# Mutational Analysis of *E. coli* maltodextrin metabolism

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Thesis presented in partial fulfilment of the requirements for the degree of Master of Science at the Institute for Plant Biotechnology, University of Stellenbosch

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March 2017

# DECLARATION

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

**Signature:** **Date:** March 2017

## Abstract

*Escherichia coli* glycogen and maltodextrin metabolism has been extensively studied; however, the roles of the enzymes involved in the mobilization of these polyglucans remains unclear. The mal enzymes; maltodextrin phosphorylase (MalP), amylomaltase (MalQ) and maltodextrin glucosidase (MalZ), which are responsible for mobilization of maltodextrins in the cytoplasm, are also examined for their effects on glycogen metabolism. Since the glycogen phosphorylase (GlgP), is structurally and functionally similar to MalP, the role of this enzyme was examined in relation to maltodextrin metabolism. To elucidate their functions, a reverse genetics approach was used, where genes which encode them were mutated in *E. coli*. Double mutant combinations of most of the mutated genes were manufactured. One double mutant combination (Δ*malQ*/Δ*malP*) proved, however, impossible to isolate. The double mutants, single mutants and wild-type strains were grown in media supplemented with either maltose or glucose and analysed for a number of parameters, including polyglucan content, growth and cell morphology. When the strains were grown with glucose supplementation, there was no effect on the growth rate of strains. Increased glycogen accumulation was observed in Δ*malP*/Δ*glgP*, Δ*malQ*/Δ*glgP* and Δ*malZ*/Δ*glgP* double mutants when compared to the Δ*glgP* single mutant indicating that mutations in the *mal* enzymes have an effect on glycogen accumulation. Scanning electron microscopy (SEM) of Δ*malP*, Δ*malP*/Δ*glgP* and Δ*malQ* strains revealed the accumulation of a few elongated cells. Since these strains also showed increased glycogen accumulation in liquid and on solid media, it may be that its accumulation is a factor which could explain the elongation of cells. Growth of strains with maltose supplementation caused variations in the growth rate of strains, however, the mechanism whereby this is affected is not evident. Polyglucan accumulation in some strains indicated an additional role for MalZ in maltodextrin as well as glycogen metabolism based on the observations made for Δ*malZ*/Δ*glgP* and Δ*malZ*/Δ*malQ* mutants. Scanning electron microscopy indicated that growth in maltose has some impact on the morphology of cultures since strains appeared to either have invaginations in the cell wall (Δ*malP*, Δ*malZ*/Δ*malP*, Δ*malP*/Δ*glgP*) or have lysed (Δ*malQ* ,Δ*malQ/glgP*). Overall, the data presented here demonstrates that MalP, MalQ, MalZ and GlgP may have additional functional roles in *E. coli* polyglucan metabolism that have not been previously elucidated.

## Opsomming

*Escherichia coli (E. coli)* glikogeen en maltodextrin metabolisme is breedvoerig bestudeer; in teen deel is die rol van hierdie poliglukane met betrekking tot mobilisering steeds onbekend. Die mal ensieme naamlik ; maltodekstrin fosforylase (MalP), amylomaltase (MalQ) en maltodekstrin glucosidase (MalZ), wat verantwoordelik is vir die mobilisering van maltodektrins in die sitoplasma, ook gebestudeer vir hul rol in glikogeen metabolisme. Aangesien glikogeenfosforilase (GlgP), struktureel en funksioneel ooreenstemming toon aan MalP, word die rol van hierdie ensiem met betrekking tot maltodekstrin metabolisme ondersoek. Dus, was 'n omgekeerde genetiese benadering gebruik om die funksie aan hierdie ensieme toe te ken d.m.v die gene wat vir dit enkodeer in *E. coli* te muteer. In meeste gevalle was dubbele gemuteerde kombinasies van die gemuteerde gene vervaardig,behalwe, een dubbele gemuteerde kombinasie (*ΔmalQ/ΔmalP*) was egter onmoontlik om te isoleer. Dubbel en enkele mutante asook wilde-tipe-stamme was opgegroei in groei media aangevul met óf maltose of glukose; waarna verskeie parameters geanaliseer was, insluitend poliglukaan inhoud, groei asook sel morfologie, gemeet was. Wanneer die groei media aangevul was met glukose, was daar geen effek op die groeikoers van stamme waargeneem nie. Verhoogde glikogeen akkumulasie was waargeneem in *ΔmalP/ΔglgP*, *ΔmalQ/ΔglgP* en *ΔmalZ/ΔglgP* dubbel mutante in vergelyking met die *ΔglgP* enkele mutante wat aandui dat mutasies in die mal ensieme dalk 'n uitwerking mag hê op glikogeen akkumulasie. Skandering elektroon mikroskopie (SEM) het getoon dat die *ΔmalP*, *ΔmalP/ΔglgP* en *ΔmalQ* stamme ‘n paar verlengde selle gevorm het. Aangesien die bogenoemde stamme ook glikogeen akkumulasie in beide vloeibare en op soliede media getoon het kan dit wees dat die akkumulasie van hierdie glikogeen moontlik die verlenging van die selle kan verduidelik. Die groei van die kulture met maltose aanvulling veroorsaak verskille in die groeikoers van stamme, maar die meganisme waardeur dit beïnvloed word, is nog steeds onbekend. Poliglukaan akkumulasie was slegs geobserveer in sekere stamme, dus mag dit 'n bykomende funksie vir MalZ in maltodekstrin en glikogeen metabolisme aandui, gebaseer op die waarnemings vir *ΔmalZ/ΔglgP* en *ΔmalZ/ΔmalQ* mutante. Skandering electron mikroskopie het ook aangedui dat die groeikoers in die teenwoordigheid van maltose ook 'n impak mag hê op die morfologie van kulture aangesien stamme naamlik invouings van die selwand (*ΔmalP*, *ΔmalZ/ΔmalP*, *ΔmalP/ΔglgP*) óf liseering (*ΔmalQ*, *Δ malQ/glgP*). In samevatting, het die data getoon dat MalP, MalQ, MalZ en GlgP dalk addisionele funksionele rolle mag hê in *E. coli* poliglukaan metabolisme wat nie voorheen beskryf is nie.

## Acknowledgements

I would like to acknowledge Dr James Lloyd for his unwavering support, guidance and patience throughout the course of this study. I am sincerely grateful for everything.

I would like to thank all academics and fellow students at the Institute for Plant Biotechnology for advice or help provided.

I would like to acknowledge Jonathan Jewell for construction of the Δ*malP/glgP* mutant strain and for all the technical assistance he provided.

Lastly, I would like to thank the National Research Foundation (NRF) for funding.

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# LIST OF ABBREVIATIONS

| | |
|---|---|
| ADP | adenosine diphosphate |
| ATP | adenosine 5'-triphosphate |
| bp | base pair |
| ˚C | degree Celsius |
| cfu/mL | colony-forming units per millilitre |
| cm | centimetre |
| dd$H_20$ | distilled water |
| DP | degree of polymerization |
| DNA | deoxyribonucleic acid |
| E. C | enzyme commission number |
| *E. coli* | *Escherichia coli* |
| EDTA | ethylenediaminetetraacetic acid |
| FLP | flippase |
| FRT | flippase recognition target |
| g | grams |
| g/mol | grams per mole |
| gDNA | genomic DNA |
| hr | hour |
| HCL | hydrochloric acid |
| kb | kilobase |
| KOH | potassium hydroxide |
| µM | micromolar |
| mM | millimolar |
| min | minute |
| µg | microgram |
| mg | milligram |
| µM | micrometre |
| µL | microlitre |
| mL | millilitre |
| $MgCl_2$ | magnesium chloride |
| M | molar |
| NAD | nicotinamide adenine dinucleotide |
| OD | optical density |

| | |
|---|---|
| OH | hydroxide |
| PCR | polymerase chain reaction |
| PMSF | Phenylmethylsulfonyl flouride |
| rpm | revolutions per minute |
| SDS | sodium dodecyl sulphate |
| SEM | scanning electron microscopy |
| Tris | trisaminomethane |
| U | units |
| v/v | volume per volume |
| *vir* | virulent |
| W | watt |
| WT | wild type |
| w/v | Weight per volume |
| xg | gravitational acceleration (9.806 $m.s^{-1}$ ) |

# CHAPTER 1: Literature Review

*Escherichia coli* serves as a model organism in a broad range of scientific disciplines including the study of biological pathways, the functions of which can be related to those found in higher organisms which are not as easy to manipulate for experimental purposes. It is a prokaryotic microorganism which is able to metabolise various compounds as carbon sources. Although it preferentially utilizes glucose (Martinez-Gomez et al., 2012), it can also assimilate maltodextrins, α1,4 linear glucose polymers. The main carbon store in *E. coli* is glycogen, a branched polyglucan consisting of linear α1,4 chains that are linked together by α1,6 branch points. This polyglucan is extremely important for bacterial survival and, in some cases, has been shown to be necessary for bacterial growth (Belanger and Hatfull, 1999).

*E. coli* can use various carbon sources other than glucose for its synthesis of glycogen; in particular it is capable of using maltose and maltodextrins for glycogen synthesis. Enzymes involved in glycogen and maltodextrin metabolism often catalyse similar reactions, so enzymes involved in the mobilization of maltodextrins in the cytoplasm are thought to be involved in glycogen degradation as well. On saying that, it is also possible that the enzymes involved in glycogen metabolism might have an effect on maltodextrin contents. The research in this thesis is aimed at answering questions regarding the functions of enzymes in maltodextrin and glycogen metabolism as well as examining connections between these closely related pathways. To do this, a reverse genetics approach was used, whereby the effect of disrupting genes encoding enzymes involved in these pathways, was investigated in *E. coli*. This research focused on the functional roles of MalQ (EC 2.4.1.25), MalP (EC 2.4.1.1), MalZ (EC 3.2.1.20) and GlgP (EC 2.4.1.1) encoded by *malQ*, *malP*, *malZ* and *glgP*, respectively. The first three enzymes are known to be involved primarily in maltodextrin metabolism while GlgP has been demonstrated to be involved in glycogen catabolism. The metabolism of glycogen and maltodextrin will be outlined below to highlight their interconnectivity.

## 1. Glycogen and maltodextrin metabolism of *E. coli*.

### 1.1 Glycogen metabolism

Glycogen is a branched polysaccharide consisting of α1, 4-linked glucose molecules with α1, 6-linked branch points (Wilson et al., 2010). It is the main carbon storage compound in *E. coli* and accumulates either during the stationary phase of growth, or in periods when carbon supply is abundant while other nutrients are limited (Preiss and Romeo, 1994). Accumulation of such carbohydrate storage molecules as energy reserves is important to allow microorganisms to survive periods of starvation (Wilson et al., 2010). For example, it has been demonstrated that the ability of yeast to accumulate glycogen is important for surviving long term nutrient deficiencies (Sillje et al., 1999) and these cells have a growth advantage over cells which are unable to do so (Anderson and Tatchel, 2001). The accumulation of glycogen is the

main strategy of *E. coli* in this regard. Several studies have linked glycogen metabolism in micro-organisms to colonization, virulence and environmental survival (Bourassa and Camilli, 2009; Chang et al., 2004; Henrissat et al., 2002; Jones et al., 2008; Marroqui et al., 2001; McMeechan et al., 2005; Sambou et al., 2008) indicating its physiological importance.

### 1.1.1 The synthesis and degradation of glycogen

The genes responsible for the synthesis (Fig 1.1) and degradation (Fig 1.2) of glycogen are situated on two tandemly arranged operons. The first of these contains the *glgC*, *glgA* and *glgP* genes and the second contains the *glgB* and *glgX* genes (Preiss and Romeo, 1994). The synthesis of glycogen is catalysed by three enzymes; ADPglucose pyrophosphorylase (EC 2.7.7.27) encoded by *glgC*, glycogen synthase (EC 2.4.1.21) encoded by *glgA* and glycogen branching enzyme (EC 2.4.1.21) encoded by *glgB* (Romeo and Preiss, 1989). GlgC catalyses the formation of ADP-glucose and pyrophosphate (PPi) from glucose-1-phosphate and ATP (Bejar et al., 2004). Although the reaction is reversible it proceeds mainly in the synthesis direction *in vivo* (Iglesias and Preiss, 1992). The second step in glycogen synthesis is the elongation of α1,4 glucan chains by GlgA through the transfer of the glucosyl residue from ADP-glucose to a growing α1,4 glucan chain (Kumar et al., 1986). Finally, GlgB introduces α1,6 branch points to the glycogen molecule by cleaving α1,4 glycoside linkages and transferring the non-reducing ends of the fragments onto the C-6 position of glucose molecules that lie internally in a α1,4 chain (Boyer and Preiss, 1977). Glycogen synthesis can also occur in the absence of GlgA, through the use of long chain maltodextrins (Park et al., 2011) are discussed in more detail below.

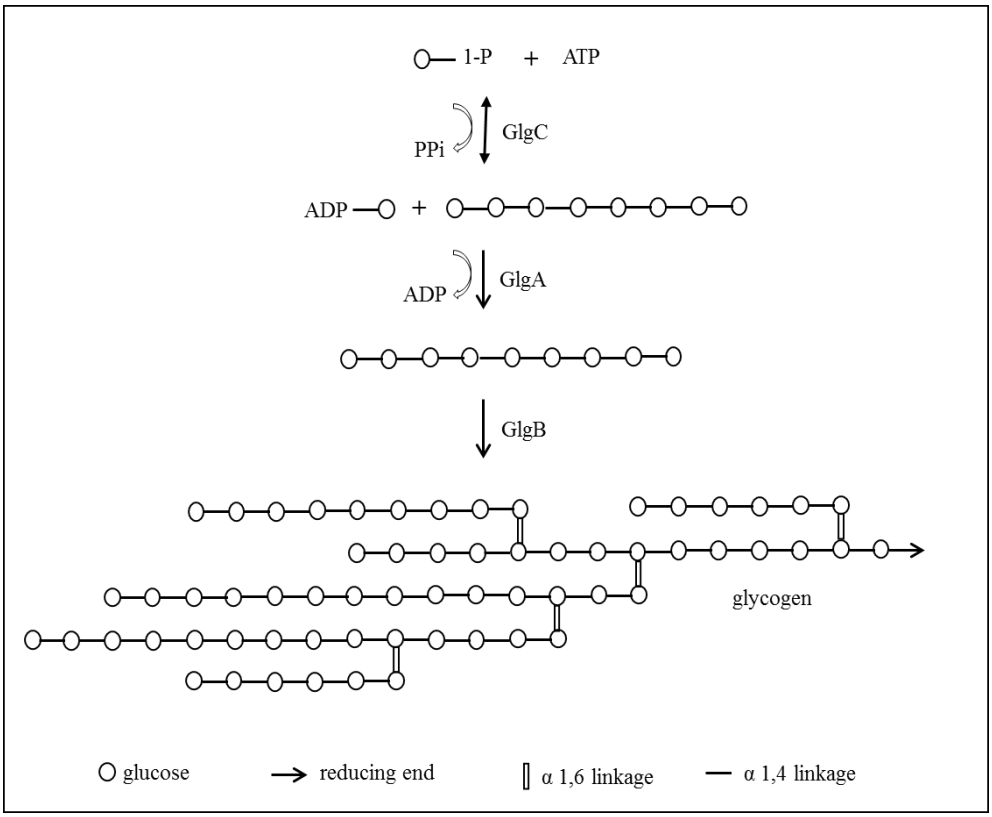

**Figure 1.1. Glycogen synthesis.** Schematic representation of *E. coli* glycogen synthesis. In the presence of glucose-1-phosphate and ATP, ADPglucose pyrophosphorylase (GlgC) catalyses the formation of ADP-glucose. Glycogen synthase (GlgA) transfers glucose from ADP glucose onto α1,4 chains. The glycogen branching enzyme (GlgB) catalyses the formation of α1,6 branches.

The degradation of glycogen is catalysed by glycogen phosphorylase (GlgP) and glycogen debranching enzyme (GlgX). GlgP phosphorolyticaly cleaves α-1, 4 glycoside linkages in the external glycogen chains until they are four glucose moieties in length after which they may be acted on by GlgX which cleaves the α-1, 6 branches to release linear maltodextrins (Dauvillée et al., 2005). In a study conducted by Strydom et al. (2017), it was observed that in the absence of GlgP and GlgX, glycogen was still being degraded. This indicates that there are additional enzymes, possibly enzymes involved in mobilization of maltodextrins, which contribute towards glycogen degradation.

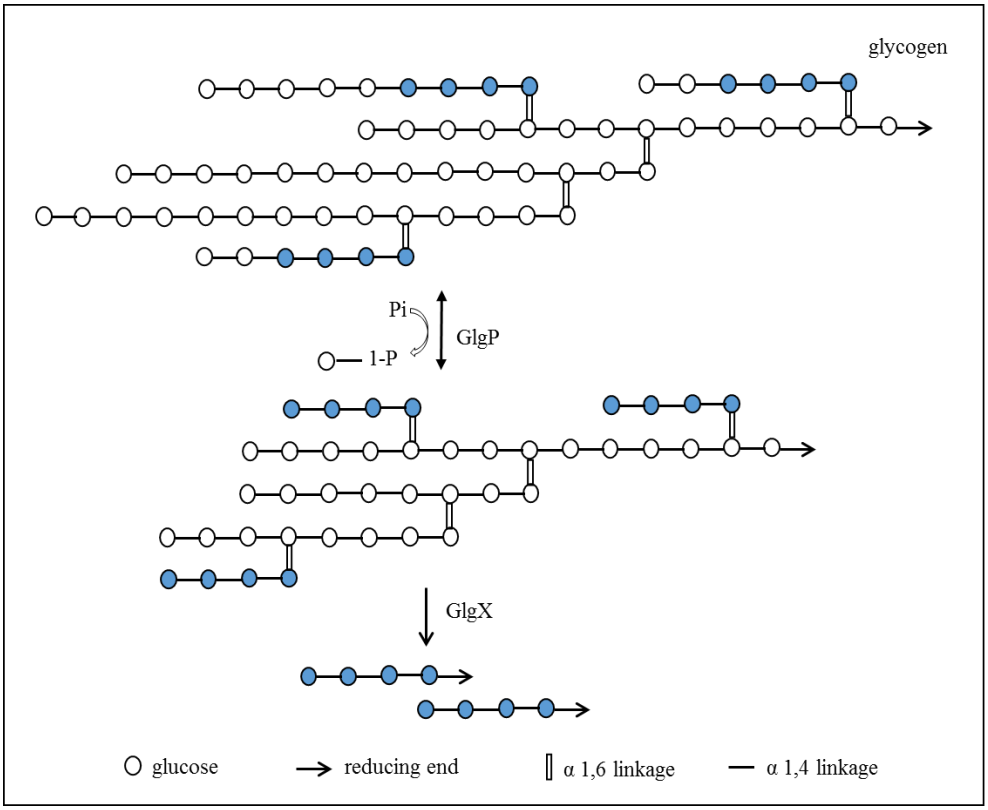


**Figure 1.2. Glycogen degradation.** Schematic representation of *E. coli* glycogen degradation. Glycogen phosphorylase (GlgP) phosphorolitically cleaves α1,4 linkages to produce phosphate limited glycogen which contains α1,6 branches which are typically four glucosyl residues long, but may also be three residues long. The glycogen debranching enzyme (GlgX) cleaves α1,6 branches, releasing maltotetraose or maltotriose. Maltotetraose (4 glucosyl residues) are blue to follow the production of linear maltodextrins due to the collective action of GlgP and GlgX.

