The Analysis of Glycogen Phosphate and Glucose-1,6-bisphosphate Metabolism in *Escherichia coli*

by
Jonathan Frederick Jewell

Thesis presented in fulfilment of the requirements for the degree of Master of Science in the Faculty of Agricultural Sciences at Stellenbosch University
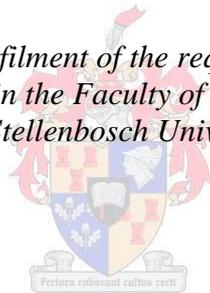
Supervisor: Dr James Lloyd

March 2015

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Jonathan F Jewell

March 2015

Abstract

This thesis examined two aspects of *E. coli* carbon metabolism, the incorporation of covalently bound phosphate into glycogen as well as the manufacture of glucose-1,6-bisphosphate (GBP).

In vitro analysis using recombinant maltodextrin phosphorylase (MalP) incubated together with maltodextrin, glucose-1-phosphate (Glc-1-P) and GBP resulted in the incorporation of phosphate into manufactured polymer at levels of 15 nmol Glc-6-P/mg polymer. No phosphate could be detected in the same incubation lacking only GBP. Moreover, higher amounts of polymer were also present in incubations where GBP was present with Glc-1-P, compared with Glc-1-P alone. Attempts were made to purify glycogen phosphorylase (GlgP), but these were unsuccessful. To examine if MalP and/or GlgP carry out this reaction *in vivo*, strains lacking them were produced. However, analysis revealed no significant difference in the phosphate content of glycogen extracted from wild type, single and double mutants lacking *glgP* and *malP*.

A protein responsible for the synthesis of a phosphoglucomutase (PGM) stimulatory compound was purified to apparent homogeneity. This was identified, through tryptic fingerprinting, as the acid glucose-1-phosphate phosphatase (AGP) protein. Using recombinant AGP protein it was demonstrated that it was able to produce GBP from Glc-1-P in a phosphotransferase reaction, where one phosphate from Glc-1-P phosphorylates the C6 position of another. However, *agp* mutant cells were unchanged in the amounts of GBP they accumulate and crude protein extracts from them were still capable of synthesizing GBP from Glc-1-P. A mutant strain lacking both *agp* and *pgm* could no longer produce a PGM stimulatory compound, indicating that PGM most likely also synthesises GBP.

Opsomming

Hierdie tesis het twee aspekte van *E. coli* koolstof metabolisme, naamlik die inkorporasie van kovalent gebonde fosfaat in glikogeen en die vervaardiging van glukose-1,6-bisfosfaat (GBP), ondersoek.

In vitro analise met behulp van rekombinante maltodekstrien-fosforilase (MalP), geïnkubeer met maltodekstrien, glukose-1-fosfaat (Glc-1-P) en GBP het gelei tot die inkorporasie van fosfaat teen vlakke van 15 nmol glukose-6-fosfaat (Glc-6-P) per milligram vervaardigde polimeer. Fosfaat was nie teenwoordig in die inkubasie waarin GBP uitgelaat was nie. Verder was hoër vlakke van polimeer vervaardig tydens die ko inkubasie van GBP en Glc-1-P as wat opgemerk was toe net Glc-1-P as substraat gedien het. Pogings wat aangewend was om glikogeen-fosforilase (GlgP) te suiwer was onsuksesvol. Om vas te stel of MalP en/of GlgP hierdie reaksie *in vivo* kan uitvoer, was mutante wat die gene ontbreek geproduseer. Daar was egter geen beduidende verskil in die fosfaat inhoud van glikogeen tussen wilde-tipe en enkel en dubbel-mutante van die *glgP* en *malP* gene opgemerk nie.

'n Proteïen wat verantwoordelik is vir die sintese van 'n fosfoglukomutase (PGM) stimulant, is gesuiwer tot oënskynlike homogeniteit. Dit was geïdentifiseer met proteïen vertering-vingerdrukking as die suurglukose-1-fosfaat fosfatase (AGP) proteïen. Deur gebruik te maak van rekombinante AGP proteïen was daar gedemonstreer dat die proteïen in staat is om GBP te vervaardig deur gebruik te maak van Glc-1-P as substraat in 'n fosfotransferase reaksie. Die reaksie behels die oordrag van 'n fosfaat van een Glc-1-P eenheid na die C6 posisie van 'n ander Glc-1-P eenheid. Die vlakke van GBP was egter onveranderd in die selle van die *agp* mutant en boonop was ru-proteïen uittreksels nog steeds in staat om GBP uit Glc-1-P te sintetiseer. 'n Dubbele mutant, ontbreek in beide *agp* en *pgm*, was nie in staat om 'n PGM-stimulerende verbinding te vervaardig nie, wat daarop dui dat PGM ook waarskynlik verantwoordelik is vir die sintese van GBP.

Acknowledgements

I would firstly like to thank my supervisor Dr James Lloyd for his devoted support and guidance throughout my masters and for continually inspiring me to reach greater heights as a student. Secondly, I would like to express thanks to Dr Daniel Vosloh for the contributions he made to my masters. I am grateful to these two mentors for their involvement that has really made my journey in science thus far, an unforgettable one!

To Professor Jens Kossmann, thank you for giving me the opportunity to do my masters at the IPB. Furthermore, I would like to acknowledge the following people that have provided me with support: Dr Stanton Hector, Dr Inonge Mulako, Dr Ebrahim Samodien, Dr Shaun Peters, Dr Paul Hills, Dr Christel van der Vyver, Marnus, Ruan, Zanele, Bianke, Emily and Anke. To the IPB academic and technical staff members who I have not mentioned, who have also in some form or another contributed, I would also like to acknowledge. I am also appreciative for the extramural activity in the form of football (James, Daniel and Ebrahim) that has provided much entertainment and physical conditioning.

Thank you to Dr John Lunn and Regina Feil, at the Max-Planck-Institute of Molecular Plant Physiology, for the quantification of GBP as well as to Fletcher Hiten, at the Central Analytical Facility, for his help in the analysis of glycogen phosphate. I would also like to acknowledge the National Research Foundation, the Institute for Plant Biotechnology and Stellenbosch University for funding.

Lastly, I would like to thank my mother for her unwavering support that has made it possible for me to be where I am today!

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List of Abbreviations

(NH4)$_2$SO$_4$	ammonium sulfate
$\times g$	gravitational acceleration
°C	degrees centigrade
ADP	adenosine diphosphate
AGP	acid
ATP	adenosine triphosphate
ddH$_2$0	deionized distilled water
DNA	deoxyribonucleic acid
DTT	dithiothreitol
E. coli	*Escherichia coli*
E.C	enzyme commission number
EDTA	ethylene diamine tetra acetic acid
FBP	fructose-1,6-bisphosphate
G1PPDM	glucose-1-phosphate phosphodismutase
G6PDH	glucose-6-phosphate-dehydrogenase
GBP	glucose-1,6-bisphosphate
GDP	guanosine diphosphate
Glc	glucose
Glc-1-P	glucose-1-phosphate
Glc-6-P	glucose-6-phosphate
GlgP	glycogen phosphorylase
GST	glutathione-*S*-transferase
HCl	hydrochloric acid
HK	hexokinase

I_2	iodine
IMAC	immobilized metal ion affinity chromatography
IPTG	isopropyl β-D-1-thiogalactopyranoside
K_2HPO_4	dipotassium hydrogen phosphate
Kan	kanamycin
KB	Kornberg
kDa	kilo-dalton
KH_2PO_4	potassium dihydrogen phosphate
KI	potassium iodide
KOH	potassium hydroxide
LB	Luria Bertani
LC-MS	liquid chromatography-mass spectrometry
M	molar
MalP	maltodextrin phosphorylase
mg	milligram
$MgCl_2$	magnesium chloride
$MgSO_4$	magnesium sulfate
ml	millilitre
mM	mill-molar
NaCl	sodium chloride
NAD	nicotinamide adenine dinucleotide
NaN_3	sodium azide
NaOH	sodium hydroxide
NaOH	sodium hydroxide
NaP_i	sodium phosphate

nm	nanometres
PCR	polymerase chain reaction
PEG	polyethylene glycol
PFK	phosphofructokinase
PGM	phosphoglucomutase
PMSF	phenylmethylsulfonyl fluoride
rpm	rounds per minute
RT	room temperature
SD	standard deviation
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
Tris	Tris(-hydroxymethyl)aminomethane
U	enzyme unit
UDP	uridine diphosphate
UTP	uridine triphosphate
v/v	volume/volume
w/v	weight/volume
WT	wild type
μg	microgram
μl	microliter
μm	micrometre

Chapter 1: General introduction

1.1   Bacterial polymers

1.1.1   History

In an ever-changing environment, bacteria have developed the ability to effectively perceive external stimuli, such as environmental stresses, and to react to these in the most energetically favourable ways to survive (Boor, 2006; Wilson et al., 2010). The production of polymers, through the utilization and conversion of various nutrients, is one of the main mechanisms that have allowed bacteria to manufacture the appropriate reserves in order to support and maintain their various metabolic activities (Wilkinson, 1963). From a historical background, the first discovery of a bacterial polymer (dextran) was by Louis Pasteur (Pasteur, 1861). The bacterium responsible for the production of this polymer was later demonstrated to be *Leuconostoc mesenteriodes* (Van Tieghem, 1878). After the contributions of Pasteur and Van Tieghem several other important polymers and polymer-producing bacteria were discovered (Fig. 1.1, Ivanovics and Erdös, 1937; Kornberg et al., 1956; Leach et al., 1957; Linker and Jones, 1966). These include bacterial cellulose (Brown, 1886), the polyamide cyanophin (Borzì, 1887) and polyhydroxybutyrate from *Bacillus megaterium* (Lemoigne, 1926). Another important polymer most likely discovered in the first half of the 20th century, was glycogen. Prior to the identification of glycogen in bacteria, the discovery of this polysaccharide was already apparent in mammalian tissue dating back to 1857 (Young, 1957). One of the earliest reports on its isolation from a microbial source, *Mycobacterium tuberculosis* (referred to as *Tubercle bacilli* at the time), was Laidlaw and Dudley (1925). Identified through iodine coloration, it was found that the isolated compound displayed similar characteristics to that of previously isolated glycogen, being a white amorphous powder that stained brown in solution with iodine. In spite of this, they were of the opinion that their finding was of little interest, partly due to similar evidences that originated from the work of Heidelberger and Avery on the isolation of a "specific substance" of polysaccharide nature from *Pneumococcus* (Heidelberger and Avery, 1923; Heidelberger and Avery, 1924). After several years, further publications on the isolation and characterisation of 'glycogen-like' polysaccharides (referred to as glycogen in some cases for conciseness) from various microbial sources emerged (Chargaff and Moore, 1944; Hehre and Hamilton, 1948; Barry et al., 1952; Levine et al., 1953). One of the first studies looking at the composition and metabolism of glycogen within *Escherichia coli* (*E. coli)* was during the 1950's and was pioneered largely through the work of Holme and Palmstierna (Holme and Palmstierna, 1955; Holme and Palmstierna, 1956; Holme et al., 1958). However, whether these 'glycogen-like' compounds were indeed glycogen in some of the reported cases is debateable.

Nevertheless, after the discovery of many of these polymers the elucidation and characterization of the biosynthetic enzymes involved in their production, along with the identification of the genes encoding them, occurred (*for review see* Rehm, 2010). The knowledge gained from these discoveries together with the scientific advancements made over the past centuries has significantly shaped the world of bacterial polymer science.
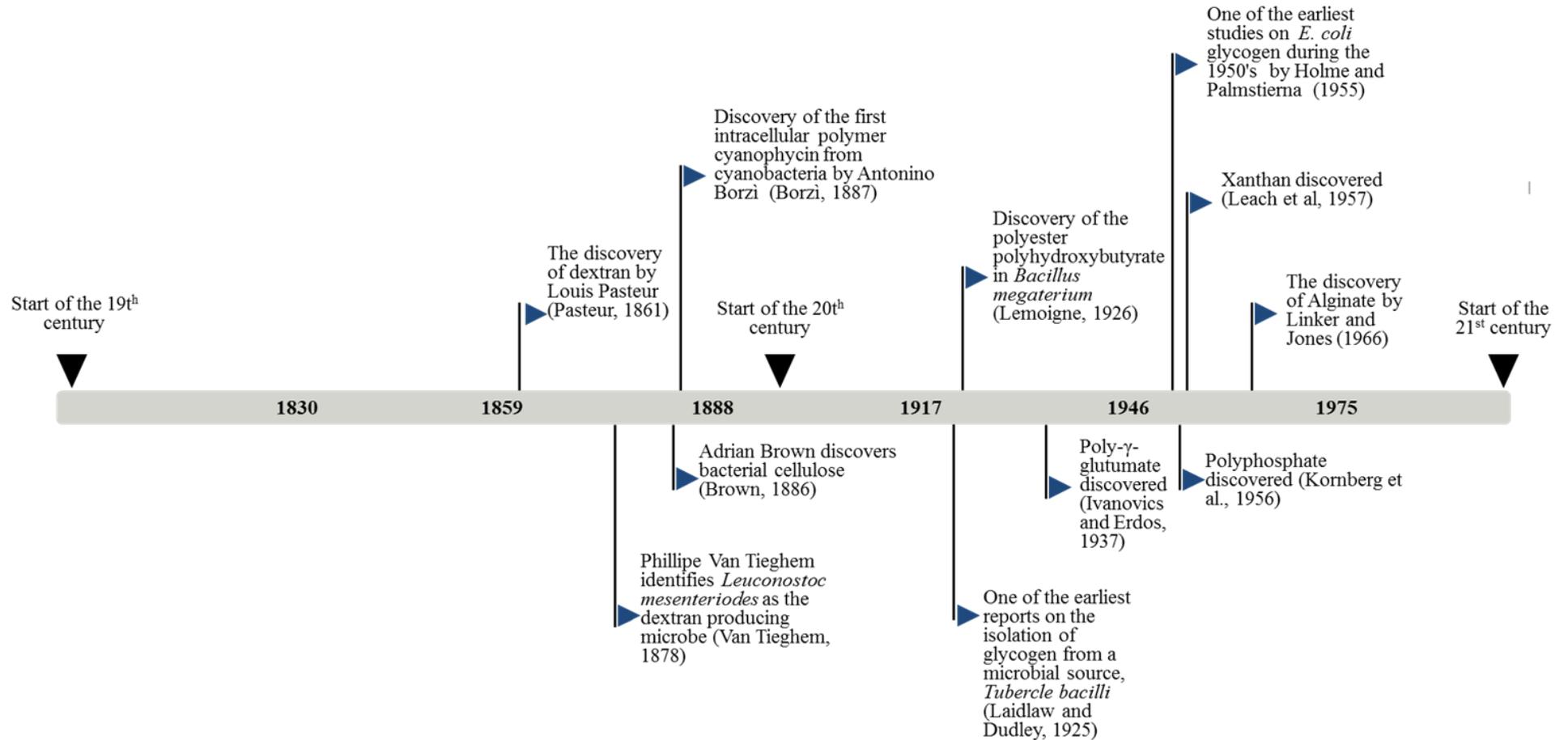
Figure 1.1 Historical overview. A timeline illustrating the history of bacterial polymers discovered during the nineteenth and twentieth century.

1.1.2   Classification and applications

Bacterial polymers can be grouped into four main classes, namely polysaccharides, polyesters, polyamides and inorganic polyanhydrides as listed below in Table 1.1 (Rehm, 2010). Given the diversity in of microbial polymer-producers, bacterial polymers possess several key characteristics (Table 1.1). These include, for example, the localization of the given polymer (whether intracellular, extracellular or capsular). As reviewed by (Rehm, 2010) all polymerizing enzymes involved in the manufacture of intracellular polymers are confined to the cytosol, while extracellular and capsular polymerizing enzymes are restricted to the cytosolic membrane (with the known exception of dextran sucrase which is secreted and fixed to the cell wall (Van Hijum et al., 2006). Another key characteristic of bacterial polymers are their structural features, which exist mainly due to the chemical configuration between the constituent monomers. These make them distinct from one another even when they are composed of the same monomer that, in turn, allows for their utilization in a wide variety of different industrial applications.

Despite their wide-ranging uses, not all bacterial polymers have an economically viable manufacturing system. For others a commercially driven industrial pipelines exist with production scales stretching towards several thousand tonnes per annum (Vandamme et al., 1996; Chen, 2009). One of the main hindrances in the use of some polymers within an industrial setting are the production costs involved which have to compete with those of established non-renewable resources (Rehm, 2010; Wang et al., 2014). The bioengineering of bacteria to produce modified biopolymers with improved properties is one of the strategies that researchers are exploring in order to overcome the various factors limiting their application and production. In spite of some of the shortcomings that exist, one of the key attributes of biopolymers are their biodegradability, making them strong competitors against finite oil-based resources (*for review on biodegradable polymers see* Doppalapudi et al., 2014). Numerous applications for the use of bacterial polymers are still emerging, illustrating the potential that these polymers possess (More et al., 2014; Tiboni et al., 2014).

Table 1.1 Polymer classification and applications. Examples of different classes of bacterial polymers with their various features (*table adapted from* Rehm, 2010). Commercially produced polymers are indicated by an asterisk (∗). Potential industrial applications are suggested for polymers that are not produced commercially.

