Elucidation of the biochemical mechanism of glycogen phosphorylation in *Escherichia coli*

by

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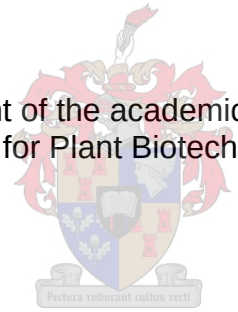

Thesis presented in fulfilment of the academic requirements for the degree Master of Science at the Institute for Plant Biotechnology, University of Stellenbosch

Supervisors: Dr James Lloyd and Prof Jens Kossmann

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DECLARATION

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

Signature: **Date:** October 2009

Abstract

Glycogen was isolated from *E. coli* and analysed for the amount of phosphate present within it. It was confirmed that a significant proportion of the glucose residues were phosphorylated at the C6 position. This glycogen phosphate was found also in both *glgb*$^-$ (glycogen branching enzyme*)* and *glgp*$^-$ (glycogen phosphorylase enzyme) mutants, demonstrating that a mechanism for phosphate incorporation that does not involve GlgP alone, and which is capable of incorporating phosphate into linear glucans could exist. The degree of phosphorylation depended on the amount of phosphate present in the media, which less being incorporated in media where phosphate was reduced. Screening for glycogen phosphorylating genes using a *E. coli* genomic library in a functional expression system identified the *malP* gene as a possible candidate for incorporation of the phosphate at the C6 position. There was no difference, however, between the glycogen phosphate content of the mutant and wild type. Efforts were made to construct a *malp*$^-$*/glgp*$^-$ double mutant, but these were unsuccessful.

In addition the influence of plants and human proteins on yeast glycogen metabolism was also investigated. These proteins have been demonstrated to have an effect on starch or glycogen in humans, plant and *E. coli*, but the data from this study indicated that this was not the case in yeast.

Opsomming

Glikogeen, wat geisoleer was uit *E.coli* was geanaliseer vir fosfaat inhoud daarin. Daar was gevind dat `n beduidende proporsie van die glukose residue gefosforileerd was op die C6 posisie. Hierdie gefosforileerde glikogeen was ook gevind in *glg*$^-$ *(*glikogeen vertakkingsensieme) en *glgp*$^-$ (glikogeen fosforileringsensieme) mutante wat daarop dui dat `n meganisme vir fosforilering bestaan was nie slegs aangewese is op die aktiwiteit van GlgP nie, en om fosfaat te inkorporeer in linêre glukane. Die graad van fosforilering was ook afhanklik van die hoeveelheid fosfaat teenwoordig in die medium, met gevolglik minder wat geinkorporeer kan word in medium waar fosfaat verminderd was. Seleksie-gebaseerde ondersoeking vir fosforileringsensieme van glikogeen deur gebruik te maak van *E. coli* genomiese biblioteke in `n funksionele uitdrukkingssisteem het die *malP* geen geidentifiseer as een van die moontlike kandidate wat verantwoordelik kan wees vir inkorporering van fosfaat in the C6 posisie. Daar was egter geen verskil in die fosfaat inhoud van glikogeen tussen die wilde tipe en die mutante. Pogings wat aangewend is om `n *malp*$^-$*/glgp*$^-$ dubbel mutant te konstrueer was onsuksesvol.

Verder is die invloed van plant en mens proteine op gis glikogeen ook bestudeer. Vroeër is aangetoon dat hierdie proteine `n invloed op stysel en glikogeen het in mense, plante en *E. coli,* maar data van hierdie studie toon aan dat dit nie die geval in gis is nie.

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I thank God. Without him I can do nothing.

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AMY	α-amylase
AGPase	adenosine 5'-diphosphate-glucose pyrophosphorylase
ATP	adenosine 5'-triphosphate
BAM	β-amylase
BE	branching enzyme
BSA	bovine serum albumin
bp	base pair
°C	degree Celsius
$CaCl_2$	calcium chloride
cDNA	complementary deoxyribonucleic acid
Cm^R	chloramphenicol resistant
dH_2O	distilled water
D-enzyme	disproportionating enzyme
DBE	debranching enzyme
DDT	dichlorodiphenyltrichloroethane
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
E.coli	*Escherichia coli*
E.C	enzyme commission number
EDTA	ethylenediaminetetraacetic acid
e.g	Example
g	Gram
xg	gravitational acceleration (9.806 $m.s^{-1}$)
GBSSI	granule bound starch sythase
G6PDH	glucose-6-phosphate dehydrogenase
gDNA	genomic deoxyribonucleic acid
glg	glycogen biosynthesis genes
GWD	glucan water dikinase
hr	Hour
HCl	hydrochloric acid
I_2	Iodine
IPB	Institute for Plant Biotechnology
ISA	Isoamylase
IWB	Institute of Wine Biotechnology
H_20	Water
KI	potassium iodide
KOH	potassium hydroxide
L	Liter
LB	luria broth
LBs	lafora bodies
Laf	Laforin
LDA	limit dextrinase
LSF	like sex four
M	Molar
malP	maltodextrine phosphorylase
Mg	Milligram
ml	Millilitre
mM	Millimolar
min	Minute
MOS	malto oligosaccharides
NaCl	sodium chloride

NA_2CO	sodium carbonate
NAOH	sodium hydroxide
NAD	nicotinamide adenine dinucleotide
NADP	reduced nicotinamide-adenine phosphate dinucleotide
P	Phosphate
PCR	polymerase chain reaction
PEG	polyethylene glycol
PGI	phosphoglucomutase isomerase
PGM	phosphoglucomutase
PPi	inorganic pyrophosphate
PVPP	polyvinypolypyrrodine
PWD	phosphoglucan water dikinase
sec	second (time unit)
SDS	sodium dodecyl sulphate
SEX4	starch excess four
SS	starch sythase
SBE	starch branching enzyme
TBE	Tris-borate/EDTA buffer
Tris	2-amino-2-(hydroxymethyl)-1,3-propanediol
U	Units
µM	Micromolar
µl	Microliter
µg	Microgram
V	Volt
v/v	volume/volume
W	Weight
Wt	wild type
w/v	weight /volume

Chapter 1: Literature review

1.1. Starch as an important polymer

1.1.1 Starch Structure

Starch is one of the important polymers produced in nature. After cellulose, it is the most abundant carbohydrate in plants (Esau, 1977) and is significant throughout the plant kingdom because it serves as a carbohydrate store. It is composed of two distinct polysaccharides: amylose and amylopectin (Fig.1.1). Amylose is a linear chain of α-1,4 linked glucose monomers interspersed with occasional α-1,6 glucosidic bonds while amylopectin is a more highly branched glucan which consists of far more α-1,6-glucosidic bonds in addition to the α-1,4 bonds (Hizukuri and Takagi, 1984; Takeda *et al.,* 1984*)* and is the major constituent of starch (Zeeman *et al*., 2002)*.* Starch is normally found in most plant organs including roots, seeds, tubers, leaves, stems and flowers. Plants synthesise starch as a semi-crystalline granule (diameter ranging from 1 µM to 100 µM depending on the species) which is insoluble in water (Fig.1.1).

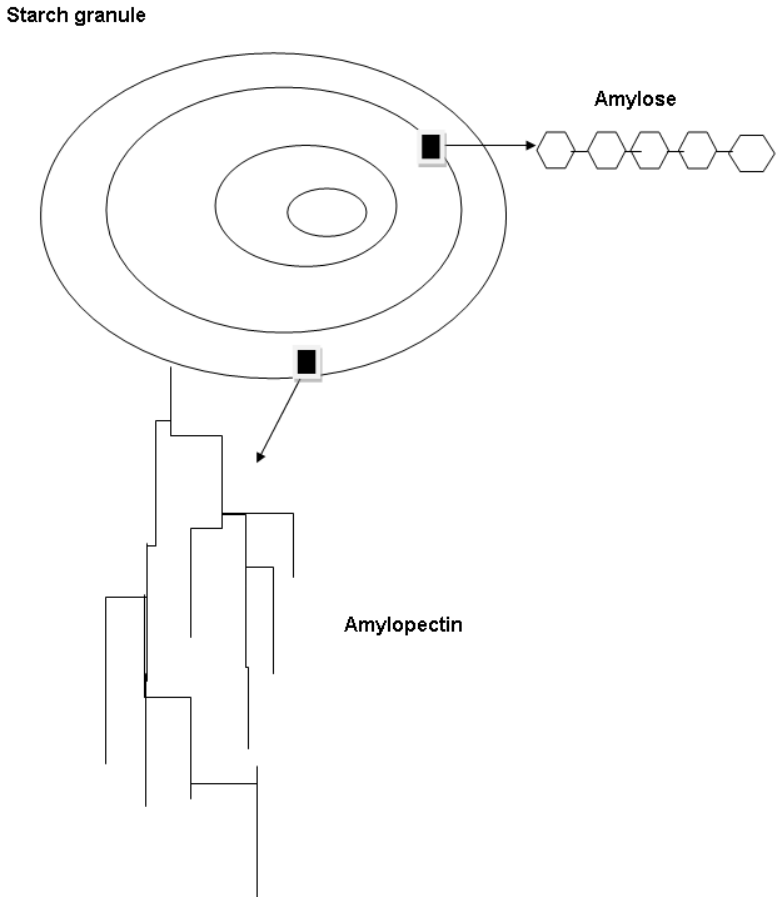

Figure 1.1 Schematic representation of a starch granule consisting of amylopectin and amylose moieties.

1.1.2 Industrial uses of starch

Starch is a very important source of carbohydrate in the human diet and serves as a major staple carbohydrate for millions of people in the world. However, it also has various industrial applications. The world starch production by plants has been estimated to be around 2,850 million tons per year (Burrell, 2003) and common uses of it in the industry is listed in Table 1.1.

Table 1.1 Examples of industrial uses of starch.

	Industry type			
	Food and drinks	Animal feed	Agriculture	Plastics
product	-Mayonnaise -Baby food -Soft drink -Meat product -Confectioner	-Pellets	-Feed coating -Fertilizer	-Biodegradable plastics

	Industry type			
	Pharmacy	Building	Textile	Paper
product	-Tablets -Dusting powder	-Mineral fibre -Gypsum board -Concrete	-Wrap -Fabrics -Yarns	-Corrugate board -Cardboard -Paper

(Source: International Starch Institute, Aarhus, Denmark web site http://home3.inet.tele.dk/starch)

Maize is the main source of starch used by industry accounting for about 75% of the total (Fig.1.2). Although other starch sources such as rice, sweet potato, cassava, sorghum, wheat and potato are also used, their industrial demand is still low in comparison (Fig. 1.2).