1.1.2 Regulation: factors influencing the genes and enzymes of glycogen metabolism

Regulation of glycogen metabolism on the level of gene expression occurs by a number of mechanisms. Expression of *glgC* and *glgA* is stimulated by guanosine 3'-bisphosphate 5'-bisphosphate (ppGpp) and cyclic AMP (cAMP), as well as by the carbon storage regulator (CsrA) protein. CsrA, a RNA-binding protein that regulates the translation of target genes as well as the stability of target transcripts (Baker et al., 2002) has been shown to regulate glycogen biosynthesis by preventing translation of *glgC* and *glgB* (Baker et al., 2002; Romeo et al., 1993) and it has also been demonstrated to affect the expression of *glgP* (Yang et al., 1996). Allosteric regulation of the pathway is thought to be controlled mainly by AMP and fructose-1,6 bisphosphate affecting ADPglucose pyrophosphorylase, the enzyme which catalyses the first step in glycogen synthesis (Yang et al., 1996). Phosphoglucomutase, an enzyme which catalyses the reversible conversion of glucose-1-phosphate to glucose-6-phosphate, has also been shown to have a more minor influence on the biosynthesis of glycogen (Adhya and Schwartz, 1971; Brautaset et al., 2000).

Montero et al (2009) presented evidence to suggest that glycogen metabolism may also be finely regulated by the concentrations of intracellular magnesium ions ($Mg^{2+}$). They observed that mutations in the *mta, phoP* and *phoQ* genes caused cells to be glycogen-deficient. This is of particular interest since these genes are induced under low extracellular $Mg^{2+}$ concentrations (Minagawa et al., 2003). PhoP-PhoQ is a regulatory system in *E .coli* which monitors the levels of extracellular $Mg^{2+}$and controls the expression of many genes, including *mta*, which encodes a $Mg^{2+}$ import system (Kato et al., 1999). When the above mentioned mutants were incubated with millimolar concentrations of $Mg^{2+}$, a substantial increase in glycogen accumulation was observed leading to the conclusion that changes in $Mg^{2+}$ concentrations in the environment influences glycogen accumulation in *E. coli*. In addition to this, *E. coli* cells grown under limiting $Mg^{2+}$ conditions (50 µM) showed a reduced glycogen content compared with cells grown in non-limiting conditions (1 mM $Mg^{2+}$). Montero et al. (2009) sought to determine whether $Mg^{2+}$ concentrations have an effect on intracellular ATP content and found that ATP increased when cells were cultured under non-limiting $Mg^{2+}$ conditions and decreased under limiting $Mg^{2+}$ conditions. Overall it was concluded that the reduced glycogen content of *E. coli* cells grown under limiting $Mg^{2+}$ concentrations could partly be due to decreased ATP levels. Moreover, their data indicated that expression of the *glgCAP* operon is under the control of the PhoP-PhoQ system, under low environmental $Mg^{2+}$ conditions. This was corroborated by Western blot analysis which showed lower amounts of GlgC protein in *phoP* and *phoQ* mutants under limited $Mg^{2+}$ concentrations, however no difference between WT and mutant cells was observed under non-limiting concentrations.

It is possible for glycogen metabolism to also be regulated by the intermediates of maltodextrin metabolism. For example, *E. coli* lacking MalP or MalQ leads to the accumulation of maltotriose and

maltotetraose residues released by GlgX (Dippel et al., 2005). This accumulation would lead to feedback inhibition of glycogen degradation. In addition, the maltodextrin products released during glycogen degradation may be mobilized by MalQ in the synthesis of longer chain maltodextrins. The maltodextrins synthesised can eventually become substrate for GlgB and in this way glycogen can be synthesized in a manner which is independent of GlgA (Park et al., 2011). To further understand these particular interconnections, maltodextrin metabolism of *E. coli* is discussed below.

## 1.2 Maltodextrin metabolism

### 1.2.1 The mal system: genes involved in transport and mobilization of maltodextrins.

The *E. coli mal* system is primarily responsible for the transport, synthesis and degradation of maltodextrins of various chain lengths to produce glucose and glucose-1-phosphate that can be metabolised in *E. coli* (Boos and Shuman, 1998). Enzymes responsible for the cycling of maltodextrins are encoded in the maltose regulon and are induced by growth of *E. coli* on media containing maltose, short maltodextrins or by the products of glycogen degradation. The regulon consists of several operons (Boos and Shuman, 1998) consisting of 14 genes. Five of these genes encode proteins which make up the sub-units of an ABC transporter; LamB, a membrane diffusion porin (or maltoporin) and λ receptor, allows for passive diffusion of maltodextrins through the cytoplasmic membrane (Freundlieb et al., 1988; Randal-Hazelbauer and Schwartz, 1973); MalF (Froshauer and Beckwith, 1984) and MalG (Dassa and Hofning, 1985) are both membrane bound permeases. In addition, MalE (Duplay et al., 1984) is a maltose/maltodextrin binding protein which is responsible for the specificity of the molecules being transferred through the transporter, and which has high affinity for maltose or longer α-1, 4 linked maltodextrins as well as α-1, 4 linked cyclodextrins. Finally, the ABC transporter also contains MalK (Gilson et al., 1982; Joly et al., 2004), the ATP hydrolysing subunit. In addition to these MalP, MalQ, MalZ and MalS are needed for anabolism and catabolism of maltodextrins, MalT is needed for transcriptional activation of the system while MalI and MalY are thought to be involved in the regulation of MalT (Schlegel et al., 2002), MalX and MalM are a PTS enzyme homologue and a periplasmic protein respectively and their functions are still unknown (Wisniewski and Rakus, 2014).

A clearly defined mode of transport for maltodextrins through the ABC transporter has not yet been elucidated, but investigations have led to several conclusions (Boos and Shuman, 1998; Dippel and Boos, 2005; Ferenci, 1980; Ferenci et al., 1977; Wandersman et al., 1979). It has been established that *E. coli* is unable to transport maltodextrins longer than maltoheptaose (Wandersman et al., 1979), irrespective of the fact that it possesses enzymes which are able to catabolize larger maltodextrins. The specificity of the size of maltodextrins must, therefore, be due to the inability of larger maltodextrins to enter the cytoplasm (Ferenci, 1980). They are thought to be cleaved in the periplasm by MalS, the periplasmic α-amylase, which is able to cleave all maltodextrins except maltose and typically releases maltohexaose as a product

(Freundlieb et al., 1988). The outer membrane of enterobacteria allows for non-specific transport of molecules with a maximum molecular weight of 600 g/mol (in terms of malto-oligosaccharides this corresponds to maltose and maltotriose), through proteins called porins while transport of larger molecules requires specific outer membrane proteins (Bavoil et al., 1977). LamB is a porin which allows for the diffusion of such molecules, but also acts as a sugar-specific porin (maltoporin) by facilitating the diffusion of maltose and maltodextrins through the outer membrane of *E. coli* (Ferenci and Boos, 1980). The maltose/maltodextrin binding protein (MBK) binds to substrates that have been transported into the periplasm and undergoes a conformational change upon binding (Quiocho et al., 1997). MBK interacts with the $MalFGK_2$ subunits of the ABC transporter located on the inner membrane, and stimulates ATP hydrolysis by MalK in order to initiate the transport process of substrates into the intracellular space (Chen et al., 2001; Oldham et al., 2013).

### 1.2.2 Mobilization of maltodextrins in the cytoplasm of *E. coli*

The research presented in this study focuses partly on the metabolism of maltose once it has been transported into the cytoplasm and, therefore, examines in part, some of the enzymes of the *mal* system which directly catalyses the utilization of maltose. Currently it is thought that maltose or longer maltodextrins enter the cell through the ABC transporter. MalQ is able to polymerize maltodextrins of various chain lengths from maltose or maltodextrins entering the cytoplasm. The maltodextrins polymerized by MalQ are either phosphorolytically cleaved by MalP or hydrolysed by MalZ (Fig 1.3). The final products of maltodextrin metabolism, glucose and glucose-1-phosphate, can be converted to glucose-6-phosphate and/or glucose-1-phosphate by glucokinase and phosphoglucomutase, respectively. These can enter other metabolic pathways such as glycolysis or the pentose phosphate pathway. These intermediates can also contribute to glycogen synthesis by affecting the formation of ADP-glucose as substrate for glycogen synthase (Park et al., 2011).

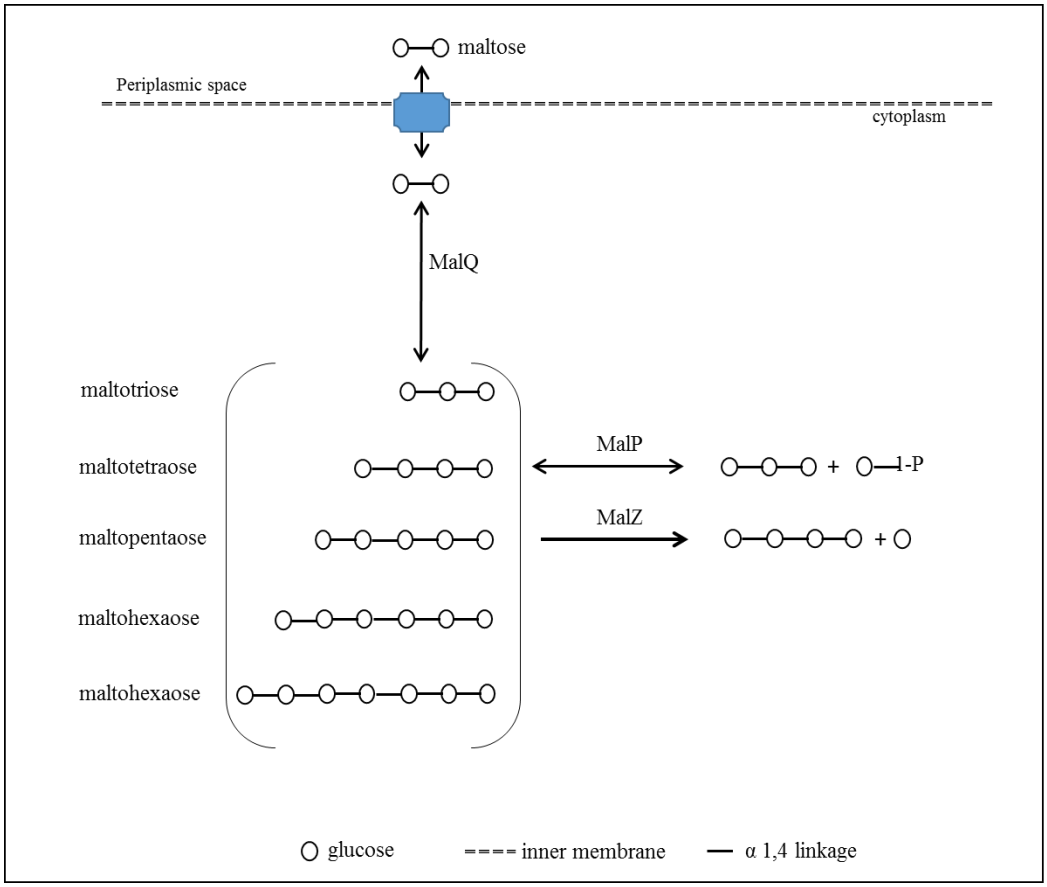


**Figure 1.3 Maltodextrin metabolism.** Schematic representation of *E .coli* maltodextrin metabolism. Maltose enters the cytoplasm *via* am ABC transporter located on the inner membrane. Amylomaltase (MalQ) polymerizes maltodextrins of various chain lengths. These maltodextrins are phosphorolytically cleaved by maltodextrin phosphorylase (MalP) resulting in the release of glucose-1-phosphate, maltotetraose is the smallest substrate recognized by MalP. Maltodextrin glucosidase (MalZ) hydrolyzes maltodextrins to yield a maltodextrin 1 DP shorter than the substrate and a glucosyl residue, maltotriose is the smallest substrate recognized by MalZ.

### 1.2.3 Regulation: factors influencing the genes and enzymes of maltodextrin metabolism

Transcriptional activation leading to expression of the genes in these operons is regulated by *malT*. This is an inducer dependent activator which is activated by maltotriose (Raibaud and Richet, 1987; Raibaud et al., 1989) with which it forms a complex. The MalT protein is able to recognize two or three direct repeats which are commonly found on all *mal* gene promoters (Vidal-Ingigliard et al., 1991). The regulatory domain of MalK, the ATP-hydrolysing sub-unit of the ABC transporter, functions as an inhibitor of MalT when there are no maltodextrins in the environment (Bohm and Boos, 2004). It is thought that MalK assumes its transport conformation in the presence of substrate to be transported, releasing MalT and leading to induction of the *mal* genes (Boos and Shuman, 1998). Its expression is also regulated by a catabolite gene activator protein/cyclic AMP (CAP-cAMP) system. The level of the CAP-cAMP complex is controlled by intracellular levels of glucose and, therefore, the *mal* system undergoes

catabolite repression by glucose on MalT (Chapon, 1982 a). In the presence of glucose, the levels of CAP-cAMP complex are decreased and, since this complex affects MalT, the decreased level of the complex in the presence of glucose lowers the affinity of MalT for its operons (Chapon, 1982). However, catabolite repression of MalT by glucose can be overcome by endogenous induction of *mal* genes by internally produced maltotriose (Boos and Shuman, 1998; Dippel et al., 2005). Schlegel et al (2002) showed that MalT is also regulated by MalY, a protein encoded by a gene present in the *malI/malXY* operon, in the absence of maltotriose. MalY binds to MalT and inactivates it. The global regulator Mlc regulates *malT* expression through repression of its transcription. The repressor function of Mlc is negatively regulated by the transport of glucose *via* the PTS which causes inactivation of Mlc and thus also relieves repression of its expression.

Death and Ferenci (1994) observed that when *E. coli* is grown on media with limited glucose, elevated expression of the *mal* genes is observed. However when it is grown in media with high concentrations of glucose, *mal* gene expression is repressed. It was hypothesised that this is due to glucose entering the cell *via* the Mgl galactose ABC transporter as opposed to the PTS (Boos and Shuman, 1998; Boos et al., 1990) which is responsible for mediating transport of hexoses and hexitols across the cytoplasmic membrane (Postma et al., 1993). Under low glucose concentrations the Mgl system is active due to the decreased levels of the CAP-cAMP complex; under these conditions the ABC maltose transporter is also able to transport glucose (Boos and Shuman, 1998). Under high concentrations of glucose, it is transported via the PTS resulting in catabolite repression of the transcription of *malT* and the expression of genes controlled by MalT.

### 1.2.4 Endogenous induction of *mal* genes

It has been observed that when *E. coli* cells are grown in media without either maltodextrins or maltose, moderate expression of *mal* genes is still observed. There must, therefore, be another method by which maltotriose, the inducer, can be produced (Boos and Shuman, 1998; Dippel and Boos, 2005; Dippel et al., 2005; Raibaud and Richet, 1987). Evidence that this is caused by maltotriose present as the result of glycogen degradation comes from the following observations; Mutants that are unable to synthesise glycogen and are also mutated in *malQ* are unable to grow on minimal media containing maltose as the sole carbon source. These mutants, however, are able to grow on minimal media containing maltodextrins with a minimum size of maltotetraose as MalP can use them to produce maltotriose and glucose 1-phosphate (Dippel et al., 2005). Since maltotriose can be derived from the catabolism of glycogen it indicates that it is possible for intermediates of glycogen degradation to be involved in inducing the *mal* system of *E. coli* and it is thought that this provides a link between glycogen and maltodextrin metabolism (Boos and Shuman, 1998; Decker et al., 1993; Dippel et al., 2005; Raibaud and Richet, 1987) (Fig 1.4). A model for glycogen-derived endogenous induction of the *mal* system would, therefore,

hypothesize that GlgP shortens glycogen side chains which are then substrates for GlgX (Dauvillée et al., 2005). Maltotetraose and very rarely, maltotriose molecules, are released during glycogen degradation, maltotetraose is acted on by MalP to also produce maltotriose, leading to MalT induction.

In the absence of glycogen, the *mal* system can still be induced provided the cell generates glucose and glucose-1-phosphate (Decker et al., 1993; Dippel et al., 2005). It has been hypothesised that these are converted into maltotriose by an, as yet unidentified enzyme, leading to induction of the *mal* genes (Decker et al., 1993).

One other possibility for how endogenous induction of maltodextrin metabolism can occur is through trehalose metabolism (Boos and Shuman, 1998). Trehalose is a disaccharide of glucose which *E. coli* synthesizes as a stress protectant. Endogenous synthesis of trehalose is increased during times of osmotic, heat or cold stress (Strom and Kaasten, 1993), but it can also be transported into the cell for use as a carbon source (Boos et al., 1990; Guiterrez et al., 1989). The final product of endogenous trehalose metabolism is glucose (Rimmele and Boos, 1994), which may then be metabolized along with glucose 1-phosphate to maltotriose by the unidentified enzyme mentioned above. Trehalose metabolism is able to induce *mal* genes to up to 30% of the fully induced level of expression (Klein and Boos, 1993), however, even in its absence *mal* genes are still expressed at very low levels (Boos and Shuman, 1998, Dippel et al., 2005). This means that maltotriose must be produced by some other means in the cell or there is another unknown substrate able to function as the inducer.