Class	Localization	Structure	Main constituents	Precursors	Polymerizing enzyme	Microbe	Industrial applications
Polysaccharides							
Glycogen	Intracellular	α-(1,6)-branched α-(1,4)-linked homopolymer	Glucose	ADP–glucose	Glycogen synthase	Bacteria and archaea	Not applicable
Xanthan*	Extracellular	β-(1,4)-linked repeating heteropolymer consisting of pentasaccharide	Glucose, mannose and glucuronate	UDP–glucose, GDP-mannose and UDP–glucuronate	Xanthan polymerase	*Xanthomonas* spp.	Food additive (for example, as a thickener or an emulsifier)
Dextran*	Extracellular	α-(1,2)/α-(1,3)/α-(1,4)-branched α-(1,6)-linked homopolymer	Glucose	Saccharose	Dextransucrase	*Leuconostoc* spp. and *Streptococcus* spp.	Blood plasma extender and chromatography media
Cellulose*	Extracellular	β-(1,4)-linked homopolymer	Glucose	UDP–D-glucose	Cellulose synthase	Alphaproteo- bacteria, Betaproteo- bacteria, Gammaproteo-bacteria and Gram-positive bacteria	Food, diaphragms of acoustic transducers and wound dressing

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Polyamides

Cyanophin Granule peptide	Intracellular	Repeating heteropolymer consisting of dipeptide units	Aspartate and arginine	(β-spartate-arginine)$_3$-phosphate, ATP, L-arginine and L-aspartate	Cyanophycin synthetase	Cyanobacteria, *Acinetobacter* spp. and *Desulfitobacterium* spp.	Dispersant and water softener

Polyester

Polyhydroxy-alkanoates[*]	Intracellular	Heteropolymer	(R)-3-hydroxy fatty acids	(R)-3-hydroxyacyl CoA	Polyhydroxy-alkanoate synthase	Bacteria and archaea	Bioplastic, biomaterial and matrices for displaying or binding proteins Polyanhydrides

Polyanhydrides

Polyphosphate	Intracellular	Homopolymer	Phosphate	ATP	Polyphosphate kinase	Bacteria and archaea	Replacement of ATP in enzymatic synthesis and flavour enhancer

1.2   Glycogen an essential polymer

1.2.1   Occurrence and structure

Glycogen is a branched storage polysaccharide that is present in organisms present within all domains, ranging from bacteria and archaea to higher organisms such as yeast and animals (Ball and Morell, 2003; Wilson et al., 2010). Structurally, this homopolysaccharide consists of glucose units that are joined via α-1,4-glycosidic bonds having branching points with α-1,6-glycosidic linkages (Fig. 1.2.B) forming a granular structure with a projected maximum size of 42 nm in diameter (Fig. 1.2.A) (Manners, 1991; Shearer and Graham, 2002). The discovery of glycogen, isolated from liver tissue, dates back to the 19th century and is accredited to Claude Bernard (Young, 1957). This preceded the finding of bacterial glycogen discussed above (Section 1.1; Holme and Palmstierna, 1955; Holme and Palmstierna, 1956). Since then, significant advances has been made in the field of glycogen metabolism within both prokaryotic and eukaryotic systems (Wilson et al., 2010; Roach et al., 2012). While glycogen is the main storage form of glucose from bacteria to humans, plants also manufacture a similar storage polymer called starch, although starch consists out of two distinct polymers, the linear amylose and more branched amylopectin (Bule et al., 1998). Both starch and glycogen share overlaps in structure and metabolism (Cenci et al., 2014).
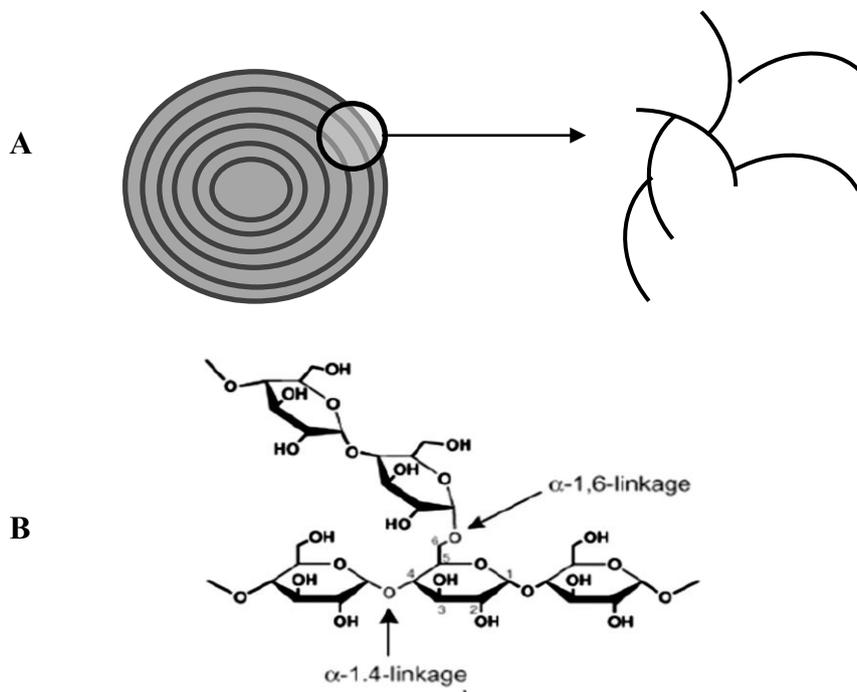

Figure 1.2 Glycogen structure. Schematic representations of A) a glycogen particle and B) glucose subunits coupled via α-1,4-glycosidic and α-1,6-glycosidic linkages (*adapted from* Ball et al., 2011; Roach et al., 2012).

Although there is currently no apparent use for glycogen in an industrial setting when compared to other polymers, it plays a fundamental role particularly in the survival and functioning of organisms manufacturing the polysaccharide. According to Wang and Wise (2011), five main energy sources namely, glycogen, triacylglycerols, wax esters, polyhydroxybutrate and polyphosphates, are present in bacteria of which glycogen has been shown to play a dynamic role in the bacterial survival. One of the key structural features of glycogen that has been shown to influence bacterial endurance is the average chain length (ACL). This, defined as the number of glucosyl units per number of branching points, is one of several other structural factors that has been created to describe glycogen structure (Manner*s*, 1991; Wang and Wise, 2011). In a review by Wang and Wise (2011), evidence from various scientific disciplines was assembled in order to support their hypothesis, that glycogen forms part of a durable energy storage mechanism (DESM). This is supported by data showing that glycogen with varying ACL's are utilized at different rates by bacteria; more specifically that the shorter the ACL, the longer it takes to degrade which increases bacterial endurance (Table 1.2).

Table 1.2 Glycogen ACL vs. bacterial survival. The association between glycogen average chain length (ACL) and bacterial endurance (*adapted from* Wang and Wise, 2011).

Bacterial name	Average chain length	50% survival time
Aerobacter aerogenes	13	45 h
Arthrobacter globiformis	6	20 d
Arthrobacter spp.	7~9	80 d
Bacillus megaterium	10	20 d
Escherichia coli	12	36 h
Klebsiella pneumoniae	11.6	2.5 d
Mycobacterium tuberculosis	7~9	52 d
Pseudomonas V-19	8	60 d
Streptococcus mitis	12	22 h
Thermococcus	7	24.5 d

1.2.2   Glycogen metabolism

1.2.2.1   Organization of structural genes

In *E. coli*, glycogen accumulation arises mainly under conditions that limit growth, when there is an excess in carbon while other nutrients are lacking (Preiss and Romeo, 1989). Genes responsible for the expression of the main enzymes involved in glycogen metabolism are located within the *glg*

operon (Romeo et al., 1988). This comprises five open reading frames in the order *glg*BXCAP where *glg*B encodes glycogen branching enzyme, *glg*X encodes glycogen debranching enzyme, *glg*C encodes ADP-glucose pyrophosphorylase, *glg*A encodes glycogen synthase and *glg*P encodes glycogen phosphorylase.

A characteristic of bacterial genome organisation is that genes with similar functions or genes involved in related biosynthetic pathways, often group into a single operon, allowing for their expression as a single unit. Several bacteria have such a system in which the genes involved in the metabolism of glycogen are contained in a single operon (Kiel et al., 1994; Ugalde et al., 1998; Marroquí et al., 2001). Despite this, much evidence (mainly from Romeo and Preiss, 1989) has led to the general acceptance that glycogen metabolizing genes in *E. coli* are clustered in two tandemly transcribed operons *glg*BX and *glg*CAP (Fig. 1.3) (Wilson et al., 2010). Recent data from Montero et al. (2011) however, suggests that *E. coli* glycogen metabolizing genes are indeed grouped in a sole transcriptional unit under the control of a main promoter located upstream of *glg*B. Furthermore, they also demonstrated the existence of an alternative sub-operonic promoter sequence situated inside *glg*C, which controls the expression of *glg*A and *glg*P (Fig. 1.3). It was hypothesized that this alternative promoter sequence might have been an evolutionary adjustment to ensure sufficient levels of expression (Montero et al., 2011).
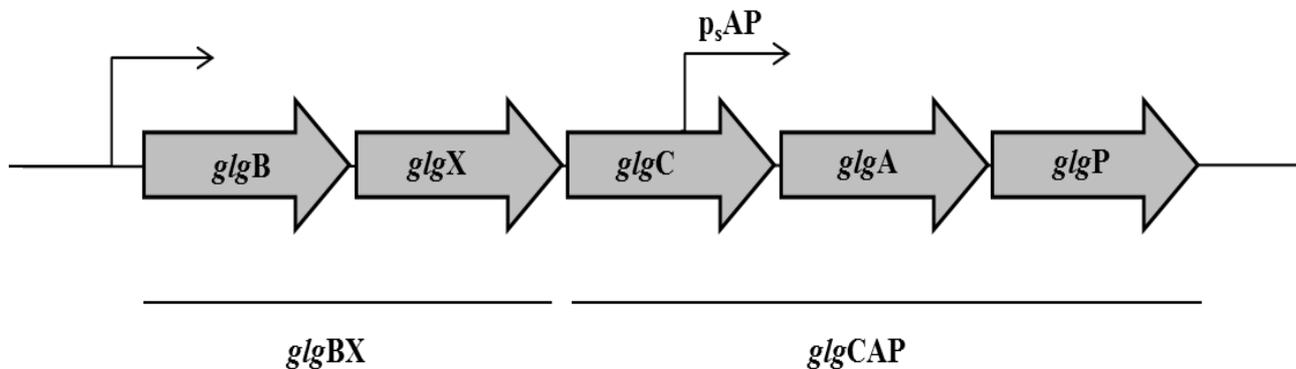
Figure 1.3 The *glg* operon in *E. coli*. Schematic depict ion of the structural organization of the *glg* operon in *E. coli*, wherein an alternative suboperonic promoter (p_sAP) is located inside *glg*C. The commonly accepted grouping of glycogen genes in two adjoining operons *glg*BX and *glg*CAP are indicated.

1.2.2.2   Enzymology

The enzymology involved in the metabolism of glycogen is highly preserved across most bacterial species highlighting the evolutionary conservation of this important process (Ballicora et al., 2003). In the proposed model of glycogen metabolism (see Fig. 1.4), the uptake of glucose and subsequent

conversion into glucose-6-phosphate (Glc-6-P) via the phosphotransferase system (PTS) is one of the first steps in its biosynthesis. The conversion from Glc-6-P to glucose-1-phosphate (Glc-1-P) is carried out by the enzyme phosphoglucomutase (PGM) and follows the PTS reaction. ADP-glucose (ADPG) is then formed from Glc-1-P in the presence of ATP by the rate-limiting enzyme GlgC (Dietzler and Leckie, 1977). The first step in production of the glycogen polymer is catalysed through the action of GlgA, where the activated sugar ADPG is used as the substrate in the transfer of glucose to an α-glucan primer forming an extended α-1,4-linked glucan. Although some species, such as *Saccharomyces cerevisiae*, can utilise a protein primer (named glycogenin) to initiate this reaction (Cheng et al., 1995), data from a study by Ugalde et al. (2003) involving the synthesis of glycogen in *Agrobacterium tumefaciens* indicates that GlgA from bacteria can form the required glucan primer through self-glycosylation. Moreover, one of the main evolutionary differences between bacterial glycogen synthesis compared to the mechanism present in yeast and mammals, is the use of ADPG instead of UDP-glucose (UDPG) (*for reviews on glycogen synthesis in yeast and mammalian tissues see* Wilson et al. (2010); Roach et al. (2012)). GlgB utilises these linear chains to introduce branched chains linked via α-1, 6-glycosidic bonds. Branching involves the cleaving and transfer of a glucan, ranging from six to nine monomers in length, to the C-6 hydroxyl group of a glucose molecule located at a different site within the glycogen molecule.
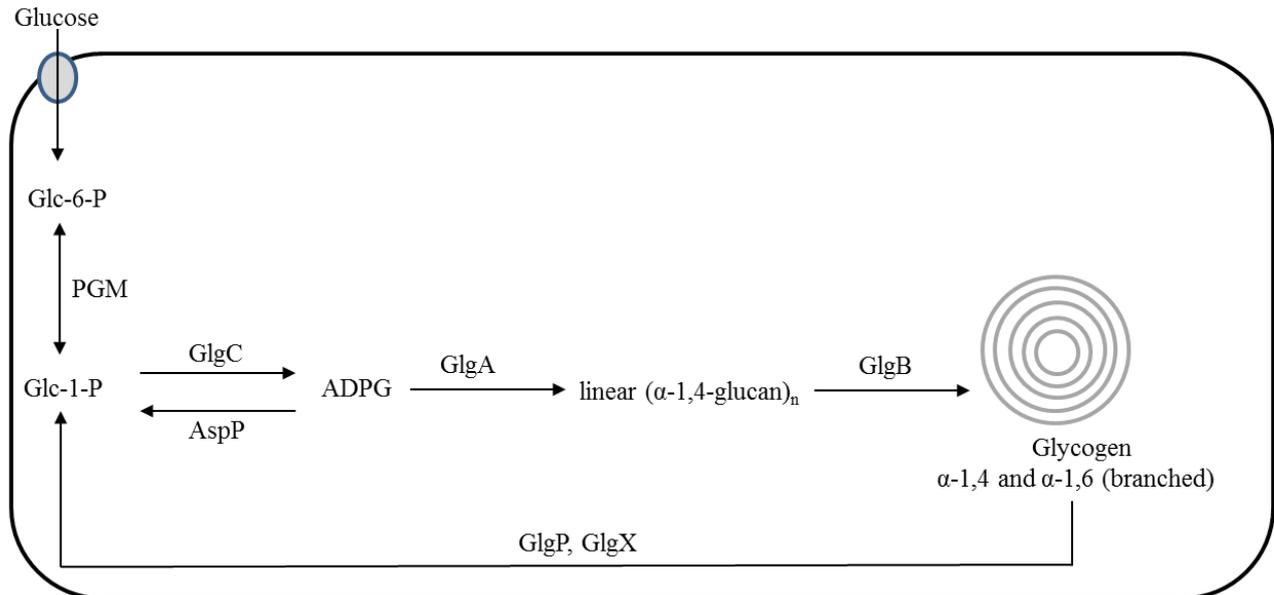
Figure 1.4 Glycogen metabolism. Schematic illustration of a proposed model for glycogen metabolism in *E. coli*. Refer to text for details (*modified from* Wilson et al., 2010).

Evidence illustrating the contribution of the genes involved in the glycogen metabolism comes from early studies involving mutants of *E. coli* some of which display unique phenotypes when exposed to iodine vapour (Damotte et al., 1968; Govons et al., 1969; Adhya and Schwartz, 1971), which

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stains polygucans different colours depending on the chain lengths within them. Short glucan chains stain red and longer ones blue. Substrate supply is clearly important in the pathway as mutations in either *pgm* (Adhya and Schwartz, 1971) or *glgC* (Govons et al., 1969) eliminate glycogen accumulation (Eydallin et al., 2007b). Mutants carrying a mutation in *glgA* also do not accumulate glycogen, whereas mutants carrying the mutated form of *glgB* stain blue indicating the accumulation of long linear glucans (Damotte et al., 1968).

In a more recent study by Eydallin et al. (2007b) involving the genome-wide screening of genes that influence the metabolism of glycogen, genes affecting glycogen formation was linked to several other cellular processes and revealed that glycogen metabolism is an interrelated process. Several other studies at the time also provided proof that GlgC is not exclusively responsible for the presence of ADPG, revealing the existence of other sources of ADPG that are connected to the biosynthesis of glycogen (Eydallin et al., 2007a; Morán-Zorzano et al., 2007a). Characterization studies of Δ*glgCAP* mutants of *E. coli* and *Salmonella enterica* revealed that, as expected, the mutant strains did not produce glycogen but, more significantly, these mutants still accumulated ADPG (Morán-Zorzano et al., 2007a). Glycogen formation was restored in Δ*glgCAP* mutants expressing a plasmid carrying *glgA* suggesting the presence of other ADPG producing mechanisms. This was confirmed in a similar study by Eydallin et al. (2007a) wherein an *E. coli glgC* mutant, expressing a truncated, and resultantly, inactive form was employed.

GlgP and GlgX are the main enzymes thought to be involved in the breakdown of glycogen (*for review see* Ball and Morell, 2003). GlgP facilitates this through the removal of glucose units from the non-reducing ends of the glycogen outer chains and the production of Glc-1-P (Alonso-Casaju et al., 2006). The chains are reduced to three to four glucosyl units in length away from the carrying chain that they are linked to. GlgX, an isoamylase-type debranching enzyme, then removes the shortened branches releasing maltotetrose and maltotriose (Dauvillée et al., 2005; Song et al., 2010). Deletion mutants of GlgP and GlgX both lead to glycogen-excess phenotypes with cells staining dark brown with iodine (Dauvillée et al., 2005; Alonso-Casaju et al., 2006). Additionally, these mutants are incapable of utilizing glycogen effectively and produce glycogen with altered structural features. The chains within glycogen from a Δ*glgP* strain are significantly longer than those within a wild-type strain (Alonso-Casaju et al., 2006) while glycogen from a Δ*glgX* strain contains shorter external chain (Dauvillée et al. 2005). This indicates that, although both enzymes are involved in degrading glycogen, they also play a role in determining glycogen structure during its synthesis.

In addition to the *glg* genes, *E. coli* also possesses genes that can act on polyglucans and which are part of the maltose (*mal*) operon. It has been demonstrated recently that these genes also can play a role in the metabolism of glycogen (Park et al., 2011). The release of maltotetrose and maltotriose, through the sequential action of GlgP and GlgX, is coupled to the maltose/maltodextrin-utilizing system. This system consists of 5 operons comprising of 10 genes coding for proteins implicated in the metabolism of maltose and maltodextrin (for *review see* Boos and Shuman, 1998; Dippel and Boos, 2005). As with glucose, both maltose (consisting of two glucose units joined via α-1,4-linkage) and maltodextrin (being longer linear chains of glucose monomers) can be utilized as substrates to produce glycogen (Jones et al., 2008). One of the main enzymes involved in the use of maltose is a 4-α-glucanotransferase, amylomaltase (MalQ) (Wiesmeyer and Cohn, 1960; Pugsley and Dubreuil, 1988). MalQ catalyses the formation of longer maltodextrin chains by the transfer of glycosyl chains (formed by the cleaving of glucose form the reducing ends of maltose and maltodexrins) to other maltodextrins. Maltodextrin phosphorylase (MalP, Schwartz and Hofnung, 1967) is another key enzyme that plays an important role in the metabolism of glycogen (Park et al., 2011). This enzyme carries out a similar reaction to GlgP, cleaving glucose from the non-reducing ends of dextrins (maltotetraose and longer maltodextrins) leading to the production of Glc-1-P and a shortened dextrin (Park et al., 2011). The combined action of MalP and MalQ produce substrates that can be fed into glycolysis. Moreover, these two enzymes enable the effective utilization of maltose and maltodextrin in *E. coli* (Park et al., 2011). In addition to these enzymes, *E. coli* also possesses two others that are implicated in the metabolism of maltose. The periplasmic α-amylase (MalS) is involved in the metabolism of longer dextrins, and is able to liberate maltohexose from linear maltodextrins present at the non-reducing ends (Freundlieb and Boos, 1986), while the cytoplasmic maltodextrin glucosidase (MalZ) successively removes glucose units from the reducing ends of maltodextrins (Tapiost et al., 1991).