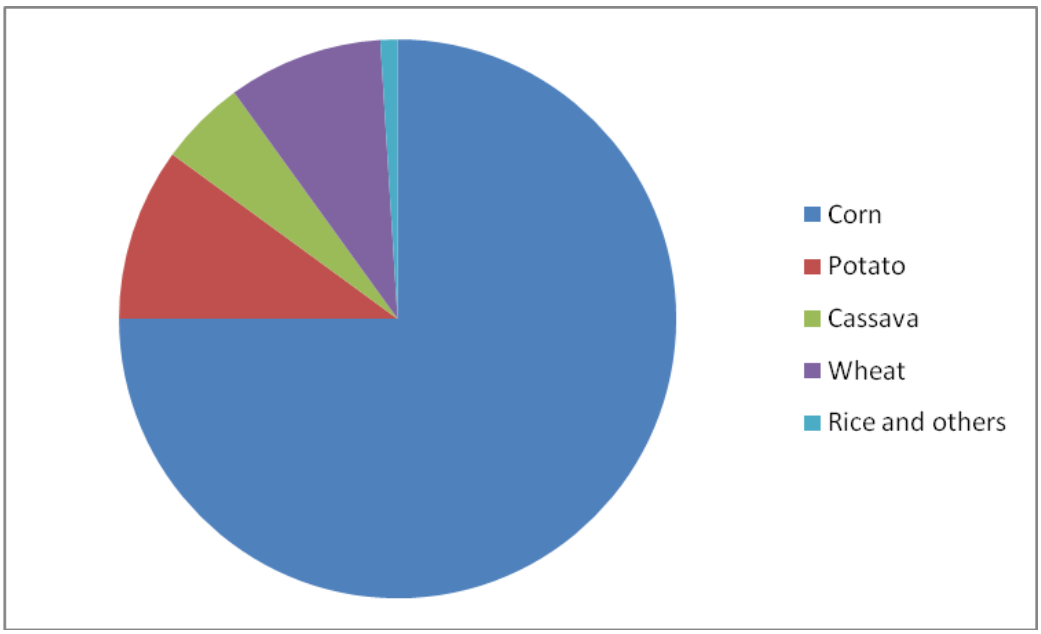

Figure 1.2. Percentage contribution of different sources of raw material for industrial starch in 1999-2001. (Source: International Starch Institute, Aarhus, Denmark. http://home3.inet.tele.dk/starch)

Starch from all these plants differ in many aspects, such as their relative proportions of amylose and amylopectin as well as starch components such as phosphate groups, lipid, proteins and the average chain length within amylopectin. All of these affect the physical properties of the starch such as paste viscosity, gelatinization, solubility, gel stability and texture (Ellis *et al*., 1998). Variation in these properties makes starch from different sources behave in different ways. Depending on the need of the specific application, industries carefully examine the characteristics of the starch in order to get the desired product. In most cases, the industrial needs are not met by native (unmodified) starches, which forces industry to look for ways to modify them to improve their properties by alteration of physical and chemical characteristics (Hermansson and Svegmark, 1996).

1.2 Starch metabolism

1.2.1 Starch synthesis

Starch is synthesized in plant leaves during the day as a product of photosynthesis and is broken down, transported, re-synthesised and stored in non-photosynthetic parts of the plants such as roots, shoots, fruits and tubers at night. Its synthesis involves three major enzymes namely, ADP-glucose pyrophosphorylase (AGPase), starch synthase (SS) and branching enzyme (BE) (Martin and Smith, 1995) (Fig.1.3).

The process of starch synthesis in leaves starts with fixation of carbon dioxide from the atmosphere by Ribulose 1,5 bisphosphate carboxylase/oxygenase (RuBisCO). The carbon is then metabolised *via* the Calvin cycle where it forms fructose-6-phosphate (Fru6P). This is converted to glucose-1-phosphate (Glc1P) by phosphoglucose isomerase (PGI) and phosphoglucomutase (PGM) and then to ADP-glucose and inorganic pyrophosphate (PPi) by AGPase in a trehalose-6-phosphate dependent redox-regulated reversible reaction (Fu *et al*., 1998; Hendriks et *al.*, 2003; Jin *et al*., 2005). AGPase is also activated by 3-phosphoglyceric acid (3-PGA) and inhibited by inorganic phosphate (Pi) (Ghosh and Preiss, 1966) (Fig.1.3).

In leaves, AGPase is located exclusively in chloroplasts, and an absolute plastidial localization was presumed to be also the case in storage organs. However, in cereal endosperm, in addition to the plastidial isoform, a cytosolic AGPase isoform is also prevalent (Denyer *et al*., 1996; Thorbjørnsen *et al*., 1996; Sikka *et al*., 2001; Tetlow *et al*., 2003) suggesting that in cereal endosperm ADP-glucose manufactured in the cytosol has to be imported into the plastid. Evidence for this is provided by a specific ADP-glucose transporter named Brittle1 (Sullivan *et al.,* 1991; Sullivan and Kaneko 1995). Within the plastid, SS isoforms use ADP-glucose as a substrate and add glucose units to the non-reducing end of a pre-existing α-1,4-glucan chain, releasing ADP in the process. There are several SS isoforms in plants, the number depending on the species, and one specific isoform is solely responsible for amylose synthesis. This is an exclusively granule bound enzyme and is known as granule bound starch synthase (GBSS) (Nelson and Rines 1962; Van Der Leij *et al.,* 1991; Denyer *et al.,* 1995; Martin and Smith 1995; Flipse *et al.,* 1994). Other isoforms tend to be present both in the soluble fraction, as well as being bound to the granule and are involved in amylopectin synthesis. BEs introduce branch points in the chains by hydrolysing α-1,4–glucosidic bonds and transferring the chain to form an α-1,6 bond (Borovsky *et al.,* 1976) leading to the formation of amylopectin.

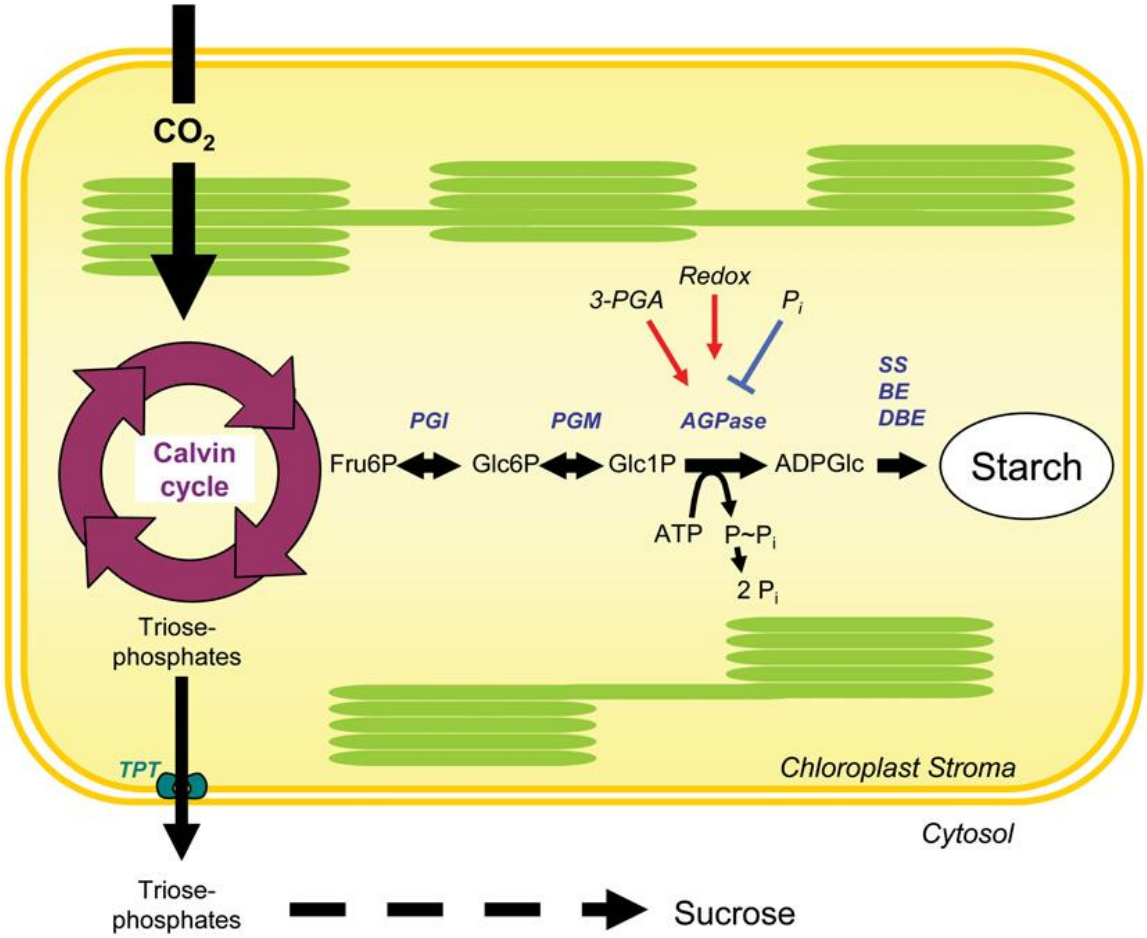

Figure 1.3 Schematic representation of the pathway of starch synthesis in chloroplasts. A portion of the carbon fixed in the Calvin cycle via Ribulose 1,5 bisphosphate carboxylase/oxygenase (RuBisCO) is utilized for starch synthesis. The first committed step towards this, ADP-glucose pyrophosphorylase (AGPase) is under redox and allosteric regulation. Abbreviations: Fru6P, fructose 6-phospate; GIc1P, glucose 1-phosphate; Gluc6P, glucose 6-phosphate; TPT, triose-phosphate/phosphate translocator. (*Figure from* Zeeman *et al*., 2007)