Park et al. (2011) discussed a mode of glycogen synthesis which was MalQ-dependent. In that study they examined glycogen content of *E. coli* mutants lacking glycogen synthase (*glgA*) both in the presence of MalQ, MalP and MalZ as well as in their absence. A *ΔglgA* mutant strain was unable to synthesize glycogen when grown on media containing either glucose or maltose, but when it was grown in media with maltodextrins as the carbon source, substantial amounts of glycogen was generated. They also found that the amount of glycogen accumulating under these conditions, as well as when maltose was included as the carbon source, increased in a *ΔmalP* mutant. From these observations they hypothesised that a *ΔglgA* strain grown on maltodextrins synthesizes glycogen due to the action of MalQ. This would polymerize long chain maltodextrins which would increase in length until they could act as substrates for glycogen branching enzyme (GlgB) leading to the synthesis of glycogen. In a strain containing MalP, the maltodextrins produced by MalQ would be reduced in length before they reached the minimum chain length for GlgB. To further support their hypothesis Park et al. (2011) incubated MalQ with maltose *in vitro* and observed the polymerization of maltodextrins up to 10 glucosyl residues long. This is only possible when glucose is continually removed from the system as at high concentrations glucose causes feedback inhibition of MalQ. When they added GlgB to this *in vitro* system, branched glycogen was formed from these long chain maltodextrins.

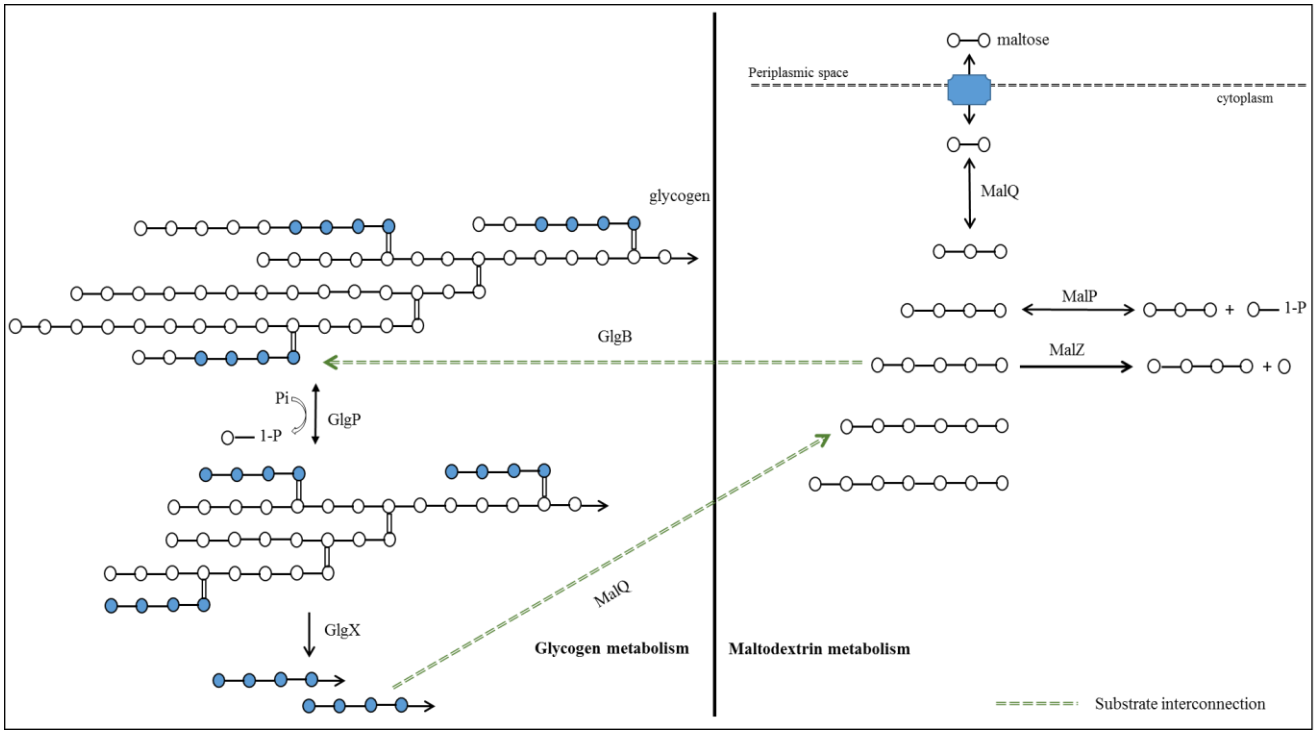


**Figure 1.4. Interconnection between glycogen and maltodextrin metabolism.** Schematic representation of the possible interconnections, between substrates involved in glycogen and maltodextrin metabolism. Maltodextrins polymerized by MalQ are able to become substrate for GlgB and be used to synthesise glycogen. Maltotetraose and maltotriose residues released by GlgX during glycogen degradation may be repolymerized by MalQ. Maltotriose released as a result of glycogen degradation also induces expression of the *mal* genes.

# 2. Polyglucan metabolism of *E. coli*: enzymes involved in mobilization of glycogen and maltodextrins.

This project examines the roles of GlgP, MalP, MalQ and MalZ, therefore, their functions and properties will be described in more detail below.

## 2.1 Glycogen phosphorylase (GlgP)

Glycogen phosphorylase (GlgP) is a polyglucan phosphorylase which phosphorolitically cleaves α1, 4 bonds at the non-reducing ends of polysaccharides such as maltodextrins and glycogen, yielding glucose-1-phosphate (Buchbinder et al., 2001). Most characterized glucan phosphorylases (including *E. coli* GlgP) are unable to act on glucan chains which are shorter than 5 glucose moieties in length (Alonso-Casajús et al., 2006; Boos and Shuman, 1998). GlgP has a greater specificity for glycogen than it does for linear maltodextrins and this distinguishes it from the maltodextrin phosphorylase (Watson et al., 1997). The physiological role of GlgP in glycogen degradation has been demonstrated. Alonso-Casajús (2006) analysed its function in *E. coli* by engineering strains to be either deficient in, or GlgP overexpressing. They observed that cells deficient in GlgP activity stained dark brown with iodine after growth on media containing glucose, indicating that the cells accumulated more glycogen than the wild-type strain. On the other hand, *glgP* overexpressing cells were glycogen free. Strains with altered *glgP* expression (both deficient and overexpressing) showed no difference in growth behaviour. This demonstrates that GlgP is involved in catalysing glycogen degradation in *E. coli*, but that does not lead to a change in its growth parameters. They also observed that during glycogen degradation, the expression of *glgP* in WT cells is several fold higher than that of genes encoding enzymes known to be involved in glycogen biosynthesis (GlgA and GlgC).

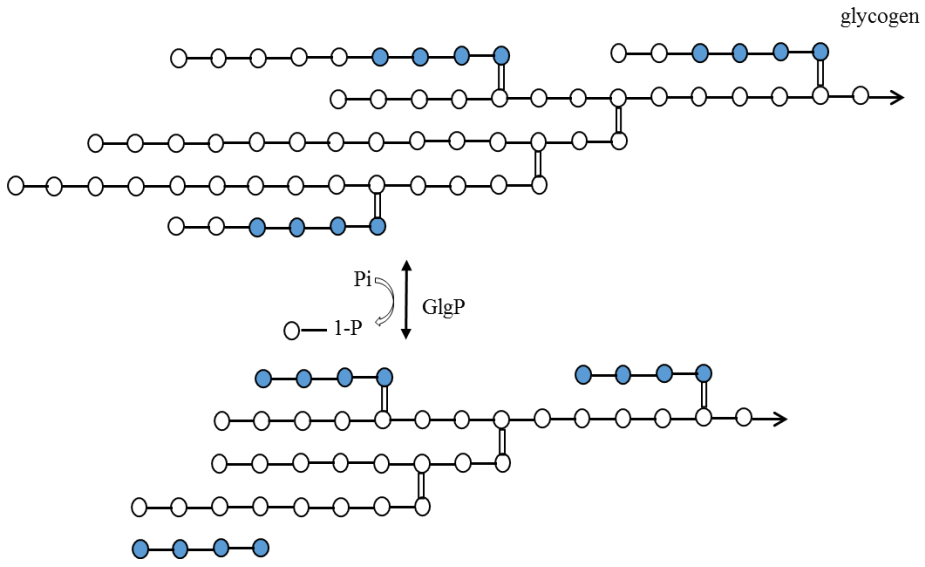

**Figure 1.5. Glycogen phosphorylase.** Schematic representation of the reaction catalysed by GlgP during the degradation of glycogen. GlgP phosphorolitically cleaves α1,4 bonds of the outer chains of glycogen, releasing glucose-1-phosphate.

To characterize the effect that an absence in GlgP activity has on the chain structure of glycogen, the chain lengths of the polymer from WT and *glgP* mutant cells were analysed (Alonso-Casajús et al., 2006). That study observed that the outer chains of glycogen, extracted from the mutant were on average 13 to 30 glucose residues longer than the chains observed for glycogen extracted from WT cells. This agrees with the model that glycogen synthase (GlgA) increases outer chain lengths of glycogen which are then degraded by GlgP. In the absence of GlgP these chains are elongated, but not degraded.

Previous models of *glgP* regulation suggested that, like other bacterial phosphorylases, it is only regulated on the level of gene expression (Hudson et al., 1993; Schinzel and Nidetzky, 1999). However, more recent studies have reported evidence which suggests that bacterial phosphorylases, including *E. coli* GlgP can be regulated post-translationally by a variety of factors.

There is evidence to suggest that GlgP is allosterically regulated by HPr (histidine phosphocarrier protein), a protein which is part of the PTS but which, has additional regulatory functions (Seok et al., 2001). HPr is a component of the PTS and in addition to three other proteins assists in phosphoryl transfer of glucose to facilitate its uptake into the cell (Seok et al., 2001). A number of factors pertaining to transport abilities of HPr, abundance in *E. coli* and the small size of the HPr protein suggested it to be a likely candidate for an enzyme regulatory factor. Seok et al (2001) utilized ligand fishing to detect any polypeptides in an *E. coli* crude protein extract which had the ability to bind to HPr and one of those was glycogen phosphorylase. The activity of GlgP was increased when bound to the HPr protein and this activation also led to an increase in GlgP affinity for glycogen. Since the concentration of HPr is present in greater excess than that of GlgP (Chen and Segel, 1968; Mattoo and Waygood, 1983), Seok et al. (2001) assumed that glycogen phosphorylase is always bound to HPr *in vivo*. The phosphorylation state of HPr varies, depending on the transport activity needed. In the absence of substrate it increases and in its presence it decreases. The ratio of phosphorylated to unphosphorylated proteins regulates processes which are influenced by the PTS systems, such as the control of metabolic pathways, gene transcription and chemosensory behaviour. GlgP showed ability to bind to both phosphorylated and unphosphorylated HPr but had a higher affinity for phosphorylated HPr *in vitro* (Seok et al., 2001). The significance of these results can be questioned as extracts of phosphorylated HPr are likely to be contaminated with the dephosphorylated form, meaning that the observed results could be artefactual. Nevertheless, it is possible that physiological changes which shift the ratio of phosphorylated HPr and dephosphorylated HPr would lead to changes in GlgP activity. HPr was found to be specific for *E. coli* glycogen phosphorylase only, regardless of the high sequence similarities between *E. coli glgP*, mammalian

phosphorylases and *E. coli* maltodextrin phosphorylase (Hudson et al., 1993). In summary, it provides evidence for a mode of regulation for GlgP and also the possible involvement of HPr and the PTS system in glycogen degradation in *E. coli*.

Another factor affecting the expression and enzyme activity of GlgP is the carbon storage regulator, CsrA (Yang et al., 1996). When the *csrA* gene was first studied by Romeo et al (1993) it was observed that mutagenesis of the gene causes increased levels of glycogen when compared to wild type cells. In a subsequent study it was found that GlgP activity increased 3-4 fold in a *csrA malP* double mutant than when *malP* alone was mutated, indicating that CsrA negatively regulates GlgP. Yang et al (1996) also reported higher levels of *glgP* gene expression in mutants which were *csrA*$^{-}$ compared with isogenic strains containing the wild-type allele, providing evidence that CsrA affects *glgP* on the level of gene expression.

**2.2 Maltodextrin phosphorylase (MalP)**

Maltodextrin phosphorylase is able to reduce any maltodextrin of up to 5 glucosyl residues to maltotetraose and then very slowly to maltotriose. This means that MalP will always be able to form maltotriose, the inducer of the *mal* system, from any endogenously synthesized maltodextrins as well as those derived from glycogen synthesis (Dippel and Boos, 2005). This substrate specificity is unique to bacterial phosphorylases, since most α-glucan phosphorylases are unable to catabolize glucan chains shorter than 5 sub-units in length (Boos and Shuman, 1998). MalP has similar enzymatic properties to that of GlgP (glycogen phosphorylase) in that they both catalyse the phosphorylation of α-1, 4 bonds between glucose residues at the non-reducing end, however they differ in substrate affinity with MalP having a high affinity for linear maltodextrins and a low affinity for glycogen (Schwartz and Hofnung, 1967) whilst GlgP has a low affinity for linear maltodextrins and a high affinity for glycogen (Hu and Gold, 1975).

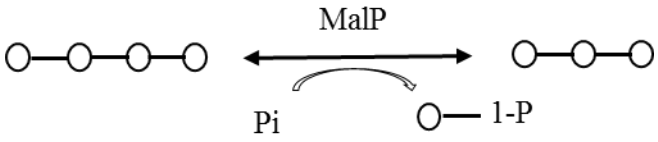


**Figure 1.6. Maltodextrin phosphorylase**. Schematic representation of the reaction catalysed by MalP on maltotetraose. MalP phosphorolytically cleaves α1, 4 bonds to form maltotriose and releasing glucose-1-phosphate.The smallest substrate recognized by this enzyme is maltotetraose, however it is also active on longer chain maltodextrins.

MalP is essential for controlling endogenous induction of the *mal* genes since it is responsible for the production of maltotriose from maltotetraose (Dippel et al., 2005). *ΔmalP* mutants grown on maltose

accumulate large amounts of linear dextrins due to the action of MalQ (Dippel and Boos, 2005). MalP enzyme also controls the formation of glycogen by shortening the length of maltodextrins polymerized by MalQ and curbing substrate availability for GlgB. In addition, the phosphorolysis of maltodextrins by MalP results in the release of glucose-1-phosphate which fuels the formation of ADP-glucose, the substrate for GlgA and, in this manner MalP can increase the rate of glycogen synthesis (Park et al., 2011).

MalP activity is catalysed by a homodimer consisting of two polypeptides (Boos and Shuman, 1998). Site directed mutagenesis of the active site of *E. coli* MalP has demonstrated that two positively charged amino acids (Lys 539 and Arg 534) play a role in the catalytic function of this enzyme (Shinzel and Dreukes, 1991). It was observed that mutagenesis of Arg 534 resulted in a decrease in $k_{cat}$ values which points to a decrease of binding of the substrate to the enzyme. Mutagenesis of Lys 539 resulted in a reduction of catalytic activity of the enzyme. This indicates that these amino acids play a role in the binding of substrate to the catalytic region of MalP. Crystallographic studies and sequence data have provided insight into the catalytic and regulatory domains of MalP (Watson et al., 1999). Overall it has been found that *E. coli* maltodextrin phosphorylase shows the basic archetype for bacterial α-glucan phosphorylases. It exhibits a conformation which promotes the degradation of maltodextrins and lacks catalytic sites which allow for allosteric regulation or inhibition of the enzyme (Schinzel and Nidetzky, 1999).

### 2.3 Amylomaltase (MalQ)

This enzyme is a dextrinyl transferase that is responsible for transferring glucosyl and maltosyl residues onto glucose, maltose and other maltodextrins (Boos and Shuman, 1998). The smallest substrate recognised by MalQ is maltose. *E. coli* strains lacking *malQ* are unable to metabolize maltose and, as a result, can accumulate large amounts of free maltose (Boos and Shuman, 1998). The products yielded as a result of the transferase reaction can be any net combination of malto-oligosaccharides, while the net number of glucosyl residues in the reactions remains conserved (Weiss et al, 2015).

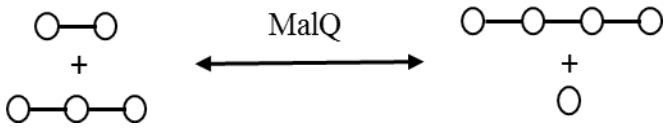


**Figure 1.7. Amylomaltase**. Schematic representation of the reaction catalysed by MalQ to polymerize maltodextrins of various chain lengths. MalQ transfers a glucosyl residue from maltose to maltotriose to yield maltotetraose and glucose.

MalQ is composed of 694 amino acids and is a member of the 4-α-glucanotransferases (E.C 2.4.1.25) (Pugsley and Dubreil, 1988). Although it is structurally and mechanistically related to α-amylases it can

be distinguished from them based on its activity (Lombard et al., 2014), while amylomaltases favour the transglycosylation (transfer of glucosyl residues from one glycoside to another) or disproportionation (the simultaneous oxidation and reduction of a glycoside to yield two products) reactions, α-amylases favour the hydrolysis reaction of glycosidic bonds (MacGregor et al., 2001).

Recently Weiss et al. (2015) studied the 3D structure of *E. coli* amylomaltase and found that it assumes three distinct conformations during the catalytic cycle. These include the structure for an enzyme which is not bound to substrate, a conformation for the binding/ release of substrate and finally the transition state during which the transferase reaction occurs and products are formed. They elucidated seven sub-sites for substrate binding which play a role in the recognition of maltodextrins. These sites are responsible for the affinity at which the enzyme forms complexes with its donor and acceptor molecules and for regulating the substrates for which amylomaltase has a preference. These analyses also revealed insights into the hydrolysis rate of the enzyme as well as the substrates which functionally inhibit amylomaltase. Weiss et al (2015) were able to demonstrate that the equilibrium of the reaction catalysed by MalQ is strongly dependent on the number of glycosidic bonds a substrate contains. The more bonds that are present, the further the equilibrium is shifted towards the production of longer chain maltodextrins and lower amounts of glucose.

MalQ undergoes feedback inhibition by glucose and so under condition of excess cytoplasmic glucose, activity of MalQ is decreased. This is most clear in a glucokinase *(glk)* mutant, since in this strain increased free glucose would be expected due to the absence of ATP-dependent phosphorylation of glucose (Lengsfeld et al., 2009). When glucokinase is overexpressed in *E. coli*, MalQ activity increases due to the removal of free glucose. This effect of glucose on maltose would also influence endogenous induction of the *mal* genes. In the presence of high concentrations of glucose, MalQ undergoes feedback inhibition and does not polymerize maltotriose to longer chains and, therefore, induction of the *mal* genes increases. In conditions where glucose is removed, MalQ will remain free of inhibition and continually re-polymerize maltotriose leading to decreased endogenous induction (Lengsfeld et al., 2009).

