpgH is obscured, and assemblage of the cell's division machinery can commence. (c-d) Growth rate-dependent size homeostasis within *E. coli* and *B. subtilis* are both regulated by UDP-glucose but by unrelated glucosyltransferases.

1.7 Production of mutants in *E. coli*

Mutations are alterations in the genetic sequence of organisms and can take on many forms. Point mutations for example alter only single nucleotides and can lead to minor changes in amino acid sequence, or premature truncation of translation. More severe changes such as insertions, deletions, inversions, duplications and translocations might affect several enzymes (Wiley et al 2011). Mutations occur spontaneously, or can be induced chemically or physically. Chemical mutagenizing agents include two main groups, alkylating agents (i.e. ethyl methane sulphonate) and base analogs (5'-bromouracil and 2'-aminopurine), whereas examples of physical mutagens include UV radiation and X-rays (Wiley et al 2011).

The use of chemical or physical mutagenesis has limitations, as these techniques do not always provide complete knockout of targeted genes, or remove several genes simultaneously. Targeted gene knockouts through homologous recombination have recently become an easy and effective way to overcome this. At the end of the last century Datsenko and Wanner (2000) developed a highly efficient method to directly disrupt chromosomal genes in *E. coli* (Fig. 1.6a). This method relies on the recombination event carried out by a recombinase isolated from λ phage. Essentially, PCR primers are designed to contain 36-50nt extensions that are homologous to the region that is adjacent to the targeted genes. These primers are firstly used to amplify an antibiotic resistance marker flanked by FRT (FLP recognition target sites) from a template plasmid. The PCR product is then transferred into a strain expressing λ Red recombinase to allow a recombination event to take place, replacing the targeted gene with the resistance marker resulting in the full disruption of chromosomal genes. Transformants are selected using plates containing the appropriate antibiotic. Finally, the resistance cassette can be eliminated using a temperature sensitive FLP expression plasmid, where the resistance marker is cut from the genome. This method was used by Baba et al (2006) to create the Keio-collection, a set of isogenic single-gene deletions of all non-essential genes in *E. coli*.

Mutations can be transferred between strains by a number of approaches. One of these entails the use of the P1 bacteriophage and is known as transduction (Fig. 1.6b; Thomason et al 2007). P1 phage is known for its sloppy packaging mechanism whereby it occasionally packages fragments of DNA of approximately 100kb from its bacterial host along with its own phage chromosome. This trait can be taken advantage of by growing the phage alongside a donor strain containing a segment of DNA that is desired to be transferred to a recipient strain. This creates a P1 lysate required to infect a second strain. DNA from the donor strain becomes

integrated into the genome of the recipient strain through homologous recombination by RecA and RecBCD. If an antibiotic resistance cassette is present in the transferred section of DNA, it can be used to easily identify the successfully transduced strain. By using a combination of P1 phage transduction and the Keio collection of *E. coli* mutants it is possible, therefore, to produce a series of isogenic *E. coli* strains lacking a number of proteins in any non-lethal process.

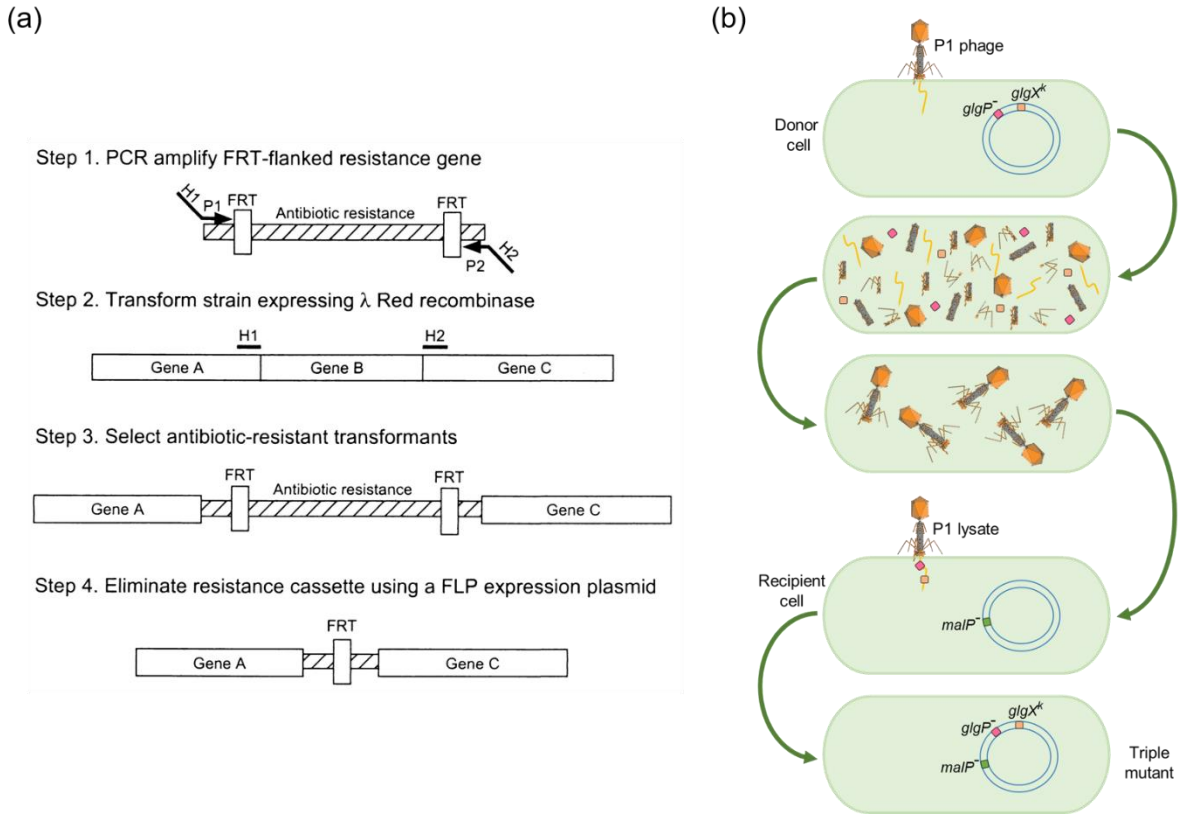

Figure 1.6 Two different approaches to produce mutants in *E. coli*. (a) PCR mediated method used to create knockout mutants by replacing the gene of interest by an antibiotic resistance marker. H1 and H2 represent the homology regions, and P1 and P2 represent the primer sites (Datsenko and Wanner 2000). (b) By using P1 lysate, mutations can be transferred from one bacterial cell (donor) to another (recipient) and incorporated into the genome of the recipient cell by homologous recombination.

1.8 Aims and Objectives

The aim of this study is to examine the roles of MalP, GlgP and GlgX in *E. coli* glycogen degradation in more detail. The objectives are:

(i) To produce a series of isogenic *E. coli* mutant strains lacking combinations of all these enzymes.

(ii) To grow the strains in different media and see if glycogen accumulation and structure is affected.

(iii) To examine if the mutations affect *E. coli* cell size and/or shape.

References

Alonso-Casajus N, Dauvillee D, Viale AM, *et al.* (2006) Glycogen Phosphorylase, the Product of the glgP Gene, Catalyzes Glycogen Breakdown by Removing Glucose Units from the Nonreducing Ends in *Escherichia coli*. *J Bacteriol* **188:** 5266-5272

Amir A (2014) Cell Size Regulation in Bacteria. *Phys Rev Lett* **112:** DOI: 10.1103/PhysRevLett.112.208102

Archer CT, Kim JF, Jeong H, *et al.* (2011) The genome sequence of *E. coli* W (ATCC 9637): comparative genome analysis and an improved genome-scale reconstruction of *E. coli*. *BMC Genomics* **12:** doi: 10.1186/1471-2164-12-9

Baba T, Ara T, Hasegawa M, *et al.* (2006) Construction of *Escherichia coli* K-12 in-frame, single-gene knockout mutants: the Keio collection. *Mol Syst Biol* **2:** 2006.0008.

Bachmann BJ (1996) Derivations and genotypes of some mutant derivatives of *Escherichia coli* K-12. In FC Neidhardt, IR Curtis, JL Ingraham, et al, eds, *In Escherichia coli and Salmonella typhimurium Cellular and Molecular Biology*, Ed 2nd. ASM Press, Washington DC, pp 2460-2488

Ballicora MA, Iglesias AA, Preiss J (2003) ADP-Glucose Pyrophosphorylase, a Regulatory Enzyme for Bacterial Glycogen Synthesis. *Microbiol Mol Biol Rev* **67:** 213-25

Bates D, Epstein J, Boye E, *et al.* (2005) The *Escherichia coli* baby cell column: a novel cell synchronization method provides new insight into the bacterial cell cycle. *Mol Microbiol* **57:** 380-91

Bates D, Kleckner N (2005) Chromosome and Replisome Dynamics in *E. coli*: Loss of Sister Cohesion Triggers Global Chromosome Movement and Mediates Chromosome Segregation. *Cell* **121:** 899-911

Bauer AP, Dieckmann SM, Ludwig W, *et al.* (2007) Rapid identi¢cation ofEscherichia coli safetyand laboratory strain lineages based on Multiplex-PCR. *FEMS Microbiol Lett* **1:** 36-40

Blattner FR, Plunket IG, Bloch CA, *et al.* (1997) The Complete Genome Sequence of *Escherichia coli* K-12. *Science* **277:** 1453-62

Blount ZD (2015) The unexhausted potential of *E. coli*, http://dx.doi.org/10.7554/eLife.05826.