Recent findings from a study by Park et al. (2011), involving the use of mutants (Δ*malP,* Δ*malQ,* Δ*malZ,* Δ*glgA*), revealed the occurrence of an alternate glycogen synthesizing mechanism via the use of maltodextrin and maltose that is thought to be controlled by MalP. The study showed the dual functioning of MalP, being able to liberate glucose in the form of Glc-1-P (fed into glycolysis) through phosphorolysis from maltodextrins as well as providing substrates that contribute to the formation of ADPG and ultimately glycogen.

1.2.2.3   Allosteric regulation

Adenosine diphosphate sugar pyrophosphatase (AspP), one of several allosterically regulated enzymes implicated in the metabolism of glycogen, is responsible for the degradation of ADPG,

leading to the inhibition of glycogen synthesis and the directing of carbon to other pathways (Moreno-Bruna et al., 2001; Morán-Zorzano et al., 2008). Furthermore, *in vitro* analysis has indicated that AspP is positively regulated by glucose-1,6-bisphosphate (GBP), nucleotide sugars for e.g. UDP-glucose and by the presence of macromolecules such as PEG (Morán-Zorzano et al., 2007b). Similarly, GBP is also a well-known allosteric activator of PGM (Beitner, 1979). Allosteric regulation of ADP-glucose pyrophosphorylase has been shown to be activated by fructose-1,6-bisphosphate (Frc-1,6-BP) and inhibited by adenosine monophosphate (AMP) and inorganic phosphate (Pi) (Gentner and Preiss, 1967). Mutational studies on *glgC* have also demonstrated the importance of its allosteric regulation in controlling flux through the pathway (Creuzat-Sigal et al., 1972). A specific point mutation of *glgC* (designated *glgC16*), where a codon change resulted in the change from arginine to cysteine on the deduced amino acid sequence (Ghosh et al., 1992), leads to the accumulation of higher amounts of glycogen with cells carrying this mutation staining dark-brown when exposed to iodine (Damotte et al., 1968). The regulation around these enzymes, illustrates how tightly controlled glycogen metabolism is, and provides a glimpse into the complex regulatory mechanisms present in *E. coli.*

1.2.2.4   Genetic regulation

Several studies mainly involving *E. coli* mutants, have shown that the metabolism of glycogen is a complex process that is exceedingly interspersed with several different cellular pathways (Eydallin et al., 2007b; Montero et al., 2009; Eydallin et al., 2010). At a gene expression level, various factors are involved in the accumulation of this polysaccharide in *E. coli* (*for detailed review see* Wilson et al., 2010). Positive regulatory factors include the RNA polymerase sigma factor (RpoS), PhoP/PhoQ regulatory system, guanosine 5'-diphosphate 3'-diphosphate (ppGpp) and the cyclic AMP/-cAMP receptor protein (-cAMP/CRP) complex. Of these factors, RpoS (the stress response sigma factor (Lange and Hengge-Aronis, 1991)) has an indirect effect by positively controlling the expression of *glgS* (Hengge-Aronis and Fischer, 1992). The formation of glycogen is positively influenced by the product of the *glgS* gene in an unknown mechanism (Montero et al., 2009; Eydallin et al., 2010). However, a recently a study by Rahimpour et al. (2013) has implicated this hydrophilic and highly charged 7.9 kDA protein (Beglova et al., 1997; Kozlov et al., 2004) in bacterial motility and biofilm formation, implying that its effect on glycogen is pleiotropic. Because of this new proposed role, it has been suggested that GlgS should be renamed as surface composition regulator (ScoR) (Rahimpour et al., 2013).

The PhoP/PhoQ system directly influences the expression of *glgCAP* genes in response to extracellular Mg^{2+} levels (Montero et al., 2009). Additionally, mutants of *phoP* and *phoQ* are

glycogen deficient (Montero et al., 2009). When nutrients are limited, *E. coli* is able to metabolically adjust and respond in order to survive. During this response the presence of ppGpp (synthesized by RelA) has been associated with an proliferation in the glycogen biosynthetic machinery (Bridger and Paranchych, 1978; Taguchi et al., 1980; Romeo and Preiss, 1989; Romeo et al., 1990; Baker et al., 2002; Traxler et al., 2008). Similarly, *in vitro* and *in vivo* experiments have demonstrated the positive regulatory effect that the cAMP/CRP complex has on the expression of glycogen phosphorylase and synthase (Dietzler et al., 1977; Urbanowski et al., 1983; Romeo and Preiss, 1989). Moreover, mutants lacking cAMP (Δ*cya*) and CRP (Δ*crp)* are glycogen deficient (Montero et al., 2009).

In contrast to the positive regulation, the carbon storage regulator CsrA negatively regulates glycogen biosynthesis by directly binding to two positions within *glgCAP* preventing the translation of *glgC* (Yang et al., 1996; Baker et al., 2002). Taking together these complex regulatory mechanisms alongside numerous other controlling factors, an integrated model has been proposed (Fig. 1.5) (Montero et al., 2009). This is continually being revised and adjusted, illustrating the complexities revolving around the understanding of this naturally occurring polymer (Eydallin et al., 2010; Wilson et al., 2010; Rahimpour et al., 2013; Tian et al., 2013).
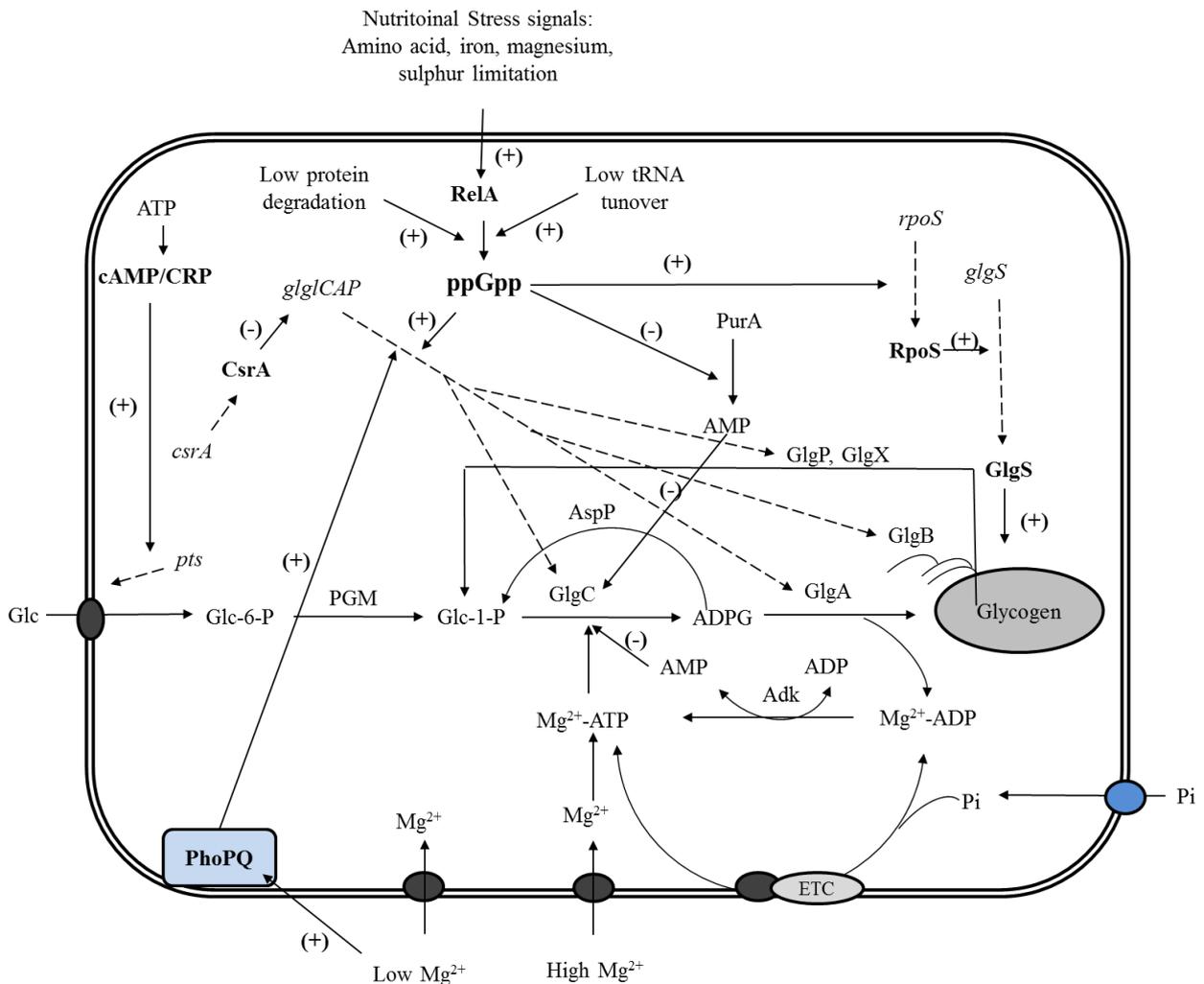
Figure 1.5 Regulation of glycogen metabolism. A proposed model of glycogen metabolism illustrating the complex interconnected regulatory mechanism at play resolving around the metabolism of glycogen (*figure adapted from* Wilson et al., 2010).

1.2.3   Glycogen phosphate

The phosphorylation of polymers is not unusual in nature, and is in fact a common occurrence that occurs, for example, in starch in plants and glycogen in animals. Starch phosphate incorporation is understood fairly well and involves two enzymes, namely the glucan, water dikinase (GWD, Ritte et al., 2002) and the phosphoglucan, water dikinase (PWD, Baunsgaard et al., 2005; Kötting et al., 2005). These enzymes respectively phosphorylate the C6 and C3 positions of glucosyl residues in starch (Ritte et al., 2006). The amount of starch bound phosphate varies between species (Blennow et al., 2000), with the most highly phosphorylated industrially important starch (form potato tubers) containing approximately one phosphorylated glucose unit out of 200-300 monomers (Hizukuri et al., 1970). The presence of phosphate in some starches leads to it being charged which is of great importance for some industries such as paper manufacture (Zeeman et al., 2010).

Studies on mammalian glycogen have also revealed that it is phosphorylated, however, to a lesser degree than starch as approximately one glucosyl unit out of every 600-1600 is phosphorylated (Fontana, 1980; Tagliabracci et al., 2007; Tagliabracci et al., 2008). In humans, the abnormal phosphorylation of glycogen, specifically hyperphosphorylation, leads to Lafora disease which is a autosomal recessive neurodegenerative disorder (myoclonus epilepsy) (Andrade et al., 2007; Delgado-Escueta, 2007). One of the characteristic features of the disease is the accumulation of Lafora bodies, which are abnormally branched glycogen-like structures that form in the affected tissue. The removal of phosphate under normal conditions is catalysed by the dual specific phosphatase laforin (Tagliabracci et al., 2007). Recently, Tagliabracci et al. (2011) was able to show the incorporation of phosphate by glycogen synthase, as well as demonstrate that phosphate is present as phosphate-monoesters bound at the C2 and C3 positions. A more recent study by Chikwana et al. (2013) has provided further evidence for the incorporation of phosphate by glycogen synthase.

In comparison with phosphate incorporation in starch in plants and glycogen in mammals, bacterial glycogen phosphate metabolism is poorly understood. Earlier studies on bacterial glycogen revealed the existence of phosphate bound covalently to the C6 position of glucosyl residues present at concentrations of approximately 0.9 nmol Glc-6-P per mg glycogen (Lorberth et al., 1998; Viksø-Nielsen et al., 2002). However, the exact mechanism for the incorporation of glycogen phosphate in *E. coli* is still unknown. A recent study at the Institute for Plant Biotechnology (IPB) has hypothesised that the combined involvement of the glucan phosphorylases, GlgP and MalP, are responsible for phosphate incorporation (Nepembe, 2009). Both these enzymes can incorporate glucose into polyglucans using Glc-1-P as substrate releasing inorganic phosphate (P_i). It was hypothesized that they could utilise GBP in place of their usual substrate, leading to the incorporation of phosphate (in the form of Glc-6-P) into a growing polyglucan (Nepembe, 2009). This mechanism was however, not demonstrated either *in vitro* or *in vivo* and the first part of the project in this thesis examines this.

1.3   Glucose-1,6-bisphosphate in *E.coli*

Glucose-1,6-bisphosphate (GBP) is a key allosteric regulator exerting control on several enzymes in both prokaryotes and eukaryotes. It controls several enzymes involved in carbohydrate metabolism, activating PGM, phosphofructokinase (PFK) and pyruvate kinase (PK) while inhibiting hexokinase (HK) (Beitner, 1979; Beitner, 1984). Since the discovery of this metabolite more than 60 years ago by the group of the Argentine Nobel laureate Luis Leloir (Leloir et al., 1948), numerous studies have attempted to identify the mechanism by which it is manufactured as well as to uncover its involvement in the metabolism of carbon (Sidbury et al., 1956; Eyer et al., 1971).

Within mammalian systems, several reactions for the synthesis of GBP have been proposed. These include the formation of GBP from fructose-1,6-bisphosphate (Fruc-1,6-BP) and Glc-6-P via PGM (Passonneau et al., 1969), the ATP-dependent phosphorylation of Glc-1-P (Eyer et al., 1971) and formation through a Glc-1-P transphosphorylase reaction (Sidbury et al., 1956). Despite these suggested reactions, the precise mechanism of synthesis for GBP has recently been revealed in mammals (Maliekal et al., 2007). In that study, it was shown that the gene, *Phosphoglucomutase2 Like1* (PGM2L1), codes for a protein that catalyses the transferal of a phosphate from 1,3-bisphosphoglycerate (1,3-BPG) to the C-6 site of Glc-1-P forming GBP and 3-phosphoglycerate (3-PG) (Fig. 1.6). Likewise, GBP is produced in *Bacillus subtilis* by ß-phosphoglucomutase via a Glc-1-P phosphodismutase reaction, in which phosphate is transferred from one Glc-1-P unit to another to the C-6 position (Mesak and Dahl, 2000).
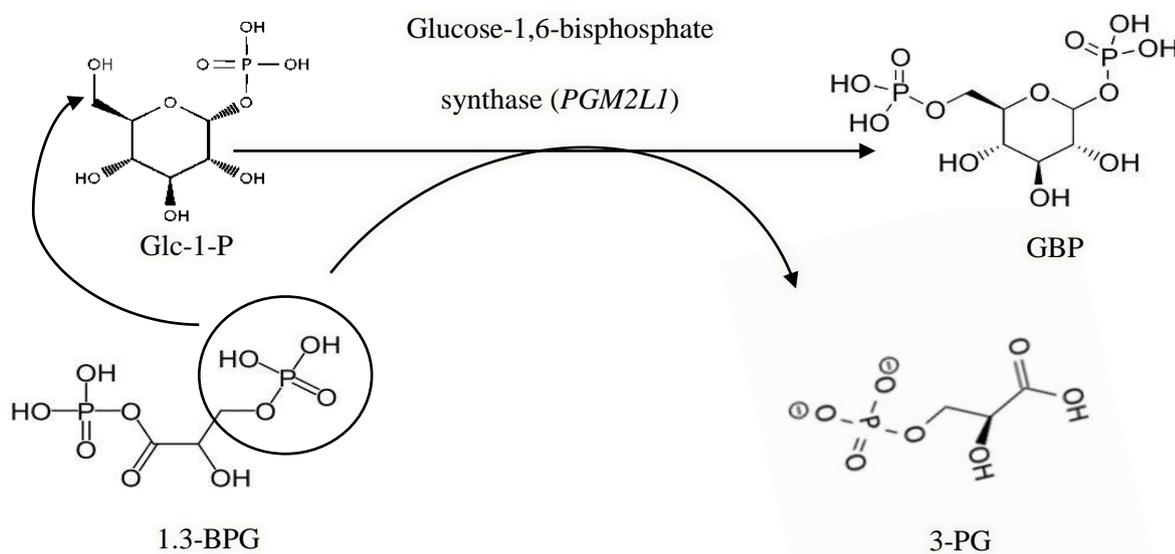
Figure 1.6 GBP synthesis in mammals. Schematic representation of the 1,3-bisphosphoglycerate (1,3-BPG) dependent synthesis of glucose-1,6-bisphosphate (GBP) in mammals (*figure adapted from* Maliekal et al., 2007).

In contrast to the mammalian systems, less is known concerning the synthesis of GBP in *E. coli*. However, the importance of GBP in the regulation of glycogen metabolism is evident in the control that it exerts over enzymes such as AspP and PGM (see section 1.2.2.3). Despite its importance in central carbon metabolism, the exact mechanism by which GBP is manufactured in *E. coli* remains to be elucidated. The only study that has examined this was published many decades ago and proposed that it was synthesised in a phosphodismutase reaction using Glc-1-P alone as a substrate

(Leloir et al., 1949), however, the enzyme involved is unknown. The other part of this project examines this in *E. coli*.

1.4   Aims of the research project

The overall goal of this research project was to examine the mechanism(s) of phosphate incorporation into *E. coli* glycogen. The study involved two main aims. Firstly, to examine the mechanism of phosphate incorporation in *E. coli* in a dual approach. This entailed investigating the involvement of two glycogen and maltodextrin phosphorylases in both *in vitro* and *in vivo* systems. Secondly, to elucidate the means by which glucose-1,6-bisphosphate, a key regulatory metabolite that is potentially involved in glycogen phosphate incorporation, is synthesized in *E. coli*.