### 2.4 Maltodextrin glucosidase (MalZ)

*MalZ* encodes a maltodextrin α-1, 4 glucosidase which catalyses the cleavage of glucose from the reducing ends of maltodextrins (Peist et al., 1996), with maltotriose being the smallest substrate recognized by MalZ (Tapio et al., 1991). The product of the action of MalZ on maltodextrins is therefore, glucose and a maltodextrin 1 DP shorter than that of its substrate (Boos and Shuman, 1998). In addition, it is able to hydrolyze γ-cyclodextrin and larger cyclodextrins (Peist et al., 1996) although the relevance of this *in vivo* is unclear as *E. coli* is unable to use them as a carbon source.

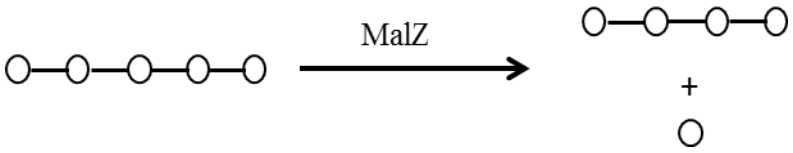


**Figure 1.8. Maltodextrin glucosidase**. Schematic representation of the reaction catalysed by MalZ on maltopentaose. MalZ hydrolyzes glucose from the reducing end of maltopentaose to yield maltotetraose and glucose. The smallest substrate recognized by MalZ is maltotriose.

*malZ* is part of a monogenic operon and its open reading frame encodes a protein with a molecular weight of 68 950 Da. Based on sequence homology and enzyme kinetics, it was deduced that MalZ is able to recognize up to seven glucosyl residues due to the observation that the enzyme contains seven sub-sites for substrate binding. In addition, its affinity for its substrate increases with increasing maltodextrin chain length (Tapio et al., 1991). It has been proposed that when a substrate binds to MalZ, each glucosyl residue occupies its own sub-site at the active site until all seven consecutive sites are occupied (Tapio et al., 1991). It has been hypothesised that while this enzyme is not needed for growth of *E. coli* on short maltodextrins, it can lead to the cellular degradation of intracellular maltotriose and, therefore, plays a role in regulating induction of the *mal* system (Raibaud and Richet, 1987).

Recently a transglycosylation reaction of MalZ was also characterized (Song et al., 2010). When MalZ was incubated with high concentrations of maltotriose (5% w/v) it catalysed the synthesis of maltosyl-alpha-1, 3-maltose, isopanose and maltosyl-alpha-1, 6-maltose. This result indicated that MalZ, while mainly exhibiting hydrolytic abilities, is also able to transfer sugar moieties to the C-2-OH OR C-6-OH of glucose (Song et al., 2010). Given the high concentrations of maltotriose required, however, it is unlikely that this observation is physiologically relevant.

Park et al. (2011) observed that overexpression of MalZ caused a 90% decrease in glycogen content in a strain lacking *glgA*, when glycogen synthesis occurs in a MalQ-dependent manner, indicating that MalQ-dependent formation of glycogen is sensitive to MalZ. This is due to MalZ shortening maltodextrin chains

produced by MalQ, curbing the amount of substrate available for GlgB for glycogen synthesis (Park et al., 2011). In contrast, a WT strain where glycogen synthesis occurred in a GlgA-mediated manner showed a 33% decrease in glycogen content when MalZ was overexpressed. It is very unlikely that the glycogen produced by GlgA is sensitive to degradation by MalZ as each glycogen molecule contains only one reducing end. Park et al. (2011) speculated that the reducing ends, onto which *glgA* transfers glucose, is occluded from MalZ for a long enough period that the chain size becomes too big for it to be a substrate for MalZ. Therefore, the mechanism whereby MalZ may affect glycogen degradation in a WT *E. coli* strain is still unclear.

Dippel et al. (2005) observed MalZ activity increased after growth in increasing osmolarity, in a strain lacking *malT*. They hypothesised that *malZ* expression was both MalT dependent but also osmo-dependent. The mechanism behind osmo-dependent regulation on *malZ* is still elusive and more research will have to be conducted to fully understand this.

## 3. Mutagenesis of *E. coli*

To gain insight into the functions of genes on the *E. coli* chromosome it is often beneficial to look at the effects that DNA mutations of these genes have on the cellular process being studied. There are various types of mutagenesis techniques which are chosen according to the effect that the mutation would have on the targeted gene or the process being studied. For example, i) random mutagenesis involves mutating genes using mutagens which cause direct damage to DNA or DNA repair processes. In this way, mutations can occur anywhere on the segment of DNA being mutated. This technique is typically used to create a library of mutants which is screened for colonies showing the desired traits. These colonies are compared with the control strain and the specific mutation(s) is/are then characterized. ii) Site-directed mutagenesis involves mutations at very specific points on the DNA; this type of mutation could involve altering base pairs in a targeted gene, deletion of entire genes or insertion of DNA at specific loci. This approach is mostly used when the focus of the research is a specific gene and the aim is to establish its function in a specific process.

Some of the most recent methodology developed to accomplish this involves the use of linear fragments of DNA generated using the Polymerase Chain Reaction (PCR), to introduce antibiotic cassettes at specific locations on the bacterial chromosome (Datsenko and Wanner, 2000). This is highly site directed and efficient, since the mutation can be achieved without the need for plasmid construction. Instead, linear fragments of DNA are transformed into *E. coli*, resulting in homologous recombination between the linear fragment and the gene of interest. Although it is inefficient to transform linear fragments of DNA into *E. coli*, due to the presence of intracellular exonucleases; such fragments are usually degraded (Lorenz and Wackernagel, 1994), it is now possible to perform this type of mutational approach using *E.*

*coli* mutants which are deficient in exonuclease V of the RecBCD recombination complex and are thus transformable with linear fragments of DNA (Cosloy and Oishi, 1973). Indeed, it has been so successful, that every non-essential gene present in *E. coli* has been knocked out using this methodology, to create a set of isogenic mutants, the Keio collection (Baba et al., 2006). One way to transfer mutations between strains utilises the P1 *vir* bacteriophage. This phage is able to package up to 100 kb segments of chromosome from the host *E. coli*. Upon infection of a second strain with the same phage, the genetic material that was previously packaged is released into the new host strain. In this way genetic material from one strain is transferred to another. Regions of homology between the host genome and the transferred segments of chromosome will recombine and the transferred segments will be permanently incorporated into the genome of the second strain (Thomason et al., 2007).

Using the Keio collection and P1 *vir* phage transduction it is possible to create a series of multiple mutants within *E. coli*. These can be used to examine the roles and interactions of genes in many processes.

## 4. Aims and Objectives

The aim of this research was to characterize the functional roles that MalQ, MalP and MalZ enzymes play in maltodextrin metabolism and possibly the functional roles that they have in *E. coli* glycogen metabolism. In addition, GlgP, an enzyme known to function in glycogen degradation, was also functionally characterized as it catalyses a very similar reaction to MalP and so there could be functional redundancy between the two enzymes in some processes. To accomplish this, genes encoding the above mentioned enzymes were deleted from the chromosome using two mutagenesis techniques. We constructed double mutants of combinations of the above mentioned genes within *E. coli* (i.e *malP*, *malQ*, *malZ*, *glgP*, *ΔmalP/ΔmalQ*, *ΔmalP/ΔmalZ*, *ΔmalP/ΔglgP*, *ΔmalZ/ΔglgP*, *ΔmalQ/ΔglgP*and *ΔmalQ/ΔmalZ*). Mutants were analysed according to malto-oligosaccharide/glycogen accumulation, growth rates on various carbon sources and cell morphology.

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# CHAPTER 2: Functional analysis of the roles of MalP, MalQ, MalZ and GlgP, on glycogen and maltodextrin metabolism in *E. coli.*

## Introduction

The utilization of polyglucans such as glycogen and maltodextrins in *E. coli* is an area that has been extensively studied and much is known about the enzymes involved in their metabolism. However, there are still uncertainties regarding the physiological importance of some of the enzymes, especially in terms of how they interact with each other within biochemical pathways.

Maltodextrin phosphorylase (MalP), amylomaltase (MalQ) and maltodextrin glucosidase (MalZ) have previously been demonstrated to play a role in the mobilization of maltodextrins in the cytoplasm of *E. coli*. When maltose is present these maltodextrins are primarily formed by MalQ and then degraded by MalP and MalZ. Maltodextrins are also produced as a result of glycogen degradation and, similarly, it has been proposed that these are mobilised by MalQ, MalP and MalZ. Glycogen phosphorylase (GlgP) is known to play a role in the degradation of glycogen and is structurally and functionally similar to MalP and therefore, might also play a role in the degradation of maltodextrins.

In this study, the functions of these enzymes in maltodextrin and glycogen metabolism in *E. coli* are examined. In order to elucidate their roles as well as any interconnections between these reactions, a reverse genetics approach was used. This chapter describes the construction of double mutant strains lacking combinations of the genes that encode those enzymes, using P1 *vir* phage transduction (Thomason et al., 2007).

These strains were examined to elucidate the roles(s) of the enzymes on growth and polyglucan metabolism when grown in media supplemented with either glucose (to stimulate glycogen synthesis) or maltose (to stimulate maltodextrin synthesis) and were compared according to polyglucan content, growth and cell morphology in media containing either maltose or glucose. The data described in this chapter indicates that these enzymes may have additional functions in the utilization of polyglucans, which have not been described previously.

## 2.2 Materials and Methods

### 2.2.1 Bacterial strains

All bacterial strains used in this research are *Escherichia coli* K-12 derivatives and form part of the isogenic 'Keio collection' (Table 1; Baba et al., 2006). Both *E. coli* strains and P1 phage (CGSC# 12133) were obtained from the Coli Genetic Stock Centre (http://cgsc.biology.yale.edu/).

**Table 2.1**. Bacterial strains used in this study

| Strain | Genotype | Reference |
|---|---|---|
| BW25113 (wild type ) | F-,Δ(araD-araB)567, ΔlacZ4787(::rrnB-3), λ-, rph-1, Δ(rhaD-rhaB)568, hsdR514 | Datsenko and Wanner (2000); CGSC# 7636 |
| Δ*malP(kan*) | BW25113 Δ*malp::kan* | Baba et al. (2006); CGSC# 11896 |
| Δ*malZ(kan*) | BW25113 Δ*malZ::kan* | Baba et al. (2006); CGSC# 8568 |
| Δ*glgP* | BW25113 Δ*glgp* | Strydom et al. (2016) |
| Δ*malQ(kan)* | BW25113 Δ*malQ::kan* | Baba et al. (2006); CGSC# 10517 |
| Δ*malQ* | BW25113 Δ*malQ* | This study |
| Δ*malP*/Δ*glgP* | BW25113 Δ*malp* Δ*glgp::kan* | Strydom et al. ( 2016) |
| Δ*malZ*/Δ*glgP* | BW25113 Δ*malZ::kan* Δ*glgp* | This study |
| Δ*malZ*/Δ*malQ* | BW25113 Δ*malZ::kan* Δ*ma lQ* | This study |
| Δ*malZ*/Δ*malP* | BW25113 Δ*malZ::kan* Δ*malP* | This study |
| Δ*malQ*/Δ*glgP* | BW25113 Δ*malQ::kan*Δ*glgp* | This study |

For the purpose of this study P1 phage transduction was used to construct double gene deletions on the *E. coli* chromosome. The inserted Kanamycin resistance genes were removed using FRT recombinase according to the protocol by Datsenko and Wanner (2000) using the vector pCP20 (Cherepanov and Wackernagel, 1995). To introduce kanamycin resistance genes, P1 *vir* phage lysates (phage titre of $10^9$ cfu/mL) were made from *E. coli* strains containing a kanamycin cassette in place of the deleted gene on the bacterial chromosome according to the protocol of Thomason et al. (2007). Phage was purified and the lysate was used to infect a recipient strain which lacked a kanamycin cassette. After transduction, cultures were plated on LB (Lysogeny Broth; 2 g/L NaCl, 2 g/L peptone, 1 g/L yeast extract) media supplemented with 50 µg/ml kanamycin (Thomason et al., 2007). PCR analysis was used to screen colonies which grew on the selection media to confirm mutation of the targeted gene using the primers listed in Table 2.2.

**Table 2.2**. Primer sequences for the screening of the constructed deletion mutants.

| Gene | Forward primer | Reverse primer |
|---|---|---|
| *malP* | 5'AAGGTCAACATCGAGCCTGG 3' | 5'ATCCACCAGCATCGCTTTGA 3' |
| *malZ* | 5' GGCTATGGCAAGGTGATCAGA 3' | 5' TCTGGCAGTTTTATCCGCGG 3' |
| *malQ* | 5' GGCACAAAAGCAGGTGGATG 3' | 5' GGCATCAACCGCACTCTACT 3' |
| *glgP* | 5'CTTCACTGTGGCGGTTTGTG 3' | 5'GGAATTACCGCAAAGCCCAC3' |

### 2.2.2 **Chemicals**

All chemicals used in this study were of analytical grade. They were ordered from Sigma-Aldrich (St. Louis, MO, USA) and Megazyme (Co. Wicklow, Ireland). Primers were ordered from Inqaba Biotech (Pretoria, South Africa).

### **2.2.3 Scanning electron microscopic imaging of mutants**

Cells were inoculated in 2 mL LB and incubated at 37$^{o}$ C overnight, shaking at 250 rpm. Fifty µL of this was used to inoculate 10 mL LB which was incubated at 37$^{o}$ C overnight, shaking at 240 rpm. Five mL of culture was centrifuged at 6500 x g at 37$^{o}$ C and the resulting supernatant discarded. Cells were suspended in 100 µL 2.5% (v/v) glutaraldehyde and kept at 4$^{0}$C for 7 hrs before being centrifuged 6488 x g for 5 min and the supernatant discarded. The cell pellet was suspended in 500 uL 0.1M sodium phosphate buffer (pH 7.0) and centrifuged at 6488 x g for 5 min and the supernatant discarded. This step was repeated twice. Cells were then suspended in 100 uL 1% (v/v) osmium tetroxide and incubated for 1 hr at 28$^{o}$ C, before being centrifuged at 6488 x g and discarding the supernatant. The pellet was suspended in 500 uL 0.1M sodium phosphate buffer (pH 7.0) and centrifuged at 6488xg for 5 min before the supernatant was discarded. This step was repeated once more with dd$H_20$. In order to dehydrate *E. coli* samples an ethanol

series was used. Cells were suspended sequentially for 10 min in 30%, 50%, 70%, 90%, 95% and 100% (v/v) ethanol with centrifugation at 1500 x g for 10 min and removal of the supernatant between steps. After dehydration pellets were left to dry overnight at 28$^{o}$ C. Samples were imaged by scanning electron microscopy (SEM) using a Zeiss Merlin™ Field Emission Scanning Electron Microscope (FE-SEM).

### 2.2.4 Protein Extractions

Crude protein extracts were prepared from *E. coli* cells in the following manner: Cells were inoculated in 5 mL LB media overnight at 37$^{o}$ C with shaking at 250 rpm. From this starter culture, 100uL was used to inoculate 50 mL LB which was incubated at 37$^{0}$C overnight with shaking at 250 rpm. Cultures were centrifuged at 7000 x g for 10 min at 4$^{0}$C. Supernatant was discarded and cell pellets were suspended in 50 mL extraction buffer (10 mM Tris-HCL (pH 7.5); 5 mM EDTA; 1.9 mM β-mercaptoethanol; 2.5 µM PMSF).

The suspension was sonicated on ice at 6W output power with three bursts of 1 min durations. Cell lysates were centrifuged at 10500 x g for 30 min at 4$^{o}$ C and the supernatant collected. Protein was quantified using a protein assay kit (Bio-Rad) based on the method of Bradford using bovine serum albumin standards.

This method of protein extraction was also used for quantification of proteins in liquid cultures used for glycogen and maltodextrin measurements (section 2.2.7.1 and 2.2.7.2, respectively). Optimizations were as follows; protein was extracted from 2 mL of cell culture instead of 50 mL and cell pellets were suspended in 500 µL extraction buffer instead of 5 mL.

### 2.2.5 Enzyme activity determinations

#### *2.2.5.1 Glycogen phosphorylase (GlgP) and Maltodextrin phosphorylase (MalP) native gel*

Approximately 400 µg of crude extract was loaded on a 10% (w/v) polyacrylamide gel lacking SDS, but containing 1% (w/v) glycogen (type 2 from oyster). The gel was electrophoresed for 4 hrs at 100V constant voltage at 4$^{o}$ C after which it was incubated overnight in 3 M acetic acid/NaOH (pH 6.0) containing 20 mM glucose-1-phosphate. After incubation, the gel was stained with Lugol's solution (0.2% (w/v) KI; 0.02% (w/v) $I_2$) for 5 min and de-stained with dd$H_20$.

#### *2.2.5.2 Amylomaltase (MalQ) assay*

Two hundred µg crude protein was used in a final volume of 1mL assay reaction mix (15 mM maltose, 200 mM potassium phosphate buffer (pH 6.0). The reaction was incubated for 2 hrs at 37$^{0}$C. After incubation samples were centrifuged for 2 min and the supernatant transferred to a new tube. Glucose was quantified in an enzyme linked assay using a mixture of hexokinase (yeast) and glucose-6- phosphate dehydrogenase (Leuconostoc) (Sigma-Aldrich). Twenty µL supernatant was incubated with 180 µL assay buffer (1 mM ATP; 1 mM NAD; 1 mM $MgCl_2$; 50 mM Tris-HCL (pH 7.5) and 5µL enzyme mix (0.42 U

Hexokinase/0.21 U glucose-6-phosphate dehydrogenase). $OD_{340}$ was measured before and after the addition of enzyme and the change in $OD_{340}$ was used to calculate the amount of glucose released.

*2.2.5.3 Maltodextrin glucosidase (MalZ) activity assay*

Maltodextrin glucosidase activity was determined using the artificial substrate *p*-nitrophenyl-α-D-maltoside (Sigma) as described by Tapio et al (1991). Two hundred µg crude protein extract was used in a 900 µL assay reaction mix containing 113 µL 0.1 M sodium phosphate buffer (pH 7.0). The reaction mix was incubated for 45 min at 37$^{o}$ C. To start the reaction, 5.5 mM *p*-nitrophenyl-α-D-maltoside was added to the reaction and incubated at 37$^{o}$ C for 45 min. The reaction was stopped by the addition of 0.5 M sodium carbonate. The change in $OD_{405}$ was used to calculate the amount of *p*-nitrophenol released using an extinction coefficient of 0.014.