Boos W, Shuman H (1998) Maltose/Maltodextrin System of *Escherichia coli*: Transport, Metabolism, and Regulation. *Microbiol Mol Biol Rev* **62:** 204-29

Boye E, Nordstrom K (2003) Coupling the cell cycle to cell growth. *EMBO Rep* **4:** 757-60

Cedergren B, Holme T (1959) On the Glycogen in *Escherichia coli* B; Electron Microscopy of Ultrathin Sections of Cells. *J Ultrastruct Res* **3:** 70-73

Chapon C (1982) Role of the Catabolite Activator Protein in the Maltose Regulon of *Escherichia coli*. *J Batcteriol* **150:** 722-29

Chen GS, Segel IH (1968a) *Escherichia coli* Polyglucose Phosphorylases. *Arch Biochem Biophys* **127:** 164-174

Chen GS, Segel IH (1968b) Purification and Properties of Glycogen Phosphorylase from Escherichia coli. *Arch Biochem Biophys* **127:** 175-86

Cheng KC, Demirci A, Catchmark JM (2011) Pullulan: biosynthesis, production, and applications. *Appl Microbiol Biotechnol* **92:** 29-44

Cohen SN, Chang ACY, Boyer HW, *et al.* (1973) Construction of Biologically Functional Bacterial Plasmids In Vitro. *Proc Natl Acad Sci U S A* **70:** 3240-44

Cooper S, Helmstetter CE (1968) Chromosome replication and the division cycle of *Escherichia coli* Br. *J Mol Biol* **31:** 519-40

Creuzet-Sigal N, Latil-Damotte M, Cattaneo J, *et al.* (1972) Genetic Analysis and Biochemical Characterization of Mutants Impairing Glycogen Metabolism in *Escherichia coli*. In R Piras, HG Pontis, eds, *Biochemistry of the Glycosidic Linkage. An Integrated view*. Vol 2. Academic Press, London, pp 647-80

Damotte M, Cattaneo J, Sigal N, *et al.* (1968) Mutants of *Escherichia coli* K 12 altered in their ability to store glycogen. *Biochem Biophys Res* Commun **32:** 916-20

Dassa E, Muir S (1993) Membrane topology of MalG, an inner membrane protein from the maltose transport system of *E. coli*. *Mol Microbiol* **7:** 29-38

Datsenko KA, Wanner BL (2000) One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *PNAS* **97:** 6640-6645

Dauvillee D, Kinderf IS, Li Z, *et al.* (2005) Role of the *Escherichia coli* glgX Gene in Glycogen Metabolism. *J Bacteriol* **187:** 1465-1473

Deutscher J, Francke C, Postma PW (2006) How Phosphotransferase System-Related Protein Phosphorylation Regulates Carbohydrate Metabolism in Bacteria. *Microbiol Mol Biol Rev* **70:** 939-1031

D'Herelle F (1918) Sur le rôle du microbe filtrant bactériophage dans la dysenterie bacillaire. *Compt Rend Acad Sci* **167:** 970-72

Dippel R, Boos W (2005) The Maltodextrin System of Escherichia coli: Metabolism and Transport. *J Bacteriol **187:** 8322-8331*

Donachie WD (1968) Relationship between Cell Size and Time of Initiation of DNA Replication. *Nature* **219:** 1077-9

Donachie WD, Blakely GW (2003) Coupling the initiation of chromosome replication to cell size in *Escherichia coli*. *Curr Opin Microbiol* **6:** 146-50

El-Hajj Z, Newman EB (2015) An *Escherichia coli* Mutant That Makes Exceptionally Long Cells. *J Bacteriol* **197:** 1507-14

Escherich T (1886) Die darmbakterien des säuglings und ihre beziehungen zur physiologie der Verdauung. F. Enke,

Eydallin G, Teresa M, Moran-Zarzano MT, *et al.* (2007) An *Escherichia coli* mutant producing a truncated inactive form of GlgC synthesizes glycogen: Further evidences for the occurrence of various important sources of ADPglucose in enterobacteria. *FEBS Lett* **581:** 4417-22

Eydallin G, Viale AM, Moran-Zarzano MT, *et al.* (2007a) Genome wide screening of genes affecting glycogen metabolism in *Escherichia coli* K-12. *FEBS Lett* **581:** 2947-2953

Figge RM, Divakaruni AV, Gober JW (2004) MreB, the cell shape-determining bacterial actin homologue, co-ordinates cell wall morphogenesis in *Caulobacter crescentus*. *Mol Microbiol* **51:** 1321-32

Freundlieb S, Boos W (1986) a-Amylase of *Escherichia coli*, Mapping and Cloning of the Structural Gene, mal& and Identification of Its Product as a Periplasmic Protein. *J Biol Chem* **261:** 2946-53

Freundlieb S, Ehmann U, Boos W, (1988) Facilitated Diffusion of p-Nitrophenyl-a-D-maltohexaosideth rough the Outer Membrane of *Escherichia coli:* Characterization of LamB as a specific and saturable channel for maltooligosaccharides. *J Biol Chem* **263:** 314-20

Froshauer S, Green GN, Boyd D, *et al.* (1988) Genetic Analysis of the Membrane Insertion and Topology of MalF, a Cytoplasmic Membrane Protein of *Escherichia coli*. *J Mol Biol* **200:** 501-11

Goh YJ, Klaenhammer TR (2014) Insights into glycogen metabolism in *Lactpbacillus acidophilus*: impact on carbohydrate metabolism, stress tolerance and gut retention. *Microb Cell Fact* **13:** doi: 10.1186/s12934-014-0094-3

Gossen JA, Vijg J (1988) *E. coli*: a convenient host strain for rescue of highly methylated DNA. *Nucleic Acids Res* **16:** 9343

Govons S, Gentner N, Greenberg E, *et al.* (1973) Biosynthesis of Bacterial Glycogen. XI. Kinetic Characterization of an Altered Adenosine Diphosphate-Glucose Synthase from a 'Glycogen-Excess' Mutant of Escherichia Coli B. *J Biol Chem* **248:** 1731-40

Griffiths AJF, Gelbart WM, Miller JH, *et al.* (1999) Bacterial conjugation. Modern Genetic Analysis. W. H. Freeman, New York

Grover NB, Woldringh CL (2001) Dimensional regulation of cell-cycle events in *Escherichia coli* during steady-state growth. *Microbiology* **147:** 171-181

Harada T, Misaki A, Akai H, *et al.* (1972) Characterization of *Pseudomonas* isoamylase by its action on amylopectin and glycogen: comparison with *Aerobacter aerogenes*. *Biochim Biophys Acta* **268:** 497-505

Harada T, Yokobayashi K, Misaki A (1968) Formation of Isoamylase by *Pseudomonas*. *Appl Microbiol* **16:** 1439-44

Hayashi K, Morooka N, Yamamoto Y, *et al.* (2006) Highly accurate genome sequences of Escherichia coli K-12 strains MG1655 and W3110. *Mol Syst Biol* **2:** 2006.0007

Hengge-Aronis R, Fischer D (1992) Identification and molecular analysis of gigS, a novel growth-phase-regulated and rpoS-dependent gene involved in glycogen synthesis in *Escherichia coli*. *Mol Microbiol* **6:** 1877-86

Hill NS, Buske PJ, Shi Y, *et al.* (2013) A Moonlighting Enzyme Links *Escherichia coli* Cell Size with Central Metabolism. *PLoS Genet* **9:** doi: 10.1371/journal.pgen.1003663

Huang JC, Lin H, Yang X (2012) Industrial production of recombinant therapeutics in *Escherichia coli* and its recent advancements. *J Ind Microbiol Biotechnol* **39:** 383-99

Jeanningros R, Creuzet-Sigal N, Frixon C, *et al.* (1976) Purification and properties of a debranching enzyme from *Escherichia coli*. *Biochim Biophys Acta* **438:** 186-99

Jones LJF, Carballido-Lopez R, Errington J (2001) Control of Cell Shape in Bacteria: Helical, Actin-like Filaments in *Bacillus subtilis*. *Cell* **104:** 913-22

Jones SA, Jorgensen M, Chowdhury FZ, *et al.* (2008) Glycogen and Maltose Utilization by *Escherichia coli* O157:H7 in the Mouse Intestine. *Infect Immun* **76:** 2531-2540

Kamionka M (2011) Engineering of Therapeutic Proteins Production in *Escherichia coli*. *Curr Pharm Biotechnol* **12:** 268-74

Kashiwabara S, Ogawa S, Miyoshi N, *et al.* (1999) Three Domains Comprised in Thermostable Molecular Weight 54,000 Pullulanase of Type I from *Bacillus flavocaldarius* KP1228. *Biosci Biotechnol Biochem* **63:** 1736-48

Katsuragi N, Takizawa N, Murooka Y (1987) Entire Nucleotide Sequence of the Pullulanase Gene of *Klebsiella aerogenes* W70. *J Batcteriol* **169:** 2301-6

Kellerman O, Szmelcman S (1974) Active Transport of Maltose in Escherichia coli K12: Involvement of a Periplasmic Maltose Binding Protein. *Eur J Biochem* **47:** 139-49

Lederberg J, Tatum EL (1946) Gene Recombination in *Escherichia coli*. *Nature* **158:** 558

Lennox ES (1955) Transduction of Linked Genetic Characters of the Host by Bacteriophage Pl. *Virology* **1:** 190-206

Leung P, Lee YM, Greenberg E, *et al.* (1986) Cloning and Expression of the Escherichia coli glgC Gene from a Mutant Containing an ADPglucose Pyrophosphorylase with Altered Allosteric Properties. *J Batcteriol* **167:** 82-88

Lieb M, Weigle JJ, Kellenberger E (1955) A Study of Hybrids Between Two Strains of *Escherichia coli*. *J Bacteriol* **69:** 468-71

Liu T, Khosla C (2010) Genetic engineering of *Escherichia coli* for biofuel production. *Ann Rev Genet* **44:** 53-69

Luria SE, Delbruck M (1943) Mutations of Bacteria from Virus Senstivity to Virus Resistance. *Genetics* **28:** 491-511

Luthkenhaus J (2007) Assembly Dynamics of the Bacterial MinCDE System and Spatial Regulation of the Z Ring. *Annu Rev Biochem* **76:** 539-62

Luthkenhaus J, Addinall SG (1997) Bacterial cell division and the Z ring. *Annu Rev Biochem* **66:** 93-116

Minamino T, Imada K (2015) The bacterial flagellar motor and its structural diversity. *Trends Microbiol* **23:** 267-74

Monahan LG, Hajduk IV, Blaber SP, et al. (2014) Coordinating Bacterial Cell Division with Nutrient Availability: A Role for Glycolysis. *Mbio* **5**: doi: 10.1128/mBio.00935-14

Montero M, Almagro G, Eydallin G, *et al.* (2011) *Escherichia coli* glycogen genes are organized in a single glgBXCAP transcriptional unit possessing an alternative suboperonic promoter within glgC that directs glgAP expression. *Biochem J* **433:** 107-17

Montero M, Eydallin G, Viale AM, *et al.* (2009) *Escherichia coli* glycogen metabolism is controlled by the PhoP-PhoQ regulatory system at submillimolar environmental Mg2+ concentrations, and is highly interconnected with a wide variety of cellular processes. *Biochem J* **424:** 129-41

Moran-Zarzano MT, Montero M, Munoz FJ, *et al.* (2008) Cytoplasmic *Escherichia coli* ADP sugar pyrophosphatase binds to cellmembranes in response to extracellular signals as the cell population density increases. *FEMS Microbiol Lett* **288:** 25-32

Morbach S, Tebbe S, Schneidert E (1993) The ATP-binding Cassette (ABC) Transporter for Maltose/Maltodextrins of *Salmonella typhimurium*. Characterization of the ATPase Activity Associated with the Purified MalK Subunit. *J Biol Chem* **268:** 18617-21

Nirenberg M, Leder P, Bernfield M, *et al.* (1965) RNA codeworks and protein synthesis, VII. On the general nature of the RNA code. *Proc Natl Acad Sci U S A* **53:** 1161-68

Palmer TN, Ryman BE, Whelan WJ (1976) The Action Pattern of Amylomaltase from *Escherichia coli*. *Eur J Biochem* **69:** 105-15