The particular objectives for each aim included:

Aim 1: Glycogen phosphate incorporation

Part 1: Analysis of *E. coli* mutants

- Creation of *E. coli* double mutant lacking both *glgP* and *malP*
- Analysis of phosphate content of mutants compared against WT

Part 2: Purification of protein derived from genes in part 1

- Production of protein purification constructs
- Purification of protein
- *In vitro* analysis of protein for the incorporation of phosphate using glucose-1,6-bisphosphate as substrate

Aim 2: Glucose-1,6-bisphosphate metabolism

- Purification of proteins involved in its synthesis
- Biochemical characterization of those proteins
- Analysis of mutant cells lacking them

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Chapter 2: Examination of the roles of glucan phosphorylases in phosphate incorporation into *E. coli* glycogen

2.1   Introduction

Within bacteria, the mechanism for glycogen phosphate incorporation is unknown. The key evidence for its presence in *E. coli* glycogen, arose from previous studies (Lorberth et al., 1998; Viksø-Nielsen et al., 2002) involving the expression of the plant phosphorylating enzyme glucan water dikinase (GWD) in that bacterium. Both of those studies demonstrated that the expression of GWD led to the production of highly phosphorylated glycogen. More importantly, covalently bound phosphate the C6 position of glucose units (of approximately 0.9 nmol Glc-6-P/mg glycogen) was found to be present in the strain containing the empty vector control (Lorberth et al., 1998; Viksø-Nielsen et al., 2002). More recently, a study by Nepembe (2009) identified the *malP* gene as being a potential candidate involved in the phosphorylation of glycogen in a functional screen using a *E. coli* genomic library. It was hypothesized that phosphate is incorporated by these enzymes using glucose-1,6-bisphosphate (GBP) as substrate instead of its usual substrate, glucose-1-phosphate (Glc-1-P). In that study it was shown that a mutant lacking MalP activity accumulates as much glycogen bound phosphate as the control, implying that a second enzyme was also involved. As both the glycogen phosphorylase (GlgP) and MalP catalyse very similar reactions it was hypothesised that GlgP is the second enzyme involved. The main difference between them is their substrate specificities with MalP preferentially using linear maltodextrins (Schwartz and Hofnung, 1967) and GlgP glycogen (Chen and Segel, 1968a; Chen and Segel, 1968b). Both GlgP and MalP are involved in the metabolism of glucans and are able to catalyse a reversible reaction in which Glc-1-P is either incorporated into or liberated from a given glucan polymer (see Chapter 1). *glgP* is located within the *glg* operon (Chapter 1, Fig. 1.3) and is one of the main enzymes involved in the degradation of glycogen through phosphorolysis (Alonso-Casaju et al., 2006). *malP* in contrast is part of the *mal* operon and was originally thought to be involved in degrading linear glucans that are the product of the action of amylomaltase (MalQ) on maltose (Park et al., 2011). More recently it has been shown to influence glycogen metabolism when *E. coli* is grown on media containing maltose and maltodextrins (Park et al., 2011). Although the precise mechanism for this is unknown, it is thought to be because MalP degrades linear glucans produced by MalQ that could otherwise be used as a substrate by glycogen branching enzyme (GlgB; Park et al., 2011).

Within this chapter, I examine the involvement of GlgP and MalP in the incorporation of glycogen phosphate in order to test the hypothesis that both are involved in incorporating phosphate into *E. coli* glycogen. Both *in vivo* and *in vitro* approaches were used in order to test this hypothesis.

2.2   Materials and Methods

2.2.1   Chemicals

All chemicals used were acquired from Sigma (St. Louis, USA) or Roche (Mannheim, Germany) unless specified otherwise. Enzymes used were from Megazyme (Bray, Ireland) and Sigma (St. Louis, USA). All chemicals and reagents were of analytical grade. Solutions were prepared with ultrapure deionized distilled H_2O (ddH20, Millipore). Histidine-tagged (His-tag) purification columns were from Macherey-Nagel (Germany). Plasmid and PCR purifications were performed using commercially available kits from Thermo Scientific unless stated otherwise. All spectrophotometric analysis was performed on the Versa-max tuneable microplate reader (Molecular Devices, USA).

2.2.2   Bacterial strains and plasmids used

Bacterial strains and plasmids utilized in this study are shown in Table 2.1. BW25113, JW0675, JW3391 and JW5689 were obtained from the Coli Genetic Stock Centre (cgsc.biology.yale.edu).

Table 2.1 Bacterial strains and plasmids used in the stud y.

Name/Number	Description	Source/Reference
Bacterial strains		
BW25113	*Δ(araD araB)567 ΔlacZ4787*(::rrnB-3) *λ⁻ rph-1 Δ(rhaD-rhaB)568*	Datsenko and Wanner, 2000
JW0675	BW25113 Δ*pgm*-736::kan	Baba et al., 2006
JW3391	BW25113 *ΔglgP-761::kan*	Baba et al., 2006
JW5689	BW25113 *ΔmalP-751::kan*	Baba et al., 2006
ΔGlgP	BW25113 *ΔglgP-761*	In this study
ΔMalP	BW25113 *ΔmalP-751*	In this study
ΔGlgP/Malp(kan)	BW25113 *ΔglgP-761 ΔmalP-751::kan*	In this study
GlgP(kan)/ΔMalP	BW25113 *ΔmalP-751 ΔglgP-761::kan*	In this study
E. coli BL21 (DE3) pLysS	F- *ompT hsdS*B (rB-mB-) *gal dcm* (DE3) pLysS(Cam^R)	Life Technologies, Invitrogen

E. coli One Shot® TOP10	F- *mcr*A *Δ*(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 *Δlac*X74 *rec*A1	Life Technologies, Invitrogen
E. coli DH5α™	F- *rec*A1 *end*A1 *hsd*R17(rk-, mk+) *sup*E44 λ- *thi*-1 *gyr*A96 *rel*A1	Life Technologies, Invitrogen
Plasmids		
pACAG	IPTG inducible expression of *glgC16,* Cam^R , Tet^R	Kossmann et al., 1999
pENTR™/D-TOPO®	Entry cloning vector, Kan^R	Life Technologies, Invitrogen
pDEST™17	IPTG inducible vector, His-tag, Cam^{R,} Amp^R	Life Technologies, Invitrogen
pDEST17.GlgP	IPTG inducible vector, His-tag, Amp^R	In this study
pDEST17.Malp	IPTG inducible vector, His-tag, Amp^R	In This study

2.2.3 Production of *E. coli* double mutant

For the generation of *E. coli* mutant lacking both *glgP* and *malP* genes, the kanamycin resistance cassettes were removed from the JW3391 and JW5689 strains using pCP20 (Cherepanov and Wackernagel, 1995), which expresses FLP-recombinase, resulting in the strains ΔGlgP and ΔMalP. The *ΔmalP-751::kan* or Δ*glgP-761::kan* alleles were transferred from either JW5689 or JW3391-donor strains to the appropriate recipient strain by P1 phage transduction (Thomason et al., 2007).

2.2.4 PCR genotyping

Genotyping was performed by PCR to confirm presence of different *glgP* alleles using forward 5'CTTCACTGTGGCGGTTTGTG3' and reverse primers 5'GGAATTACCGCAAAGCCCAC3', while for detection of *malP* alleles (forward 5'AAGGTCAACATCGAGCCTGG3' and reverse primers 5'ATCCACCAGCATCGCTTTGA3' were used. An additional PCR using a primer (5'CTGATCGACAAGACCGGCTT3') that binds to the kanamycin resistance marker, was utilised alongside the respective forward primer to confirm the *ΔglgP-761::kan* and *ΔmalP-751::kan* alleles.

2.2.5 Non-denaturing gel electrophoresis

E. coli colonies from WT, single mutants of *glgP* and *malP* together with the double mutants were inoculated in 50 ml liquid Kornberg (KB) medium (1.1% (w/v) K_2HPO_4, 0.85% (w/v) KH_2PO_4, 0.6% (w/v) yeast extract) containing either 0.1% (w/v) glucose or maltose and grown overnight at 37°C with shaking at 200 rpm. All *E. coli* growth conditions throughout the study took place under these parameters unless indicated otherwise. Cells were harvested by centrifugation for 15 min at

5000g and 4°C (all subsequent steps in the extraction of protein were performed at this temperature). The resulting cell pellets were re-suspended in 5 ml pre-chilled extraction buffer (50 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 2 mM EDTA, 10% (v/v) glycerol, 0.1% (v/v) Triton X-100, 1 mM PMSF, 1 mM DTT). Cells were lysed by means of sonication on ice (five times bursts of 15 s each with 5 s cooling in between; VirSonic 100, VirTis). The resultant cell mixtures were clarified by centrifugation for 20 min at 10 000g and supernatants were used in the polyacrylamide gel electrophoresis.

Soluble proteins were separated by polyacrylamide gel electrophoresis on non-denaturing gels containing 10% (w/v) polyacrylamide and 1% (w/v) glycogen (from *Credipula fornicata*) in the resolving gel. Protein separation was performed at 4°C and at a constant voltage of 100V at 4°C. Gels were subsequently incubated overnight in an incubation buffer containing 3 M acetic acid-NaOH pH 6.0 and 20 mM Glc-1-P as described by Nepembe (2009). After the removal of the incubation buffer, the gels were stained for 1 min with Lugol's solution (4% (w/v) KI, 2% (w/v) I_2) and subsequently de-stained with distilled H_20 (dH_2O).

2.2.6 Competent cell preparation

To increase the amount of glycogen manufactured by the *E. coli* they were transformed with the plasmid pACAG (Kossmann et al. 1999), which contains the *glgC16* gene that encodes an allosterically unregulated ADP-glucose pyrophosphorylase (Creuzat-Sigal et al., 1972).

2.2.7 Glycogen extraction

Three colonies of each transformation ΔGlgP::pACAG, ΔMalP::pACAG, GlgP(kan)::pACAG, MalP(kan)::pACAG, ΔGlgP/MalP(kan)::pACAG, ΔMalP/GlgP(kan)::pACAG were grown overnight in 2 ml liquid KB medium containing 34 μg/ml chloramphenicol (Cam) and 15 μg/ml tetracycline (Tet). A 50 μl aliquot of each of the cultures was transferred to 5 ml liquid Kornberg medium (containing 1% (w/v) glucose and antibiotics). The cells were cultured for approximately 5 h before being plated onto solid KB medium containing 1% (w/v) bacterial agar, 1% (w/v) glucose, 34 μg/ml Cam, 15 μg/ml Tet and incubated overnight. Each plate had an approximate growth surface area of 500 cm^2.

E. coli cells were harvested from the bacterial plates and transferred to a 50 ml centrifuge tube. Glycogen isolation was performed as previously described by Park et al. (2011). The collected cells were washed with cold water and suspended with vigorous vortexing in 5 ml of 50 mM sodium acetate pH 4.5. Cell suspensions were boiled for 10 min and then sonicated (three times for 5 min each, 60% maximum output, at RT). The lysed cell suspension was centrifuged at 10 000g for 20

min and RT. Glycogen was precipitated from the supernatant by the addition of two volumes of ethanol and collected by centrifugation at 10 000g for 20 min at 4°C.

2.2.8   Determination of glucose-6-phosphate in glycogen

Glycogen within the pellet was hydrolysed by re-suspending it in 400 µl of 0.7 M HCl and heating for 3 h at 95°C with shaking (800 rpm). After digestion, the suspension was neutralized by the addition of 400 µl of 0.7 M KOH and centrifuged at 10 000g for 10 min and RT. As a control, glycogen of the respective *E. coli* strains was re-suspended in 800 µl of ddH$_2$0 heated at 95°C for 5 min with shaking, following centrifugation (as described above for the digested treatment).

The amounts of Glc and Glc-6-P equivalents were determined by spectrophotometric analysis similar to Nepembe (2009). For the determination of Glc, 10 µl of the samples were combined with 290 µl assay buffer (300 mM Tris-HCl pH 7.5, 1 mM MgCl$_2$, 1 mM NAD$^+$, 1 mM ATP). The reaction was started by the addition of the coupled enzyme-mix (Megazyme) containing 0.42U hexokinase (from yeast) and 0.21U glucose-6-phosphate-dehydrogenase (G6PDH; from *Leuconostoc mesenteroides*). The increase in absorbance at 340 nm was determined. Glc-6-P was assayed by combining 100 µl of sample with 200 µl of assay mix (300 mM Tris-HCl pH 7.5, 1 mM MgCl$_2$, 1 mM NAD$^+$). 1 U G6PDH from *L. mesenteroides* (Megazyme) was added to start the reaction which was followed at 340 nm. The increases in absorbance after addition of enzyme(s) in the water heated blank sample was deducted from those in the HCl treated sample and used to calculate the amounts of glucose or glucose-6-phosphate.

2.2.9   Production of GlgP and MalP protein expression vectors

PCR was used to amplify the *glgP* (forward primer: 5'CACCATGAATGCTCCGTTTACATA3'; reverse primer: 5'ACTTACAATCTCACCGGATCGA3') and *malP* (forward primer: 5'CACCATGTCACAACCTATTTTTAA3'; reverse primer: 5'TCCTTAGCGTTTTGCCTGC3') genes from *E. coli* genomic DNA (strain JW0675) using Phusion DNA polymerase (Thermo Scientific). Amplicons were visualised on a 0.8% (w/v) agarose gel following extraction and purification. Purified PCR products were directionally cloned into pENTR™/D-TOPO® according to the manufacturer's instructions. The genes were recombined into pDEST™17 using LR Clonase® II enzyme mix (Invitrogen) and transformed into *E. coli* DH5α, producing pDEST17.GlgP and pDEST17.MalP. The presence of the *glgP* and *malP* genes was confirmed with PCR. Plasmid mini-preps were performed on positive colonies; the isolated plasmids were stored at - 20°C until further use.

2.2.10 Purification of recombinant protein

Both pDEST17.GlgP and pDEST17.Malp were transformed into *E. coli* BL21(DE3) by heat shock. Positive transformants were selected on Luria Bertani (LB, Bertani, 1951) agar plates (1% (w/v) tryptone, 1% (w/v) NaCl, 0.5% (w/v) yeast extract, 1.5% (w/v) bacteriological agar) containing 50 µg/ml ampicillin. Single colonies were picked and inoculated into 2 ml liquid LB and cultured overnight at 37°C. The 2 ml starter cultures were then transferred to 200 ml auto-inducing media (ZYP-5052: 1% (w/v) N-Z-amine, 0.5% (v/v) glycerol, 0.05% (w/v) glucose, 0.2% (w/v) α-lactose, 50 mM Na_2HPO_4, 50 mM KH_2PO_4, 25 mM $(NH4)_2SO_4$, 1 mM $MgSO_4$) to allow for growth and the expression of the target genes (Studier, 2005). The cultures were grown for 3-4 h at 37°C and then at 28°C overnight. Cells were harvested by centrifugation at 5000*g* for 10 min and 4°C and pellets re-suspended in 10 ml buffer containing 50 mM NaH_2PO_4, 300 mM NaCl and 10 mM imidazole (pH 8). The cells were disrupted by sonication followed by centrifugation for 30 min at 10000*g* and 4°C and the resulting supernatant was further clarified by filtration through a 0.45 µm cellulose acetate membrane. Protein purification was achieved by immobilized metal ion affinity chromatography (IMAC), using a 1ml nitrilotriacetic acid (NTA) agarose-linked column according to the manufactures instructions. The supernatant was passed through the column and washed with 10 column volumes of washing buffer (50 mM NaH_2PO_4, 300 mM NaCl 20 mM imidazole, pH 8). The protein of interest was eluted in 1 ml fractions using 50 mM NaH_2PO_4, 300 mM NaCl 250 mM imidazole, pH 8. Glycogen and maltodextrin phosphorylase activity assays were performed on the respective collected fractions to test for activity. Fractions containing the highest activity were pooled together, dialyzed against 50 mM Tris-HCl pH 7.6 and frozen at -20°C until use.

2.2.11 Glycogen and maltodextrin phosphorylase activity assay

To test for GlgP and MalP activities, their ability to liberate G1P (in the presence of inorganic phosphate-(P_i)) from their respective substrates was determined spectrophotometrically in an enzyme-coupled assay. The reaction was started by the addition of 10 µg protein to 200 µl assay buffer containing 50 mM potassium phosphate buffer (pH 7), 2 mM NAD^+, 20 µM GBP, 5.6 U/ml G6PDH (*L. mesenteroides*), 1 U/ml PGM (Rabbit muscle, Sigma) and 1% (w/v) substrate (glycogen (*C. fonicata*) for GlgP and maltodextrin (Dextri-maltose, MP Biomedicals) for MalP). The increase in OD at 340 nm was followed.

2.2.12 Protein quantification

Protein concentrations were determined using a kit (BioRad, Munich) based on the method of Bradford (1976). Bovine serum albumin (BSA) was used as a standard.

2.2.13 Incorporation of glucan bound phosphate by recombinant protein MalP and GlgP

5 µg of recombinant protein was incubated in 1 ml incubation buffer (50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 0.1% (w/v) NaN_3, 1 mg/ml maltodextirn (Dextri-maltose, MP Bio medicals) containing either 5 mM Glc-1-P or 2.5 mM Glc-1-P and 2.5 mM GBP. Incubations were performed at 37°C for 30 min, after which the resulting glucan polymer was precipitated by the addition of 1ml 80% (v/v) ethanol to 200 µl of the incubations and centrifugation (20 min, 10000g at 4°C). The polymer was washed twice in 80% (v/v) ethanol at 80°C for 1 h with shaking. After each wash step, the sample was centrifuged for 5 min at 10000g and room temperature. Glucose and Glc-6-P contents were determined as described in Section 2.2.8.

2.2.14 Statistical analysis

Statistical analysis of the data was performed using a two-tailed homoscedastic *Students t-test* (Microsoft Excel software, 2010).