**2.2.6 Visualization of polyglucan accumulation**

Strains were inoculated in 2 mL LB media and incubated at 37$^{o}$ C overnight. Ten µL of each culture was spotted on solid LB media supplemented with either 1% (w/v) glucose or 1% (w/v) maltose, before being incubated 37$^{o}$ C overnight. Cells were exposed to iodine vapour until sufficient staining was visible.

**2.2.7 Polyglucan accumulation determined from liquid cultures**

*2.2.7.1 Glycogen accumulation*

Strains were inoculated in 2 mL LB media and grown overnight at 37$^{o}$ C. From this starter culture, 50 µL was used to inoculate 50 mL LB media containing 1% (w/v) glucose and incubated for 16 hrs at 37$^{o}$ C. Triplicate 2 mL samples were removed from the culture at 4 hrs, 8 hrs, 16hrs, 24 hrs and 48 hr time points, and these were centrifuged at 13000 x g for 7 min at room temperature. The supernatant was discarded and cell pellets were suspended in 1mL 70% (v/v) ethanol and incubated at 80$^{o}$ C for 2 hrs. Samples were then centrifuged at 13000 x g for 7 min at room temperature and the supernatant removed. This step was repeated twice. Pellets were suspended in 0.2 M KOH and incubated at 95$^{o}$ C for 1 hr. Seventy µL 1M acetic acid was added to neutralise each sample. Ten µL of each sample were combined with 10 µL of digest buffer (50 mM sodium acetate (pH 4.6); 10 U α-amylase from *Bacillus amyloliquefaciens* (Megazyme); 10 U amyloglucosidase from *Aspergillus niger* (Megazyme) and incubated at 37$^{o}$ C for 2 hrs. Two hundred µL assay buffer (1 mM ATP; 1 mM NAD; 1 mM $MgCl_2$; 100 mM Tris-HCL (pH 7.5) and 5 µL enzyme mix ( 0.42 U hexokinase / 0.21 glucose-6- phosphate dehydrogenase) was added to samples. The change in $OD_{340}$ was determined and used to calculate the amount of glycogen.

*2.2.7.2 Maltodextrin accumulation*

Strains were inoculated in 2 mL LB media and incubated at 37$^{o}$ C overnight before 50 µL was used to inoculate 50 mL LB containing 1% (w/v) maltose. Cultures were incubated at 37$^{o}$ C with shaking at 250 rpm for 48 hrs. At 4 hour intervals 2 mL samples of culture were removed from the flask and centrifuged at 13000 x g for 7 min at room temperature. Cell pellets was suspended in 70 % (v/v) ethanol and heated at 80$^{o}$ C for 2 hrs before being centrifuged at 13000xg for 7 min. The supernatant and pellet were separated with the supernatant being used to determine polyglucans soluble in 70% (v/v) ethanol and the pellet used to determine polyglucans that are insoluble.

i) Insoluble polylucans

The pellet was assayed for insoluble polyglucans as described in section 2.2.7.1.

ii) Soluble polyglucans

Twenty µL of the 70% (w/v) ethanol extract was assayed for glucose in the system by adding 180µL assay buffer (1 mM ATP; 1 mM NAD; 1 mM MgCl2; 100 mM Tris-HCL (pH 7.5) and 5µL enzyme mix (0.42U hexokinase / 0.21U glucose-6- phosphate dehydrogenase). The change in $OD_{340}$ was determined and used to calculate glucose concentrations. To determine polyglucans, 20µL of the ethanol extract was combined with amyloglucosidase by adding 25 mM sodium acetate (pH 4.5) and 1.2U amyloglucosidase and incubating this at 55$^{o}$ C for 15 min. Eighty µL 0.1M potassium phosphate buffer (pH 6.0) and 1.5U maltase from yeast (Megazyme) was then added and further incubated at 25$^{o}$ C for 30 min. The reaction was stopped by incubating the final reaction mix at 100$^{o}$ C for 3 min. Twenty µL of the final reaction mix was assayed for glucose as described above. Soluble polyglucans were calculated as the difference between the amounts of glucose in the samples with and without digestion.

## 2.3 Results

### 2.3.1 Mutant construction

Double mutant strains were constructed by removing antibiotic resistance markers using FLP recombinase to excise antibiotic cassettes at the FRT sites found either side of the marker. Mutant alleles were transferred between isogenic strains using P1 *vir* phage transduction. Successful construction of these strains was confirmed by PCR analysis on *E. coli* gDNA using primers (Table 2.2) that bind upstream and downstream of the genes targeted for mutation (Fig 2.1). It proved impossible to construct a *ΔmalP/ΔmalQ* double mutant strain due to their proximity in the *E. coli* genome where they are separated by only 10 base pairs. Due to this close linkage almost all P1 phage that come from infection with the single Δ*malP(Kan)* or Δ*malQ(Kan)* mutant, will carry the wild type allele of the second gene. To overcome this *ΔmalP/ΔmalQ* was ordered from a commercial service; however, they were also unable to manufacture this double mutant. It proved impossible to amplify the *malQ* gene in a Δ*malP* mutant background (Fig 2.1c) and a *malP* gene in a Δ*malQ* (Fig 2.1d) mutant background as one of the primer binding sites of each was destroyed during the construction of the mutants (Baba et al., 2006).

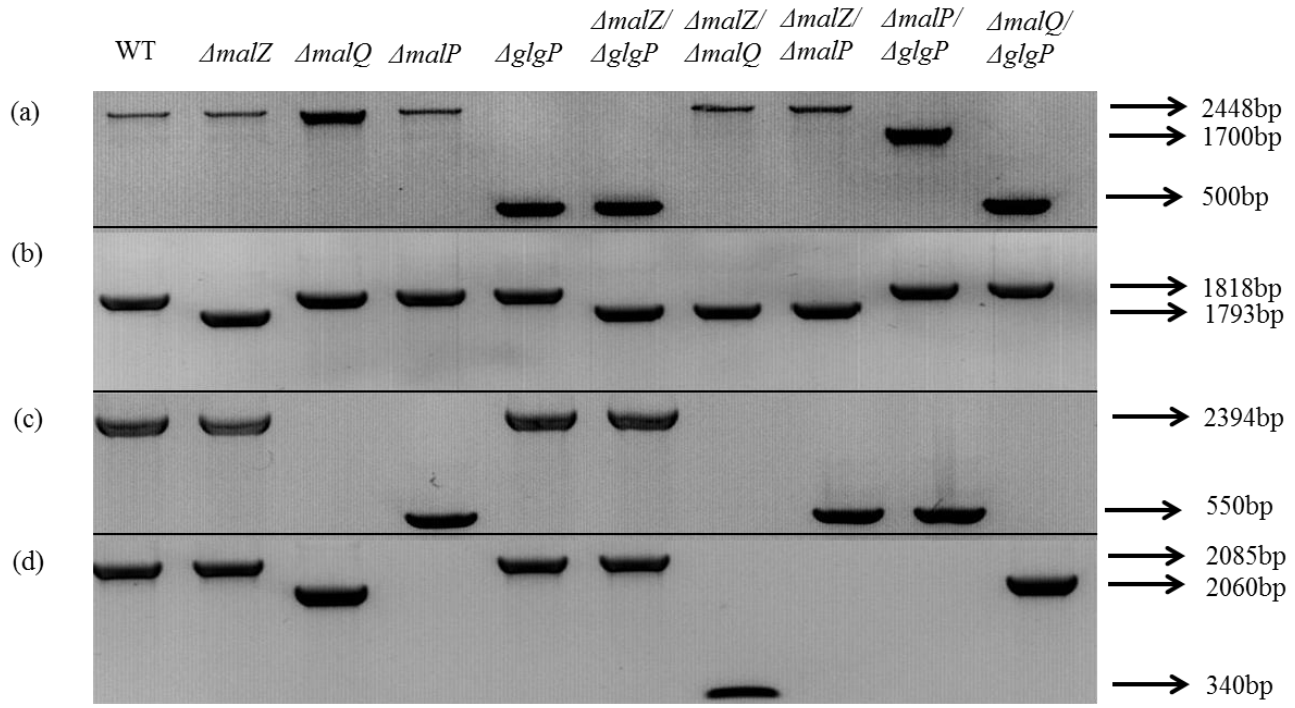



**Figure 2.1**. **PCR confirmation of constructed mutants.** (a) Strains amplified with *glgP* primers, 2488bp, 1700bp and 500bp represent glgP WT allele, *ΔglgP* (kan) and *ΔglgP* respectively (b) strains amplified using *malZ* primers, 1818bp and 1793bp represent *malZ* WT allele and *ΔmalZ* (kan) respectively, (c) strains amplified with *malP* primers, 2394bp and 550bp represent *malP* WT allele and *ΔmalP* respectively, (d) strains amplified with *malQ* primers, 2085bp, 2060bp and 240bp represents *malQ* WT allele, *ΔmalQ* (kan) and *ΔmalQ* respectively.

### 2.3.2 Enzyme activity assays

To confirm that the mutagenesis approach led to decrease in enzyme activities, enzyme assays or native gels were performed.

#### 2.3.2.1 Phosphorylase native gel

To visualize active MalP and GlgP enzymes a zymogram was used. Crude protein was extracted from all strains and separated in a non-denaturing gel containing glycogen and incubated in glucose-1-phosphate. Active glucan phosphorylases incorporate glucose-1-phosphate into linear glucan chains using glycogen as the primer. The linear chains stain blue when exposed to iodine, while the glycogen stains brown. Strains which are expected to contain active MalP and GlgP enzymes (WT, *ΔmalZ*, *ΔmalQ*, *ΔmalZ/malQ*), demonstrated three phosphorylase activity bands, one strong one at the top of the gel and two weaker ones below. MalP activity is represented by the highest band as it is lacking in all strains with a mutant *malP* allele. The other two are not present in strains with a mutant *glgP* allele and, therefore, represent GlgP activity (Fig 2.2). In addition to the bands representing MalP and GlgP activity, a clear band was also visualized in every strain, just underneath the MalP band.

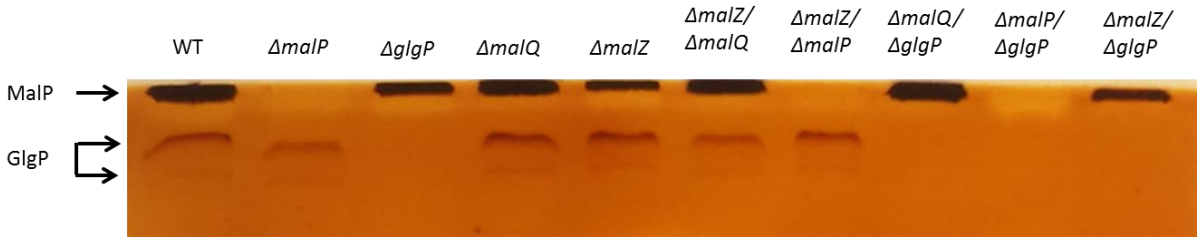



**Figure 2.2. Glucan phosphorylase activities in crude extracts of *E. coli* strains**. Non-denaturing native gel assay of various *E. coli* strains. Activity bands representative of MalP and GlgP enzyme activities are indicated by arrows.

#### 2.3.2.2 Amylomaltase enzyme activity

MalQ enzyme activity was assayed by examining the release of glucose from maltose. The WT activity measured was 6 µmol/min/µg while almost no activity was found in any strain containing a mutant *malQ* allele. No significant difference was seen between WT, *ΔmalZ, ΔglgP* and *ΔmalZ/*Δ*malP* strains. Some of the mutant strains that contain a wild-type malQ allele showed activities intermediate between the WT and Δ*malQ* mutant strains, specifically the *ΔmalP*, *ΔmalP/ΔglgP* and *ΔmalZ/ΔglgP* strains (Fig 2.3).

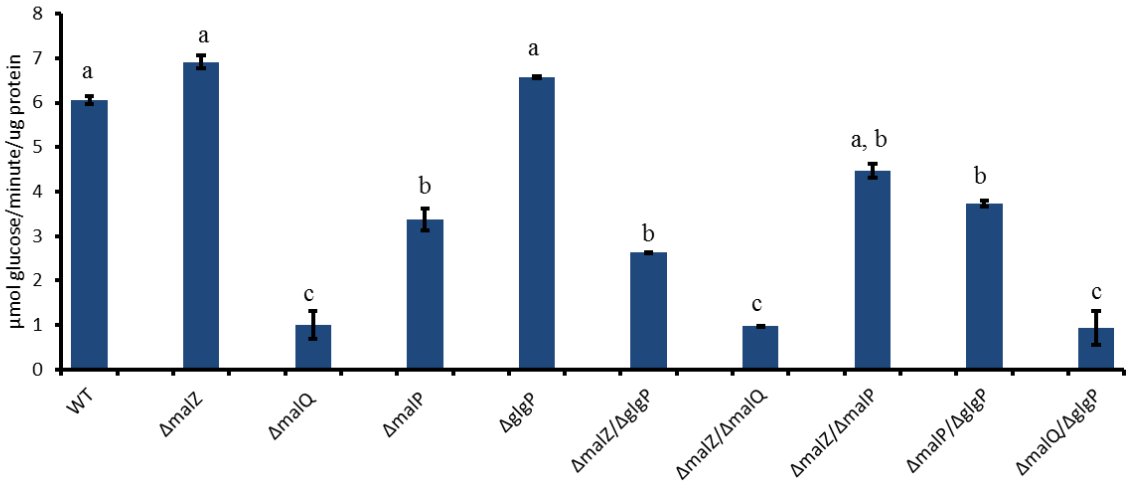



**Figure 2.3. Amylomaltase activity.** Glucose release form maltose was determined using crude protein extracted from each strain. Data represent means ± SEM of 3 biological replicates. Bars with the same letters are similar at the 5% level as determined by a Bonferroni-Holm test after a one way analysis of variance.

2.3.2.3 Maltodextrin glucosidase enzyme activity

MalZ activity was assayed using an artificial substrate, p-nitrophenol α-D-maltoside. Δ*malZ* mutant strains showed no release of *p*-nitrophenol and were, therefore significantly different to all strains containing a WT *malZ* allele (Fig 2.4). Some strains (Δ*malQ,* Δ*malP* and Δ*malQ*/Δ*glgP*) showed increased activity compared to the WT, while the Δ*glgP* strain was reduced in MalZ activity.

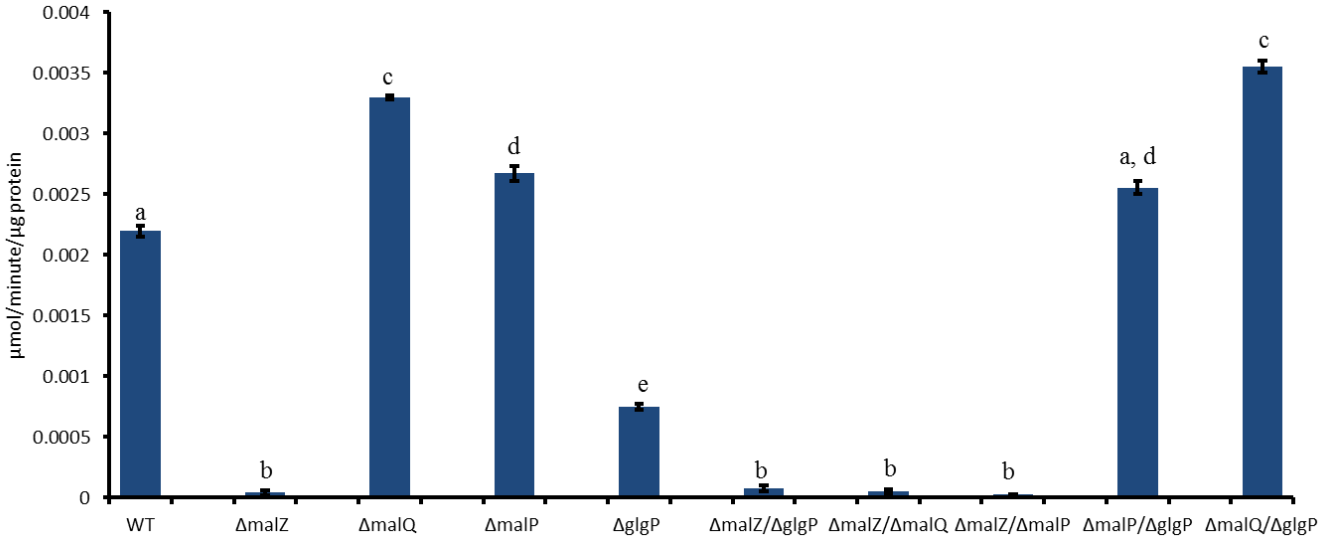



**Figure 2.4. Maltodextrin glucosidase activity.** Activity was determined by the release of *p*-nitrophenol from *p*-nitrophenol-α-D-maltoside using crude protein extract. Data represent means ± SEM of 3 biological replicates. Bars with the same letters are similar at the 5% level as determined by a Bonferroni-Holm test after a one way analysis of variance.

### 2.3.3 Semi-quantitative measurement of polyglucan accumulation

To determine the effect that mutations eliminating the targeted enzymes had on maltodextrin and glycogen metabolism the mutant strains were initially plated onto solid media containing either 1% (w/v) glucose or 1% (w/v) maltose. These were grown overnight and stained for polyglucans using iodine vapour. Brown staining is indicative of branched glycogen, while blue staining is indicative of maltodexrin accumulation (Roehl and Vinopal, 1979).

#### 2.3.3.1 Polyglucan staining on LB containing 1% (w/v) glucose

When grown on media supplemented with glucose, all strains stained brown. *ΔglgP* and *ΔmalQ* single mutants stained slightly darker brown than the WT strain, whilst *ΔmalP* and *ΔmalZ* mutant strains stained slightly lighter. All double mutants (*ΔmalZ/malQ, ΔmalZ/ΔglgP, ΔmalZ/ΔmalP, ΔmalP/ΔglgP and ΔmalQ/ΔglgP*) stained darker brown compared to the WT strain as well as the single mutants except *ΔmalQ* (Fig 2.5).