Palmer TN, Wober G, Whelan WJ, (1973) The Pathway of Exogenous and Endogenous Carbohydrate Utilization in *Escherichia coli*: A Dual Function for the Enzymes of the Maltose Operon. *Eur J Biochem* **39:** 601-12

Park JT, Shim JH, Tran PL, *et al.* (2011) Role of Maltose Enzymes in Glycogen Synthesis by *Escherichia coli*. *J Bacteriol* **193:** 2517-2526

Preiss J (1984) Bacterial Glycogen Synthesis and its Regulation. *Ann Rev Microbiol* **38:** 419-58

Preiss J (2014) Glycogen: Biosynthesis and Regulation. *EcoSal Plus* **6:** doi: 10.1128/ ecosalplus

Preiss J, Romeo T (1989) Physiology, biochemistry and genetics of bacterial glycogen synthesis. *Adv Microb Physiol* **30:** 183-238

Preiss J, Romeo T (1994) Molecular Biology and Regulatory Aspects of Glycogen Biosynthesis in Bacteria'. *Prog Nucleic Acid Res Mol Biol* **47:** 299-329

Proft T, Baker EN (2009) Pili in Gram-negative and Gram-positive bacteria – structure, assembly and their role in disease. *Cell Mol Life Sci* **66:** 613-35

Qu Q, Lee SJ, Boos W (2004) TreT, a Novel Trehalose Glycosyltransferring Synthase of the Hyperthermophilic Archaeon *Thermococcus Litoralis*. *J Biol Chem* **279**: 47890-7

Raha M, Kawagishi I, Muller V, *et al.* (1992) *Escherichia coli* Produces a Cytoplasmic a-Amylase, AmyA. *J Batcteriol* **174:** 6644-52

Rahimpour M, Montero M, Almagro G, *et al.* (2013) GlgS, described previously as a glycogen synthesis control protein, negatively regulates motility and biofilm formation in *Escherichia coli*. *J Biochem* **452:** 559-73

Robert L, Hoffmann M, Krell N, *et al.* (2014) Division in *Escherichia coli* is triggered by a size-sensing rather than a timing mechanism. *BMC Biology* DOI: 10.1186/1741-7007-12-17

Romeo T, Gong M, Liu MY, *et al.* (1993) Identification and Molecular Characterization of *csrA*, a Pleiotropic Gene from *Escherichia coli* That Affects Glycogen Biosynthesis, Gluconeogenesis, Cell Size, and Surface Properties. *J Batcteriol* **175:** 4744-55

Romeo T, Kumar A, Preiss J (1988) Analysis of the *Escherichia coli* glycogen gene cluster suggests that catabolic enzymes are encoded among the biosynthetic genes. *Gene* **70:** 363-76

Romeo T, Preiss J (1989) Genetic Regulation of Glycogen Biosynthesis in *Escherichia coli*: In Vitro Effects of Cyclic AMP and Guanosine 5'-Diphosphate 3'-Diphosphate and Analysis of In Vivo Transcripts. *J Bacteriol* **171:** 2773-82

Rose AH, Tempest DW (1989) *Advances in Microbial Physiology*. Academic Press **30:** 276

Sauls JT, Li D, Jun S (2016) Adder and a coarse-grained approach to cell size homeostasis in bacteria. *Curr Opin Cell Biol* **38:** 38-44

Schaechter M, Maaloe O, Kjeldgaard NO (1958) Dependency on Medium and Temperature of Cel Size and Chemical Composition during Balanced Growth of *Salmonella typhimurium*. *J Gen Microbiol* **19:** 592-606

Scheutz F, Strockbine NA (2005) *Escherichia*. In GM Garrity, DJ Brenner, NR Krieg, JT Staley, eds, Bergey's manual of systematic bacteriology. Springer, New York, NY, pp 607-24

Schneider E, Freundlieb S, Tapio S, *et al.* (1992) Molecular Characterization of the MalT-dependent Periplasmic alpha Amylase of *Escherichia coli* Encoded by *malS*. *J Biol Chem* **267:** 5148-54

Sebald M (2012) *Genetics and Molecular Biology of Anaerobic Bacteria*, Ed 1st. Springer Science & Business Media, New York

Seok YJ, Koo BM, Sondej M, *et al.* (2001) Regulation of *E. coli* Glycogen Phosphorylase Activity by HPr. *J Mol Microbiol Biotechnol* **3:** 385-93

Seok YJ, Sondej M, Badawi P, *et al.* (1997) High affinity binding and allosteric regulation of *Escherichia coli* glycogen phosphorylase by the histidine phosphocarrier protein, HPr. *J Biol Chem* **272:** 26511-21

Shephard VA (2006) The Cytomatrix as a Cooperative System of Macromolecular and Water Networks. Curr Top Dev Biol **75:** 171-223

Shiomi D, Mori H, Niki H (2009) Genetic mechanism regulating bacterial cell shape and metabolism. *Commun Integr Biol* **2:** 219-20

Shiomi D, Sakai M, Niki H (2008) Determination of bacterial rod shape by a novel cytoskeletal membrane protein. *EMBO J* **27:** 3081-91

Shuman HA, Silhavy TJ (1981) Identification of the *malK* Gene Product. A Peripheral Membrane Component of the *Escherichia coli* Maltose Transport System. *J Biol Chem* **256:** 560-2

Song HN, Jung TY, Park JT, *et al.* (2010) Structural rationale for the short branched substrate specificity of the glycogen debranching enzyme GlgX. *Proteins* **78:** 1847-55

Strange RE (1968) Bacterial 'Glycogen' and Survival. *Nature* **220:** 606-607

Taheri-Araghi S, Bradde S, Sauls JT, *et al.* (2015) Cell-Size Control and Homeostasis in Bacteria. *Curr Biol* **25:** 385-91

Tanouchi Y, Pai A, Park H, *et al.* (2015) A noisy linear map underlies oscillations in cell size and gene expression in bacteria. *Nature* **523:** 357-60

Thomason LC, Constantino N, Court DL (2007) *E. coli* genome manipulation by P1 transduction. *Curr Protoc Mol Biol* **Unit 1.17:** DOI: 10.1002/0471142727.mb0117s79

Ugalde JE, Lepek V, Uttaro A, *et al.* (1998) Gene Organization and Transcription Analysis of the *Agrobacterium tumefaciens* Glycogen (glg) Operon: Two Transcripts for the Single Phosphoglucomutase Gene. *J Batcteriol* **180:** 6557-64

Ugalde JE, Parodi AJ, Ugalde RA (2003) De novo synthesis of bacterial glycogen: *Agrobacterium tumefaciens* glycogen synthase is involved in glucan initiation and elongation. *Proc Natl Acad Sci U S A* **100:** 10659-63

Voorn WJ, Koppes LJH (1998) Skew or third moment of bacterial generation times. *Arch Microbiol* **169:** 43-51

Wang L, Wise MJ (2011) Glycogen with short average chain length enhances bacterial durability. *Naturwissenschaften* **98:** 719-29

Weart RB, Lee AH, Chien AC, *et al.* (2007) A metabolic sensor governing cell size in bacteria. *Cell* **130:** 335-47

Wiley JM, Sherwood LM, Woolverton CJ (2011) *Prescott's Microbiology*, Ed 8th. McGraw-Hill, New York

Wilson WA, Roach PJ, Montero M, *et al.* (2010) Regulation of glycogen metabolism in yeast and bacteria. *FEMS Microbiol* Lett **34:** 952-85

Yang H, Liu MY, Romeo T (1996) Coordinate Genetic Regulation of Glycogen Catabolism and Biosynthesis in *Escherichia coli* via the CsrA Gene Product. J Batcteriol **178:** 1012-17

Yokobayashi K, Misaki A, Harada T (1970) Purification and properties of Pseudomonas isoamylase. Biochim Biophys Acta **212:** 458-69

Chapter 2

2. The roles of GlgP, GlgX and MalP in Glycogen Metabolism

2.1 Introduction

Glycogen is a branched homopolysaccharide consisting of linear α-1,4-linked glucose chains linked by α-1,6-glucosyl branches occurring approximately every 8-12 glucose moieties. *E. coli* synthesises glycogen as a carbon store when external conditions are rich in carbon but limited in other nutrients (Preiss and Romeo 1994).

Five genes, g*lgA*, *glgB*, *glgC*, *glgP* and *glgX* operate concurrently to promote glycogen metabolism (Preiss and Romeo 1994; Montero et al 2011). During its synthesis, linear chains are formed through the actions of GlgC (ADP-glucose pyrophosphorylase; EC 2.7.7.27), and GlgA (Glycogen synthase; EC 2.4.1.21). GlgB (EC 2.4.1.18), a branching enzyme introduces α-1,6-glucosyl branches to the chains *via* a glucanotransferase reaction (Park et al 2011). Once carbon becomes limiting, endogenous degradation of this polymer occurs. Glycogen phosphorylase (GlgP; EC 2.4.1.1) releases glucose-1-phosphate (G1P) by targeting the non-reducing ends of external chains until they are three to four glucosyl units in length (Alonso-Casjús et al 2006). This resultant phosphorylase limit dextrin is the optimal substrate of GlgX (EC 3.2.1.33), a debranching enzyme which liberates either maltotriose or maltotetraose (Dauvillée et al 2004). Mutations in either *glgP* or *glgX* lead to *E. coli* cells that accumulate increased amounts of glycogen as they lack the ability to mobilize this polyglucan efficiently (Dauvillée et al 2004; Alonso-Casjús et al 2006). Furthermore, the glycogen accumulated is altered in structure, with *glgX* mutant glycogen enriched in short chains (DP 3-4) and *glgP* mutant glycogen enriched in longer chains (≥DP 15).

GlgX and GlgP are not only involved in glycogen degradation but also provide a link to the endogenous induction of maltose/maltodextrin metabolism (Park et al 2011). It has been suggested that this is the result of glycogen-derived maltodextrins produced by the action of GlgX which bind to the transcriptional activator MalT (Dippel and Boos 2005), thereby inducing other genes that are part of the *mal* regulon. One of these, *malP* (maltodextrin phosphorylase; EC 2.4.1.1) has been characterized to release G1P as it cleaves glucosyl residues from the non-reducing ends of linear maltodextrins through a phosphorylase reaction (Park et al 2011). MalP and GlgP thus catalyse similar reactions, the most important difference

being their substrate specificities. GlgP has a high affinity for glycogen, but can also act on maltodextrins that contain five or more glucose moieties (Boos and Schuman, 1998). MalP, in contrast, prefers short chain maltodextrins but can also act on glycogen although it has less affinity for it than GlgP (Schwartz and Hofnung 1967; Chen and Segel 1968; Schinzel and Nidetzsky 1998). Either GlgP or MalP could, therefore, mobilize maltodextrins produced by the action of GlgX on glycogen. Although it has been suggested that MalP is the main enzyme involved (Dauvillée et al 2004), there is no experimental evidence to support this.