2.3   Results and Discussion

2.3.1   *In vitro* analysis of phosphate incorporation

To demonstrate the ability of GlgP and MalP to incorporate phosphate into a polyglucan, the genes encoding them were transferred to the vector pDEST™17 and expressed in an *E. coli* system. It was attempted to purify the resultant recombinant protein by IMAC. After washing, 15 fractions were eluted and assayed for MalP activity, which was found in the first ten fractions. These were visualized via SDS-PAGE (Fig. 2.1) which showed that some of the fractions contained contaminating bands (*e.g*. Fig. 2.1, lane 7). All the fractions (Fig. 2.1, lanes 9, 10 and 11) containing a single band were selected and combined. Several attempts were made to purify GlgP but these were unsuccessful in producing suitable amounts of protein, as the tagged-protein did not bind to the column, possibly indicating that the histidine tag folded internally to the protein. Nonetheless, additional steps were taken to optimize the expression of GlgP in a suitable protein expressing system, however due to time limitations this will not be reported.
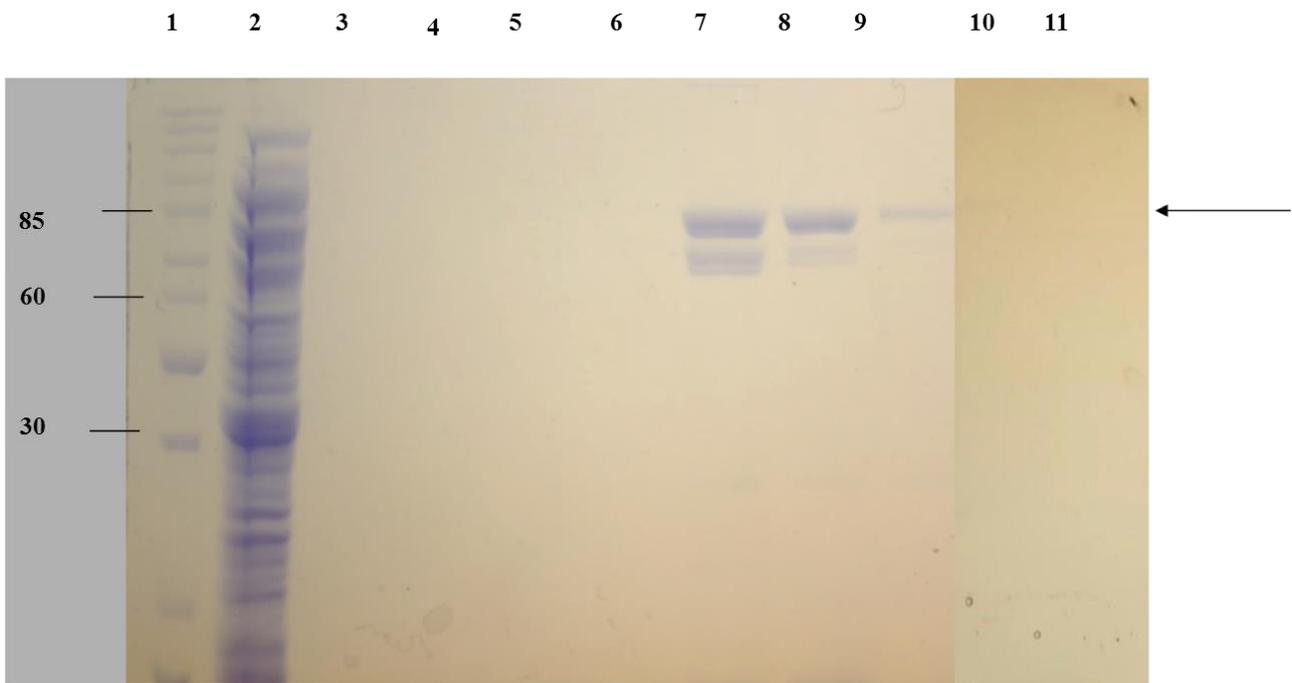
Figure 2.1 Purification of MalP. A 10% (w/v) SDS-PAGE resolving gel of the purification of maltodextrin phosphorylase (MalP) visualized with Coomassie brilliant blue. Lanes 1: protein ladder, lanes 2: crude protein extract, lanes 3-9: fractions 1-7 and lanes 10-11: fractions 8-9. The arrow indicates the expected band size of roughly 80 kDa.

Using maltodextrin as glucan primer together with recombinant MalP protein, phosphate incorporated into the produced polymer was determined after incubation with different combinations of Glc-1-P and GBP. As expected, no phosphate could be detected when Glc-1-P alone was the substrate. When a combination of Glc-1-P and GBP was used, however, approximately 15 nmol Glc-6-P/mg polymer was found after 2 h (Fig. 2.2). This result clearly indicates that MalP, as was hypothesized, is able to utilise GBP in order to incorporate Glc-6-P into a growing glucan meaning that it would have the potential to incorporate phosphate into glycogen *in vivo*. Interestingly, the polymer forming activity was higher in the incubation containing GBP indicating the preferential utilization of GBP over Glc-1-P (Fig. 2.2).
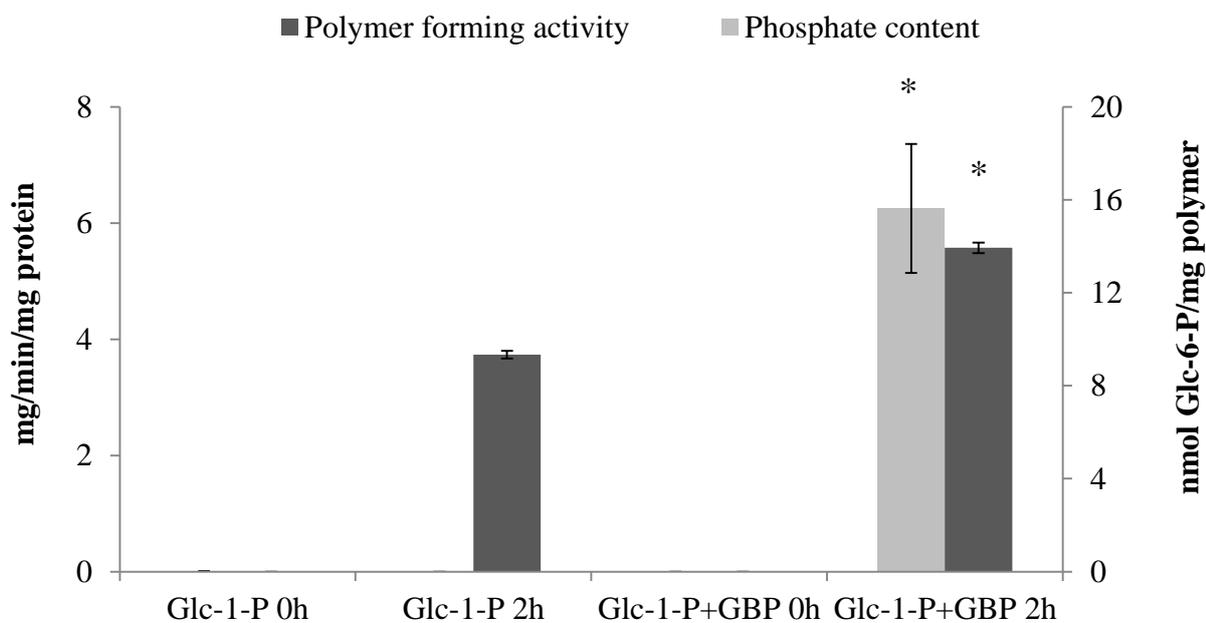
Figure 2.2 Phosphate incorporation. The phosphate content (nmol Glc-6-P/mg polymer) and polymer forming activity (mg/min/mg protein) determined from incubations containing recombinant MalP protein incubated with Glc-1-P (5 mM) and Glc-1-P (2.5 mM) in combination with GBP (2.5 mM). Values are indicated as the mean ± SD (n=3). Significant differences (*Student's t-test*) between the two substrate incubations after a 2 h incubation are indicated by an asterisk (*).

In spite of this result, this experiment will have to be repeated and supplemented with several changes to biochemically characterize the protein. Firstly, a proper series should be set up performing incubations over several time points. Secondly, varying concentrations and proportions of the two substrates needs to be tested. The protein of interest could potentially be incubated with different glucan primers (glycogen vs. maltodextrin) in combination with the various substrates. Likewise, GlgP will have to be purified and similar experiments performed to assess its ability to catalyse the same reaction.

2.3.2   Production and analysis of an *E. coli* double mutant lacking both *glgP* and *malP*

To demonstrate whether glycogen is phosphorylated through the combined actions of GlgP and MalP *in vivo* an *E. coli* double mutant was constructed. By making use of the P1 bacteriophage's ability to infrequently package DNA of its host instead of its own genetic material, the *glgP(kan)* and *malP(kan)* allelic regions from the single mutant donor *E. coli* strains (JW3391 and JW5689 respectively) were transferred to a second recipient mutant *E. coli* strain lacking *malP* or *glgP* (Thomason et al., 2007). The two putative double mutants were first examined using PCR alongside the WT (BW25113) strain and single mutant strains (JW3391, JW5689, ΔGlgP, ΔMalP).

Genotyping of *E. coli* colonies selected on media containing kanamycin revealed that the mutant *glgP* or *malP* allele carrying the marker was successfully transferred to the recipient strain (Fig. 2.3 and Fig. 2.4). Additionally, PCR analysis to validate the presence of the kanamycin selection marker, using a specific downstream kanamycin binding primer in conjunction with a gene specific primer (Fig. 2.4) was performed.

Although the *E. coli* genotyped as double mutants, it was decided to further analyse them to demonstrate that this led to a reduction in GlgP and MalP activities. To demonstrate this, bacteria were grown in the presence of two different carbon sources. It has previously been shown that MalP is negatively influenced by the presence of glucose (Chao and Weathersbee, 1974) whereas maltose induces its activity (Schwartz and Hofnung, 1967). GlgP on the other hand, has been found to be constitutive and not dependent on the carbon source present (Chen and Segel, 1968a). However, based on GlgP's involvement during glycogen degradation it is reasonable to consider glucose as an inducer seeing that glucose utilization ultimately leads to glycogen accumulation under the appropriate conditions (Chen and Segel, 1968b). Protein extracts from *E. coli* grown in media containing glucose or maltose were subjected to non-denaturing gel electrophoresis. Native GlgP and MalP activities were determined by distinct banding patterns that occur, following iodine staining, based on their activities ability to incorporate Glc-1-P into a glucan polymer. This was achieved by subsequently incubating the resulting gels with a high concentration Glc-1-P that drives the reaction in the direction of Glc-1-P incorporation.
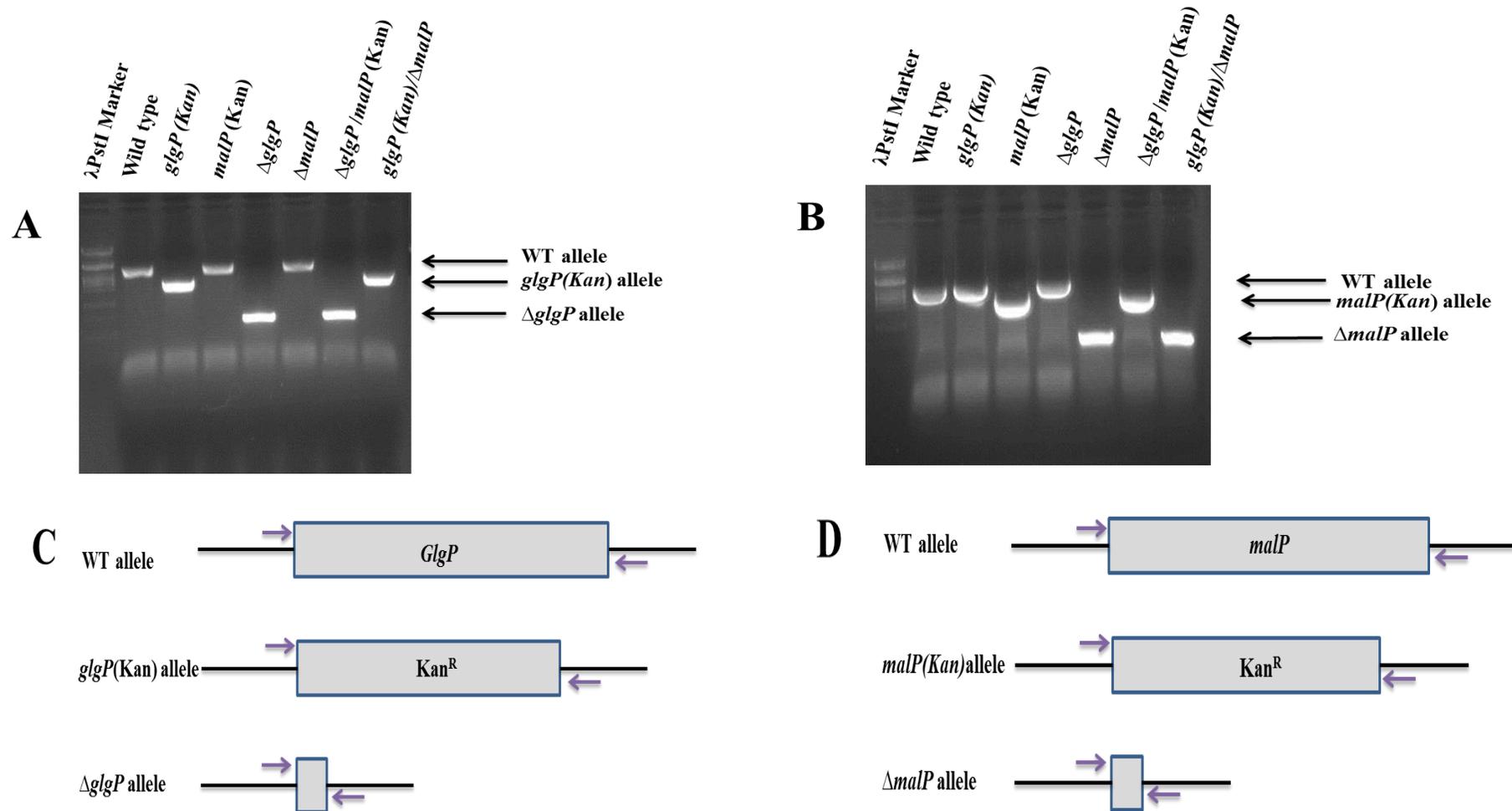
Figure 2.3 PCR genotyping of *E. coli* mutants. Agarose gels depicting A) the particular *glgP* allele (indicated by the arrows) and B) the particular *malP* allele (indicated by the arrows) present in the respective *E. coli* strain. Schematic representation of C) the WT *glgP* allele along and D) WT *malP* allele along with the two mutant variants; arrows indicate primer binding sites.
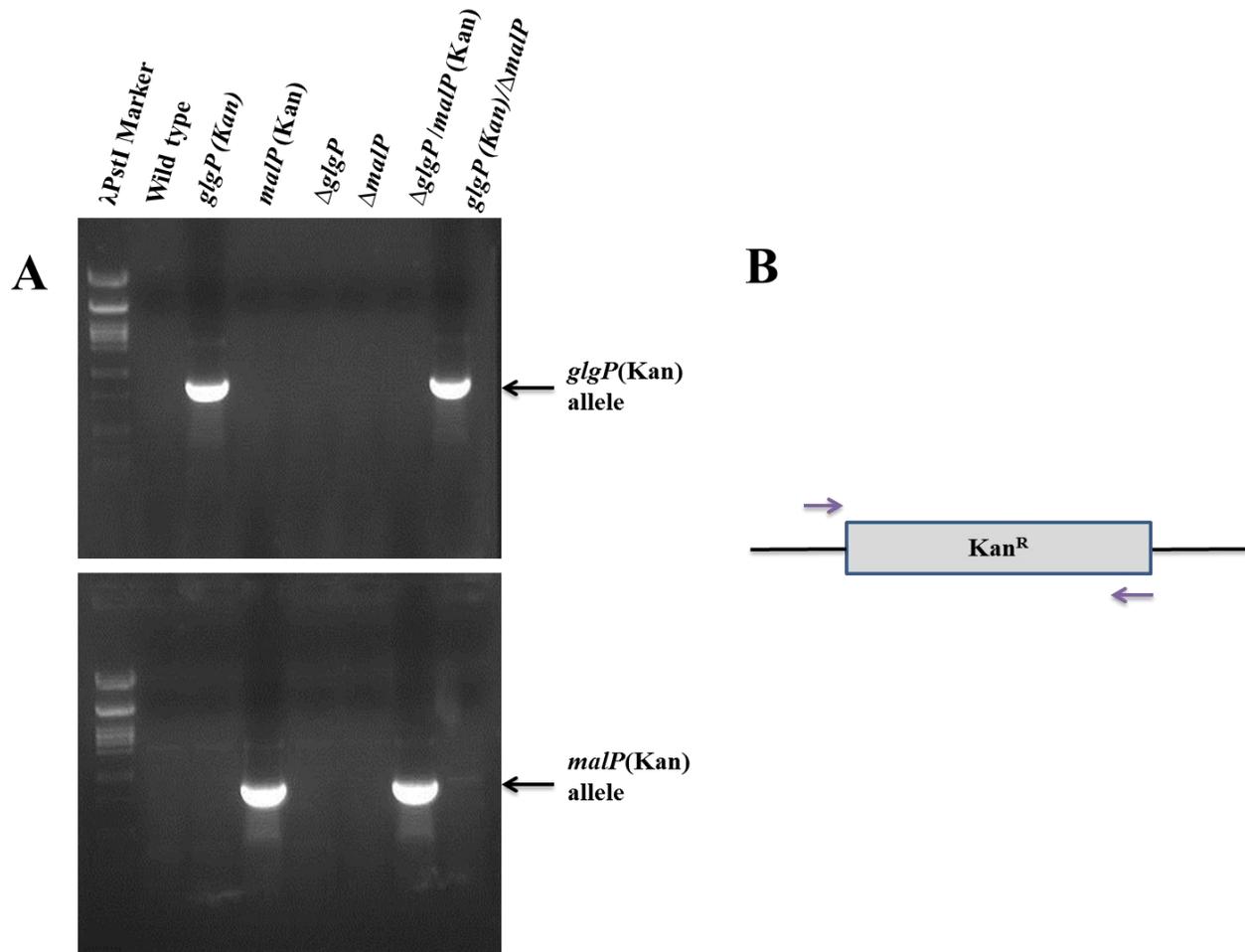
Figure 2.4 A) PCR analysis of *E. coli* mutants. Agarose gel showing the *glgP(kan)* and *malP(kan)* alleles (indicated by the arrows) present in the respective *E. coli* strains that contain the kanamycin cassette. B) Schematic representation of the Kan^R gene insert; arrows indicate the binding of the primers.