Debranching enzymes (DBE) are able to cleave α-1,6 bonds and there are several isoforms of these, which are generally divided into isoamylase and limit dextrinase (LDA or pullulanase-type) classes, depending on their substrate specificities. In Arabidopsis there are three isoamylase (ISA1-3) isoforms and one LDA. ISA1 and ISA2 have been shown to be involved in starch synthesis as, when the genes coding for them are mutated, the plants accumulate a uncrystalline polyglucan known as phytoglycogen, as well as starch (Zeeman *et al.,* 1998; Myers *et al.,* 2000; Bustos *et al.,* 2004; Delatte *et al.,* 2005). It is speculated that DBEs are involved in tailoring the branched glucans into a form capable of crystallization, although the precise

mechanism for this remains unclear (*for reviews see* Ball and Morell, 2003; Zeeman *et al*., 2007; Streb *et al.,* 2008)

1.2.2 Starch phosphorylation

The presence of small amounts of mono-esterified phosphates have been reported in potato starch since the early twentieth century (Fernbach, 1904). These phosphate groups are bound as mono-esters at the C3 and C6 positions of glucose residues within amylopectin, but not amylose (Posternak 1951; Hizukuri *et al*., 1970; Takeda and Hizukuri, 1982; Blennow *et al*., 2002). Phosphate has been found in starch extracted from several plant species, which indicates that many (if not all) plant starches are phosphorylated (Kasemsuwan and Jane 1996; Blennow *et al*., 2002). In potato (S*olanum tuberosum* L.) tuber starch, about 0.1% to 0.5% of the glucose residues are phosphorylated (Ritte *et al*., 2002), whereas less than 0.01% of those in cereal endosperm starch contain phosphate (Tabata *et al*., 1971; Kasemsuwan and Jane, 1996).

The mechanism by which phosphate is incorporated into starch was unknown until the discovery of a 157 kDa starch-granule-bound protein, originally named R1 (Lorberth *et al.,* 1998). The *R1* gene was first identified in potato and its antisense inhibition resulted in approximately a 90% reduction of starch bound phosphate, indicating a role of this protein in starch phosphorylation (Lorberth *et al.,* 1998). Interestingly, the antisense potato plants also displayed an inhibition of starch degradation in both cold stored tubers and leaves. However, the reason why decreased levels of starch phosphate affect its degradation has remained unclear until recently (discussed further in Section 1.2.3).

It has been demonstrated that the R1 protein phosphorylates glucose moieties in starch at the C6 position. This was shown firstly by expressing the full length potato cDNA in *E. coli,* which then produced glycogen (a storage polyglucan similar to starch) containing increased amounts of covalently bound phosphate (Lorberth *et al*., 1998). More recently, the mechanism by which the R1 acts was elucidated (Ritte *et al*., 2002; 2006, Mikkelsen *et al*., 2004). The enzyme utilizes ATP as a phosphate donor in a dikinase mechanism, transferring the γ-phosphate to water and the β-phosphate to the C6 position on glucose monomers within amylopectin. The enzyme was thus renamed glucan, water dikinase (GWD). The phosphorylation at the C3

position of amylopectin is performed by a similar enzyme, but this enzyme only phosphorylates amylopectin which has been pre-phosphorylated by the GWD (Kötting *et al.,* 2005; Ritte *et al*., 2006; Hejazi *et al*., 2008). This second enzyme is therefore named phosphoglucan, water dikinase (PWD) (Baunsgaard *et al*., 2005; Kötting *et al*., 2005).

1.2.3 Starch degradation

As previously mentioned, starch accumulates in chloroplasts during the day as a product of photosynthesis. During the night, it is degraded and converted to sucrose before being exported to non-photosynthetic parts of the plant. Starch degradation involves a number of enzymes, all of which have multiple isoforms. Over the past decade much effort has been spent into understanding the roles of these various enzymes. This has led to a general model for Arabidopsis where most starch degradation is accomplished by β-amylases (BAM) with maltose being the main sugar being exported from the chloroplast (Fig.1.4). The evidence for this is reviewed in the rest of the section.

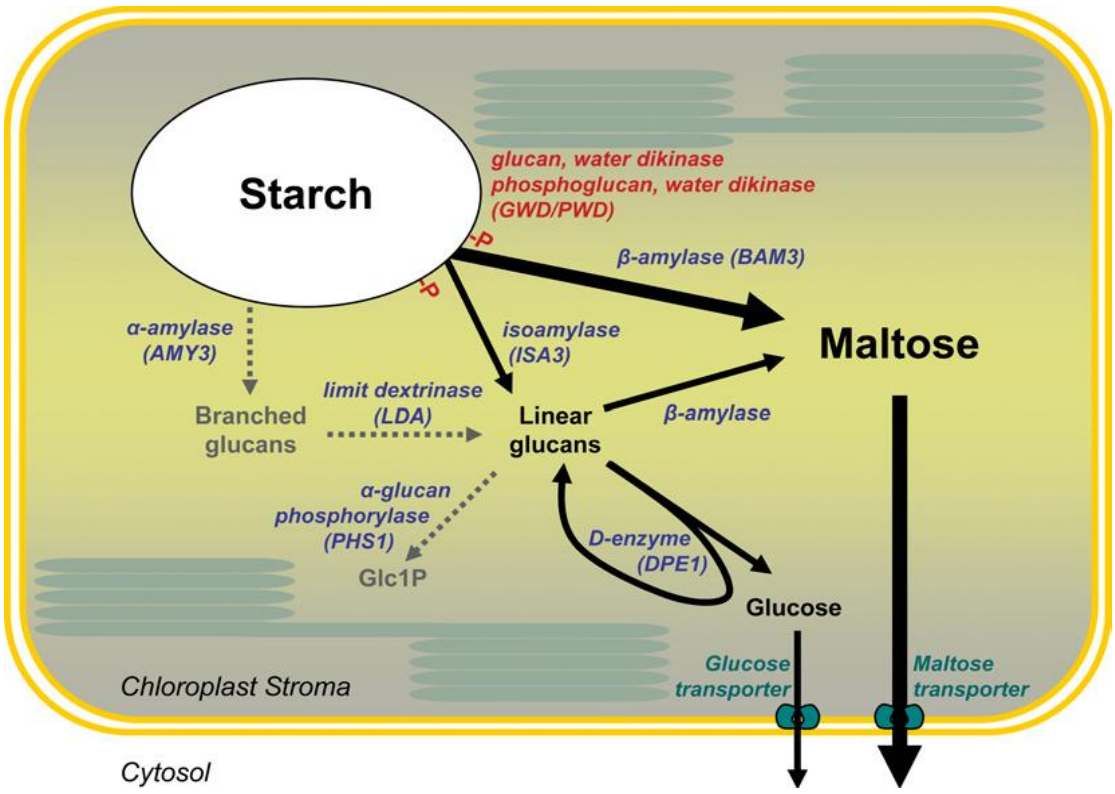

Figure 1.4 A generalised model for the pathway of starch degradation in Arabidopsis leaves. Starch, hydrolysed to maltose and glucose during the dark, is converted to sucrose before being exported to heterotrophic tissue. Refer to text for further details. (*Figure from* Zeeman *et al*., 2007)

Initially, it was thought that α-amylase (AMY) proteins, endohydralases capable of cleaving α-1,4 bonds within the amylopectin molecule, is the key enzyme in starch degradation (Fig.1.4). However, a recent mutational study questions this and further suggests that it may even be involved in leaf starch synthesis (Yu *et al.,* 2005). There are three genes that code for α-amylase isoforms in the Arabidopsis genome (Yu *et al.,* 2005). One of these, AMY3 (At1g6930), has been demonstrated to be localised in the chloroplast (Stanley *et al*., 2002) but an insertion mutation that was isolated showed no reduction in starch degradation in Arabidopsis leaves (Yu *et al*., 2005). On the other hand, the other two α-amylases (AMY1 and AMY2) are not predicted to have transit peptides, suggesting that they are not chloroplastidic. In addition, the triple mutant of *amy1/amy2/amy3* showed no effect on starch metabolism (Yu *et al*., 2005), suggesting that AMYs are not essential for starch degradation in Arabidopsis.

Recently, an Arabidopsis mutant was manufactured that lacked all DBE activities (Streb *et al.,* 2008). As was discussed above (Section 1.2.1) some debranching

enzyme isoforms appear to be involved in starch synthesis. When all four debranching enzyme isoforms are mutated in Arabidopsis, starch synthesis in leaves is abolished (Streb *et al.,* 2008). However, when *AMY3* is mutated in addition to that, starch accumulation is restored (Streb *et al.,* 2008) demonstrating that starch synthesis can be accomplished without DBEs. Based on this data Streb *et al.* (2008) proposed a model for starch synthesis where amylopectin is produced by starch synthases and branching enzymes which is capable of crystallization to form starch granules. The process is enhanced by ISA1/ISA2 enzymes which remove the branch points. Glucans produced by starch synthases and starch branching enzymes cannot be debranched in the absence of ISA1 and/or ISA2. This delays the formation of secondary structures which leads to the formation of phytoglycogen. In the *isa1/isa2* double mutant short chains can be removed by ISA3 and/or LDA, which leads to the production of some abnormal amylopectin although the majority of glucan remain soluble in the form of phytoglycogen (Streb *et al.,* 2008). In the absence of all DBEs the glucans produced cannot be degraded by debranching enzymes and are subjected to additional α-amylolysis and β-amylolysis, leading to the formation of limited glycogen–like structure. In the absence of all DBEs and AMY3, amylopectin is only subjected to β-amylolysis, which allows crystallization of the glucan and, therefore starch accumulation is restored.

BAM isoforms, on the other hand, are exoamylases, that can degrade the outer amylopectin chains, producing maltose, until they reach an α-1,6 branch point after which degradation is terminated. There are nine β-amylase's in Arabidopsis assigned *BAM1* to *BAM9* (Smith *et al*., 2004). Four of the nine isoforms (*BAM1,-2, -3,* and *-4*) in Arabidopsis are predicted to be chloroplastidially localised (Fulton *et al.,* 2008). The repression of one chloroplast-localised β-amylase in potato and Arabidopsis (BAM3) leads to a reduction in starch degradation in leaves, indicating a significant involvement of this isoform in starch degradation (Scheidig *et al*., 2002; Kaplan and Guy, 2005). Recently, Fulton *et al.* (2008) further demonstrated that while a mutation in BAM4 impairs starch breakdown, that BAM1 is necessary for starch breakdown in the absence of BAM3, and that BAM2 shows no function in starch degradation. The roles of the other BAM isoforms remains unknown.