This study examines the roles of MalP, GlgP and GlgX in glycogen degradation in more detail by producing and analysing a series of mutant strains lacking combinations of all three enzymes. These mutants are also observed under scanning electron microscopy to determine whether glycogen accumulation within *E. coli* affects cell morphology. We demonstrate that all three genes contribute to glycogen mobilization, but also provide evidence that at least one additional enzyme must participate. In addition, we observe elongated cell lengths for strains containing a mutant *malP*-allele.

2.2 Materials and Methods

2.2.1 *E. coli* strains and growth conditions

The *E. coli* single mutants and the corresponding wild-type (WT) strain used in this study (Table 2.1) were part of the Keio collection obtained from the Coli Genomic Stock Centre (cgsc.biology.yale.edu). Cultures were grown in Lysogeny Broth (LB) (1% ($^{w}/_{v}$) NaCl, 1% ($^{w}/_{v}$) peptone and 0.5% ($^{w}/_{v}$) yeast extract; 1% ($^{w}/_{v}$) glucose was added where applicable with gyratory shaking (250 rpm) at 37°C. For cells grown on solid LB media, 1.5% ($^{w}/_{v}$) agar was added. Antibiotic selection was carried out with 50 μg.ml^{-1} kanamycin when needed.

2.2.2 Construction of *E. coli* double and triple mutants

Antibiotic resistance genes were eliminated where necessary using the plasmid pCP20 (Cherapanov and Wackernagel 1995). Strains containing multiple mutant alleles were created via P1 phage transduction as previously described (Thomason et al 2007). Mutant alleles were assessed by PCR using gDNA as a template and primers that bind either side of the gene (Table 2.1).

2.2.3 Assessment of growth

Strains were cultured in 100 mL Erlenmeyer flasks containing 50 mL LB at 37°C with shaking at 250 rpm. Samples were taken at multiple time points and the absorbance at 600 nm determined.

2.2.4 Glycogen determination

Two-mL aliquots from cells grown in liquid culture were harvested by centrifugation (17,000 x g) at 22°C for 5 min, and each cell pellet was re-suspended in 1 mL of 70% ($^{v}/_{v}$) ethanol and heated at 80°C for 2 h to remove soluble sugars. This step was repeated and any remaining liquid removed. Pellets were re-suspended in 400 μL of 0.2 M KOH and heated at 95°C for 1 h, before being neutralized with 70 μL of 1 M acetic acid. Ten-μL aliquots were digested at 37°C for 2 h with 10 μL of 50 mM sodium acetate buffer (pH 5.6) containing 10 U.ml^{-1} of amyloglucosidase (*Aspergillus niger* from Megazyme) and 10 U.ml^{-1} of α-amylase (*Bacillus amyloliquefaciens* from Megazyme). Two hundred-μL of 10 mM Tris-HCl (pH 7.5), 5 mM Mg_2Cl, 1 mM NAD and 1 mM ATP was added and the absorbance at 340 nm determined. One U.ml^{-1} of both glucose-6-phosphate dehydrogenase (*Leuconostoc* from

Megazyme) and hexokinase (yeast from Megazyme) was added to each of the samples and the increase in absorbance at 340 nm determined.

Table 2.1 Primers and bacterial strains used in this study.

***E. coli* strains**	**Description**	**Reference**
BW25113	Δ*(araD-araB)567*	Datsenko and Wanner (2000)
	Δ*lacZ4787(*::rrnB-3*) λ⁻ rph-1*	
	Δ*(rhaD-rhaB)568 hsdR514*	
Δ*malP(kan*)	BW25113Δ*malp::kan*	Baba et al (2006)
Δ*glgP(kan*)	BW25113Δ*glgp::kan*	Baba et al (2006)
Δ*glgX(kan)*	BW25113Δ*glgx::kan*	Baba et al (2006)
Δ*malP*	BW25113Δ*malp*	Kind gift from Dr James Lloyd (SU)
Δ*glgP*	BW25113Δ*glgp*	Kind gift from Dr James Lloyd (SU)
Δ*glgX*	BW25113Δ*glgx*	Kind gift from Dr James Lloyd (SU)
Δ*malP*Δ*glgP*	BW25113Δ*glgp::*Δ*malp::kan*	Kind gift from Mr. Jonathan Jewell (SU)
Δ*malP*Δ*glgX*	BW25113Δ*malp::*Δ*glgx::kan*	This study
Δ*glgP*Δ*glgX*	BW25113Δ*glgp::*Δ*glgx::kan*	Kind gift from Mr. Michael A. Meier (SU)
Δ*malP*Δ*glgP*Δ*glgX*	BW25113Δ*malp::*Δ*glgp::*Δ*glgx::kan*	This study
Gene	**Forward primer**	**Reverse Primer**
malP	5'AAGGTCAACATCGAGCCTGG3'	5'ATCCACCAGCATCGCTTTGA3'
glgP	5'CTTCACTGTGGCGGTTTGTG3'	5'GGAATTACCGCAAAGCCCAC3'
glgX	5'GGCAGTAATGCAGGCAATGG3'	5'TGATGCAGTTAGACAGCGCA3'

2.2.5 Iodine staining

Twenty-µL aliquots of overnight cultures from the strains were spotted onto solid media and incubated overnight at 37°C. The colonies were stained using iodine vapor.

2.2.6 Enzyme determination

Total protein was extracted by sonicating cell pellets in 17 mM Tris-HCl (pH 8.8), 5 mM EDTA, 2 mM β-Mercaptoethanol and 1 mM Phenylmethylsulfonyl fluoride and the lysates collected by centrifugation (3400 x g) at 4°C for 30 min. Protein content was measured

by the Bradford method using a kit (BioRad). To examine the relative activities of MalP and GlgP, 240 µg protein from each extract were separated at 4°C for 6 h (110 V) in a 10% ($^{w}/_{v}$) polyacrylamide gel containing 1% ($^{w}/_{v}$) glycogen (Oyster from Sigma). The gel was incubated overnight with gyratory shaking at 22°C in 3 M acetic acid/NaOH (pH 6.0) containing 20 mM glucose-1-phosphate and stained with Lugol's solution.

GlgX activity was determined as described by Dauvillée et al (2004), however 10 mg of oyster glycogen (Sigma) was used as substrate and, a second set of boiled extracts represented negative controls. Glucose was used as standard in the reducing ends assay.

2.2.7 Glycogen isolation and determination of its chain length distribution

Strains were cultured in 50 mL LB at 37°C for 16 h with shaking (250rpm). Glycogen was isolated as previously described by Park et al (2011) and constituent chains were produced and analyzed by HPLC using the method of Streb et al (2008).

2.2.8 Data analysis

Data were compared using a one way analysis of variance. A *post hoc* Bonferoni-Holm test was used to examine the significance of differences between means using Daniels XL Toolbox (Kraus 2014).

2.2.9 Electron microscopy (EM)

Strains were cultured in liquid LB medium without any glucose supplementation for 16 h. Five-mL aliquots were harvested by centrifugation (5000 x g) at 22°C for 5 min and the pellets fixed after re-suspending in 100 µL of 2.5% ($^{v}/_{v}$) gluteraldehyde in 0.1 M phosphate buffer (pH 7.4) and incubated for 8 h at 4°C. Cells were washed twice with 0.1 M phosphate buffer (pH 7.4) and fixed with 1% ($^{v}/_{v}$) osmium tetroxide for 1h at 22°C. After one wash with 0.1 M phosphate buffer (pH 7.4) and a second wash with distilled water, the cells were dehydrated by exposure to an ethanol series of 30% ($^{v}/_{v}$), 50% ($^{v}/_{v}$), 70% ($^{v}/_{v}$), 90% ($^{v}/_{v}$), 95% ($^{v}/_{v}$) and 100%. Cells were left overnight to air dry and were then observed under a Zeiss Merlin FEG SE microscope.

2.3 Results

2.2.1 Production of *E. coli* strains lacking MalP, GlgP and GlgX activities

We produced a series of double and triple mutants (Fig. 2.1) by phage transduction using isogenic strains from the Keio collection (Baba et al 2006). To demonstrate that the mutant alleles affected the enzymes they encode we examined MalP and GlgP activities in crude extracts in a zymogram (Fig. 2.2a). Three bands representing phosphorylase activities were distinguishable. The band representing the slowest migrating protein is the product of MalP, as it is missing in all mutants lacking the *malP* gene. The other two bands are missing in mutants lacking a wild-type *glgP* allele and therefore, are both the product of GlgP. Interestingly a band of clearing, indicating a glycogen degradation activity, was present in all strains between the MalP and GlgP bands.

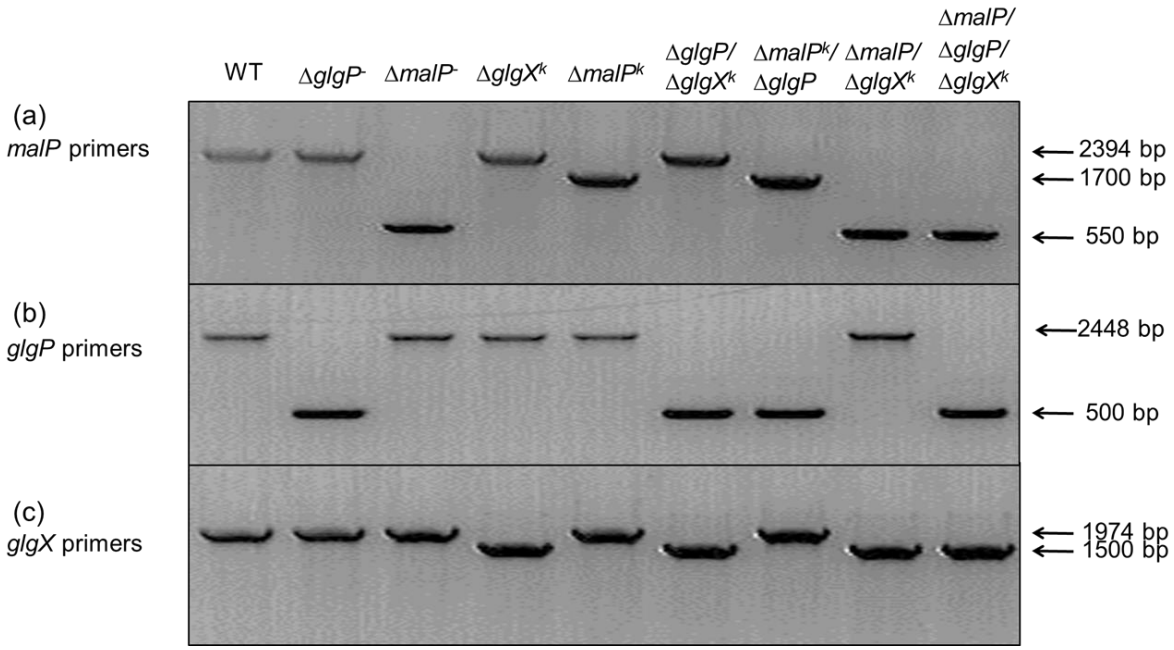

Figure 2.1 PCR analysis of mutant and wild type alleles in the *E. coli* strains. Alleles were amplified using gDNA and primers described in Table 1. *malP* primers should amplify a 550bp amplicon for the Δ*malP*, 1700bp for the Δ*malP(kan)* and 2394bp for the WT alleles. *glgP* primers should amplify a 500bp and 2448bp amplicons for the Δ*glgP* and WT alleles respectively. *glgX* primers should amplify a 1500bp and 1974bp amplicons for the Δ*glgX(Kan)* and WT alleles respectively. Superscript k denotes the presence of a kanamycin resistance gene in a specific allele.