From the zymogram analysis (Fig. 2.5) it was clear that both double mutants lacked the GlgP and MalP activities as they contained no bands indicating incorporation of glucans, in comparison to the WT and single mutants, were present for both glucose and maltose induction. Moreover, when grown in the presence of maltose a MalP activity appeared to be more pronounced (thicker band) in the WT, Δ*glgP* and Δ*glgP(kan) E. coli* strains. This is not surprising, as maltose is known to induce MalP activity. Unexpectedly, no clear reduction in MalP activity was observed in the presence of glucose. This is most likely due to the formation of maltose and maltotriose from glycogen degradation, which would help overcome catabolic repression by glucose (Decker et al., 1993). It also demonstrates that MalP can use glycogen as a substrate for incorporation of glucose from Glc-1-P adding further credence to its proposed role in incorporating phosphate into glycogen.
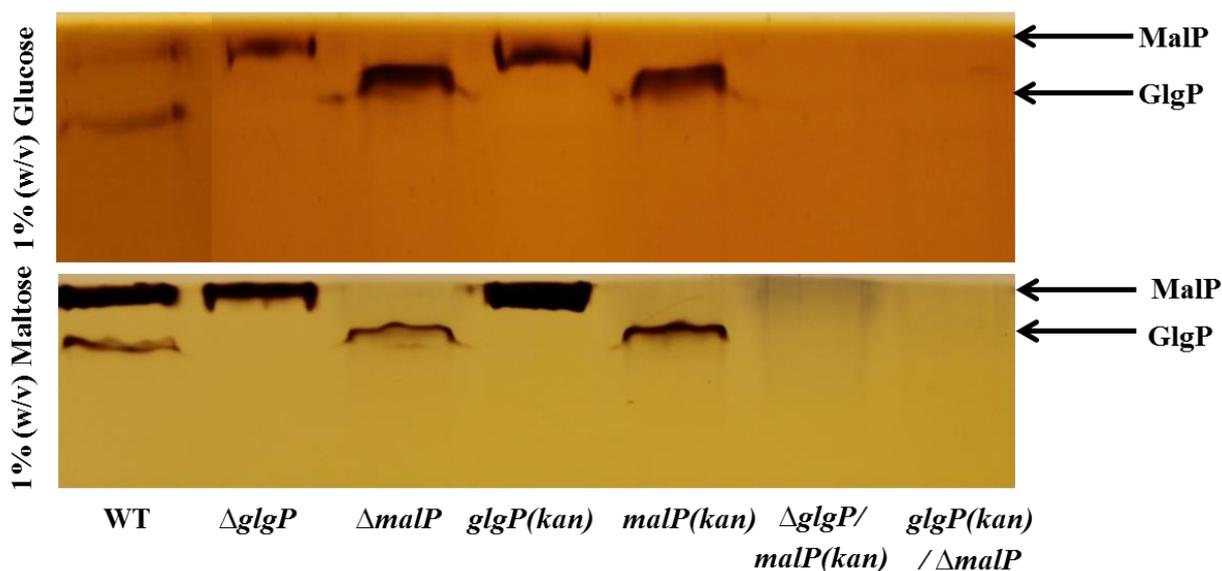
Figure 2.5 Non-denaturing gel electrophoresis. A 10% (w/v) non-denaturing polyacrylamide resolving gel containing 1% (w/v) glycogen to visualize glycogen and maltodextrin phosphorylase activities in WT and mutant *E. coli* grown in media containing 1% (w/v) glucose or 1% (w/v) maltose. Arrows indicate the bands indicative of GlgP and MalP.

2.3.3 *In vivo* analysis of glycogen phosphate content

In order to examine the involvement of *glgP* and *malP* in the incorporation of phosphate an *in vivo* based approach was developed using the double mutants. Due to reports on the occurrence of glycogen bound phosphate at low concentrations in *E. coli* (Lorberth et al., 1998; Viksø-Nielsen et al., 2002), it was decided to manipulate the available mutants and WT *E. coli* in such a way to increase their glycogen content. To achieve this, the strains were transformed with pACAG (Kossmann et al. 1999) carrying the *glgC16* gene that encodes an unregulated ADP-glucose pyrophosphorylase that is not allosterically regulated (Creuzat-Sigal et al., 1972) and which leads to the accumulation of large amounts of glycogen. The cells were cultured on media rich in phosphate and glucose, both of which stimulate glycogen accumulation in *E. coli* (Yang et al., 1996). Glycogen was isolated and subjected to acid hydrolysis. To account for any background, undigested water controls were included. The increase in absorbance at 340 nm of the control was subtracted from the treated sample to allow calculation of the amounts of both Glc and Glc-6-P present. Glc is indicative of the amount of glycogen, whereas Glc-6-P of the amount of phosphate within it. Several attempts were made to determine whether there were any significant differences between the mutants and WT *E. coli*, however, none could be detected (Fig. 2.6).
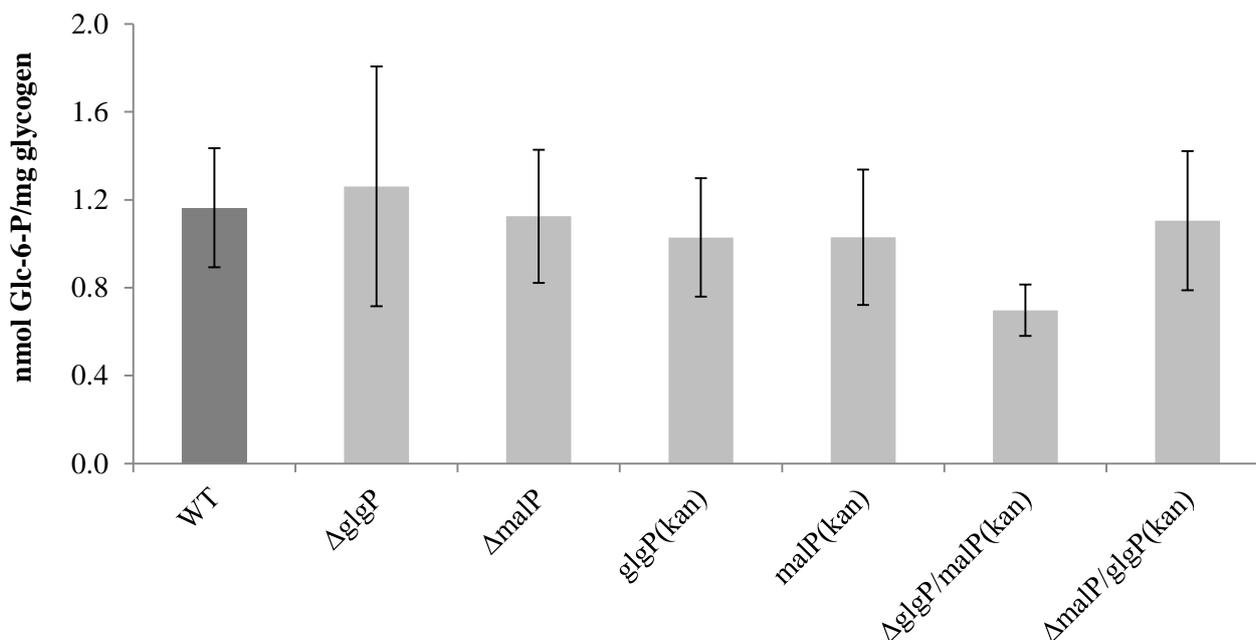
Figure 2.6 Glycogen phosphate content. The glycogen phosphate content of WT (parental strain BW25113) and seven *E. coli* mutants. Values are displayed as the mean ± SD (n=3). No significant difference (p<0.05) was detected between the mutant strains in comparison with the WT (*Students t-test*).

One of the possible reasons failing to detect significant differences in the glycogen phosphate content revolves around the relative insensitivity of the reader at the measured wavelength of 340 nm. Bearing in mind that WT *E. coli* has been shown to have low amounts of phosphate (Lorberth et al., 1998; Viksø-Nielsen et al., 2002) and that the generated double mutants along with the obtained single mutants is hypothesised to contain less, any differences observed could have been attributed to 'noise' in the signal of the machine. Based on the amount of glycogen present in the sample tested (approximately 0.3 mg) and reported values of glycogen phosphate (Lorberth et al., 1998; Viksø-Nielsen et al., 2002) the change in OD for glycogen from the wild type strain would be expected to be around 0.006. The sensitivity of plate reader is such that it only determines up to three decimal places and as such, it is extremely difficult to measure lower amounts of glycogen phosphate using this instrument. It may be possible to overcome this by increasing the amount of glycogen in the sample to increase the amount of phosphate. This has proven to be difficult due to difficulty in purifying large amounts of glycogen from *E. coli*, even despite the utilization of the pACAG plasmid (Kossmann et al., 1999) as well as the use of a large amounts of cells. Additionally, approaches that are more sensitive, such as LC-MS, could be used. However, this is sensitive to the presence of chloride ions, so alternative digestion methods to the one employed in this study (HCl-hydrolysis) would have to be employed. It was attempted to use both dilute trifluoroacetic acid (TFA) hydrolysis as well as enzymatic digestion, using α-amylase and

amyloglucosidase, but both these approaches led to phosphate removal from a Glc-6-P positive control (data not shown). As a result of this, it can be concluded that although MalP (and probably GlgP) have the potential to incorporate Glc-6-P into glycogen, their role(s) *in vivo* have not been confirmed and need to be assessed further in future.

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Chapter 3: The production of glucose-1,6-bisphosphate (GBP) in *E. coli*

This chapter is intended to be submitted to the *Journal of Biological Chemistry* and has been written up in that format. The relative contributions of the authors on the paper are listed below:

Daniel Vosloh and Jonathan Jewell

- Purification and Biochemical characterization of recombinant AGP protein

- Analysis of *E. coli* mutants

- Writing of first draft

Margaretha J van der Merwe

- General advice

Benedicte Leboutillée

- Construction of AGP-expressing vector

John Lunn and Regina Feil

- Determination of absolute concentrations of GBP (Triple Quadrupole MS - AEC-MS/MS)

Jens Kossmann

- Funding

James R Lloyd

- Supervisor

- Funding

- Writing

- Initial identification and purification of protein responsible for GBP synthesis

- Manufacturing of double mutant lacking *agp* and *pgm*

Glucose-1,6-bisphosphate is produced by a combination of the acid glucose-1-phosphate phosphatase and phosphoglucomutase in *Escherichia coli* [*]

Daniel Vosloh[1], Jonathan Jewell[1], Margaretha J van der Merwe[1,2], Benedicte Leboutillée[1], John Lunn[3], Regina Feil[3], Jens Kossmann[1], James R Lloyd[1]

[1]From the Institute for Plant Biotechnology, Department of Genetics. University of Stellenbosch, South Africa

[2]ARC Centre of Excellence in Plant Energy Biology, University of Western Australia, Australia

[3]Max-Planck-Institute of Molecular Plant Physiology, Golm, Germany

*Running title: *Glucose-1,6-bisphosphate production in E. coli*

To whom correspondence should be addresses: James R. Lloyd, Institute for Plant Biotechnology, Department of Genetics, Private Bag X1, Matieland 7602, Stellenbosch, South Africa, Tel.: +27 (21) 808-3837; Fax: +27 (21) 808-3835; E-mail: lloyd@sun.ac.za

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Background: The enzymes responsible for manufacturing GBP in *E. coli* are unknown.

Results: AGP catalyses a GPPDM activity producing GBP. Mutant *agp* cells still produce GBP. Mutant cells lacking both *agp* and *pgm* do not produce GBP.

Conclusions: Two enzymes synthesize GBP in *E. coli*.

Significance: These findings reveal a novel mechanism for the formation of glucose-1,6-bisphosphate in *E coli.*

3.1 Abstract

In order to identify the protein responsible for glucose-1-phosphate phosphodismutase (GPPDM) activity in *E coli* we purified it to apparent homogeneity. Tryptic fingerprinting demonstrated it to be the acid glucose-1-phosphate phosphatase (AGP) protein (EC 3.1.3.10). Using purified recombinant AGP we show that, in addition to its phosphatase activity, it also possesses the catalytic function of transferring a phosphate unit from one glucose-1-phosphate (G1P) molecule to the C6 position of another. This yields glucose-1,6-bisphosphate (GBP), an important regulatory signal in central carbon metabolism. *agp* mutant cells are, however, unaltered in GBP amounts, and were still able to manufacture GBP from G1P. A strain mutated in both *agp* and phosphoglucomutase (*pgm*) was unable to produce GBP, indicating that both enzymes contribute towards its manufacture *in vivo*.

The first report of glucose-1-phosphate phosphodismutase (EC 2.7.1.41; G1PPDM) activity in *E. coli* was published in 1949 (1). This enzyme catalyses the transfer of the phosphate moiety from one glucose-1-phosphate molecule to the C6 position of another, resulting in the production of glucose-1,6-bisphosphate (GBP), a molecule that acts as a cofactor for several enzymes involved in carbon metabolism. Its role as cofactor includes the stimulation of phosphoglucomutase (PGM), phosphofructokinase (PFK) and pyruvate kinase (PK), while exerting an inhibitory effect on hexokinase (HK) (2, 3). Furthermore, this regulating metabolite has been connected to several other primary metabolic pathways essential to the cellular functioning within both prokaryotic and eukaryotic organisms (2–5). Despite the time since the initial description of the activity, a gene encoding G1PPDM has not been identified from *E coli*. In contrast to that, enzymes responsible for GBP formation in other biological systems have been studied more intensively. Although several different glucose-1,6-bisphosphate forming activities have been identified in crude extracts, only two genes have been isolated which encode them. Firstly, a mammalian PGM-like isoform phosphorylates the 6-position of G1P using bisphosphoglycerate (6) and secondly, in *Bacillus subtilis,* GBP is produced in a G1P phosphodismutase reaction catalyzed by β-phosphoglucomutase (7).

The *AGP* gene from *E coli* is annotated as a periplasmic acid glucose-1-phosphatase (8–10). *E. coli* are unable to import G1P and, therefore, its primary function is thought to be to the conversion of external G1P to an

assimilable sugar. It has been shown, however, that it is not essential for growth when G1P is the sole carbon source, as other enzymes can compensate for its loss of function (11). Structure elucidation of AGP (12) demonstrated additional binding capacity for various phosphorylated inositol substrates, consistent with a reported phytase activity (13). In this paper, we demonstrate that the AGP protein has a further catalytic function as it is able to manufacture GBP from G1P. In addition, we show that an additional GBP manufacturing activity that is catalysed by PGM.

3.2 Experimental procedures

Materials — All chemicals used in this study were of analytical grade and were purchased from Sigma (St. Louis, USA) or Roche (Mannheim, Germany). Solutions were prepared with ultrapure H_2O (Millipore). Enzymes were either from Sigma (St. Louis, USA) or from Megazyme (Bray, Ireland). The ion exchange and hydrophobic interaction protein purification columns used were from GE Healthcare Life Sciences (Uppsala, Sweden) while GST purification columns were from Macherey-Nagel (Dueren, Germany). pET41a was obtained from Merck KGaA (Darmstadt, Germany).

Bacterial strains and Plasmids — E. coli strains and plasmids used in this study are listed in Table 1. *agp* and *pgm* mutant strains were obtained from the Yale *E coli* Genetic Stock Center.

Table 3.1 Bacterial strains and plasmids.

	Description	Ref.
E. coli **Strains**		
BW25113	*Δ(araD-araB)567 ΔlacZ4787(*::rrnB-3*) λ⁻ rph-1 Δ(rhaD-rhaB)568 hsdR514*	Datsenko and Wanner (14)
JW0675-1	BW25113 Δ*pgm-736::kan*	Baba et al (15)
JW0987-4	BW25113 Δ*agp-746::kan*	Baba et al (15)
PGM	BW25113 Δ*pgm-736*	This study
PGM-AGP	BW25113 Δ*pgm-736 Δagp-746::kan*	This study
E. coli BL21 (DE3 pLys)	F- *omp*T *hsd*SB(rB-, mB-) *gal dcm* (DE3) pLys (Cam^r)	Life Technologies
Plasmids		
pET41a	IPTG-inducible expression vector, GST-tag	Merck KGaA
pET41a-AGP	IPTG-inducible expression vector, GST-tag	This study
pCP20	Temperature sensitive plasmid controlling FLP-recombinase	Cherepanov and Wackernagel (16)

Purification of Protein Responsible for G1PPDM Activity from E. coli *crude Extracts* — *E. coli* (JW0675-1) *pgm* mutant cells were grown overnight in liquid culture at 37°C before being spread onto solid Luria-Bertani (LB) media. The bacteria were grown overnight at 37°C and harvested by scraping before being re-suspended in 50 mM Tris-HCl (pH 7.0) and lysed by sonication. The cells were clarified by centrifugation at 10 000g and 4°C for 10 min after which the supernatant was taken. Ammonium sulfate was added to this at a final concentration of 50% (w/v) and the solution was left at 4°C for 1 h before being centrifuged again at 10 000g and 4°C. The supernatant was dialyzed overnight against 50 mM Tris-HCl (pH 8.8) and then separated by anion exchange chromatography. Approximately 200 mg of *E. coli* protein was loaded onto 10 ml of HiTrapQ resin, washed with 100 ml of 50 mM Tris-HCl (pH 8.8) after which bound protein were eluted over 90 ml with a linear gradient of Tris-HCl (pH 8.8), 1 M NaCl. Fractions demonstrating activity were pooled and dialyzed overnight against 50 mM NaP$_i$ (pH 7.0), 1 M ammonium sulfate. These were loaded onto a HiPrep 16/10 Phenyl FF (high sub) column in the same buffer and eluted using 50 mM NaP$_i$ (pH 7.0). Fractions demonstrating activity were dialyzed overnight against 50 mM acetic acid/NaOH (pH 4.5) before being loaded onto a 5ml Hi-TrapSP column in the same buffer. Proteins were eluted using 50 mM acetic acid/NaOH (pH 4.5), 1 M NaCl and fractions demonstrating activity were pooled and frozen at -20°C.

Protein Identification —Peptide mass fingerprinting was performed. Sample preparation was achieved by SDS-PAGE following proteolysis using trypsin. The resulting peptide digest was analyzed and the protein identified through a commercial service.

Production of the AGP expression vector — *AGP* was amplified from genomic DNA using a forward primer (5'-AT<u>AGGCCT</u>TAATGCAAACCGTACCGGAAGGCTATCA-3') flanked by a *Stu*I site (underlined) and reverse primer (5'-AT<u>CTCGAG</u>TTATTTCACCGCTTCATTCAACACGCT-3') flanked by an *Xho*I site (underlined) and ligated into pET41a between the *Stu*I and *Sal*I sites producing pET41a-AGP.

Purification of Recombinant G1PPDM protein —pET41a-AGP was transferred into BL21 DE3 *E. coli* cells by means of heat shock. Positive transformants were selected for on agar plates containing 50 μg ml^{-1} ampicillin. Single colonies were selected, inoculated into 2 ml LB and cultured overnight at 37°C. The starter cultures were used to inoculate 200 ml LB and grown at 37°C with shaking.

Protein expression was achieved by the addition of Isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 1 mM, the cultures were subsequently grown at 25°C overnight with shaking. The cells were collected by centrifugation (3000g for 5 min and 4°C) and re-suspended in 5 ml of a buffer containing 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 2 mM EDTA, 10 mM $MgCl_2$, 10% (v/v) glycerol, 0.1% (v/v) Triton X-100 and 1 mM PMSF. The cells were lysed by sonication and centrifuged for 30 min at 4°C and 10 000g. Protein purification was performed by using a 1ml glutathione agarose-column. Supernatant containing approximately 100 mg total protein was passed through the column, which was washed with 10 column volumes of phosphate buffered saline (PBS). The protein of interest was eluted in ten to fifteen 1 ml fractions using 50 mM Tris-HCl pH8.0 containing 10 mM reduced glutathione. Aliquots of these were denatured and separated by SDS-PAGE on a 10% (w/v) resolving gel. Protein amounts were visualized by staining with stain solution (0.4% (w/v) Coomassie brilliant blue R350, 20% (v/v) methanol and 10% (v/v) acetic acid) detaining with destain solution (50% (v/v) methanol and 10% (v/v) acetic acid). Fractions with the highest protein content were combined and frozen at -20°C for further experiments. The same procedure was used for the purification of the glutathione-S-transferase (GST) protein from the pET41a empty vector control. Thrombin CleanCleave™ Kit (Sigma-Aldrich) was used to cleave the GST tag according to the manufactures instructions. All proteins were then frozen and stored at -20°C until use.