#### Polyglucan staining on LB containing 1% (w/v) maltose

When maltose was present in the media two of the mutants lacking *malQ* (*ΔmalQ* and *ΔmalQ/ΔglgP*) did not stain indicating no accumulation of polyglucans, while the *ΔmalZ/ΔmalQ* strain appeared to stain very slightly brown. All mutants lacking *malP* (*ΔmalP, ΔmalP/ΔglgP* and *ΔmalZ/ΔmalP)* stained dark blue indicating significant accumulation of linear maltodextrins. A dark brown phenotype was observed for WT and *ΔglgP* mutants, indicating glycogen accumulation while *ΔmalZ* and *ΔmalZ/ΔglgP* mutants stained, brown, but with less intensity than either the WT or Δ*glgP* strains. (Fig 2.5)

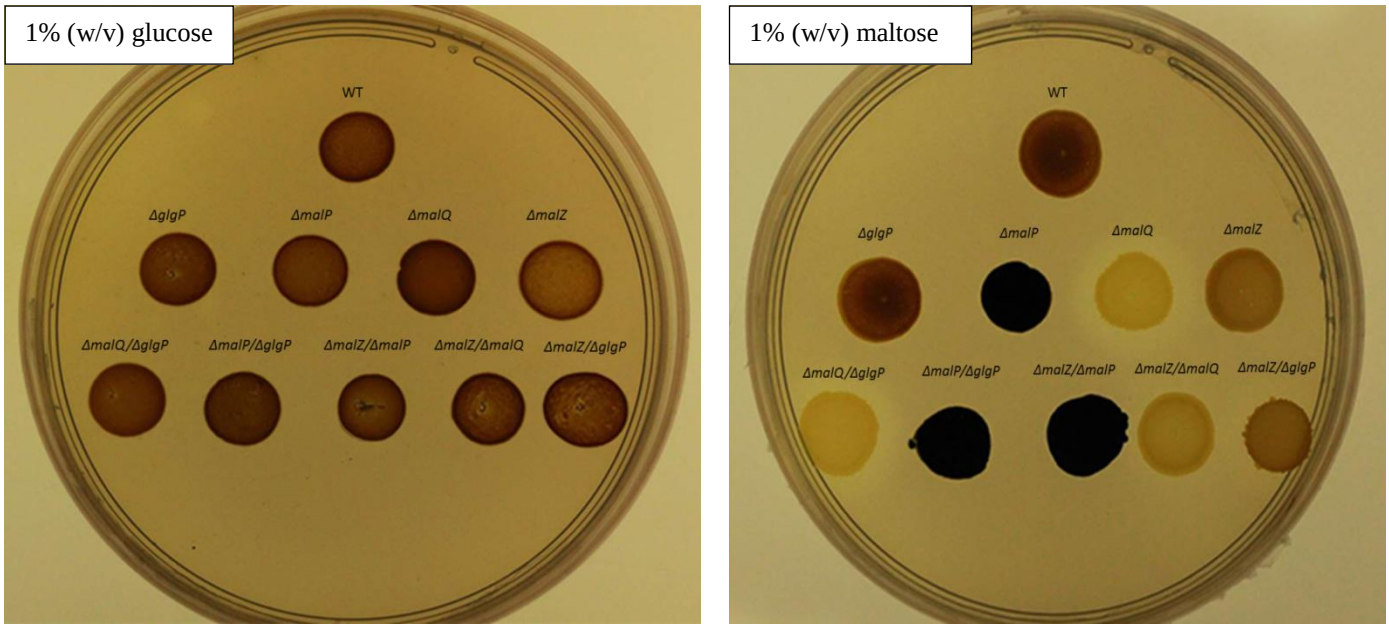



**Figure 2.5. Polyglucan staining**. Cells were grown on solid LB media containing either 1% (w/v) glucose or 1% (w/v) maltose before being stained with iodine vapour.

### 2.3.4 Growth properties of mutants

The growth properties of mutants, including the WT strain was measured by growth in rich media containing either 1% (w/v) glucose or 1% (w/v) maltose. $OD_{600}$ readings were taken at 4 hrs, 8 hrs, 12 hrs, 16 hrs, 24 hrs and 48 hrs.

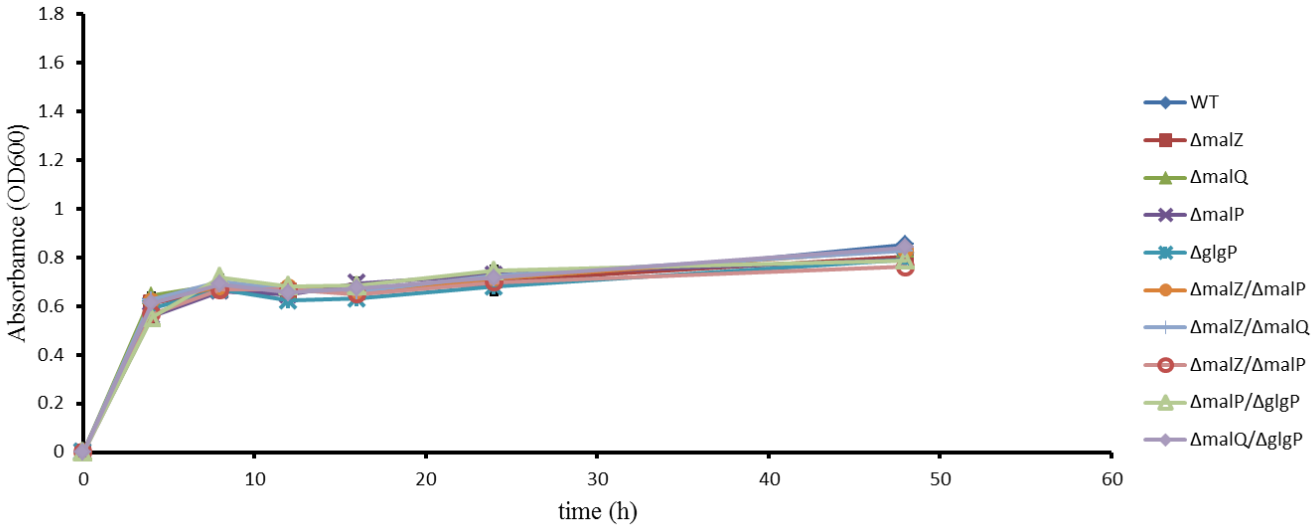
2.3.4.1 Growth properties of mutants in 1% (w/v) glucose

There were no significant differences in the growth properties of mutant strains to WT over a 48 hr period (Fig 2.6a)

2.3.4.2 Growth properties of mutants in 1% (w/v) maltose

All strains grew at the same rate for the first 4 hours but deviated significantly after that. The *ΔmalZ/ΔmalP* double mutant reached the highest $OD_{600}$ of approximately 1.6 after 48 hours, which was significantly ($p \leq 9.72^{-06}$) greater than any of the other strains. The Δ*malZ*/Δ*malQ* double mutant grew to a final $OD_{600}$ of approximately 1.2, which was significantly ($p \leq 0.0012$), lower than that of the Δ*malZ*/Δ*malP* double mutant at 24 hrs, but significantly greater ($p \leq 1.41^{-05}$) than all the other strains except Δ*malQ* and Δ*malP*. In those the $OD_{600}$ ranged between 0.8-1.0 and they did not differ significantly at any time point. Double mutants containing mutations in *malZ* and either *malQ*, *malP* or *glgP* remained significantly different to all strains at 8 hrs, 12 hrs, 16 hrs and 24 hr time points and show the highest $OD_{600}$. Growth of the Δ*malQ*/Δ*glgP* strain was significantly different at 8hrs, 12hrs and 24 hrs from all strains except Δ*malZ*, Δ*malQ* and Δ*glgP* (Fig 2.6b).

**a)**



**b)**

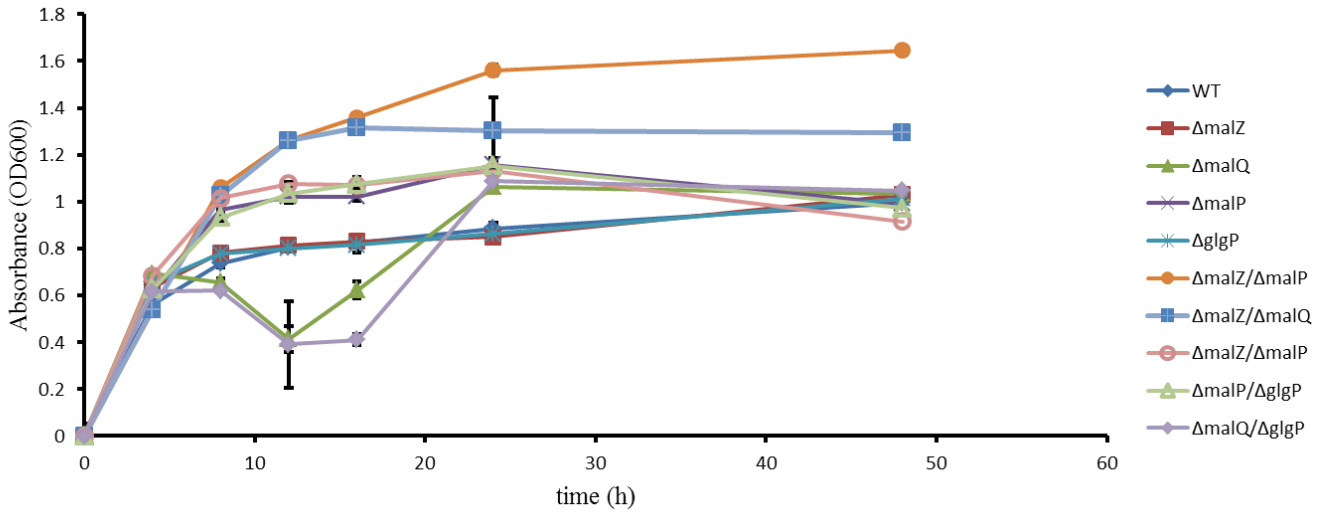



**Figure 2.6. Growth of *E. coli* strains with (a) glucose of (b) maltose.** $OD_{600}$ readings of all strains were determined at different time points over 48 hr when cells were grown in LB supplemented with 1% (w/v) maltose. Data represent means ± SEM of 3 biological replicates.

### 2.3.5 Polyglucan contents of strains grown in liquid media

Polyglucans (glycogen and maltodextrins) were measured in samples at different time points taken during the growth experiment. Cells grown in media supplemented with glucose should produce glycogen which is insoluble in 70% (v/v) ethanol. Therefore, for those samples polyglucan amounts were determined in the cell pellet only, not the 70% (v/v) ethanol extracts. Cells grown on maltose may produce linear polyglucans of various sizes. It is known that shorter chain maltodextrins with a degree of polymerisation of approximately 3-10 are soluble in 70% (v/v) ethanol (Balto et al., 2016) and, therefore, the polyglucan amounts in both the ethanol extract (soluble polyglucans) and pellets were determined for cultures grown in maltose.

#### 2.3.5.1 Cells grown with glucose supplementation

No significant differences in $OD_{340}$ were noted between strains in the first 16 hours of growth. At that point, glycogen contents ranged between approximately 0.002-0.03 µg glycogen/ µg protein. The most significant differences were only seen after 24 hr. At 24 hr only the *ΔmalP* mutant had a glycogen content that was significantly ($p \leq 5.19^{-05}$) different from the WT and this was also significantly ($p \leq 0.001$) greater than all the other mutant strains except *ΔmalP/ΔglgP* and *ΔmalZ/ΔmalP*. Strains with a mutation in *malQ* (*ΔmalQ, ΔmalZ/ΔmalQ, ΔmalQ/ΔglgP)* never showed any significant difference to WT, however *ΔmalQ/ΔglgP* and *ΔmalZ/ΔmalQ* exhibited significantly ($p \leq 6.64^{-05}$ and $p \leq 0.002$ respectively) lower glycogen contents compared with the *ΔmalP* mutant at 24 hours. *ΔmalZ* did not show any significant difference in glycogen content when compared to WT (Fig 2.7)

2.3.5.2 Cells grown with maltose supplementation

The cells of strains grown in media supplemented with maltose showed differences from early time points. All strains lacking *malP* (*ΔmalP, ΔmalZ/malP, and ΔmalP/glgP*) accumulated significantly ($p \leq 0.001$) increased polyglucans compared with all other strains at 4 hours after culture initiation. The amounts decreased over time until, after 16 hours, the difference between these strains and the others was not consistently significantly altered. Strains mutated in *malQ* (Δ*malQ,* Δ*malQ*/Δ*glgP*) accumulated very little polyglucan at all time points and were significantly reduced compared with all strains. However, the Δ*malZ*/Δ*malQ* strain accumulated more polyglucans than Δ*malQ* at 8 hours, 12 hours and 48 hours ($p \leq 0.0015$, $p \leq 4.09^{-05}$, $p \leq 0.004$) and more polyglucans than Δ*malQ*/Δ*glgP* at 8 hours, 12 hours and 16 hours ($p \leq 0.002$, $p \leq 5.12^{-05}$, $p \leq 0.003$). Interestingly, while the Δ*malZ* and Δ*glgP* strains accumulated almost identical amounts of polyglucan to the WT at all time points, the Δ*malZ*/Δ*glgP* double mutant accumulated more glycogen than WT at 16 hours ($p \leq 0.0025$) and more than Δ*glgP* and Δ*malZ* at 8 hours and 24 hours ($p \leq 0.0015$, $p \leq 0.0011$ ) (Fig 2.8).

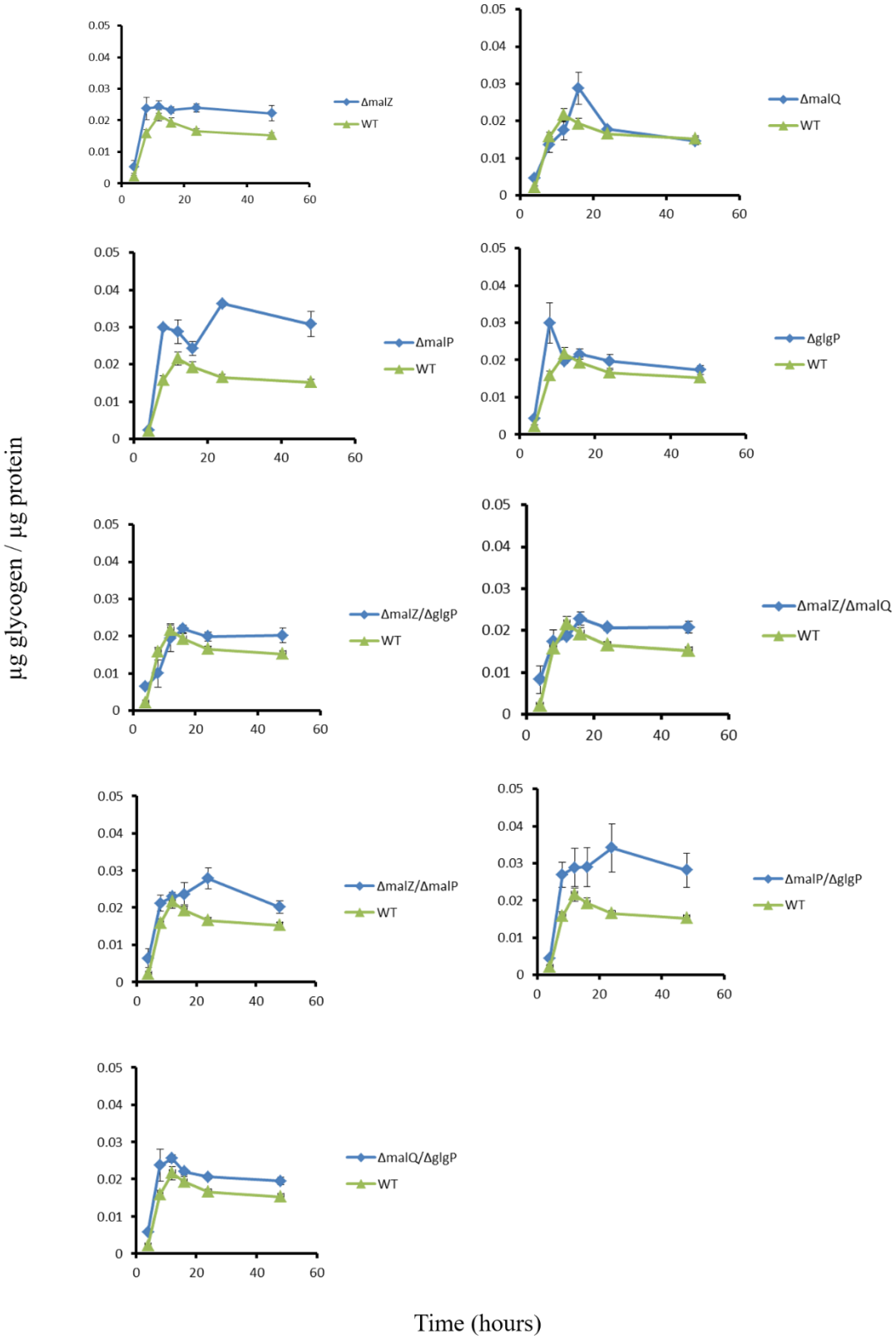



**Figure 2.7. Polyglucan content of strains with glucose supplementation**. Amounts of glycogen accumulating in strains grown in media supplemented with 1% (w/v) glucose. Data represent means ± SEM of 3 biological replicates.