GlgX activity was determined by examining the release of reducing ends from glycogen by crude protein extracts (Fig. 2.2b). A three-fold lower enzyme activity was observed for Δ*glgX* and Δ*malP*/Δ*glgX* cells. The other strains lacking *glgX* also demonstrate significantly reduced activity in comparison with the WT strain, but increased when compared with Δ*glgX* and Δ*malP*/Δ*glgX*. Evidently, there is another enzyme present capable of releasing reducing ends from glycogen, potentially the activity responsible for the zone of clearing observed in the zymogram (Fig 2.2a).

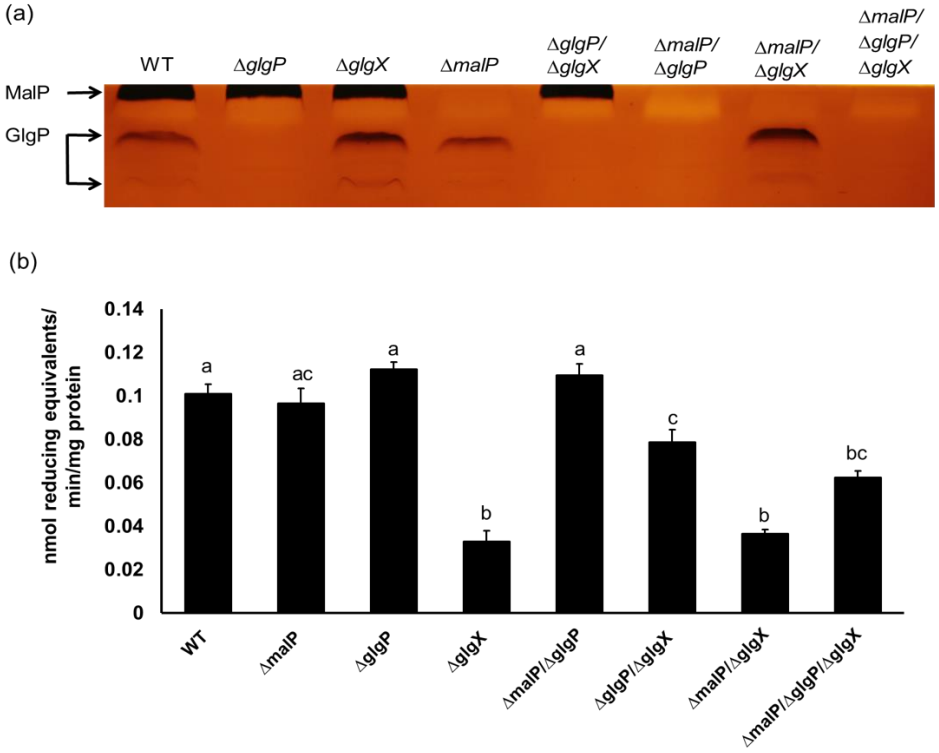

Figure 2.2 MalP, GlgP and GlgX activities. (a) MalP and GlgP activities were examined following separation of crude protein extracts in a polyacrylamide gel containing glycogen and incubation with G1P. Incorporation of linear glucans by the different phosphorylases was visualized using Lugol's solution. (b) Release of reducing ends from glycogen by crude protein extracts from different *E. coli* strains. Bars represent means ± SEM of three biological replicates. Statistically significant differences at the 5 % level are indicated by different letters over the appropriate bars and were calculated using a *post hoc* Bonferroni-Holm test following a one-way analysis of variance.

2.2.2 Effect of mutating *malP*, *glgP* and *glgX* on glycogen accumulation

To determine the effect of mutating these three genes on glycogen, we compared the glycogen contents of the strains when grown on solid or in liquid LB media supplemented with or without 1% ($^{w}/_{v}$) glucose.

Staining by iodine vapour of cells grown overnight on solid media demonstrated that more glycogen accumulated in cells of most of the mutant strains. As expected there was more glycogen present when cells were grown on media supplemented with glucose, than on media without. In the absence of added glucose, all strains, other than the Δ*glgP* single mutant stained darker than the wild-type, with the Δ*malP*/Δ*glgX* and Δ*malP*/Δ*glgP*/Δ*glgX* strains staining darkest (Fig. 2.3a). Some differences were noted in the staining of these strains when glucose was included in the medium (Fig. 2.3b). Under these conditions, the Δ*malP* single mutant stained similarly to the wild type, while all the other mutant strains stained darker. Colonies of the Δ*glgP* and Δ*malP*/Δ*glgP* strains stained relatively uniformly light brown while the Δ*glgX* and Δ*malP*/Δ*glgX* strains stained light brown in the centre and dark brown at the periphery. Both Δ*glgP*/Δ*glgX* and Δ*malP*/Δ*glgP*/Δ*glgX* mutant strains stained very dark brown.

The glycogen contents in these cells were determined through measurement following growth in liquid LB medium. Cultures were grown with and without glucose for a period of 48 h and samples taken at multiple time points for glycogen determination. Growth curves (Fig. 2.3e, f) showed no differences in growth rates between any of the mutants within a specific media, however those grown in media supplemented with glucose reached a higher cell density. Cultures grown without glucose (Fig. 2.3c) demonstrated no significant differences in glycogen content between any of the strains for the first 24 h, after which both the wild-type and Δ*malP* mutant strains contained less than the others. After 48 h, these two strains contained almost undetectable levels of glycogen compared with the others, differing significantly ($p \leq 0.001$) from the Δ*malP*/Δ*glgP*, Δ*glgP*/Δ*glgX* and Δ*malP*/Δ*glgX* strains. After 12 h growth in liquid media supplemented with 1% ($^{w}/_{v}$) glucose (Fig. 2.2d), all strains containing a *glgX* mutant allele contained more glycogen than all other strains ($p \leq 0.003$). There were, however, no significant differences between the strains mutated in *glgX* at any time point. After 48 h all *glgX*-deficient strains accumulated approximately 2.5-3 times more glycogen than any of the others. No significant differences were observed between the Δ*malP*, Δ*glgP*, Δ*malP*/Δ*glgP* and wild-type strains at any time point.

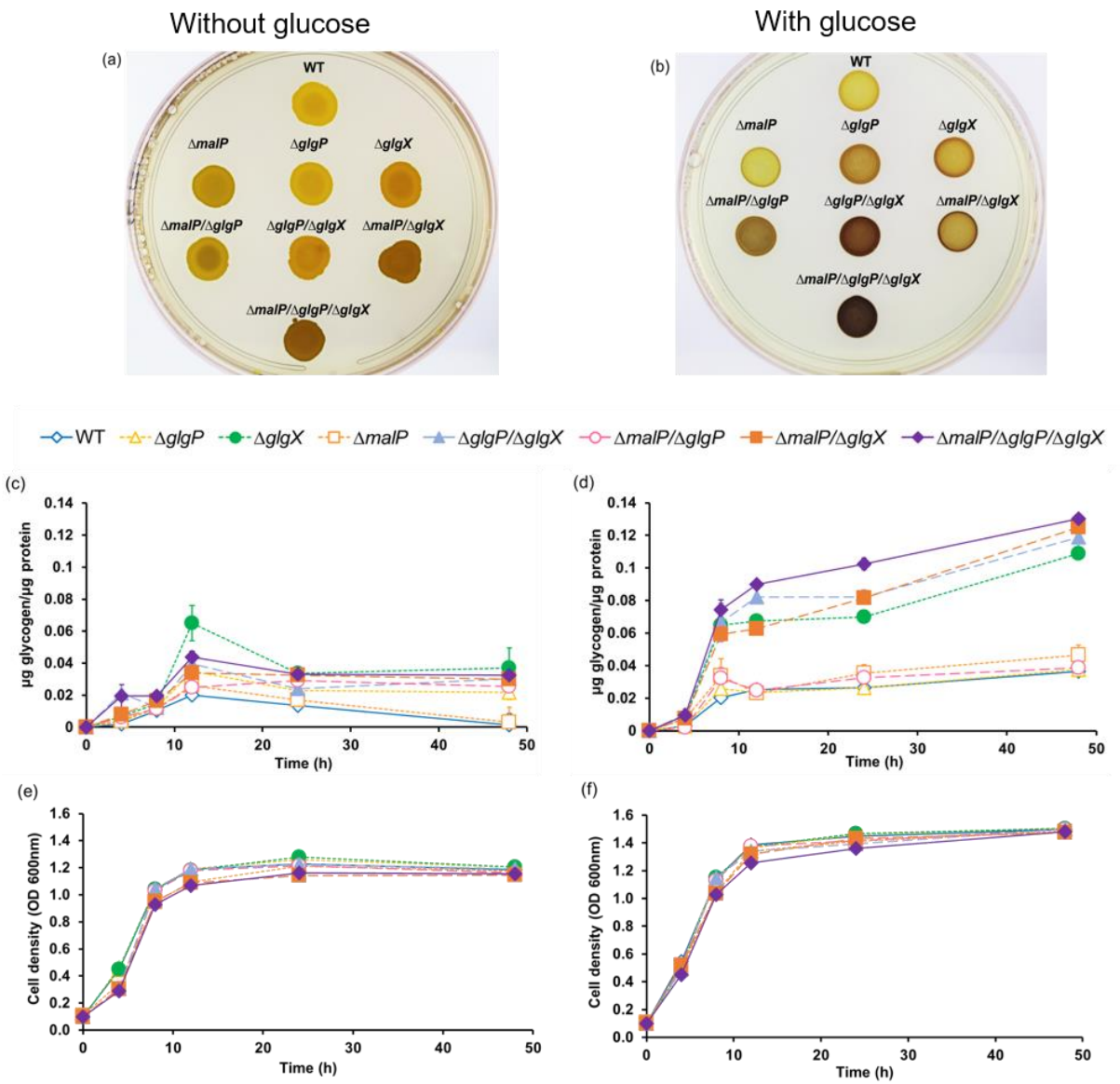

Figure 2.3 Accumulation of glycogen in the *E. coli* strains and their growth rates. Cells were grown for 16 h on solid media either without (a) or with (b) supplementation by 1 % ($^{w}/_{v}$) glucose. Glycogen was visualized by exposure to iodine vapour. (c) Glycogen accumulation and (e) OD_{600} of strains grown in LB over 48 h. (d) Glycogen accumulation and (f) OD_{600} of strains grown in LB supplemented with 1% ($^{w}/_{v}$) glucose over 48 h.