Although it is clear that β-amylase isoforms are the main route for starch degradation in Arabidopsis leaves, other enzymes are also necessary for the complete catabolism

of amylopectin. This is due to the fact that β-amylases are unable to digest α-1,6 branch points. As discussed above there are four enzymes in Arabidopsis known to be able to digest α-1,6 bonds, namely three isoamylases and one limit dextrinase. ISA1 and ISA2 are involved in starch synthesis (Zeeman *et al.,*1998; Myers *et al.,* 2000; Bustos *et al.,* 2004; Delatte *et al.,* 2005), but ISA3 and LDA have been demonstrated to be involved in starch degradation (Wattebled *et al*., 2005; Delatte *et al.,* 2006). Loss of ISA3 causes a reduction in starch degradation but when LDA is mutated there is no significant change (Wattebled *et al.,* 2005). However an *isa3/lda* double mutant leads to a greater repression of starch degradation than in the single *isa3* mutant, suggesting that in the absence of ISA3 LDA is required. An *isa3/lda* double mutant also leads to the accumulation of soluble branched oligosaccharides and an increase in AMY3 activity (Wattebled *et al.,* 2005).

Although β-amylases produce maltose exclusively, debranching enzymes will lead to the production of longer malto-oligosaccharides (MOS). These are minor in comparison with the production of maltose and can be degraded by β-amylase to maltose and maltotriose. Maltotriose cannot be catabolised by β-amylase, and is further metabolised by disproportionating enzyme (D-enzyme) (Critchley *et al.,* 2001). This enzyme transfers α-1,4 bonds from one linear polyglucan to another. A mutation in D-enzyme leads to a minor impairment of starch degradation, and plants which accumulate maltotriose and other longer MOS (Critchley *et al.,* 2001). Consistent with the proposed major role of β-amylase during starch degradation, it has been found that maltose (the product of β-amylase) is the major metabolite exported from the chloroplast. This was first found by *in vitro* experiments performed on isolated chloroplasts from different plants (Neuhaus and Schulte, 1996; Ritte and Raschke, 2003; Servaites and Geiger, 2002; Weise *et al*., 2004). Later the gene coding for the maltose transporter was also identified. This was done by isolating a mutant, *maltose excess 1* (*mex1),* from Arabidopsis which accumulates excess amount of maltose. Map based cloning of the mutated gene led to the identification of a protein that is present in the chloroplast membrane and which is able to transport maltose (Niittylä *et al.,* 2004*).* The *mex1* mutant not only accumulates maltose, but is unable to degrade starch demonstrating that maltose is the major sugar produced during starch degradation. Interestingly, a putative glucose transporter has also been characterised in spinach chloroplasts (Schäfer *et al*., 1977) and further cloned from spinach,

tobacco, tomato, Arabidopsis as well as maize (Weber *et al.,* 2000). The role of this in regards to starch degradation, however, is unknown.

The GWD protein incorporates phosphate into starch, and its removal in mutant and transgenic plants leads both to a decreased accumulation of starch bound phosphate and to a repression of starch degradation (Lorberth *et al*., 1998, Yu *et al*., 2001; Nashilevitz *et al*., 2009). According to Ritte *et al.* (2004), starch in the green algae *Chlamydomonas reinhardtii* and potato leaves is mainly phosphorylated while it is being degraded. In addition, higher levels of phosphate were observed on the outer surface of potato granule at night than during the day (Ritte *et al*., 2004). This indicates a link between starch phosphorylation and its degradation. Yu *et al.* (2001) suggested that the starch phosphorylation leads to an increase in hydrophilicity of the starch particles, which makes it more accessible to degradative enzymes. Recently, it was discovered that incubating starch with β-amylase (BAM1) and GWD leads to a starch degradation rate three times greater than with BAM1 alone (Edner *et al*., 2007). It is therefore hypothesised that β-amylase (BAM1) first degrades starch, which provides space for GWD to attack the neighbouring double-helix within the amylopectin. This enables the GWD to unwind the double helix and phosphorylate one strand at a time. BAM1 then degrades the individual chains up to the phosphorylated residue (Edner *et al*., 2007; Hejazi *et al*., 2008) (Fig.1.5).

Until recently, many aspects of starch degradation were not well understood. One of these is what happens to the phosphate covalently bound to the amylopectin. A clue as to the enzyme involved in this comes from a study of the recently identified Starch Excess 4 (SEX4) protein, mutations in which lead to a starch excess phenotype in Arabidopsis leaves (Kerk *et al*., 2006; Niittylä *et al.,* 2006; Sokolov *et al.,* 2006). SEX4 contains both carbohydrate binding and dual specificity-phosphatase domains, is plastidial targeted, binds and dissociates to starch granules during the day and night, respectively (Niittylä *et al.,* 2006; Sokolov *et al.,* 2006). *sex4* mutants decrease the rate of starch degradation in Arabidopsis, however, the phenotype is complex as it also leads to the reduction of the activity of an α-amylase isoform (Zeeman e*t al.,* 1999). One proposed role of SEX4 is to dephosphorylate starch. This has been demonstrated through incubation of SEX4 with starch granules leading to their dephosphorylation and through the demonstration that *sex4* mutants accumulate phosphorylated oligosaccharides (Kötting *et al*., 2009). It is assumed that starch

phosphate has to be removed prior to its degradation, possibly as a signal for starch catabolism to begin, or to make the starch molecule more accessible for starch degrading enzyme(s) (Edner *et al*., 2007). Evidence for this comes from the work done by Kötting *et al.* (2009) where they incubated the SEX4 protein and starch granules with ISA3, BAM3 and GWD, resulting in increased *in vitro* granule degradation. Since BAM cannot degrade a glucan chain past a phosphate group or a branched α-1,6 chain, there is a limitation in maltose release (Edner *et al*., 2007). Removal of branched points by ISA3 or the removal of phosphate by Sex4 would enable further degradation of the glucan chain by BAM (Kötting *et al*., 2009). This demonstrates that Sex4 is required for starch degradation and confirms early speculation that phosphate has to be removed prior to degradation for some enzymes to function (see also Fig. 1.4 and Fig. 1.5 for proposed models of starch degradation).

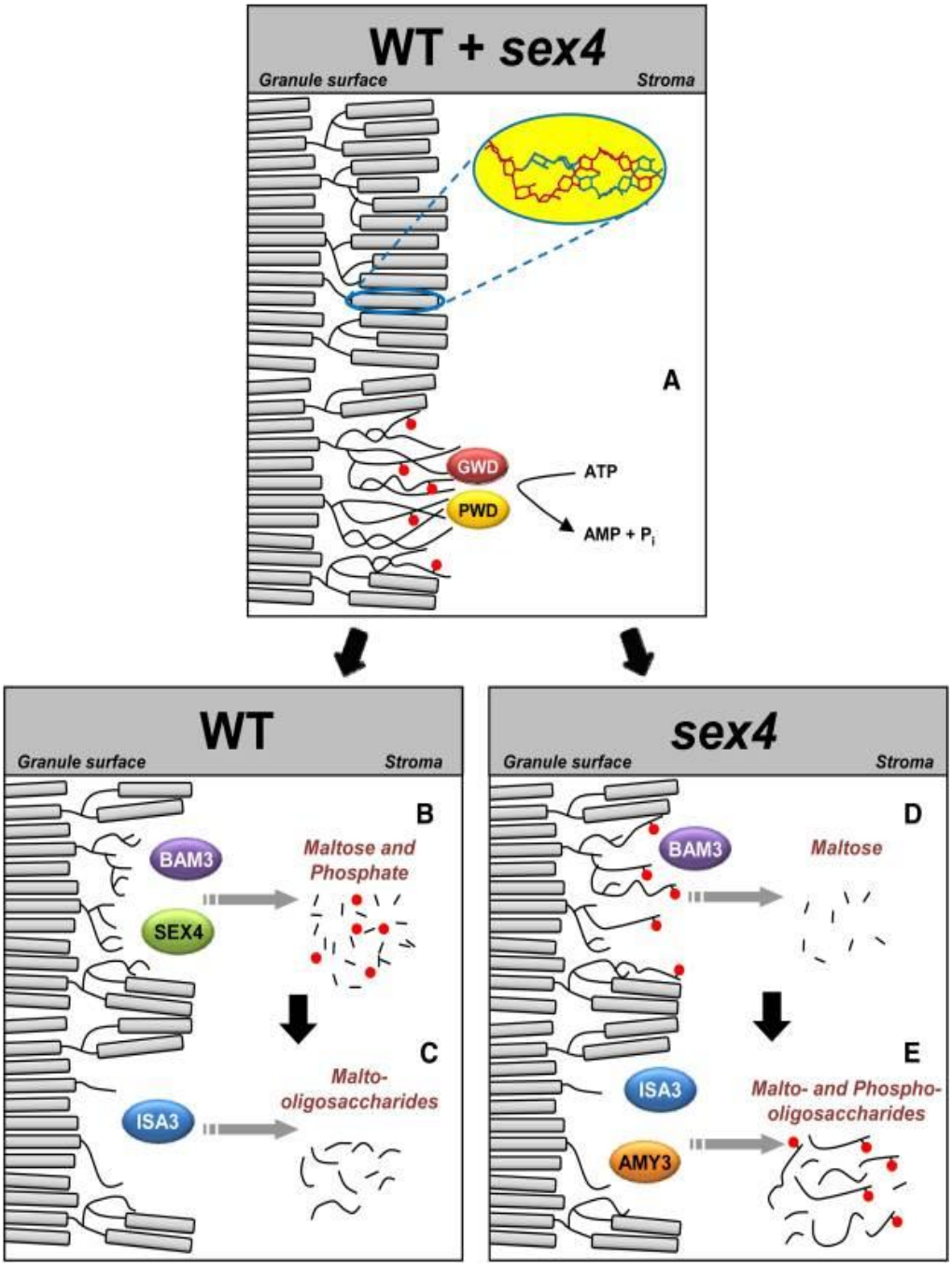

Figure 1.5 Proposed model for the involvement of phosphorylation and dephosphorylation events during the initial phases of starch breakdown. Starch catabolism is dependent on phosphorylation of GWD and PWD of the starch granule (top panel). This allows the amylopectin to partially unwind, and BAM3 and SEX4 can release maltose and phosphate, respectively. ISA3 hydrolyses branch points and releases malto-oligosaccharides (bottom left panel). Without SEX4 phosphate is not removed and less maltose is released by BAM3 (bottom right panel). (*Figure from* Kötting *et al*., 2009)

PTPKIS2 (At3g01510) is a protein found in Arabidopsis which has a very similar sequence to SEX4 (Fordham-Skelton *et al*., 2002). Recent work by Comparot-Moss *et al.* (2009) showed that SEX4 and PTPKIS2 (which has been renamed Like Sex Four 1; LSF1), has a function in starch degradation also. *sex4/lsf1* double mutants demonstrated a greater accumulation of starch than individual mutants. However, LSF1 cannot replace SEX4 in starch degradation. It might be that LSF1 acts as a glucan phosphatase but on different groups of phosphate than those removed by SEX4, or that it acts as a protein phosphatase which activates one or more enzymes involved in starch degradation. A third locus is also found in the Arabidopsis genome which is highly similar to *SEX4* and is known as *LSF2* (At3g10940). It isn't known if this codes for a protein involved in starch degradation and, if so, what its specific role is.