Kinetics of the Purified Recombinant Protein — Approximately 10 µg of the protein containing a mixture of AGP GST was incubated in 1 ml of reaction buffer (50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$ and 0.1% (w/v) NaN_3) containing either 5 mM G1P or 5 mM G1P and 5 mM ATP. As controls the same reactions were incubated either with heat inactivated AGP and GST, or with thrombin alone. Samples were incubated at 37°C for different time periods (0 min, 30 min, 1 h, 2 h, 4 h, 8 h and 24 h) after which they were heated for 10 min at 95°C. The samples were frozen at -20°C until analysis. To test for the effect of various compounds on the activity of AGP, protein reactions were set up as described above containing 5 mM G1P and ADP, AMP, UTP or FBP at an end concentration of 1 mM. Reactions were stopped after 4 h by heating for 10 min at 95°C.

In order to determine the K_m of the AGP protein for G1P incubation conditions as above were chosen and the G1P concentration in the assay mix was increased to 1, 5, 10 and 50 mM. Reactions were stopped after 4 h as above.

To test for the minimal concentration of the activation effect of ATP above described incubation conditions were chosen and 5 mM G1P was incubated with the following ATP concentrations: 5 mM, 2 mM, 1 mM, 500 µM, 100 µM and 10 µM. Reactions were stopped after 4 h as above. AGP protein was incubated with 1 mM GBP and 1 mM glucose for 4 h at 37°C and the reaction was stopped as above.

Extraction of crude proteins and metabolites from E. coli — Wild type (WT) and *agp* mutant strains were grown overnight in LB broth at 37°C. Protein was extracted in 50 mM Tris-HCl (pH 7.5) after the cells were lysed by sonication. The protein supernatant was taken after centrifugation at 10 000g and 4°C for 10 min and was desalted before being used to test for GBP production by incubation with 5 mM G1P for 4 h at 37°C followed by heating at 95°C for 10 minutes. Protein extracts that were heated for 10 minutes at 95°C before the incubation acted as negative controls. Similarly, for the extraction of metabolites, WT and *agp* mutant strains were cultured in LB at 37°C and metabolites extracted in 50 mM Tris-HCl (pH 7.5). The resulting supernatant after sonication and centrifugation (as described above), was passed through a 3 kDa size exclusion column (Merck KGaA). The GBP content was determined in the flow-through.

Enzyme Activity and Metabolite Assays —Stimulation of rabbit muscle PGM (17) was examined as an estimate for GBP production (see Fig. 3.5-Supplementary material). The reaction mixture contained 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$; 1 mM β-mercaptoethanol; 1 mM NAD^+; 1 mM G1P; 0. 034 U ml^{-1} PGM and 5.7 U ml^{-1} G6PDH. PGM activity was followed by measuring absorbance at 340 nm. Stimulation of PGM was examined following addition of samples by increases in absorbance at 340 nm.

Detection of GBP was achieved by anion exchange chromatography – Triple Quadrupole MS - AEC-MS/MS as described in Lunn et al. (18).

The presence of glucose was assayed using hexokinase and G6PDH. The assay mixture contained 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 1 mM β-mercaptoethanol, 1 mM ATP, 1 mM NAD^+, 5.6 Unit ml^{-1} G6PDH (*Leuconostoc*) and 1 Unit ml^{-1} hexokinase (yeast). Following the addition of sample, the reaction was monitored at 340 nm until completion.

G1P was assayed by coupling of G6PDH and phosphoglucomutase. The assay mixture consisted of 50 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 1 mM β-mercaptoethanol, 1 mM NAD^+, 5.6 Unit ml^{-1} G6PDH (*Leuconostoc*) and 1

U ml^{-1} PGM (Rabbit muscle). Following the addition of sample, the reaction was monitored at 340 nm until completion.

ADP amounts were measured by the coupled oxidation of NADH by lactate dehydrogenase (LDH). The assay mixture consisted of 50 mM Tris-HCl (pH 7.5), 10 mM MgCl$_2$, 1 mM β-mercaptoethanol, 1 mM phosphoenolpyruvate (PEP), 1 mM NADH, 1 mM Glc1P, 2 U ml^{-1} pyruvate kinase (PK; Rabbit muscle) and 1 U ml^{-1} lactate dehydrogenase (LDH; Rabbit muscle). Following the addition of sample, the reaction was monitored at 340 nm until completion.

Phosphate production was measured using the EnzChek® Phosphate Assay Kit (E-6646) from Molecular Probes following the company's instructions.

Determination of Protein Concentrations – Protein amounts in crude extracts were determined using a commercially available kit based on the method of Bradford (19). A dilution series of known amounts of bovine serum albumen was used to quantify the amount of recombinant protein.

Production of E. coli Double Mutant – The kanamycin resistance cassette inserted in the *pgm* gene of strain JW0675-1 was removed using the plasmid pCP20, producing the strain PGM (16) A double mutant containing the Δ*agp746::kan* and deleted *pgm* alleles was produced by transferring Δ*agp746::kan* from JW0987-4 to PGM by P1 phage transduction (20), leading to the production of the strain PGM-AGP. The presences of both mutant alleles were confirmed using PCR (data not shown). For the *pgm* allele the primers used bind immediately upstream and downstream of the excision points (Forward: GCGTTTACTTTGCGGCAGAT; Reverse: GACGACCAGACTCCTGTTGG). The *agp* allele was detected using a forward primer (CAGGTGCAATTATCAGCGGC) that binds upstream of the insertion site and a reverse primer (CTGATCGACAAGACCGGCTT) that binds within the kanamycin resistance gene.

3.3 Results

AGP converts G1P to a PGM stimulatory compound – To identify the protein that demonstrates G1PPDM activity in *E. coli* extracts, classical protein purification methods were used. A *pgm* mutant strain was utilized to overcome potential   with the detection system, which examined stimulation of the activity of rabbit muscle PGM. Crude extracts were fractionated using a series of ion exchange and hydrophobic interaction resins until we obtained fractions with one major polypeptide of approximately 35 kDa (Fig. 3.1). This was identified by tryptic fingerprinting as being AGP.

Purification of AGP from E. coli BL21 cells expressing pET41a-AGP leads to a single band of 70 kDa -

To achieve the production recombinant protein the *agp* gene was cloned into pET41a and purified based on the GST-gene fusion system. Separation was achieved on a SDS-PAGE gel and revealed the purification of a single 70 kDa band (Fig. 3.1) in the eluted fractions.

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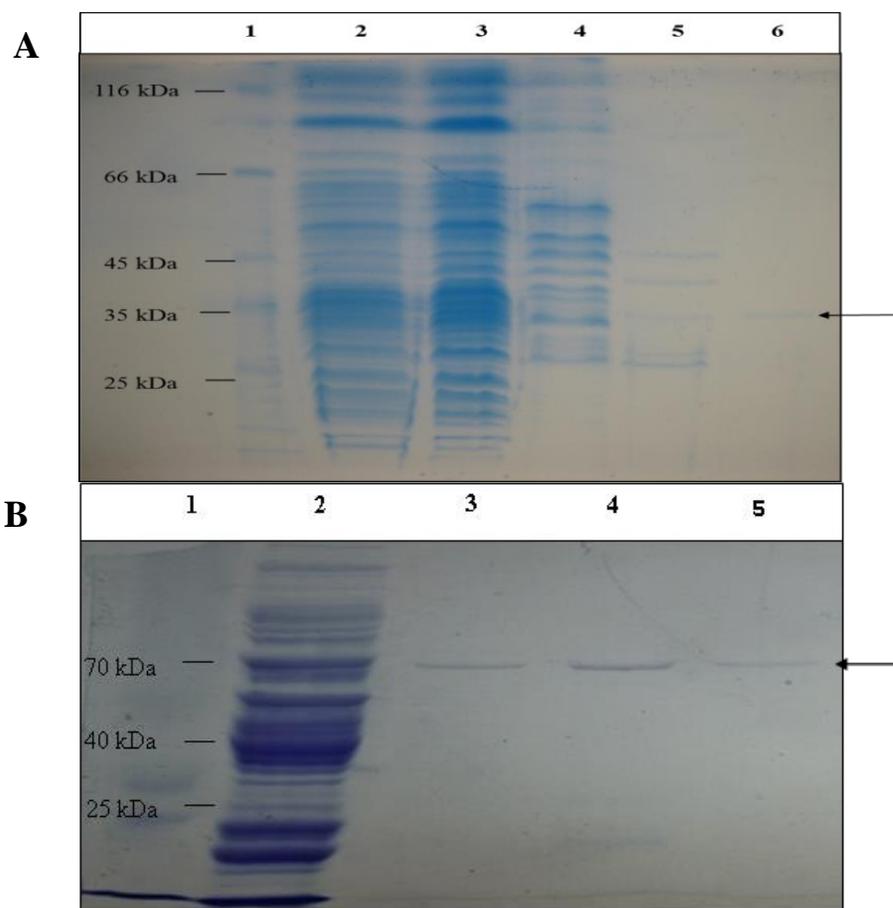
Figure 3.1 Purification of AGP. Coomassie brilliant blue stained SDS-PAGE gels of A) different protein fractions present after each purification step used to fractionate AGP from *E. coli* crude extracts for its identification; lane 1: protein marker, lane 2: crude extract, lane 3: ammonium sulphate separation, lane 4: anion exchange, lane 5: hydrophobic interaction and lane 6: cation exchange and B) for isolation of purified recombinant protein; lane 1: protein marker, lane 2: crude protein extract, lanes 3-5: eluted protein. Arrows indicate the anticipated protein band size of the approximately 35 kDa native (A) and 70 kDa recombinant polypeptides (B). Visualization was achieved with Coomassie brilliant blue.

Incubation of Recombinant AGP Protein with G1P produces both Glucose and GBP – To examine the reaction in more detail we purified recombinant AGP protein and cleaved and removed the GST tag, leaving a mixture of AGP and thrombin. Purified AGP was assessed by testing for the production of glucose after incubation with G1P. 10 µg/ ml of protein was incubated with 5 mM G1P and, after 1 h, approximately 10% of the G1P had been converted to glucose. Heat inactivated AGP protein or thrombin incubated with 5 mM G1P did not lead to any glucose production. Following incubation with AGP, the G1P demonstrated the ability to stimulate the activity of PGM, indicative of the production of GBP in addition to glucose (data not shown).

A timecourse was then performed. Recombinant protein was incubated with 5 mM G1P and samples were taken over a 24 h period. An increase in the stimulation of PGM was observed over time (Fig. 3.2A) reaching up to sixteen fold higher values at the final time points compared to the controls. Examination of the annotated function of AGP, namely glucose-1-phosphatase activity was performed by measuring the production of both glucose and inorganic phosphate (P_i) as well as the consumption of G1P (Fig. 3.2C-E). These experiments demonstrated that AGP consumed almost all the G1P after 24 h, producing approximately 4 mM of glucose. The amount of P_i produced was less than that of glucose, reaching approximately 3 mM after 24 h. In order to confirm the production of GBP the samples from the different timepoints were analyzed by LC-MS/MS (Fig. 3.2F). This demonstrated that GBP was also present in the samples and, therefore, that it is synthesised by the AGP in the presence of G1P. A clear time dependent pattern can be observed here. The specific activity for the phosphatase activity was approximately 0.55 µmol/min/mg protein while that of the GPPDM reaction was 0.06 µmol/min/mg protein.

To examine if AGP also acts in the reverse direction the protein was incubated with 1 mM G1P, GBP and 1 mM glucose to see if G1P was produced. Neither consumption of glucose nor production of G1P could be detected (data not shown).
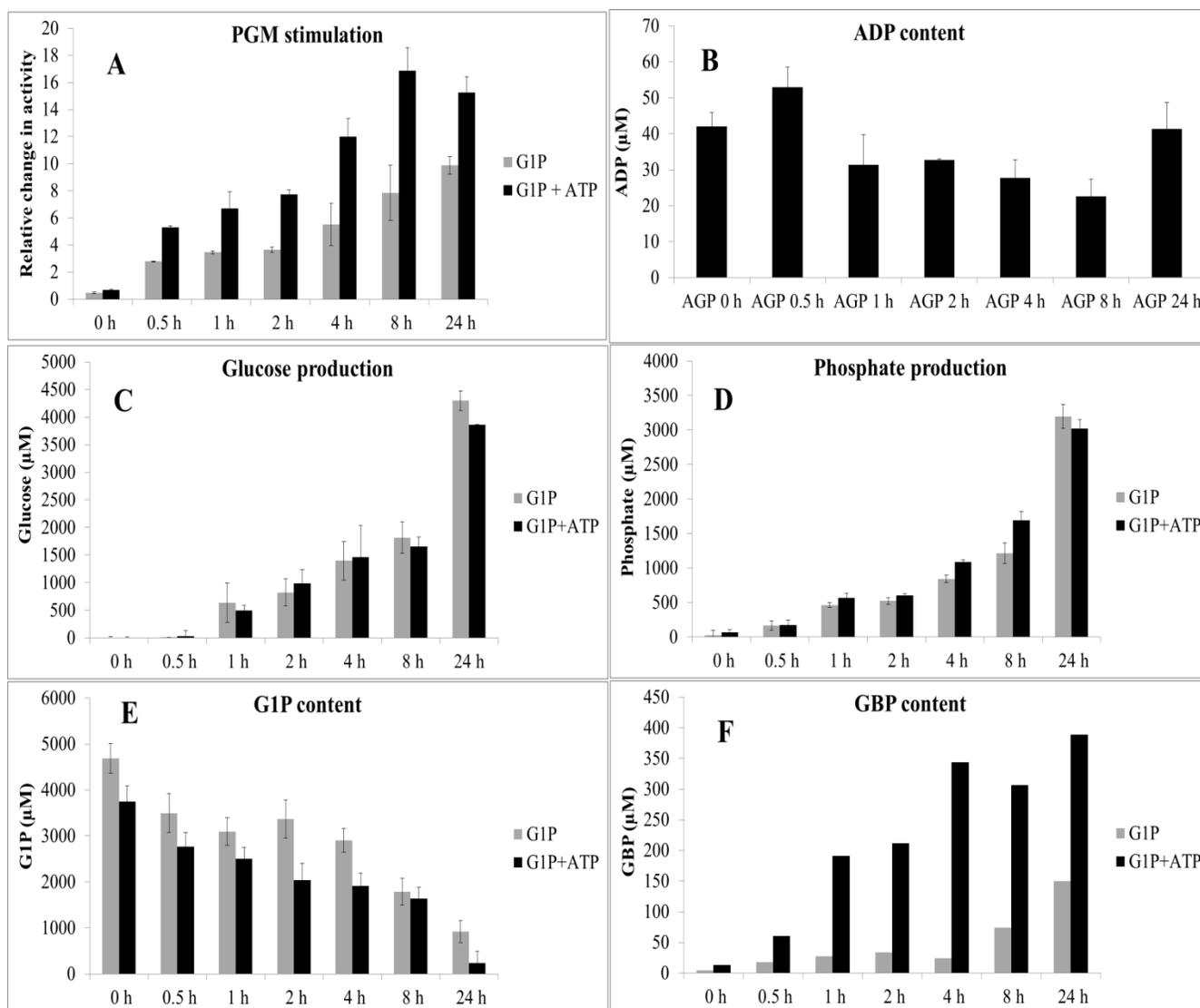
Figure 3.2 PGM stimulation, ADP content, Glucose production, G1P consumption, phosphate Production and GBP content of the AGP protein. Roughly 10 µg of the purified AGP protein were incubated with either 5 mM G1P or 5 mM G1P and 5 mM ATP at 37°C. Samples were removed after 0 min, 30 min, 1 h, 2h, 4 h, 8 h and 24 h aliquots and heated for 30 min at 95°C to inactivate the protein. These aliquots were used to quantify ADP (B), glucose (C) and G1P (E) content via coupled assays. GBP content was estimated indirectly using the PGM stimulation assay (A) and directly via LC-MS/MS (F). Phosphate production was monitored using a phosphate assay kit (D). All measurements were performed in triplicates except the phosphate production which was done in duplicate and the LC-MS/MS which was performed on a single sample. Error bars represent standard deviations (n=3).

GBP production is not reduced in an agp *mutant* – To examine if AGP is responsible for producing GBP *in vivo* we firstly incubated crude extracts from WT and an *agp* mutant with G1P. After 4 h incubation with 5 mM G1P, GBP was indirectly detected in both via PGM stimulation (data not shown). WT and mutant strains were then grown in LB media to an OD of 1. Metabolic extracts were made from them and the GBP content determined. The amounts of GBP present were normalized on the volume of extract used. The GBP content was found to be invariable between the two strains (Fig. 3.3).
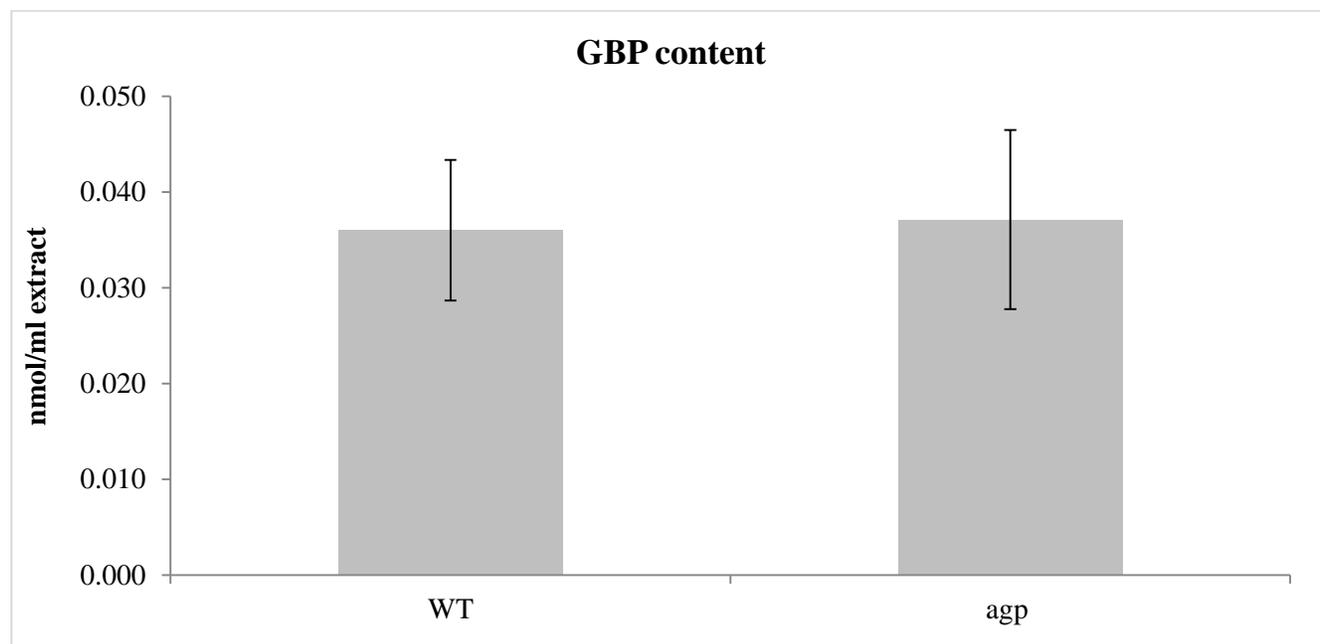

Figure 3.3 GBP content of WT and the *agp* mutant *E. coli* strains grown in LB. Metabolites were extracted from the cells and the amounts of GBP determined. The amount of GBP was normalized on the extract volume. Samples were measured in triplicate by LC-MS-MS with error bars representing standard deviations.