2.2.3 Analysis of glycogen chain length distribution

In order to elucidate the effect of mutating several genes involved in glycogen degradation on glycogen structure, the constituent chains isolated after debranching of purified polymer were separated by high performance anion exchange chromatography. Differences

were only noted in glycogen isolated from strains where the *glgX* gene had been mutated (Fig. 2.4). Similar to Dauvillée et al (2004), glycogen from strains lacking GlgX, contained significantly more shorter chains of DP4 (Fig 2.4c; *Δglgx* and *ΔmalP/ΔglgX* strains). Surprisingly, our analyses did not reveal a major difference in the chain length distribution of glycogen from the *glgP* mutant, in contrast to the findings of Alonso Casjús et al. (2006).

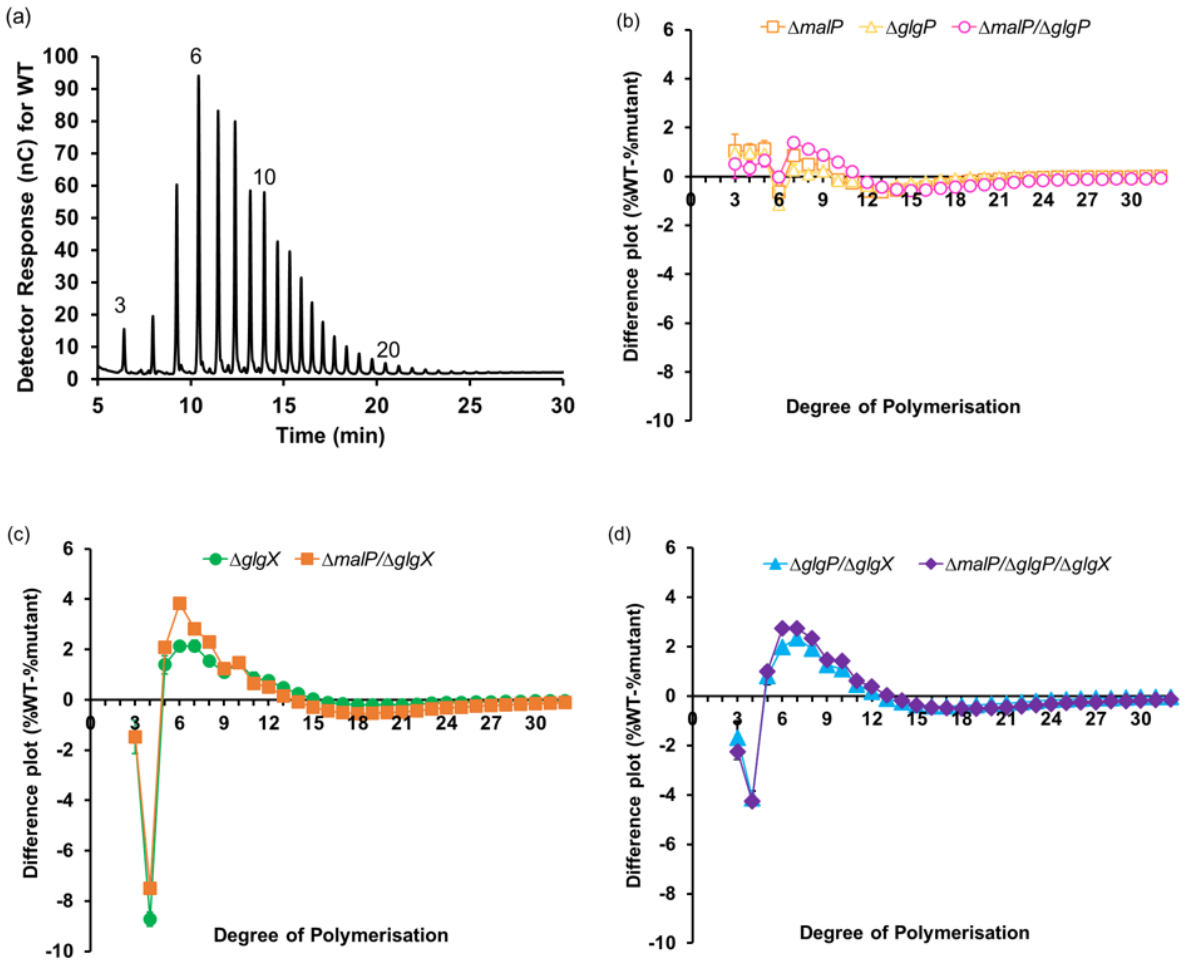

Figure 2.4 Distribution of chain lengths present in glycogen isolated from each strain as determined by high performance anion exchange chromatography following debranching by isoamylase. (a) Chain length distribution profile of the wild type. (b-d) Difference plots generated by subtracting the mol % value of the mutant at each chain length from the corresponding mol % of the WT at the corresponding chain length. Each point represents the means of three biological replicates ± 95 % confidence intervals.

Interestingly, when both GlgX and GlgP were lacking (Fig 2.4d; *ΔglgP/ΔglgX* and *ΔmalP/ΔglgP/ΔglgX* strains) an intermediate phenotype was observed where the proportion of DP4 chains were less than in the glycogen from *ΔglgX* and *ΔmalP/ΔglgX* mutants, but greater than in the glycogen from all the other strains suggesting that another enzyme besides GlgP produces these DP4 chains.

2.2.4 Visualization of *E. coli* cells by scanning electron microscopy (SEM)

EM analysis of all the strains, grown in liquid LB medium without glucose, were carried out to determine whether glycogen accumulation within *E. coli* affects cell morphology. Interestingly, cells from strains containing a mutant *malP* allele (Fig. 2.5d, f, g & h; Δ*malP*, Δ*malP*/Δ*glgP*, Δ*malP*/Δ*glgX*, and Δ*malP*/Δ*glgP*/Δ*glgX)* contained a number of cells that were elongated. This was not seen for any of strains containing the wild-type *malP* allele (Fig. 2.5a, b, c & e; wild-type, Δ*glgP*, Δ*glgX* and Δ*glgP*/Δ*glgX*).

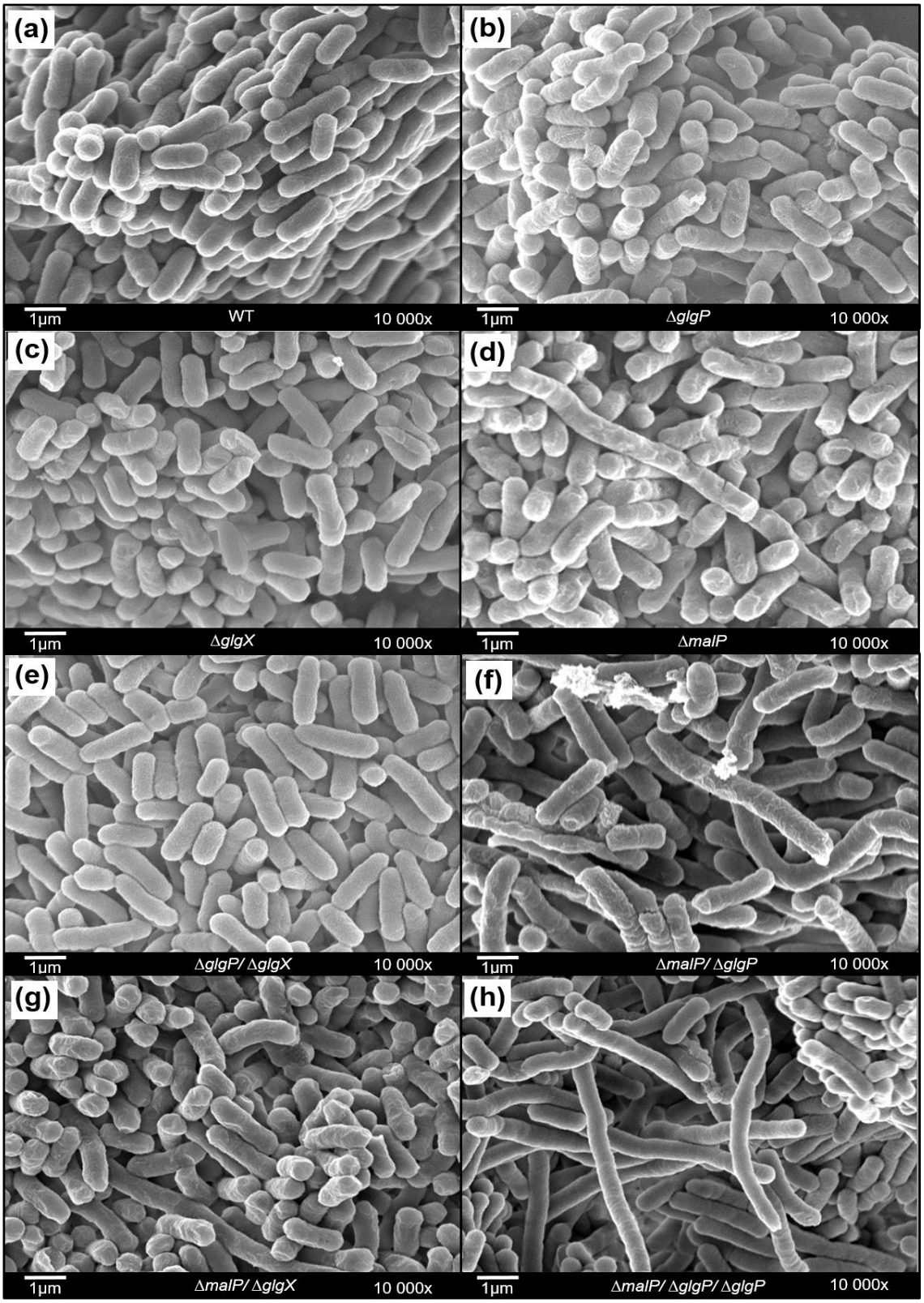

Figure 2.5 SEM analysis of all strains. Cells were cultured in liquid LB for 16 h before preparation. (a) wild-type, (b) Δ*glgP*, (c) Δ*glgX*, (d) Δ*malP* (e) Δ*glgP*/Δ*glgX*, (f) Δ*malP*/Δ*glgP*, (g) Δ*malP*/Δ*glgX*, and (h) Δ*malP*/Δ*glgP*/Δ*glgX*. Bars, 1µM.