1.3 Starch and glycogen are storage polyglucans with similar biosynthetic pathways

While starch is a storage form of glucose in many plants, glycogen is the storage form of glucose in animals, bacteria, and fungi. Glycogen is a branched polysaccharide made of α-1,4-glucose subunits with a few α-1,6 glucose branch points but differs from starch in that it is uncrystalline and water soluble. It is synthesised by glycogen synthases from ADP-glucose in bacteria and UDP-glucose in mammals and fungi (Greenberg and Preiss, 1964).

Glycogen accumulates under conditions of limited growth when carbon sources are in excess (Preiss and Romeo, 1989). Enzymes involved in glycogen metabolism in *E. coli* are encoded in the *glg* operon (Romeo *et al.,* 1988) which consists of five open reading frames. These are named *glgA* (encoding glycogen synthase), *glgB* (encoding glycogen branching enzyme), *glgC* (encoding ADP-glucose pyrophosphorylase), *glgP* (encoding glycogen phosphorylase) and *glgX* (encoding glycogen debranching enzyme).

The organization of the gene cluster shows that the *glg* genes may be transcribed as two tandomly arranged operons, *glgBX* which consist of *glgB* and *glgX* and *glgCAP* which consist of *glgC, glgA* and *glgP* genes (Preiss and Romeo, 1989) (Fig.1.6). At the transcriptional level, *glgCAP* is positively regulated by both guanosine 5'-diphosphate 3'-diphosphate (ppGpp), which is synthesised by *relA* (Bridger and

Paranchych, 1978; Romeo and Preiss 1989; Taguchi *et al*., 1980; Romeo and Preiss, 1990; Traxler *et al*., 2008), and cyclic AMP (cAMP) (Dietzler *et al*., 1977; Dietzler *et al*., 1979; Urbanowski *et al*., 1983) (Fig.1.6). Recent work by Montero *et al.* (2009) also demonstrated that the transcriptional unit *glgCAP* is influenced by the *PhoP-PhoQ* genes which, in turn, are controlled by Mg^{+} concentrations When these genes were mutated it led to less glycogen accumulating in *E. coli* (Montero *et al.,* 2009). However at the post-transcriptional level synthesis is negatively regulated by the C*srA* gene which binds to two positions within *glgCAP* and this prevents *glgC* translation (Baker *et al*., 1992; Romeo *et al*., 1993; Yang *et al*., 1996; Liu and Romeo, 1997).

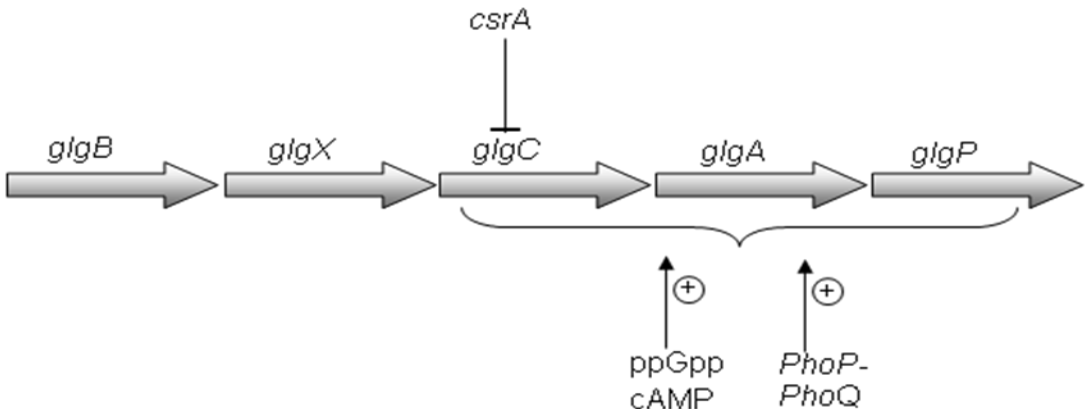

Figure 1.6 Schematic representation of the organization and transcriptional regulation of the *glg* operon in *E. coli.* Refer to text for details.

Knockout mutations in *glgC* lead to *E. coli* that cannot accumulate glycogen as they are unable to produce ADP-glucose (Creuzat-Singal *et al*., 1972). One specific *glgC* mutation (*glgC16*) affects the metabolic regulation of the glgC protein suggesting that it is no longer inhibited by its normal allosteric repressor (Pi). Cells carrying this mutation accumulate large amounts of glycogen and stain dark–brown with iodine (Damotte *et al*., 1968). Mutations in the *glgA* gene further leads to a lack of glycogen synthase activity and these mutants form colonies that do not stain brown when exposed to iodine as they do not accumulate glycogen despite the presence of ADP-glucose pyrophosphorylase (Damotte *et al*., 1968) (Fig.1.7). Furthermore, mutation of the *glgB* gene leads to the accumulation of linear polysaccharides which do not stain brown when exposed to iodine, but rather blue (Damotte *et al*., 1968). When *glgP* is mutated, *E. coli* colonies stain brown with iodine in comparison to the wild type, indicating that they accumulate more glycogen than usual. This has been demonstrated in *glgp*$^{-}$ mutants to be due to reduced glycogen breakdown (Alonso-

Casajús *et al*., 2006). Similarly, disruption of the g*lgX* gene by homologous recombination leads to *E. coli* that are less able to degrade glycogen (Dauvilleé *et al.,* 2005).

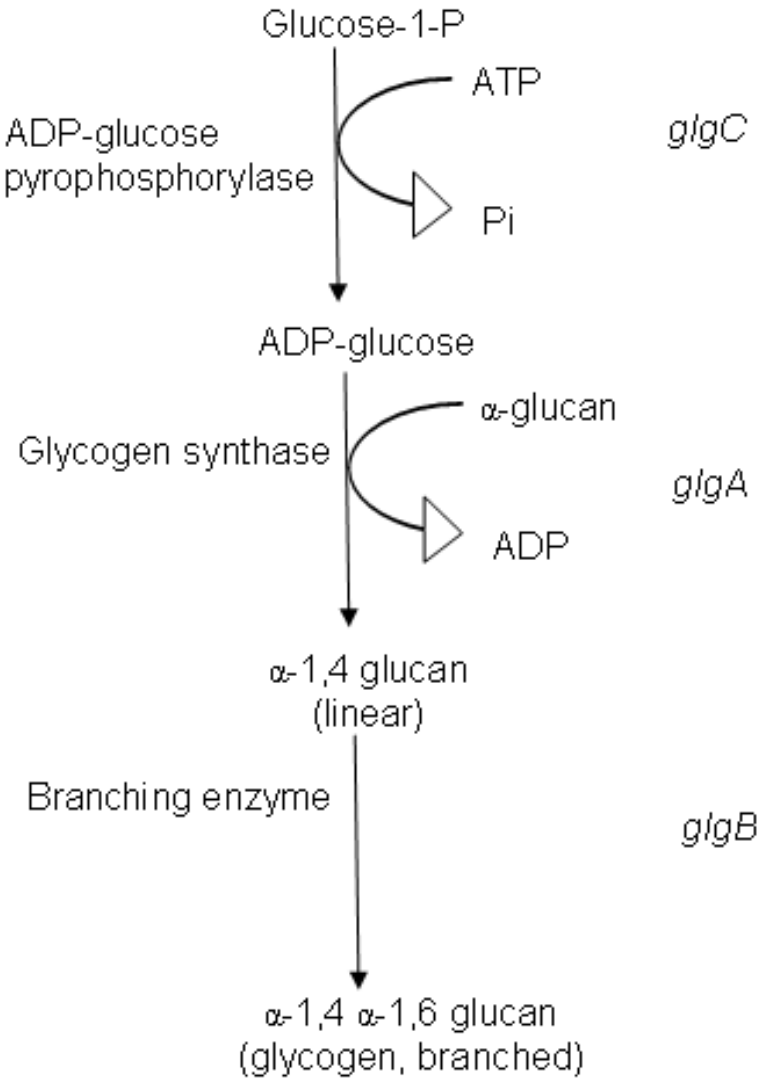

Figure 1.7 Schematic representation of glycogen synthesis in *E. coli*. Refer to text for details.

1.4 Lafora disease

Laforin is a dual-specificity phosphatase which was originally thought to be conserved in vertebrates (Ganesh *et al*., 2004) and which is essential for normal glycogen metabolism. However, it was demonstrated recently that Laforin orthologues are present in five protists (Gentry *et al*., 2007) as well as invertebrates (Gentry and Pace, 2009). In addition Laforin shows significant homology to the Arabidopsis SEX4 protein (Edner *et al*., 2007).