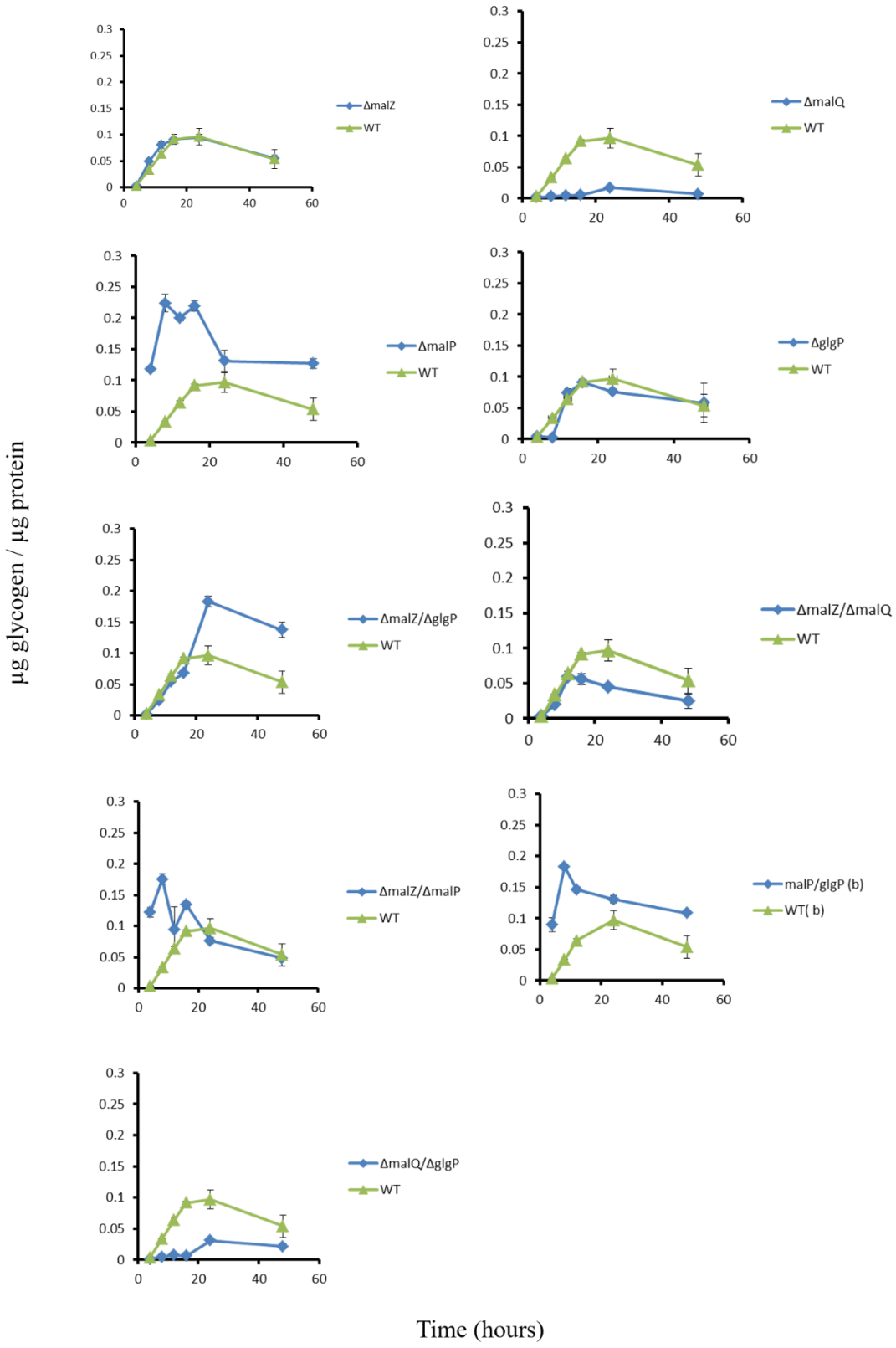



**Figure 2.8. Polyglucan content of strains with maltose supplementation.** Amounts of polyglucan present in strains grown in media supplemented with 1% (w/v) maltose. All single and double mutants are compared to polyglucan contents of the WT strain. Data represent means ± SEM of 3 biological replicates.

2.3.5.3 Soluble maltodextrin content of strains grown with maltose supplementation

To examine the accumulation of short chain polyglucans in the strains, the 70% (v/v) ethanol extract was assayed for polyglucan content. There was a great deal of variation during the time course that could be due to maltose contamination from media residue. Although there were some significant differences at a number of time points, these were not consistent between strains and therefore no conclusions were made from this data.

**Scanning electron imaging**

All strains were imaged by scanning electron microscopy after 16-18 hours of incubation in liquid media supplemented with either 1% (w/v) glucose or 1% (w/v) maltose. When strains were grown in media containing glucose, cells from *ΔmalZ*, *ΔglgP, ΔmalZ/ΔmalQ*, *ΔmalZ/ΔglgP, ΔmalZ/ΔmalP* and *ΔmalQ/ΔglgP* did not show any phenotypic differences compared with WT. Samples from *ΔmalP*, *ΔmalQ,* and *ΔmalP/ΔglgP* strains, however, contained a few elongated cells (Fig 2.9).

When strains were grown in media containing maltose, the *ΔmalZ, ΔmalZ/ΔglgP* and *ΔmalZ/ΔmalQ* strains were phenotypically identical to each other and the WT strain. The *ΔglgP* mutant showed a small number of elongated cells. Imaging of *ΔmalP, ΔmalQ, ΔmalZ/ΔmalP, ΔmalP/ΔglgP, ΔmalQ/ΔglgP* showed cells which appeared morphologically distinct form the other strains, appearing either to be split, or to have an altered cell wall morphology containing many invaginations (Fig 2.10). These morphological differences were noted in three separate experiments.

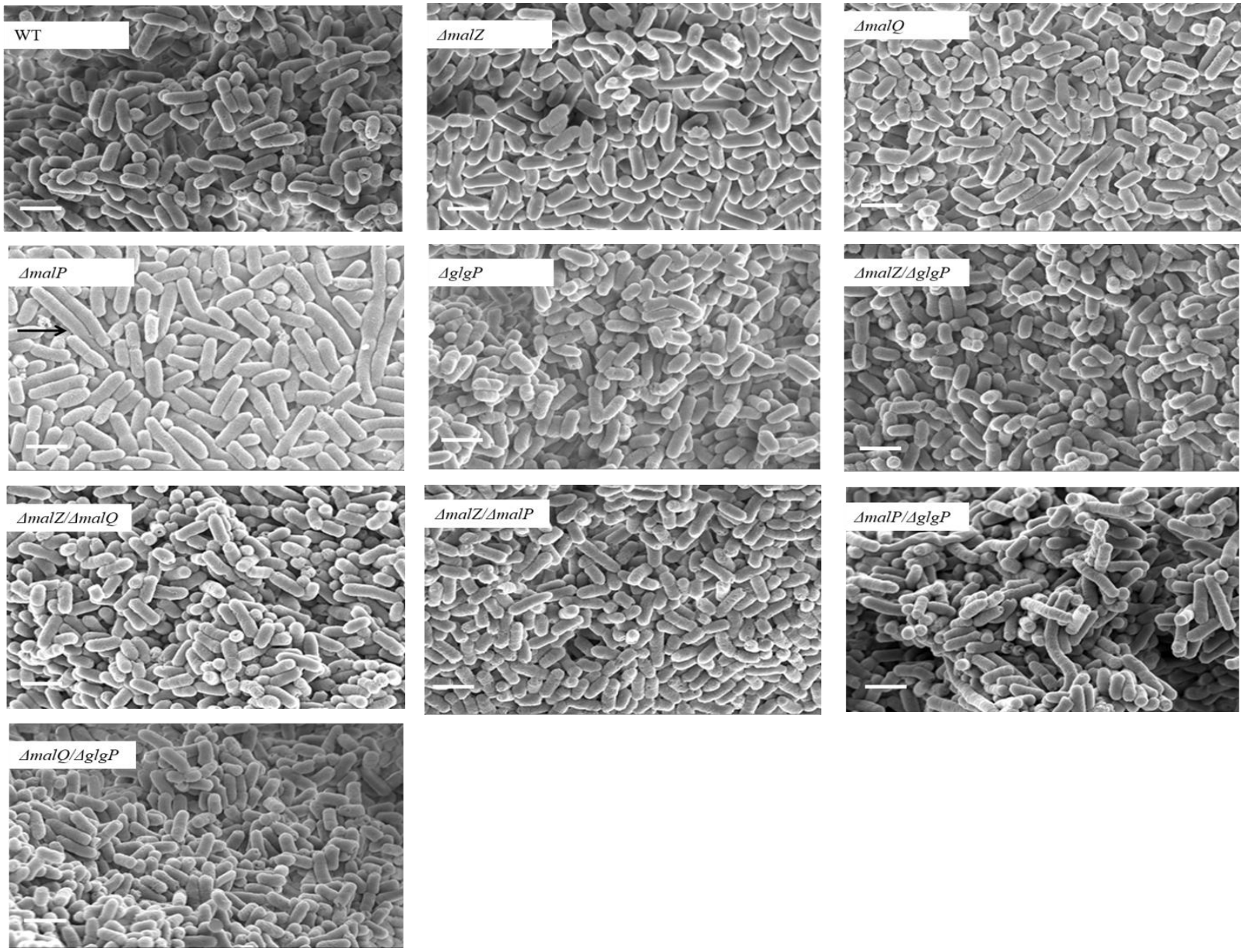



**Figure 2.9. Scanning electron microscopy**. Cells were grown in LB media supplemented with 1% (w/v) glucose for 16 h before being prepared for imaging. Scale: 1cm= 1µm. Black and white arrows indicate elongated cells.

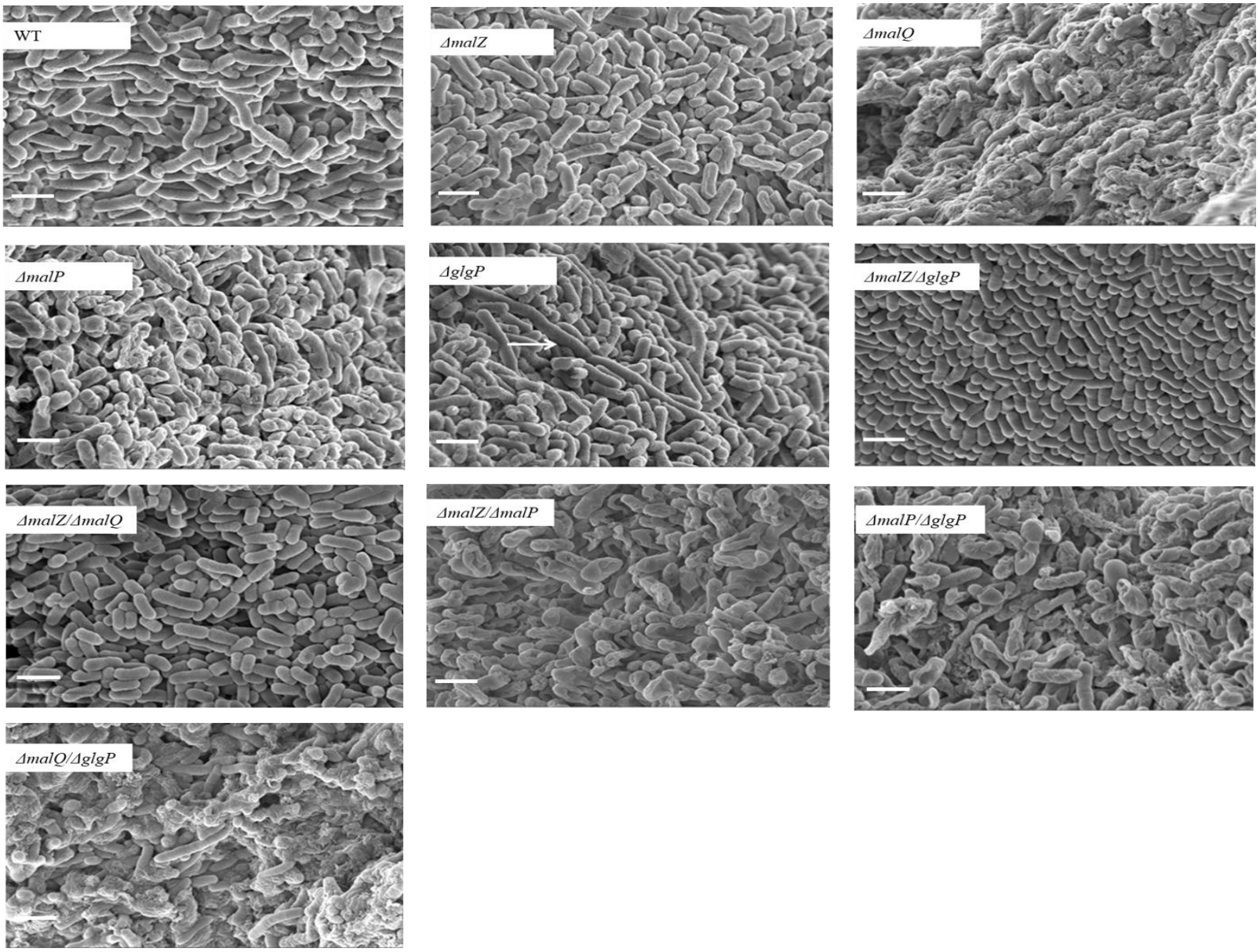



**Figure 2.10 Scanning electron microscopy.** Cells were grown in LB media supplemented with 1% (w/v) maltose for 16h before being prepared for imaging. Scale: 1cm= 1µm. Black and white arrows indicate elongated cells.

## 2.4 Discussion

This study has sought to elucidate the functions of four enzymes (MalP, MalQ, MalZ and GlgP) in maltodextrin and glycogen metabolism in *E. coli*, as well as examining their roles in interconnecting these two pathways. To do this, a series of *E. coli* mutants were constructed where combinations of the genes encoding these enzymes were deleted from the chromosome. These mutants were analysed by examination of polyglucan content, growth properties and cell morphology under growth conditions where either glucose or maltose was present.

### 2.4.1 Construction of double mutants

A series of double mutants lacking *malP*, *malQ*, *malZ* and *glgP* were created (Fig 2.1). Unfortunately it proved impossible to construct a *ΔmalP/ΔmalQ* double mutant strain due to the close proximity of the loci of these two genes. P1 *vir* phage transduction packages approximately 100 kb segments of bacterial gDNA so, if genes are adjacent to each other, it is likely that they will be co-transduced (Thomason et al., 2007). In this way recipient strains containing a mutation in one gene (e.g. Δ*malP)* are likely to have that mutation replaced by the WT allele during transduction with phage lysates from a donor strain, where the second mutation (e.g. Δ*malQ*) lies close by in the genome. To overcome this, the double mutant strain was ordered to be constructed by a commercial service, but they have also been unable to manufacture this in time to complete the project and so it was decided to proceed without it. It seems unlikely that the reason for the lack of progress for construction of this double mutant is that it is lethal, as another group has constructed it previously (Jones et al., 2008), albeit in a different genetic background.

We assayed the enzymatic activities of the products of all genes mutated in all strains to ensure that the mutations resulted in the loss of activity. A native gel examining glucan phosphorylase activities demonstrated three bands (Fig 2.2). Although only one activity band was found that represents MalP, there were two that were the result of GlgP. It is tempting to speculate that the two GlgP bands are the result of differential binding to Hpr. This is known to affect its affinity for glycogen and has been shown to cause structural changes in GlgP (Seok et al., 2001).

In addition to the bands seen for GlgP and MalP activities, a clear band which migrated just below the MalP activity was noted. This band is likely due to the activity of an enzyme which is able to hydrolyse glycogen. One candidate for this would be GlgX, the glycogen debranching enzyme; however, this cannot be the case as the same activity was noted in Δ*glgX* mutants (Strydom et al., 2017). Other possibilities would be AmyA or MalS, the two known α-amylases in *E. coli*; however, both of these have been demonstrated to have very little activity on glycogen as a substrate (Raha et al., 1992; Schneider et al., 1991). Mutants lacking these two proteins could be used to examine whether or not they are responsible for this activity.

Amylomaltase activity varied between the strains and the amount of activity is similar to MalQ activity noted previously (Weismeyer and Cohn, 1960). As expected, cells containing a mutant *malQ* allele showed significantly lower amounts of activity than all others (Fig 2.3), however, there was also significantly reduced activity in *ΔmalP*, *ΔmalP/ΔglgP* and *ΔmalZ/ΔglgP* mutant strains. MalQ not only releases glucose from maltose, but also synthesizes longer maltodextrins, which are substrates that can be utilized by GlgP, MalP and MalZ (Boos and Shuman, 1998; Dippel and Boos, 2005; Palmer et al., 1976; Park et al., 2011; Pugsley and Dubreil, 1988). Therefore, the activity seen in WT extracts may be a combination of MalQ's activity on maltose to release glucose, as well as the products of the activities of the other enzymes acting on longer maltodextrins, that have been synthesized by MalQ, and would help explain the reduced MalQ activity in mutant strains lacking those enzymes. MalP has a higher affinity for maltodextrins (Schwartz and Hofnung, 1967) than GlgP (Hu and Gold, 1975). Therefore, extracts from cells mutated in *malP* should produce less glucose than those from cells mutated in *glgP*. This is indicated by the observation that *ΔglgP* shows a higher amylomaltase activity than *ΔmalP* and *ΔmalP/ΔglgP*. MalZ would release glucose from the longer maltodextrins produced by MalQ, while GlgP and MalP would release glucose-1-phosphate. It could be argued that, as glucose was the sugar measured in the assay, the products of the reactions catalysed by MalP and GlgP should not contribute towards activity. However, a periplasmic glucose-1-phosphate phosphatase exists in *E. coli* (Pradel and Bouquet, 1988) which would convert any G1P released to glucose.

Maltodextrin glucosidase activity was assayed by measuring the release of *p*-nitrophenol from *p*-nitrophenol-α-D-maltoside. MalZ typically hydrolyzes α-1, 4-glucosyl residues from the reducing ends of substrates and is capable of hydrolyzing this artificial substrate (Tapio et al., 1991). The activity observed in the WT strain is consistent with previous data and the specificity of this substrate in *E. coli* is demonstrated by the lack of activity in any mutants containing a mutant *malZ* allele (Fig 2.4). *ΔmalQ* and *ΔmalQ/ΔglgP* mutant strains showed increased activity compared with WT. Mutations in *malQ* lead to increased accumulation of the products of glycogen degradation (maltotriose and maltotetraose) which induce expression of *malP* and *malZ* (Dippel and Boos, 2005) which may help explain this. On the other hand, mutations in *malP* are known to decrease endogenous induction. The observation that the *ΔmalP* mutant strain also showed increased MalZ activity is unlikely to be due to increased expression of *malZ* and is most probably due, therefore, to biological variation.

Mutations in *glgP* are known to increase the average chain length within glycogen (Alonso-Casajús et al., 2006), making it a worse substrate for GlgX. This could lead to reduced release of maltotetraose and maltotriose by GlgX, resulting in reduced induction of *malT* and, consequently, *malZ* and may help explain why MalZ activity was significantly reduced in a *ΔglgP* mutant strain.

### 2.4.2 The effects of deletions in *malZ, malP, malQ* and *glgP* on *E. coli* grown in media containing glucose.

No significant differences were seen in the growth properties of all strains grown in rich media supplemented with 1% (w/v) glucose (Fig 2.6a) demonstrating that, when glucose is present in high concentrations the mutations do not alter growth. Although some studies have indicated that the *mal* system should be repressed under these conditions (Chapon, 1982), more recent data has demonstrated that *mal* genes are expressed in the presence of glucose due to glycogen derived endogenous induction (Boos and Shuman, 1998; Decker et al., 1993; Dippel et al., 2005; Raibaud and Richet, 1987). Indeed, all the enzymes mutated in this study are thought to be directly involved in glycogen degradation by helping to mobilize the products of GlgX (Dippel and Boos, 2005). To gain a better understanding of the effect that the mutations have on glycogen metabolism, the polyglucan contents of strains were assessed both semi-quantitatively on solid media as well as measured in liquid culture.