2.3 Discussion

In this study we have examined the combined effects of *malP*, *glgP* and *glgX* on glycogen metabolism in *E. coli*. Both *glgP* (Alonso-Casjús et al 2006) and *glgX* (Dauvillée et al 2004) have been studied individually in this process, while the function of *malP* has mainly been examined in the context of maltodextrin metabolism (Boos and Schuman, 1998; Dippel and Boos 2005). MalP has, however, been demonstrated to influence glycogen synthesis under some circumstances through the production of glucan substrates for GlgB (Dippel and Boos 2005). It is currently thought that GlgP is the main enzyme that removes glucose from the non-reducing ends of external glycogen chains. As can be seen in Fig. 2.2a, however, MalP is also capable of utilising glycogen as a primer, and has been demonstrated to release G1P from glycogen, albeit slowly (Schwartz and Hofnung 1967). GlgX is the only enzyme present in *E. coli* known to be capable of cleaving α-1,6 branch points. According to the current models of glycogen catabolism, removal of combinations of *malP, glgP* and *glgX* should progressively limit degradation of the glycogen polymer *in vivo*. Indeed, the triple mutants should be incapable of glycogen degradation.

The mutant strains produced (Fig. 2.1) grew at identical rates in each of the media types (Fig. 2.3e, f), indicating that the genetic manipulations had little effect on bacterial proliferation under each condition. It would be expected that mutations in these genes could affect growth under other circumstances, for example under carbon limitation, where mobilisation of glycogen may be crucial for survival. Indeed mutations in genes involved in glycogen metabolism, including the genes involved in this study, have been demonstrated to be important for colonisation of mouse guts (Jones et al 2008). Nevertheless, the lack of differences in growth rates demonstrated that we could use these strains to examine glycogen accumulation over a time course without any complications of the cells being in different growth stages.

Semi-quantitative staining to examine glycogen accumulation in cells grown on solid media indicated that a number of mutant strains accumulated more glycogen than the control (Fig. 2.3a, b). Importantly, the Δ*malP*/Δ*glgP*/Δ*glgX* triple mutant stained darker than the Δ*glgP*/Δ*glgX* double mutant (Fig. 2.3a, b), which indicates that a mutation in the *malP* gene can, under some circumstances, affect glycogen accumulation. However, this trend was not observed in liquid media. When grown without glucose all strains except the wild type and Δ*malP* mutants contained similar amounts of glycogen after 48 hours (Fig. 2.3c) indicating that, in these conditions, mutations in both *glgP* and *glgX* influence glycogen degradation, but

that their effect is not additive. In media supplemented with glucose all strains containing a mutant *glgX* allele accumulated more glycogen than ones with the wild-type *glgX* allele and similar amounts to each other (Fig. 2.3d), consistent with previous data indicating that glycogen is continuously turned over within *E. coli* cultures (Dauvillée et al. 2004). Overall these data illustrate the relative importance of each of these enzymes for glycogen turnover under distinct environmental conditions and demonstrate that they can influence glycogen accumulation differently depending on those conditions.

Mutations in either *glgP* or *glgX* have been demonstrated to alter the constituent chain lengths within glycogen; a lesion in *glgX* leads to accumulation of shorter chains (DP3-4; Dauvillée et al 2004) while a lesion in *glgP* leads to fewer chains between DP4-10 and a greater number between DP15-30 (Alonso-Casjús et al 2006). For *glgX* mutants, we obtained comparable results to Dauvillée et al (2004): glycogen was enriched in short chains of DP3 and especially DP4 (Fig. 2.4c). However, unlike the study of Alonso-Casjús et al (2006) we found no major differences in the glycogen chain length distribution of the Δ*glgP* single mutant relative to the WT (Fig. 2.4b). This is most likely due to differences in growth conditions between experiments rather than strain differences as Alonso-Casajús et al (2006) also used a BW25113 derived mutant in their experiments, but grew it in medium containing additional glucose. Glycogen for our experiment was isolated from media without carbon supplementation. Mutants where both GlgP and GlgX are lacking (*ΔglgP/ΔglgX* and Δ*malP/*Δ*glgP/*Δ*glgX*), contain glycogen with approximately half the amount of DP4 chains compared with that from Δ*glgX* or Δ*malP/*Δ*glgX* mutants, demonstrating that GlgP affects glycogen structure when GlgX is lacking. This is consistent with the roles of GlgP and GlgX proposed by Alonso-Casjús et al (2006) and Dauvillée et al (2004) with GlgP removing glucose moieties from the outer chains of glycogen, leaving short chains that GlgX releases as maltodextrins. However, this cannot be the whole story. It remains unclear what is responsible for the production of short chains in the *ΔglgP/ΔglgX* double mutant, however, it cannot be MalP as comparable amounts of short chains remain in the triple mutant as in *ΔglgP/ΔglgX*.

These data provide three lines of evidence to suggest that there may be another enzyme involved in glycogen catabolism in *E. coli*. First, *glgX* mutations do not eliminate glycogen hydrolytic activity in terms of reducing end production (Fig. 2.2b). Second, an as-yet unidentified glycogen-metabolising activity is visible on zymograms (Fig. 2.2a). Third, our analysis of glycogen structure indicates that an enzyme other than GlgP and MalP can shorten

chains for release by GlgX (Fig. 2.4). This is consistent with the fact that, on solid media the Δ*malP*/Δ*glgP*/Δ*glgX* triple mutant appears to accumulate more glycogen than the Δ*glgP*/Δ*glgX* strain (Fig. 2.3a, b) and can be interpreted to mean that another enzyme may be producing substrate for MalP. One explanation could be the involvement of an α-amylase. Previously, Raha et al (1992) reported on a cytoplasmic α-amylase (AmyA) that effectively digested MOS consisting of six glucose units or larger. Although that study demonstrated that glycogen is a poor substrate for AmyA, the authors nevertheless hypothesized that it may act on cellular glycogen. A role for it in glycogen metabolism has, however, never been tested so its role and the possible involvement of other enzymes will need to be the subject of further investigation.

Furthermore, a mutation in the *malP* gene was demonstrated to lead to cells exhibiting an increase in cell length (Fig 2.5). It has been demonstrated by Park et al (2011) that *malP* mutants grown on maltose displayed an enlarged cell phenotype which could be linked to maltooligosaccharide (MOS) accumulation. In this study only strains lacking MalP demonstrated this phenotype and so it could be argued that the change in size would be caused by accumulation of glycogen derived MOS. However, this cannot be the case in the Δ*malP*/Δ*glgP*/Δ*glgX* triple mutant as this is unable to remove external chains from glycogen and, yet, it is the strain where the most extreme phenotype was observed.

A more likely, explanation, therefore, is that a product downstream of the action of MalP is responsible and that its lack of production is exacerbated by the lack of GlgP and GlgX. This could be G1P, or a metabolite manufactured from it, such as UDP-glucose. Clearly lack of MalP and GlgP would eliminate G1P production during glycogen degradation. However, GlgX would still be able to release linear maltodextrins that could be acted upon by MalQ, MalZ and AmyA, releasing glucose that would become phosphorylated by hexokinases leading to G6P, G1P and UDP-glucose production. The triple mutant lacking all three of these enzymes would, therefore, be expected to be severely reduced in the amounts of these metabolites. It would clearly be helpful to demonstrate this through determination of the concentrations of these metabolites and future work examining this should, therefore, include such measurements.

Hill et al (2013) proposed a model for nutrient dependent control of cell division that involves sensing of G1P and UDP-glucose (Fig 2.6b). Cells grown on nutrient rich medium accumulates G1P leading to an increase in UDP-glucose, which promotes binding between OpgH and FtsZ, inhibiting cell division and leading to cell elongation. Alternately when cells

are grown on nutrient poor medium, there would be less G1P and UDP-glucose meaning that there would be reduced binding between OpgH and FtsZ, allowing Z-ring formation and cell division. If the change in cell size is caused by a decrease in UDP-glucose affecting OpgH, we would expect to see reduced cell sizes, not increased ones. Therefore, it seems unlikely that the phenotype noted is caused through an effect on OpgH.

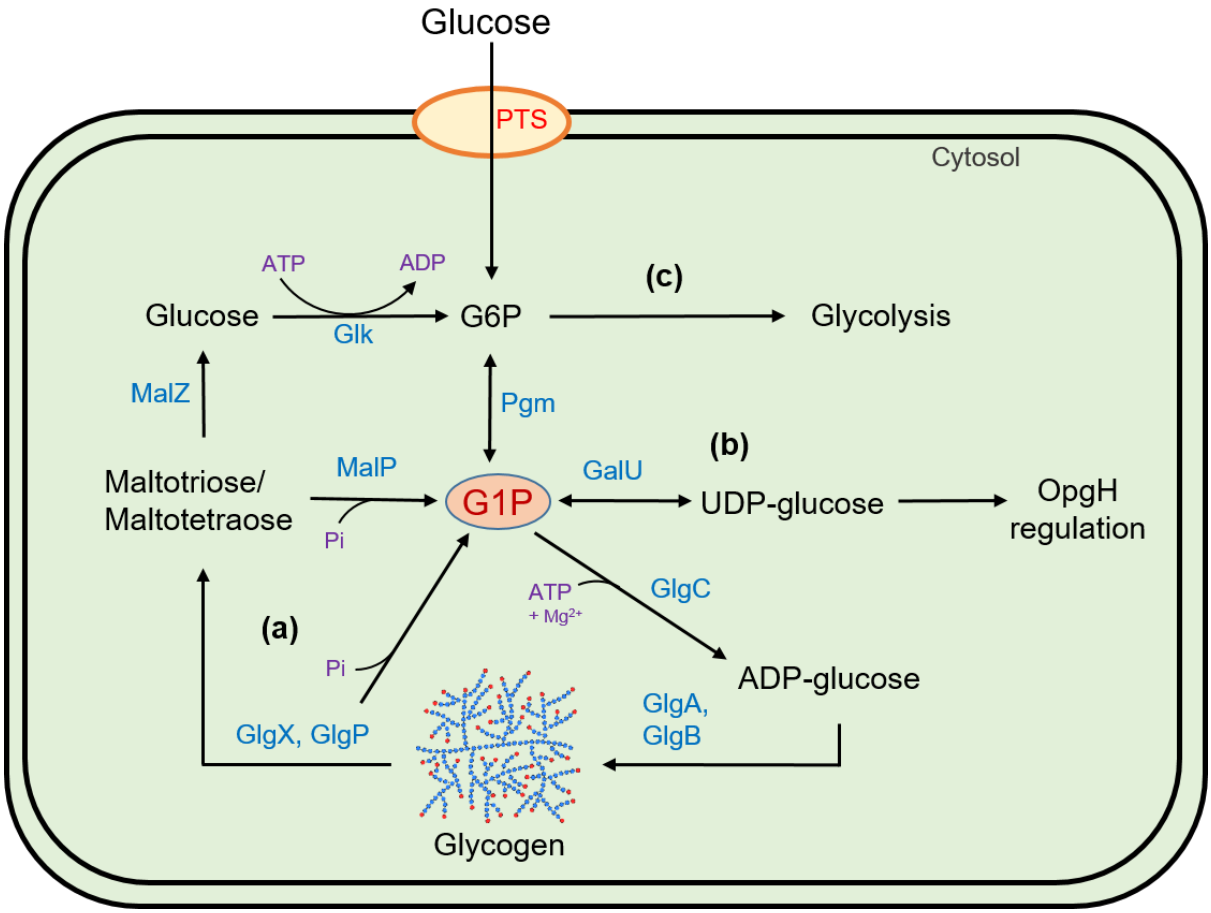

Figure 2.6 Schematic diagram outlining the pathways that produce and utilise G1P. (a) Glycogen metabolism, G1P is the product of GlgP and MalP catalytic activities. (b) OpgH regulation, UDP-glucose is produced from G1P by GalU. (c) Glycolysis, G1P is converted into G6P by Pgm.