It is the only known phosphatase in animals with a highly conserved polysaccharide binding domain (Worby *et al*., 2006). In humans, mutations in the *laforin* gene contributes to the Lafora disease, which is a neurodegenerative disorder that results in severe epilepsy and death (Lafora and Gluck, 1911; Minassian *et al*., 1998; Serratosa *et al*., 1999). The Lafora disease is characterised by abnormal accumulation of glycogen. Patients that are suffering from this disease accumulate Lafora bodies (LBs) which are poorly branched glycogen-like polyglucans located in the cytoplasm of the cells of most organs that normally accumulate little glycogen, like liver, neurones and skin (Harriman *et al*., 1955; Schwarz and Yanoff, 1965) and are essentially an insoluble form of glycogen (Lafora and Gluck, 1911; Minassian *et al*., 1998). The LBs more closely resemble plant starch than glycogen (Yokoi *et al*., 1968a; Yokoi *et al*., 1968b; Sakai *et al*., 1970). Current research has indicated that Laforin can dephosphorylate glycogen and amylopectin *in vitro,* which led to the hypothesis that Laforin is a glucan phosphatase (Worby *et al*., 2006; Gentry *et al*., 2007). Glycogen from mammals contains significant amount of phosphate (Lomako *et al*., 1993). This was demonstrated in recent studies where glycogen-bound phosphate has shown a 4-fold elevation in the liver and muscle of Laforin deficient mice (Tagliabracci *et al*., 2007; Tagliabracci *et al*., 2008).

1.5 Is polyglucan phosphorylation a general phenomenon?

The fact that dual specific phosphatases involved in polyglucan metabolism are present in both mammals and plants indicates that this process might be evolutionarily very ancient. As such it might also be present in other organisms. The yeast genome contains several genes coding for such proteins, but their role is not well understood. Although phosphate has been reported to be present in *E. coli* glycogen there are no obvious genes within its genome that code for proteins that play a similar role to SEX4 and Laforin.

1.6 Summary

Starch often has to be chemically modified before use, for example by incorporation of phosphate. Phosphorylation of starch, therefore, is necessary for some industrial utilization. Increased phosphorylation, for example, prevents the crystallization of the final product (Ellis *et al*.,1998) and increases the hydration capacity of starch after gelatinization, which influences both paste viscosity and gel formation (Lorberth *et al.,* 1998). If such modifications can be carried out *in planta*, the need for expensive

and environmental damaging chemicals would be reduced. One way of doing this would be by identifying genes from other organisms that can phosphorylate polyglucans and use them to produce genetically modified plants which express the proteins coded for by these genes in plant plastids. *E. coli* glycogen has been reported to contain low levels of covalently bound phosphate (Lorberth *et al.,* 1998; Viksø-Nielsen *et al*., 2002). The first aim of this project was to confirm the presence of phosphate in *E. coli* glycogen as reported in the previous two studies. After confirmation of this the second aim was to identify the gene(s) that incorporate the phosphate. The mechanism for incorporation of phosphate into glycogen is, however, unknown and, therefore the third aim of this study was to establish the mechanism of phosphate incorporation in *E. coli* glycogen In addition, the data discussed above about the Lafora protein indicates that mammalian glycogen is also phosphorylated. It is thus possible that glycogen from other species might also contain phosphate. The fourth aim was to try and evaluate whether yeast glycogen also contains phosphate by examining the effect of enzymes involved in polyglucan phosphate metabolism on yeast glycogen accumulation.

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Chapter 2: Identification of glycogen phosphorylating genes from *E. coli*.

2.1 Introduction

Starch phosphorylation is a common phenomenon in plants that has been well documented over the past few years. It is phosphorylated by two enzymes, the glucan water dikinase (GWD) (Lorberth *et al.,* 1998; Ritte *et al*., 2002) and phosphoglucan water dikinase (PWD) (Baunsgaard *et al*., 2005; Kötting *et al*., 2005) (see Chapter 1, section 1.2.2). One of the first pieces of evidence that GWD was able to phosphorylate polyglucans was the observation that when it was expressed in *E. coli,* the glycogen within the *E. coli* became highly phosphorylated (Lorberth *et al.,* 1998). Interestingly, both in that study and in a similar second one (Viksø-Nielsen *et al.,* 2002) small amounts of phosphorylated glucose moieties at the C6 position were determined in the glycogen from the strain containing the empty vector as a control. This amount ranged between approximately 0.2-0.9 nmol glucose-6-phosphate.mg^{-1} glycogen (Lorberth *et al*., 1998; Viksø-Nielsen *et al*., 2002).

Further evidence for the presence of phosphate in *E. coli* glycogen comes from a study of Scheidig (2006) who employed an *E. coli* functional expression system to identify plant genes that can degrade starch. Bacteria that were engineered to accumulate large amounts of linear glucans stain blue with iodine vapour and, when transformed with a potato cDNA library, the isolated colonies that stained white contained plant genes that code for proteins which was able to degrade the glucans (Scheidig, 2006). Several of the genes isolated did indeed code for starch degradative enzymes (Scheidig *et al*., 2002; Scheidig, 2006); however, one of them turned out to be a potato homolog of the Arabidopsis *Like Sex4-2* (*Lsf2)* gene. As was discussed in the general introduction (Section 1.2.2) the predicted primary protein sequence of Lsf2 shows high similarity to the SEX4 protein and SEX4 has been demonstrated to act by dephosphorylating starch (Edner *et al*., 2007; Kötting *et al*., 2009). The precise role of Lsf2 has yet to be elucidated; however, given its similarity to SEX4 it is reasonable to assume that it also acts in a polyglucan dephosphorylating manner.

Furthermore, studies within the Institute for Plant Biotechnology (IPB) have demonstrated that expression of *AtSEX4* in *E. coli* leads to bacteria that are not able to accumulate glycogen (James Lloyd, unpublished data). It is not yet clear why expression of these two proteins in *E. coli* would have this effect, however, one hypothesis would be that they act by removing phosphate from the glycogen, either stopping it being manufactured, or leading to it being degraded quickly.

Genes coding for proteins involved in glycogen metabolism in *E. coli* are present within the *glg* operon (Chapter 1, Fig.1.6). The enzymes involved in glycogen synthesis are GlgA, GlgB and GlgC, (Latil-Damotte and Lares, 1977) while GlgP and GlgX are known to be involved in its degradation (Dauvillée *et al.,* 2005; Alonso-Casajús *et al*., 2006). The question arises that, if phosphate is indeed present in *E. coli* glycogen, by which mechanism it gets incorporated? The only known enzymes able to do this are GWD and PWD, but there appears to be no genes in the *E. coli* genome coding for proteins which show significant similarities to these. One theoretical possibility, based on the enzymes coded for in the *glg* operon, is that GlgP could incorporate phosphate in the C6 position of the glucose monomers of glycogen. Glucan phosphorylases catalyse the reversible reaction where the glucose moiety of glucose-1-phosphate is either incorporated into, or liberated from, a polyglucan. Enzymes in this class are generally named due to their substrate specificity, for example glycogen phosphorylase uses glycogen as its preferred polyglucan substrate while starch phosphorylase utilises starch. It has been hypothesised (Lorberth *et al.,* 1998) that these enzymes could also use glucose-1,6-bisphosphate as a sugar donor instead of glucose-1-phosphate. Glucose-1,6-bisphosphate is known to be present in *E. coli* as it is a required allosteric activator of phosphoglucomutase (Joshi and Handler, 1964) and is thought to be produced in *E. coli* in a phosphodismutase reaction (Leloir *et al*., 1949).

Here we postulate that, if GlgP uses glucose-1,6-bisphosphate in the polymerization direction, it would lead to incorporation of phosphate in the C6 position of the glucosyl moiety in glycogen. In order to test this, the phosphate content in *E. coli* glycogen was first re-examined by determining the amount of glucose 6-phosphate present in glycogen of three bacterial strains. Secondly, a functional expression screen was performed to identify several putative *E. coli* proteins that might be

involved in glycogen phosphorylation. The results of this will be discussed in the context of current models of glycogen metabolism.

2.2 Materials and methods

2.2.1 Chemicals

All oligo-nucleotides used in this study were purchased from Inqaba Biotech (Pretoria, South Africa) and Integrated DNA Technology (IDT) (Coralville, USA). All the chemicals used in enzyme assays were purchased from Roche Biochemicals (Mannheim, Germany) or Sigma Aldrich Fluka (SAF) chemical company (St. Louis, MO, USA), unless stated otherwise. All reagents and chemicals were of analytical grade.

2.2.2 *E. coli* strains and plasmid used in this study

Different *E. coli* strains were obtained to study glycogen phosphorylation in this project and are listed in Table 2.1. In addition, the plasmids used are also listed (Table 2.1).

Table 2.1. *E. coli* strains and plasmids used in this study with their genotypes and source or reference.

Strain no./name	Genotype/relevant characteristic	Source or reference
	***E.coli* strain**	
DH5α	*fhuA2 Δ(argF-lacZ)U169 phoA glnV44 Φ80 Δ(lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17*	Invitrogen
BW25113	*Δ(araD-araB)567* , *ΔlacZ4787* (::rrnB-3), lambda$^-$, *rph-1* , *Δ(rhaD-rhaB)568* , *hsdR514*	Datsenko and Wanner, 2000
JW5689-1	ΔmalP751::kan^R,rph-1	Baba *et al.,* 2006
JW3395	ΔglgB765::kan^R,rph-1	Baba *et al.,* 2006
JW3391	ΔglgP761::kan^R,rph-1	Baba *et al.,* 2006
JW3483	ΔgadW::kan^R,rph1	Baba *et al.,* 2006
JW3484	ΔgadX::kan^R,rph1	Baba *et al.,* 2006
KV832	ΔglgB::kan^R,rph1	Kiel *et al* ., 1987
	Plasmids	
pKD46	Amp^R	Datsenko and Wanner, 2000
pACYC184	Cm^R and Tet^R	New England Biolabs, Frankfurt am Main, Germany
pACAG	Cm^R and Tet^R,	Kossmann *et al.,* 1999
pBluescript SK(+)	Amp^R	Stratagene, La Jolla, CA, USA

2.2.3 Growth of *E. coli,* and measurement of glucose-6-phosphate and glucose content in glycogen

Either Kornberg liquid media (Romeo and Preiss, 1988; 1.1% (w/v) K_2HPO_4, 0.85% (w/v) KH_2PO_4, 0.6% (w/v) yeast extract, 0.5% (w/v) glucose), or ½ phosphate Kornberg media (0.55% (w/v) K_2HPO_4, O.425% (w/v) KH_2PO_4, 0.6% (w/v) yeast extract, 0.5% (w/v) glucose) containing appropriate antibiotics was prepared. 10ml of media was inoculated with *E. coli* and incubated at 37^{o}C overnight with shaking. Cells from the cultures were harvested by centrifugation at 20 000**g** for 2 minutes at room temperature.