All strains stained brown with iodine when grown on solid media containing glucose, indicating that they accumulate glycogen (Fig 2.5). Although the structure of polyglucans accumulating in strains, grown in liquid culture, were not determined, it is assumed to be glycogen as this has been demonstrated in many other studies (Alonso-Casajús et al., 2006; Dauvillée et al., 2005; Strydom et al., 2017 ).

Glycogen appeared to be increased in some of the mutant strains, which is in agreement with previous studies which indicate that the process of glycogen degradation may be affected by additional enzymes such as MalP, MalQ and MalZ (Park et al., 2011; Strydom et al., 2017). It was observed that there were differences in glycogen accumulation when strains lacking MalP, MalQ, MalZ or GlgP, depending on whether they were plated on solid media or cultured in liquid media; most likely due to the differences in growth conditions.

GlgP has previously been demonstrated to play a role in the degradation of glycogen by phosphorolytic cleavage of glycosidic bonds on glycogen outer chains (Alonso-Casajús et al., 2006; Strydom et al., 2017). Strains mutated in *glgP* would, therefore, be expected to stain darker brown when exposed to iodine vapour due to increased accumulation of glycogen, indeed, all mutants containing a Δ*glgP* allele (*ΔglgP, ΔmalP/ΔglgP, ΔmalZ/ΔglgP, ΔmalQ/ΔglgP*) demonstrated increased glycogen accumulation both in cells grown in liquid culture as well as on solid media. Interestingly, *ΔmalP/ΔglgP*, *ΔmalQ/ΔglgP* and *ΔmalZ/ΔglgP* strains stained darker brown than both WT and *ΔglgP* mutant strains on solid media, indicating that deletions in *malP*, *malQ* and *malZ* affect glycogen synthesis or degradation.

MalP is thought to phosphorolytically cleave maltodextrins produced by GlgX (Boos and Shuman, 1998). Its elimination should, therefore, lead to increased accumulation of maltodextrins during periods of glycogen degradation and feedback inhibition of that process. The data from this study indicate that this

hypothesis is true as strains containing a mutation in *malP* (*ΔmalP, ΔmalZ/ΔmalP*, *ΔmalP/ΔglgP*) show increased glycogen accumulation both on solid and in liquid media. Although MalZ is also capable of removing those substrates, if it does so *in vivo*, it is clearly not as effective as MalP since *ΔmalZ* mutants appear to show decreased glycogen accumulation on solid media, but no significant difference to WT in liquid culture.

SEM imaging of *ΔmalP* and *ΔmalP/ΔglgP* strains showed that they contained a small number of elongated cells when compared to the WT strain, with the *ΔmalP/ΔglgP* strain demonstrating many more elongated cells than *ΔmalP* (Fig 2.9). As this occurred in mutants lacking MalP, it might be thought that this is caused by increased accumulation of maltodextrins produced by GlgX resulting in feedback inhibition of glycogen degradation. Recent data from another study indicates that is, however, unlikely to be the case. Strydom et al. (2016) observed that both *ΔmalP/ΔglgP* and *ΔmalP/ΔglgP/glgX* strains accumulated elongated cells with the triple mutant producing cultures where almost all cells were extremely elongated. As the triple mutant is unable to produce maltodextrins from glycogen due to the lack of GlgX, the reason for the change in cell length must be different. It is possible perhaps that the accumulation of glycogen has some effect on the ability of these cells to divide, but this needs to be examined in more detail.

During glycogen degradation it has been hypothesized (Dippel and Boos, 2005) that cells containing MalQ would synthesise longer chain maltodextrins from those produced by GlgX and that these are mobilized by MalP and/or MalZ. Lack of MalQ would therefore be expected to lead to the accumulation of maltotriose and maltotetraose and lead to feedback inhibition of glycogen degradation. Strains containing mutations in *malQ* (*ΔmalQ, ΔmalZ/ΔmalQ, ΔmalQ/ΔglgP)* stained darker brown than WT on solid media, providing support for this hypothesis. To provide more evidence, the *ΔmalP/ΔmalQ* and Δ*malZ*/Δ*malQ* mutants could be examined to see if it accumulates increased amounts of maltodextrins, alongside a more extreme inhibition of glycogen degradation. Unfortunately, the construction of the Δ*malP*/Δ*malQ* strain was not possible and this will have to be a target for future work. SEM imaging of *ΔmalQ* showed a small number of elongated cells. As this was seen only in the Δ*malQ* mutant, and not any of the double mutant combinations that include the Δ*malQ* allele, the most likely explanation for this is that it was caused by biological variation.

MalZ is known to be capable of hydrolysing maltodextrins with a minimum chain length of maltotriose *in vitro.* It has been proposed that it cleaves the maltodextrins polymerized by MalQ, curbing the length of these glucan chains and releasing glucose in the process (Tapio et al., 1991); although this has never been demonstrated conclusively *in vivo*. Park et al. (2011) observed that a WT strain overexpressing MalZ showed a 33% decrease in glycogen when it was grown in media containing glucose which indicates that MalZ can affect glycogen metabolism, but the reason for this is unclear. It is unlikely that MalZ acts on

glycogen directly, as it only degrades glucans at the reducing end and glycogen only has one reducing end. The observations in this study indicate that MalZ affects glycogen accumulation, although it is not clear if this is due to altered synthesis or degradation of glycogen, based on the observation that both *ΔmalZ/glgP* and *ΔmalZ/malP* double mutants stain darker than either the Δ*glgP* or *ΔmalP* single mutants, respectively. This demonstrates that a mutation in *malZ* affects glycogen metabolism in a background where either *malP* or *glgP* have already been mutated. One explanation for these observations is that MalZ may decrease the amount of glycogen initiation by GlgA through degradation of the reducing ends, of the initial α1, 4 linked chains manufactured by this enzyme. This should cause a decrease in glycogen synthesis and, therefore, in its absence glycogen accumulation would be expected to increase.

### 2.4.3 The effect of deletions in *malZ, malP, malQ* and *glgP* on *E. coli* grown in media containing maltose.

The presence of maltose should fully induce the *mal* system and lead to a higher expression of the enzymes encoded by *malZ, malP* and *malQ*. In addition, when maltose is the carbon source, glycogen synthesis is also possible via the formation of long chain maltodextrins by MalQ, which can be used as substrate for GlgB, in addition to the glucan chains synthesised by GlgA (Park et al., 2011). Interestingly, the amount of polyglucans accumulating in cells grown on maltose was approximately five fold greater than when the same cells were grown on glucose (Figs 5.1 and 5.2) as has been observed previously. The physiological reason for an increase in polyglucan content under maltose supplementation is unclear. It is possible that accumulation of soluble sugars in the cytoplasm is detrimental to the bacterium and, the maltose needs to be removed. Unlike growth on glucose, where there are several pathways that can be used to metabolise that sugar directly (e.g. glycolysis), the only pathway that can mobilise maltose involves polymerising it to linear maltodextrins by MalQ. The potential detrimental effect of maltose accumulation in *E. coli* will be discussed in more detail below.

In the presence of maltose, the growth of *ΔmalZ/ΔglgP* and *ΔmalZ/ΔmalQ* mutants differed significantly from the other strains leading them to reach a higher $OD_{600}$ (Fig 2.6b), but the reason for this difference is not obvious. $OD_{600}$ does not differentiate between live and dead cells in a culture, so it is possible that some of the strains which did not reach such a high $OD_{600}$ were detrimentally affected by the presence of high concentrations of maltose, while the *ΔmalZ/ΔglgP* and *ΔmalZ/ΔmalQ* strains are not, and that this would only be noted by examining the number of live cells in a culture. As a mutation in *malZ* is present in both these strains it could also be argued that the lack of MalZ is a factor in the increased $OD_{600}$. However, one of the strains is the Δ*malZ*/Δ*malQ* double mutant that is unable to synthesise substrate for MalZ, as it also lacks MalQ, and so it is difficult to understand how the lack of MalZ activity could affect metabolism and growth in this mutant. On saying that, this particular double mutant appeared to accumulate some polyglucans, as shown both by the appearance of slightly brown colonies when it was

grown on solid media and stained with iodine (Fig 2.5) as well as when polyglucans were measured in cells grown in liquid culture (Fig 2.8).Therefore this strain must be metabolising maltose and producing polyglucans. The reason for this is unclear and will be discussed below.

When strains lacking *malQ* were grown with maltose supplementation, no polyglucans should be synthesised and these strains would be expected to accumulate maltose (Szmelcman et al., 1976). Neither, *ΔmalQ* nor Δ*malQ*/Δ*glgP* strains stained when exposed to iodine vapour (Fig 2.5) and both also show very low polyglucan levels that were significantly reduced compared with the WT strain (Fig 2.8). Intriguingly, as was stated above, the *ΔmalZ*/*ΔmalQ* double mutant appeared to accumulate some maltodextrins or branched polyglucans. It stains very slightly brown with iodine (Fig 2.5) which could be due to the presence of either glycogen or linear maltodextrins of a DP of approximately 10-30 (Banks and Greenwood, 1975), and also contains significantly increased amounts of insoluble polyglucan compared with either of the other two strains mutated in *malQ* (Fig 2.8). It is, however, difficult to understand the mechanism whereby this strain could accumulate polyglucans when their growth media is supplemented with maltose.

Unlike the other strains mutated in *malQ*, cells from the *ΔmalZ*/*ΔmalQ* strain showed no morphological difference to WT (Fig 2.10). Strains containing mutations in *malQ* appeared to have lysed, either before, or during their preparation for electron microscopy. This observation, alongside alterations in growth and polyglucans accumulation, suggests that the Δ*malZ*/Δ*malQ* double mutant strain differs in some way from the strains containing mutations in *malQ*. In order to formulate a more informed hypothesis as to why that is, analysis of the polyglucans accumulating in this particular mutant needs to be conducted to elucidate whether maltodextrins, glycogen or both accumulates. Knowledge of the type of polyglucan present will allow a more informed investigation into their method of biosynthesis.

Although GlgP is known to function in the degradation of glycogen when *E. coli* cells are grown on glucose (Alonso-Casajús et al., 2006), its role in glycogen degradation in cells grown on maltose has not been examined. MalZ is known to be partly responsible for the degradation of maltodextrins (Tapio et al., 1991), alongside MalQ and MalP (Dippel and Boos, 2005). Analysis of single mutants lacking only GlgP or MalZ grown on maltose indicated that neither GlgP nor MalZ strongly influence glycogen metabolism as both the amounts of polyglucans measured in liquid cultures (Fig 2.8) as well as the degree of staining on solid plates (Fig 2.5) were similar to WT. Unexpectedly, the *ΔmalZ*/*ΔglgP* strain stained darker brown than either of the respective single mutants on solid media and accumulated nearly double the amount of polyglucans in liquid culture when compared to WT. It is likely that this is due to functional redundancy between GlgP and MalZ in some process since increased polyglucan accumulation is only observed when neither is present. It could be, that they both influence the length of chains synthesised by MalQ, potentially altering the affinity of GlgB to them, but could also be during glycogen catabolism.

Comparison of the amounts and lengths of malto-oligosaccharides that accumulate during times of active glycogen synthesis or degradation in the single and double mutants would help elucidate their roles. Interestingly, SEM imaging of *ΔglgP* cultures showed some elongated cells, if this is caused by the accumulation of polyglucans, then the same morphology should be seen for *ΔmalZ/ΔglgP*, which shows a greater polyglucan accumulation in liquid culture and on solid media. Since the morphology of *ΔmalZ/ΔglgP* is similar to the WT strain, the elongated cells observed for *ΔglgP* must be due to factors unrelated to the accumulation of polyglucans in this strain, or the additional deletion of *malZ* causes the polyglucans accumulating in a *ΔglgP* strain to be different to those accumulating in the *ΔmalZ/ΔglgP* strain.

The known physiological function of MalP is to phosphorolytically cleave linear chains produced by MalQ from maltose and longer chain maltodextrins (Schinzel and Nidetsky, 1999). In its absence *E. coli* strains accumulate large amounts of linear dextrins (Boos and Shuman, 1998) as was shown by the observation that strains mutated in *malP* (*ΔmalP, ΔmalP/ΔglgP, ΔmalZ/ΔmalP)* stained dark blue when exposed to iodine (Fig 2.5). High amounts of polyglucan accumulation were also observed at early growth stages in liquid culture (Fig 2.8). The observation that *malP* mutants stains blue, while *malZ* and *glgP* mutants stain brown, highlights the important role that MalP has in controlling the size of MalQ produced linear maltodextrins in comparison with MalZ or GlgP. It is possible that MalZ and/or GlgP can affect the lengths of chains that accumulate in the Δ*malP* mutant, but further evidence is needed to examine this, for example by examination of the chain lengths that accumulate in the Δ*malP*/Δ*glgP* and Δ*malP*/Δ*malZ* double mutants.

SEM imaging of all strains containing mutations in *malP* also showed a distinctly different morphology to WT (Fig 2.10). The cell wall appears to be disrupted having a number of invaginations, but the reasons for this are unknown. They are clearly caused by the presence of maltose as they were not noticed when these strains were grown in media containing glucose (Fig 2.6b). Unfortunately, the determination of maltose and soluble maltodextrin accumulation in the strains showed too much variation to allow any conclusions to be drawn. It is possible that accumulation of long chain maltodextrins affects the cells internal osmotic pressure, or that growth in maltose causes cells from these strains to react differently to the preparation used for imaging. Future work to clarify this could be to express an enzyme which is able to degrade maltose (such as maltase from yeast), in order to eliminate the accumulation of maltose in the cytoplasm of strains mutated in *malQ*. This would also prevent the synthesis of long chain maltodextrins. Alternatively, a different preparation method could be used for imaging of cells grown in media containing maltose.

## Conclusion

In conclusion, the research presented here demonstrates the involvement of the *mal* enzymes; MalP, MalQ, MalZ as well as GlgP in both glycogen and maltodextrin metabolism. In particular, the identification of a new physiological role for MalZ in maltodextrin metabolism and glycogen degradation is implied based on observations made for polyglucan accumulation in a *ΔmalZ/ΔglgP* strain when grown on media containing maltose. This strain accumulated significantly more glycogen than WT whilst *ΔmalZ* and *ΔglgP* single mutant strains did not; in addition, this strain stained brown, indicating accumulation of glycogen. This observation must be caused by both GlgP and MalZ affecting either the synthesis or degradation of glycogen when it is manufactured by glucans produced by MalQ. Future work would involve examining the structure of polyglucans accumulating in a *ΔmalZ/ΔglgP* strain, compared to glycogen accumulating in *ΔmalZ* and *ΔglgP* strains, to determine whether MalZ is able to affect glycogen content via degradation of the glycogen molecule, or if MalZ causes a decrease in glycogen content by affecting its synthesis. Future work could involve flux analysis using radioactive pulse chase experiments to examine the velocity of glycogen synthesis and degradation in this mutant.

A role of MalZ in maltodextrin metabolism is also implied based on the observation of polyglucan accumulation within cells of a *ΔmalZ/ΔmalQ* double mutant strain, which should be incapable of doing so due to the lack of MalQ. This strain differed to all other strains containing mutations in *malQ* as cells from it showed increased cell density, and polyglucan accumulation in liquid culture and no morphological difference to WT. It is currently believed that the metabolism of maltose in *E. coli* is only possible when MalQ is present, but since polyglucans accumulated in a strain lacking *malQ*, it can be proposed that another, as yet unknown enzyme is able to utilize maltose in a reaction which leads to the synthesis of polyglucans. This may, for example, be the enzyme measured by Decker et al. (1993) which synthesises maltose and longer chain maltodextrins using glucose 1-phosphate, but a completely novel activity cannot be ruled out. Analysing the type of polyglucans accumulating in this strain, to elucidate whether they are maltodextrins or glycogen would help in the identification of enzymes which are involved in the production of these polyglucans.

The cell morphology of strains investigated in this study was examined using a scanning electron microscope. The role of polyglucans such as glycogen and maltodextrins in cell morphology is still unclear. The observations here would suggest, based on growth in glucose, that the accumulation of glycogen is not the only possible factor influencing cell size and morphology. Cells which differ in morphology to WT were found both in strains that accumulate large amounts of glycogen as well as those which do not. The presence of elongated cells was observed in specific strains grown in media containing glucose. The reason for this is unclear, but recently Hill et al. (2013) identified a mechanism where nutrient availability affected *E. coli* cell size. They hypothesised that high concentrations of UDP-glucose

cause a structural change in the glucosyltransferase, OpgH, which allows it to bind and inhibit FtZ, a cell division protein, resulting in delayed cell division. Glucose-1-phosphate in the presence of UTP can be converted to UDP-glucose by phosphoglucomutase. Thus, strains which produce less G1P, such as those deficient in phosphorylases like GlgP and MalP should, contain reduced levels of UDP-glucose and should not be hindered in cell division. Since elongated cells were observed for strains lacking *malP* and *glgP*, this mechanism cannot explain the morphology or the impact of maltodextrins or glycogen on cell size seen in this study. Recently, Monahan et al. (2014) showed that glycolysis and cell division were linked in *Bacillus subtilis*. They described a mechanism which suggests that a decrease in glycolysis should lead to a decrease in cell division and, therefore, elongated cells. A decrease in intermediates for glycolysis is expected in strains which produce less G1P, such as the *ΔmalP, ΔglgP* and *ΔmalP/ΔglgP* mutants examined in this study and could thus explain the cell morphology observed for these strains. Clearly more research needs to be done to examine this, perhaps by introducing mutations in genes known to affect *E. coli* cell division, or by examining proteins involved in that process using specific antibodies.

Cells from some strains grown in maltose demonstrated altered morphology. It was not possible to validate whether it is the accumulation of maltose or maltodextrins, within these strains, causing the observed morphology or whether growth in maltose, causes sensitivity to the manner in which these strains are prepared for imaging.

This thesis suggests additional roles for MalP, MalQ, MalZ and GlgP, other than those previously defined and provided insight pertaining to interconnections between glycogen and maltodextrin metabolism. The observations made in this study have led to questions being raised regarding the understanding of polyglucan metabolism in *E. coli* and will stimulate the production of hypotheses for future work.

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