Phosphoglucomutase can also manufacture GBP – We hypothesised that PGM may also be responsible for manufacturing the remaining GBP for two reasons. Firstly it has been demonstrated previously that mammalian PGM can catalyse this reaction (6) and secondly, because when we purified AGP as a GBP producing enzyme, we did so from a *pgm* mutant strain and only observed one fraction of activity throughout (Data not shown). This indicated that any remaining GBP forming activity is most likely as the result of the PGM activity. To examine this we manufactured an *E. coli* strain containing mutant alleles of both both *agp* and *pgm*. Desalted crude extracts containing 250 μg of total protein from these were then incubated with 5 mM G1P

and the amounts of PGM stimulatory product as well as glucose, G1P, G6P and Pi were determined in a timecourse. As expected the strains containing an active PGM were able to convert G1P to G6P (Fig. 3.4B), while those containing an active AGP were able to convert G1P to phosphate (Fig. 3.4C) and glucose (Fig. 3.4D). A compound that is able to stimulate the activity of rabbit muscle PGM was identified in protein extracts from all the strains, except the one mutated in both *agp* and *pgm* genes (Fig. 3.4A). Interestingly the amount of stimulation decreased over time (Fig 3.4A). As PGM is the only enzyme in *E. coli* capable of converting G1P to G6P we wished to eliminate the possibilty that PGM itself was not making the stimulatory compound, but rather another enzyme that utilises G6P. To that end we also incubated the crude extracts with G6P, however no stimulatory compound was produced in the double mutant (data not shown).
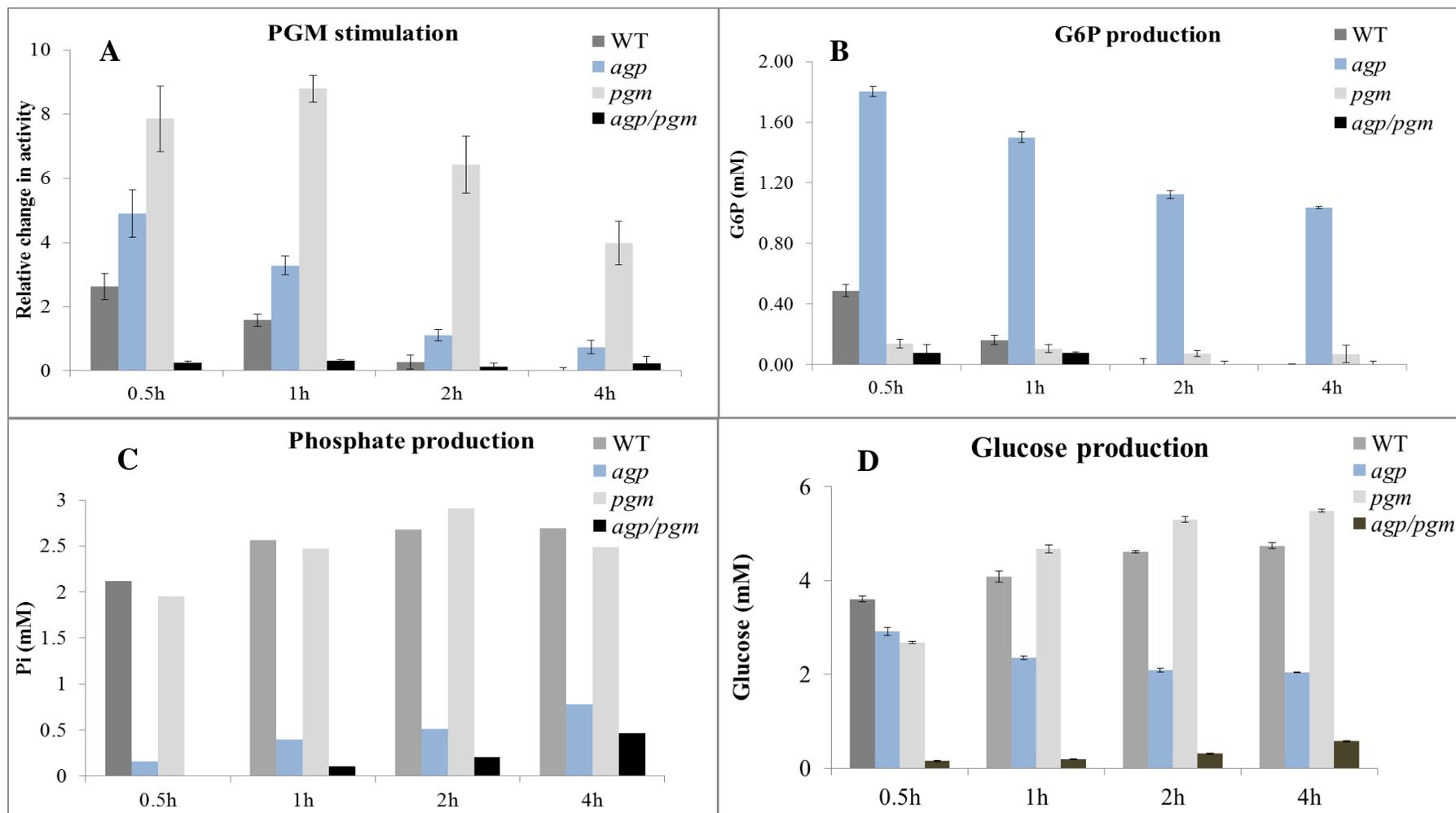
Figure 3.4 PGM stimulation, G6P production, phosphate production and glucose production for WT, *agp*, *pgm* and *agp/pgm* mutants. Crude extracts containing approximately 250 μg of protein were incubated with 5 mM G1P and aliquots removed and boiled after 0.5 h, 1 h, 2 h and 4 h. The aliquots were used to determine G6P (B) and glucose (D) content via enzyme-coupled assays. The presence of GBP was indirectly determined using a PGM stimulation assay (A), a relative change above 1 is indicative of the presence of GBP. Phosphate content (C) was measured using a phosphate assay kit. All measurements were performed in triplicate except for the quantification phosphate. Error bars represent standard deviation (n=3).

3.4 Discussion

The mechanism by which *E. coli* manufactures GBP through transfer of phosphate from one G1P molecule to the C6 position of another has been assumed for more than 60 years (1). However, the enzyme(s) responsible for this activity has, until now, remained undiscovered. In this paper, we show for the first time that two enzymes, glucose 1-phosphate phosphatase and phosphoglucomutase synthesize this compound. AGP was first discovered based on its ability to cleave a synthetic phosphatase substrate at low pH (8) and was shown to be important for utilization of G1P by *E. coli* grown on minimal media containing that sugar as a sole carbon source (9). More recently it has been demonstrated to also exhibit phosphatase activity against various inositol substrates (13). It isn't surprising that *E. coli* genes encode proteins with multiple functions as its genome consists of only approximately 5000 open reading frames (21). In this case, the novel phosphotransferase activity that we detected is very different to the phosphatase activities previously described. AGP is part of branch 2 of the histidine phosphatase superfamily (22), which includes a number of enzymes that are also mutases. Phosphatase and mutase activities are somewhat similar. In both cases, phosphate is transferred from the substrate to the 'phosphate pocket' within the enzyme. In the case of phosphatases, nucleophilic attack hydrolyzes the phosphate, whilst in mutases either the substrate re-orientates itself within the active site and the phosphate is transferred to a new position within it, or a new substrate is bound to the primed phosphoenzyme, which then receives the phosphate. We propose in the case of AGP that, prior to nucleophilic attack, a G1P binds within the active site with the 6-carbon orientated to the 'phosphate pocket' whereupon the phosphate becomes transferred to the 6-position. Clearly the phosphatase reaction is favoured as we found that the specific activity of this reaction was tenfold greater than that of the mutase reaction.

It seems unlikely that AGP would function as the main source of GBP that is be involved in allosteric regulation of central carbon metabolism due to its known periplasmic localization (8). No transport mechanisms are known that could allow it to enter the cytoplasm, so if any is manufactured it would likely be degraded by periplasmic phosphatases. Although we demonstrated that AGP would be unable to accomplish that function, other non-specific phosphatases are known to be present in the periplasm (23) which might degrade GBP. Indeed our measurements of GBP contents in an *agp* mutant indicate that it plays no overall role in its accumulation, as the amounts present were unaltered compared to WT (Fig. 3.3).

Analysis of the activities found in crude extracts from WT as well as *agp, pgm* and *agp/pgm* mutant strains incubated with G1P indicated that PGM is the most likely other enzymes to be involved. Although the presence of a PGM stimulatory compound (most probably GBP) was identified in WT and both *agp* and *pgm* single mutants after incubation with G1P, the double mutant lacking both activities was unable to produce any stimulatory product (Fig. 3.4A). Two conceivable mechanisms for PGM in the manufacture of GBP have previously been proposed. Firstly, it is known to be an intermediate in the mutase reaction (24, 25) and it is conceivable that GBP could be released from PGM prior to transfer of phosphate back to the enzyme. Secondly, some PGM's have been shown to be able to manufacture GBP from G1P and either fructose 1,6-bisphosphate or 1,3-diphosphoglycerate (26, 27). Given the fact that the crude extracts were desalted prior to the incubations and that the only available substrate present was G1P, these experiments would indicate that the first mechanism is correct in *E. coli*. Nonetheless, the use of the other suggested substrates is currently being tested using purified *E. coli* PGM. The results from the experiment furthermore reveal the occurrence of other enzymes that are able to degrade GBP, as a marked decrease in the relative stimulation of PGM over time was observed in WT, *pgm* and *agp* mutant strains (Fig. 3.4A). This decrease could be due to the proposed utilization of GBP by glycogen and/or maltodextrin phosphorylase in the incorporation of phosphate into glucans (Chapter 2), although it could also be due to the action of unknown phosphatases.

Taken together, our study clearly demonstrates that AGP and most probably PGM are responsible for the synthesis of GBP within *E. coli*. Nonetheless, experiments using purified PGM protein will have to be conducted to validate that process.

In conclusion this study has identified and biochemically characterized a new biochemical activity for the AGP protein. It appears to be unimportant in determining GBP concentrations *in vivo*, with the remaining activity most likely coming from PGM. The strains produced in this study will help to elucidate the role of GBP in *E. coli* metabolism.

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**FOOTNOTES**

3.6 Supplementary material
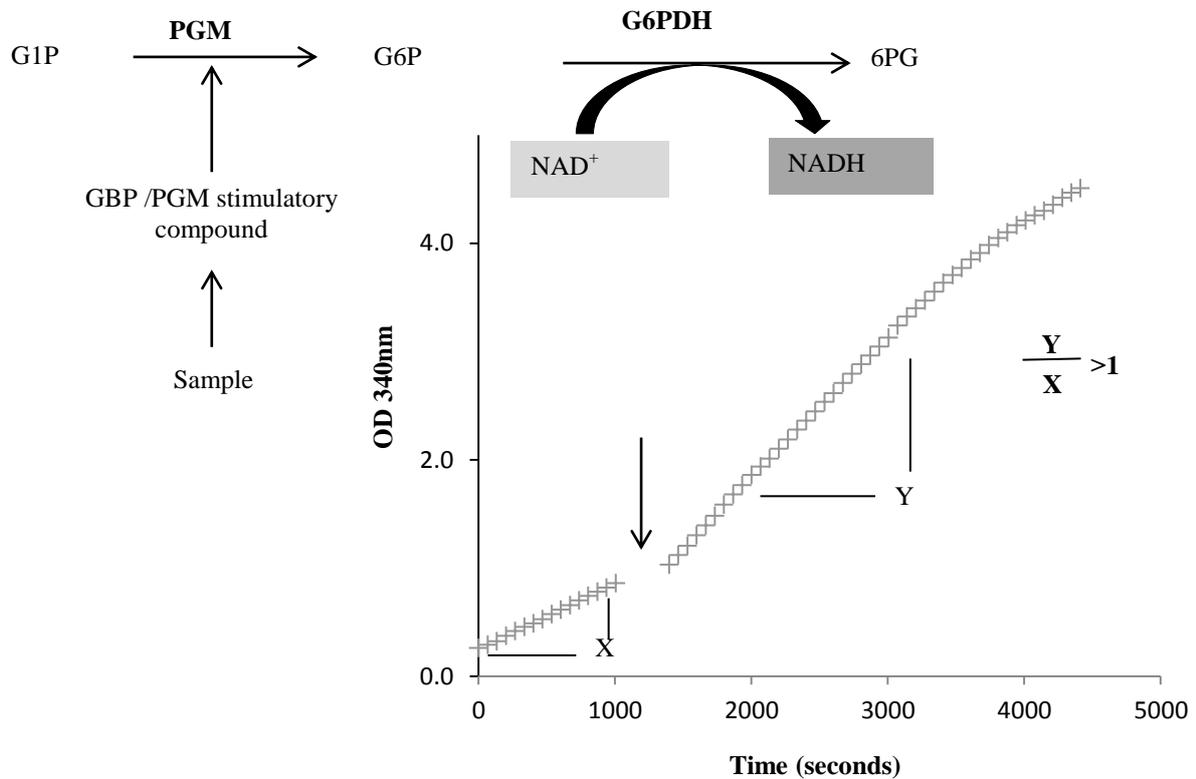
Figure 3.5 A phosphoglucomutase stimulation. A change in the absorbance (OD- 340 nm) is plotted against time (seconds). The stimulation of PGM is apparent when the relative change in the absorbance increases after the addition of the sample (specified by the downward arrow on the graph). A ratio above 1 is indicative of a PGM stimulatory compound present in the sample.

Chapter 4: General conclusion

Glycogen is an important storage polysaccharide present in organisms across all domains of life. Within bacteria, it is one of several polymers that can be synthesized and is considered to be one the most important components involved in the survival of bacteria (Wang and Wise, 2011). Since the discovery of this important energy reserve, several scientific advancements have been made in the understanding of its metabolism in bacteria, specifically in the model organism *E. coli* (see Chapter 1). Despite this, there is still some knowledge lacking about some aspects of its metabolism (Wilson et al., 2010). Two examples of this are the mechanism of incorporation of covalently bound phosphate and the synthesis of glucose-1,6-bisphosphate (GBP), a key regulatory metabolite.

The main aim of this research project was to look at these two aspects.

4.1   The incorporation of phosphate into glycogen

In chapter 2, the involvement of the glycogen and maltodextrin phosphorylases, previously implicated in the incorporation of phosphate into glycogen (Nepembe, 2009), was investigated using a dual approach. Firstly, *in vitro* analysis using maltodextrin as glucan primer together with recombinant MalP protein revealed the incorporation of phosphate into a manufactured polymer using GBP in combination with Glc-1-P as substrates. Approximately 15 nmol Glc-6-P/mg polymer was present after 2 h. However, no phosphate could be detected when Glc-1-P alone was used as substrate. This result clearly proves that MalP has the potential to incorporate phosphate into glycogen *in vivo*, and is the first *in vitro* demonstration of this mechanism. Significantly, higher amounts of polymer were also produced when the incubations composed of GBP and Glc-1-P in comparison to those containing only Glc-1-P. This indicates a direct stimulatory effect that GBP has on polymer production. It is not clear if this is due to some allosteric activation of the enzyme by GBP, or because glucans containing incorporated phosphate act as a better substrate for MalP. These possibilities need to be tested in future. Attempts were made at purifying GlgP, however, they were unsuccessful and will have to be repeated in the near future so that similar experiments can be performed.

The second approach involving *in vivo* analysis, a double *E. coli* mutant lacking both *glgP* and *malP*. Due to earlier reports of extremely low levels of covalently bound phosphate in *E. coli* glycogen of approximately 0.9 nmol Glc-6-P/mg glycogen (Lorberth et al., 1998; Viksø-

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Nielsen et al., 2002), the mutant strains were modified to manufacture higher amounts of glycogen. However, analysis of glycogen phosphate revealed no significant difference between the strains. The assay used those experiments relies on detection of increased absorbance at 340 nm due to the reduction of NAD^+ in an enzyme-coupled assay. Given the anticipated low amount of phosphate, failure to detect any significant reductions is most likely due to the insensitivity of the detection instrument. To overcome this, approaches that are more sensitive will have to be employed. Nonetheless, if the hypothesis is correct, mutants lacking either *glgP* or *malP* should have reduced amount of phosphate compared to the WT. More importantly, glycogen in a mutant lacking both these genes should then, given the assumption that there are no other role players involved, should be devoid of covalently bound phosphate.

Additional future studies could possible involve the targeting of the GlgP and MalP proteins into plants such as potato, wherein phosphate plays an integral part during the metabolism of starch (see Chapter 1).

4.2   The synthesis of GBP in *E. coli*

The second part of the project involved examining the reaction mechanism of the production of GBP. The rationale behind this was that if MalP and GlgP incorporate phosphate into glycogen using GBP, then eliminating its production would also eliminate glycogen bound phosphate. In addition, the project was also scientifically interesting due to the current lack of knowledge about its manufacture and role in metabolism. Based on the data in Chapter 3 it seems that two activities are likely involved in manufacturing GBP, namely AGP and PGM. Unfortunately it will not be possible to use the *agp/pgm* double mutant to examine the effect of eliminating it on glycogen phosphate incorporation as *pgm* mutants are glycogen free (Eydallin et al., 2007). However, GBP is known to affect the activities of several glycolytic enzymes and the production of this double mutant should start to allow the identification of its role on control of flux through glycolysis.

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