In order to remove soluble sugars, *E. coli* pellets were re-suspended in 1ml of 80% (v/v) ethanol and incubated at 80°C for 1 hour. The cells were harvested by

centrifugation at 20 000**g** for 10 minutes and the supernatant discarded. Glucose-6-phosphate (Glc6P) amounts within the glycogen were determined by the method of Nielsen *et al.* (2004). Glycogen was digested by incubating the bacterial pellet in 400µl of 0.7M HCl for 4 hours at 95°C. Following neutralization with an equal volume of 0.7M KOH, a half spatula of polyvinylpolypyrollidine (PVPP) was added. This was vortexed briefly and centrifuged at 20 000**g** for 10 minutes. Glc6P was determined by combining 200µl of assay buffer (300mM Tris-HCl (pH 6.8), 10mM $MgCl_2$, 1mM NAD) and 100µl of the digest. 1U of glucose-6-phosphate dehydrogenase (from *Leuconostoc mesenteroides)* was added and the increase in absorbance was followed at 340nm. Glucose determination was achieved by combining 290µl of assay buffer (300mM Tris-HCl, pH 6.8, 10 mM$MgCl_2$, 1mM NAD, 1mM ATP) and 10µl of the digest. Determination was done by adding 0.34U/0.17U of glucose-6-phosphate dehydrogenase/hexokinase from yeast, and the increase in absorbance was followed at 340nm.

2.2.4 Construction of *E. coli* libraries

Several *E. coli* (DH5α strain) genomic libraries were prepared in-house in a pBluescript SK(+) (Stratagene, La Jolla, California) vector. This was done by means of partial digestion of gDNA with three independent restriction enzymes, namely *Sau3A (*average insert size *688bp)*, *Rsa*I *(*average insert size *1034bp)* and *Hae*III *(*average insert size *888bp*) which were ligated into the pBluescript SK(+) vector using the *Bam*HI site for *Sau3A* digested DNA and the *EcoR*V site for the DNA digested with the other two enzymes.

2.2.5 Library screening

Libraries were transformed into *E. coli glgb*$^-$*::pACAG* cells. The *glgb*$^-$ mutant was from the Keio collection (CGSC# 10528; Baba *et al.,* 2006) and was obtained from the Yale *E. coli* Genetic Resource Center. These were grown on solid Luria broth (LB) media containing 2% (w/v) glucose at 37°C overnight and stained for accumulation of glycogen by exposure to iodine vapor. Colonies accumulating increased amounts of glycogen were isolated and plasmid DNA isolated.

2.2.6 Plasmid sequencing and gene identification

Inserts within plasmids were sequenced at the Stellenbosch University Central Analytical Facility (CAF).

2.2.7 Preparation of *E. coli* protein extracts

Protein extracts were prepared by inoculating *E. coli* in 5ml of LB media containing either 2% (w/v) glucose or 2% (w/v) maltose, followed by incubation at 37°C overnight with shaking. The culture was then transferred to 150ml of LB media containing the same sugars and incubated for 37°C for 4 hours with shaking. The culture was harvested by centrifuging at 7875**g** at 4°C for 15min and the pellet re-suspended in 5ml of buffer solution containing 50mM Tris-HCl (pH 6.8), 10% (v/v) glycerol, 5mM $MgCl_2$, 2mM EDTA and 2mM DDT. The cells were disrupted by sonication. Protein extract was clarified by centrifugation at 8507**g** for 15min at 4°C, frozen in aliquots in liquid nitrogen and stored at -80°C until further use.

2.2.8 Non-denaturing gel electrophoresis

Discontinuous gels containing 10% (w/v) polyacrylamide and 1% (w/v) rabbit muscle glycogen in the separation gel were prepared without sodium dodecyl sulfate (SDS) according to the method of Sambrook *et al*. (1989). Protein was separated at 120V and 4°C. The gels were subsequently incubated in 3M acetic acid-NaOH (pH6.0) containing 20mM glucose-1-phosphate at room temperature overnight with shaking. The solution was discarded and the gel was stained with Lugol's (0.2% (w/v) KI, 0.02% (w/v) I_2) solution.

2.2.9 Protein determination

Protein quantification was performed as described by Bradford (1976) with bovine serum albumin used as a standard.

2.2.10 Maltodextrin phosphorylase activity

Maltodextrin phosphorylase (MalP) activity was measured in a kinetic assay at room temperature with maltoheptaose as a substrate, with the method adapted from Xavier *et al.* (1999). The assay mix contained 50mM potassium phosphate buffer (pH 7.0), 2mM NADP, 2.5mM maltoheptaose, 4.41U phosphoglucomutase (rabbit muscle), and 1U glucose-6-phosphate dehydrogenase (*Leconostoc mesenteroides).* The reaction was started by adding 10µl of protein extract to 200µl assay buffer and glucose-1-phosphate production was followed at 340nm.

2. 2.11 Generation of PCR fragments for insertional mutagenesis in *E. coli*.

A PCR product was generated by using forward (5'-AACTAGCGATAA CGTTGTGTTGAAAATCTAAGAAAAGTGGAACTCCTATGATGGAGAAAAAAATCAC TGGAT-3') and reverse (5'-TCCAGACGTTTGCTTTCCATCGAGCTTCCTTAGCGTT TTGCCTGCCAGATTTACGCCCCGCCCTGCCACTCA-3') primers that include homology extensions encoding the beginning and end of the *malp* gene (underlined) and flanking sequences homologous to the *chloramphenicol acetyltransferase* gene (not underlined). PCR was performed using the vector pACYC184 (New England Biolabs) as a template. The PCR product was separated using 1% (w/v) agarose gel electrophoresis and then purified using the QIAquck^{R} purification kit according to the manufacturer's recommendations (Qiagen).

2.2.12 Transformation of pKD46 into the *glgp*$^{-}$ mutant.

glgp^{-} mutants were grown on LB media with kanamycin (40μg.ml^{-1}) to an OD_{600} of 0.6-0.8. The red helper plasmid pKD46 (Datsenko and Wanner, 2000) was transformed into electrocompetent *glgp*$^{-}$ cells which were prepared exactly as described previously (Sambrook *et al.,* 1989). Cells were electroporated according to the manufacturer's instructions using a Gene Pulser Xcell^{TM} Electroporation system (BioRaD) and incubated at 28°C for 26hr before plating on a media containing 20μg/ml ampicillin and kanamycin (40μg.ml^{-1}) for selection.

2.2.13 Transformation of the PCR product and selection of putative double mutants

The *glgp*$^{-}$ mutant carrying pKD46 was grown in liquid LB media containing 20μg.ml^{-1} ampicillin and 20mM L-arabinose at 30°C to an OD_{600} of 0.6-0.8, and electro-competent cells were prepared as described (Sambrook *et al*., 1989). The PCR product of the chloramphenicol resistance gene flanked by m*alp* regions was electroporated into the competent cells according to the manufacturer's instructions. After incubation for 26hr at 28°C cells were spread on solid LB media containing chloramphenicol (25μg.ml^{-1}).

2.2.14 Confirmation of loss of pKD46 plasmid

In order to make sure that the cells lose pKD46, colonies were inoculated in LB media without ampicillin and grown at 42°C. The media was streaked on plates

lacking ampicillin and individual colonies were tested for ampicillin sensitivity by streaking on plates that either did, or did not, contain ampicillin.

2.2.15 Confirmation of the double mutant

PCR using 5'-GCGATAACGTTGTGTTGAAAA-3' and 5'-ATCACAGACGGCA TGATGAA-3' primers was used to examine inserts into the *malp* gene. The first (forward) primer anneals to gDNA just upstream of the first ATG in the *malp* gene, while the second (reverse) primer anneals to *chloramphenicol acetyltransferase* gene and should lead to the production of a 636bp fragment if the gene has been successfully disrupted.

2.3 Results and Discussion

2.3.1. Measurement of glycogen phosphate content in *E. coli* strains DH5α, CGSC7451 (*glgp*$^-$) and KV832 (*glgb*$^-$)

To confirm earlier reports of phosphate in *E. coli* glycogen (Lorberth *et al*.,1998; Viksø-Nielsen *et al.,* 2002) a high-throughput method for measuring glycogen phosphate in bacterial pellets from liquid cultures was developed. This involved growing *E. coli* cultures and sedimenting the pellets by centrifugation. The pellets were washed with ethanol and the remaining glycogen digested to its constituent monomers by heating with 0.7M HCl. Resulting sugars were then used for enzymatic determination of glucose and glucose-6-phosphate content.

Three strains of bacteria present within the Institute of Plant Biotechnology were used for this initial experiment. These were DH5α (Invitrogen), KV832 (Kiel *et al*., 1987) and CGSC7451 (Singer *et al.,* 1989). DH5α is a general laboratory strain which contains the entire *glg* operon while KV832 is a *glgb*$^-$ mutant and CGSC7451 is a *glgp*$^-$ mutant. The reason for using the two mutant strains were firstly to test whether any phosphate is incorporated into linear glucan chains produced in the KV832 *glgb*$^-$ mutant and secondly to examine whether GlgP is the protein incorporating phosphate into glycogen.