Alternatively, a recent study by Monahan et al (2014) demonstrated that cell division within *B. subtilis* is influenced by the glycolytic pathway. They showed that pyruvate, formed by pyruvate kinase in the final step of the glycolytic pathway, can influence cell division by promoting the formation of the Z-ring in *B. subtilis* and that a knockout mutant containing a deletion in the gene encoding pyruvate kinase (*pyk*), rescued the inability of an *ftsZ*-deletion

mutant to divide. Similarly, a wild-type strain disrupted in its *pyk* gene fails to divide normally. It is reasonable to assume that a similar link between pyruvate and Z-ring formation exists in *E. coli*. It is possible that Δ*malP*-mutants experience elongated cell size because there is less glycogen-derived G1P that enters glycolysis (Fig. 2.6c), affecting the concentration of pyruvate within the cell and, in turn, causing aberrant Z-ring based cell division.

To test this, future work should include, (1) manufacture of quadruple mutants, lacking MalP, GlgP and GlgX and either Pgm, GlgC or GlgA (which would all eliminate glycogen synthesis). If such mutants accumulate normal sized cells, then it will demonstrate that the elongated cell phenotype is indeed caused by glycogen derived-G1P. (2) Analysis of FtsZ assembly of cell division machinery through the use of immunoblots utilizing FtsZ antibodies. (3) Study of FtsZ assembly using an FtsZ::GFP protein fusion in the mutant cells. (4) Examination of cell lengths should be examined over a time course to determine at what stage during the growth phase cell length starts increasing.

It should be remembered, however, that before a cell can divide many processes and environmental factors need to be in place including, but not limited to, the cell division machinery, growth rate, cell size, DNA replication, nutrient availability, temperature and pH (Monathan et al 2014). Therefore it is possible that the change in cell length observed in these mutants might be caused by a combination of factors. Nevertheless, the analysis of the suite of mutants produced in this study should help to elucidate novel factors involved in determining cell size in *E. coli*.

References

Alonso-Casajús N, Dauvillée D, Viale AM, *et al.* (2006) Glycogen phosphorylase, the product of the *glgP* gene, catalyzes glycogen breakdown by removing glucose units from the nonreducing ends in *Escherichia coli*, *J Bacteriol* **188**: 5266-72

Baba T, Ara T, Hasegawa M, *et al.* (2006) Construction of *Escherichia coli* K-12 in-frame, single-gene knockout mutants: The Keio collection. *Mol Syst Biol* **2**: 2006.0008

Boos W, Shuman H. (1998) Maltose/maltodextrin system of *Escherichia coli*: Transport, metabolism, and regulation. *Microbiol Mol Biol Rev* **62***:* 204-29

Chen GS, Segel IH. (1968) *Escherichia coli* polyglucose phosphorylases**.** *Arch Biochem Biophys* **127**: 164-74

Cherapanov PP, Wackernagel W. (1995) Gene disruption of *Escherichia coli*: Tc^R and Km^R cassettes with the option of Flp-catalyzed excision of the antibiotic-resistance determinant. *Gene* **158**: 9-14

Datsenko KA, Wanner BL (2000) One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *PNAS* **97:** 6640-6645

Dauvillée D, Kinderf IS, Li Z, *et al.* (2004) Role of the *Escherichia coli glgX* gene in glycogen metabolism. *J Bacteriol* **187**: 1465-73

Dippel R, Boos W. (2005) The maltodextrin system of *Escherichia coli*: Metabolism and Transport. *J Bacteriol* **187**: 8322-31

Hill NS, Buske PJ, Shi Y, *et al.* (2013) A Moonlighting Enzyme Links *Escherichia coli* Cell Size with Central Metabolism. *PLoS Genet* **9:** doi: 10.1371/journal.pgen.1003663

Jones SA, Jorgensen M, Chowdhury FZ, *et al.* (2008) Glycogen and maltose utilization by *Escherichia coli* O157:H7 in the mouse intestine. *Infect Immun* **76**: 2531-40

Kraus D. (2014) Consolidated data analysis and presentation using an open-source add-in for the Microsoft Excel® spreadsheet software. *Med. Wri.* **23**: 25-28

Monahan LG, Hajduk IV, Blaber SP, et al. (2014) Coordinating Bacterial Cell Division with Nutrient Availability: A Role for Glycolysis. *Mbio* **5**: doi: 10.1128/mBio.00935-14

Montero M, Almagro G, Eydallin G *et al*. (2011) *Escherichia coli* glycogen genes are organized in a single *glgBXCAP* transcriptional unit possessing an alternative suboperonic promoter within *glgC* that directs *glgAP* expression. *Biochem. J.* **433**: 107-117

Park JT, Shim JH, Tran PL, *et al*. (2011) Role of maltose enzymes in glycogen synthesis by *Escherichia coli*. *J Bacteriol* **193**: 2517-26

Preiss J, Romeo T. (1994) Molecular biology and regulatory aspects of glycogen biosynthesis in bacteria. *Prog Nucleic Acid Res Mol Biol* **47**: 299-329

Raha M, Kawagishi I, Muller V, *et al*. (1992) *Escherichia coli* produces a cytoplasmic α-Amylase, AmyA. *J Bacteriol* **174**:6644-52

Schinzel R, Nidetzky B. (1998) Bacterial α-Glucan Phosphorylases. *FEMS Microbiol Lett* **15**: 73-9

Schwartz M, Hofnung M. (1967) La maltodextrine phosphorylase d'*Escherischia coli*. *Eur J Biochem* **2**:132-145

Streb S, Delatte T, Umhang M, *et al*. (2008) Starch granule biosynthesis in Arabidopsis is abolished by removal of all debranching enzymes but restored by the subsequent removal of an endoamylase. *Plant Cell* **20**: 3448-66

Thomason LC, Constantino N, Court DL. (2007) *E. coli* genome manipulation by P1 transduction. *Curr Protoc Mol Biol* **1**: 1.17

Chapter 3

3. General conclusion

The project aimed to examine the role of MalP, GlgP and GlgX in glycogen degradation by creating and analysing a series of knockout mutants lacking combinations of all three enzymes. Glycogen accumulation and structure was compared between all the strains. Importantly, a few key findings were made. First, it was shown that MalP can effect glycogen accumulation but that this is greatly affected by the environment. Second, consistent with the work presented by Dauvillée et al (2004) and Alonso-Casjús et al (2006), there is a constant flux of glycogen synthesis and degradation within *E. coli* in nutrient rich conditions. Third, glycogen structure was unaltered between Δ*glgP*/Δ*glgX* and Δ*malP*/Δ*glgP*/Δ*glgX* mutants. This indicates that MalP is not responsible for the shortening of external glycogen chains and begs the question, therefore, which enzyme is? Fourth, an activity that metabolises glycogen was visible on a zymogram but which is of unknown origin. Could this possibly be the link?

A reasonable suggestion is that this activity is caused by the cytoplasmic α-amylase, AmyA. Although it has been reported that this enzyme acts preferentially on oligosaccharides, as opposed to glycogen (Raha et al 1992), it cannot be ruled out that it is involved. Biochemical characterisation of AmyA on glycogen has only been performed on commercially obtained samples of unspecified biological origin (Raha et al 1992) and so it is possible that it can utilise glycogen with external chain lengths differing form the ones used in that study. Future research should involve the manufacturing of a Δ*malP*/Δ*glgP*/Δ*glgX*/Δ*amyA* quadruple mutant and analysis of its ability to degrade glycogen under similar circumstances as in this project. If AmyA is not involved in this process then another possibility includes MalQ which could potentially transfer glucan chains from glycogen to glucose or malto-oligosaccharides.

Interestingly, elongated cell sizes were observed for all the strains containing a mutated *malP* allele. A recent study (Monahan et al 2014) found that glycolysis plays a very important role in determining cell division in *B. subtilis*. Given that parallel pathways within these two microorganisms has previously been established in control of cell division (Hill et al 2013; Weart et al 2007), it is possible that glycolysis also effects this process within *E. coli*. Based on this hypothesis, reduced G1P release from glycogen in Δ*malP*-mutants should reduce pyruvate synthesis, interfering with Z-ring assembly and limiting cell division. Future work

here should include ways to elucidate the involvement of glycogen-derived G1P in the glycolytic pathway, possibly by producing quadruple mutants and growing them on minimal media supplemented with glucose and observing whether the increase in cell size is reversed. Cell division could also be observed within these mutants by performing immunoblots with FtsZ antibodies and constructing FtsZ::GFP fusion proteins. Cell size measurements and how it relates to the four growth phases could be determined microscopically by staining and observing mutant cells on glass slides. It is evident however, that multiple factors play a role in glycogen metabolism as well as cell growth and division and further investigation in this field would increase our understanding of these intricate processes.

References

Alonso-Casajús N, Dauvillée D, Viale AM, *et al.* (2006) Glycogen phosphorylase, the product of the *glgP* gene, catalyzes glycogen breakdown by removing glucose units from the nonreducing ends in *Escherichia coli*, *J Bacteriol* **188**: 5266-72

Dauvillée D, Kinderf IS, Li Z, *et al.* (2004) Role of the *Escherichia coli glgX* gene in glycogen metabolism. *J Bacteriol* **187**: 1465-73

Hill NS, Buske PJ, Shi Y, *et al.* (2013) A Moonlighting Enzyme Links *Escherichia coli* Cell Size with Central Metabolism. *PLoS Genet* **9:** doi: 10.1371/journal.pgen.1003663

Monahan LG, Hajduk IV, Blaber SP, et al. (2014) Coordinating Bacterial Cell Division with Nutrient Availability: A Role for Glycolysis. *Mbio* **5**: doi: 10.1128/mBio.00935-14

Raha M, Kawagishi I, Muller V, *et al.* (1992) *Escherichia coli* produces a cytoplasmic α-Amylase, AmyA. *J Bacteriol* **174**:6644-52

Weart RB, Lee AH, Chien AC, *et al.* (2007) A metabolic sensor governing cell size in bacteria. *Cell* **130:** 335-47