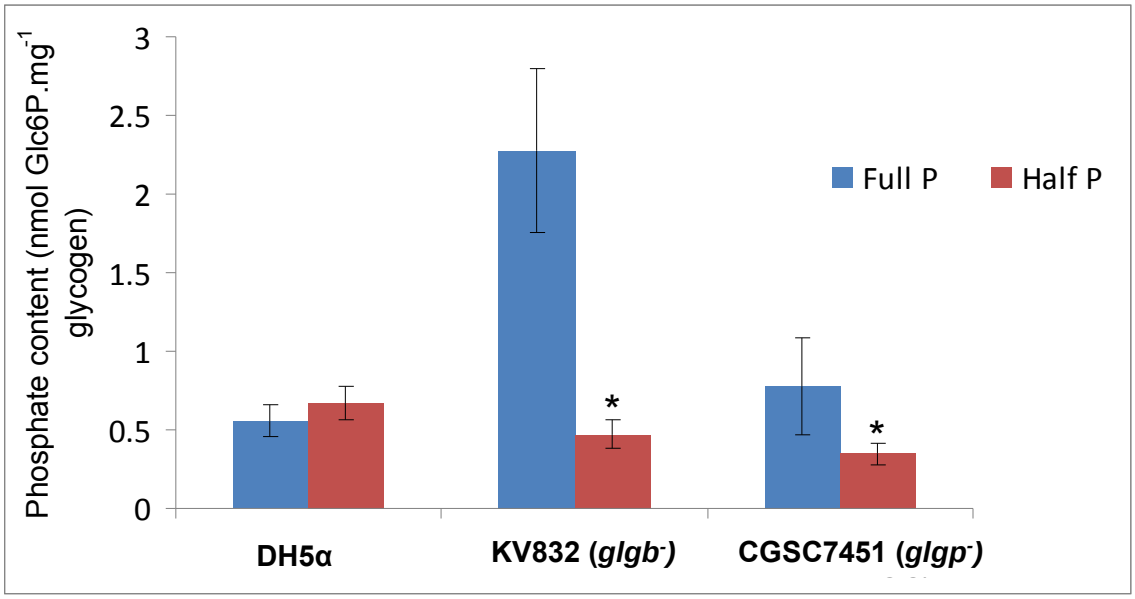

Figure 2.1 Glycogen phosphate content from three *E. coli* strains (DH5α, KV832 (*glgb*$^{-}$) and CGSC7451 (*glgp*$^{-}$), grown under high (blue bar) and moderate (magenta bar) phosphate conditions. Values are presented as mean ± SE (n=5) and an asterisk (*) indicates a value that were determined by *Students t*-test to be significantly different (P<0.05) from the respective full-strength phosphate control.

The data shown in Fig. 2.1 indicates that that there are indeed significant amounts of phosphate in *E. coli* glycogen in all three of the strains used. There appears to be a reduction in the amount of phosphate in the glycogen when grown under moderate phosphate conditions in the KV832 and CGSC7451 strains in comparison with the high phosphate conditions. In contrast, the DH5α strain had similar phosphate content in the glycogen between high and moderate phosphate conditions (Fig.2.1). Phosphate was also present in the CGSC7451 strain, which is mutated in the *glgp* gene, indicating that GlgP may not be solely involved in incorporating phosphate into glycogen. Finally, the KV832 strain, which lacks glycogen branching enzyme, contained the highest amount of phosphate. This is important as in starch it is only the branched amylopectin fraction that contains phosphate (Posternak 1951; Hizukuri *et al*., 1970; Takeda and Hizukuri, 1982; Blennow *et al*., 2002), due to the substrate specificity of the GWD protein (Mikkelsen *at al.,* 2004). It would be interesting, from a biotechnological point of view, to introduce phosphate into the amylose fraction as well and, as the unbranched chains in KV832 contain phosphate, the elucidation of the mechanism of phosphate incorporation in *E. coli* may provide a valuable tool to do this in plants.

2.3.2 *E. coli* library screening

In order to identify candidate genes that might be involved in phosphorylating *E. coli* glycogen a functional screening approach was taken. This is because it was noted that when a specific *glgb*$^-$ mutant strain (CGSC# 10528) was transformed with the potato *GWD* gene it started to accumulate glucans (Fig.2.2; James Lloyd, *unpublished data*). Using the assumption that any other glycogen phosphorylating protein would induce the same phenotype it was decided to transform an *E. coli* gDNA library into the same *glgb*$^-$ mutant and isolate colonies that accumulate glucans.

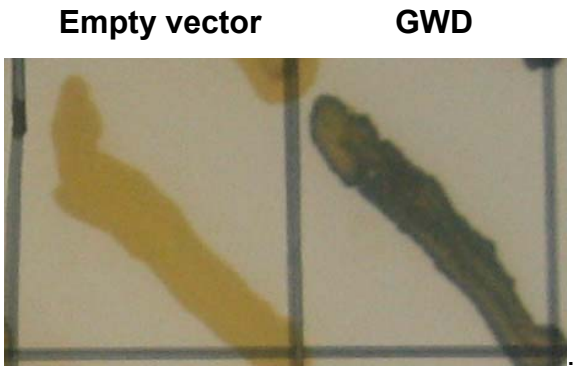

Figure 2.2 Expression of glucan water dikinase (GWD) in a *glgb*$^-$ (strain CGSC# 10528) mutant. Plates were exposed to iodine vapour to visualise glucan accumulation. The construct expressing GWD (right panel) accumulated significantly more glucans (as observed from the darker stain) than the strain containing only the empty vector (left panel).

This indicated that glucan phosphorylation stimulated glucan accumulation in the particular strain. Subsequently, three *E. coli* gDNA libraries were transformed independently into this mutant which had been engineered to contain the pACAG plasmid (Kossmann *et al*., 1999). pACAG allows expression of the *glgC16 E. coli* gene, which is an unregulated form of ADP-glucose pyrophosphorylase. This allows large accumulation of significant amounts of glycogen in *E. coli* (Creuzat-Sigal *et al,* 1972). Plasmids were isolated from positive staining colonies and re-transformed into the same strain to confirm the phenotype before sequencing. Approximately 80 000 colonies were screened and 40 were confirmed to demonstrate a positive phenotype, of which 10 were randomly chosen for further analysis. Fig.2.3 shows an example of a plate exposed to iodine vapour. The colonies indicated with red ticks are examples of those selected for sequencing.

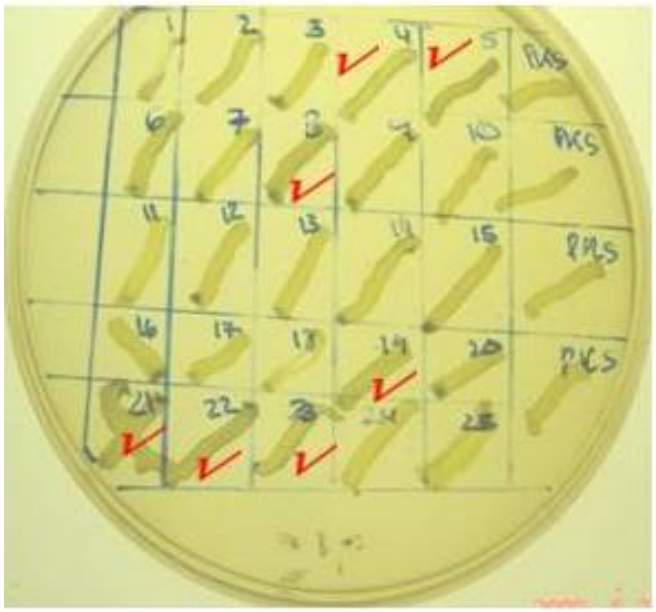

Figure 2.3 Iodine staining of *glgb*⁻::pACAG *E. coli* colonies expressing glycogen genes from an *E. coli* genomic library. Colonies were grown overnight on media supplemented with 2% (w/v) glucose at 37°C and were then exposed to iodine vapour. Numbers represent independent positive staining clones while PKS denotes the same strain containing the empty vector (pBluescript KS(+)).

2.3.3 Analysis of gene sequences

Sequence data was analysed using the Basic Alignment Search Tool (BLAST) (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and demonstrated that the 10 sequences obtained encoded 6 genes. Table 2.2 shows the BLAST search results. Some of the genes encode proteins with known functions, but which are likely to be false positives. These include the cytoplasmic α-amylase (AmyA) which breaks down maltodextrins that have entered the cytoplasm. In can also digest polyglucans although starch has been proven to be a better substrate for this enzyme than glycogen (Raha *et al.,* 1992). AmyA can also act as a 4-α-glucan transferase, an enzyme that transfers α-1,4 bonds from one linear polyglucan to another, leading to the production of longer malto-oligosaccharides (Raha *et al*., 1992).

Table 2.2 Proteins encoded by genes identified in the functional screen.

Gene name /protein	Gene symbol	NCBI accession number	E-value	Max identity	References
Glutamatic acid decarboxylase transcription factor	*gadW*	EBT991GX01N	0.00E+00	99%	Tramonti *et al.,* 2002
Glutamatic acid decarboxylase transcription factor	*gadX*	EBUVPRBT01S	0.00E+00	97%	Tucker *et al.,* 2002
cytoplasmic alpha-amylase	*amyA*	EBNGDU7T01N	9.00E-50	97%	Raha *et al.,* 1992
carbon storage regulator	*csrB*	EBPPDRUR01S	0.00E+00	100%	Lui *et al.,* 1997
Transcriptional regulator MalT	*malT*	EBR2N7RS01S	0.00E+00	99%	Chapon, 1982
maltodextrin phosphorylase	*malP*	BRNFHDF01N	3.00E-134	100%	Schwartz and Hofnung, 1967

One of the identified gene is *csrB*, which is bound to *csrA* gene a negative regulator of *glgCAP* operon (Liu *et al*., I1997). Overexpression of *csrB* gene leads to glycogen accumulation in *E. coli* (Liu *et al*., 1997) Similar to the observed phenotype in this experiment. It is involved in transcript stability of RNA coming from the *glg* operon (Liu *et al*., 1997) so is unlikely to be directly involved in glycogen phosphorylation.

GadX and GadW are araC-like transcription factors which are 42% similar to each other. They are involved in activating glutamate metabolic genes which allow *E. coli* to acclimatise to stomach acid (Tucker *et al.*, 2002; Hommais *et al.*, 2004; Weber *et al.*, 2005; Sayed *et al*., 2007). In one study Tucker *et al.* (2003) demonstrated that both regulate expression of the *glgS* gene. *glgS* has been reported to influence glycogen accumulation as a mutation in it reduces glycogen amounts while its overexpression increases its amounts in *E. coli* (Hengge-Aronis and Fischer, 1992). The expression of GadX and GadW thus agrees well with the observed phenotype. The function and role that *glgS* plays, however, remains unknown.

malT is a positive regulatory gene that controls the maltose operons in *E. coli,* namely *malPQ*, *malK-lamB* and *malEFG* (Hatfield et al., 1969; Debarbouille and Schwartz 1979). Danot and Raibaud (1994) reported a detailed biochemical

characterization of the interaction of MalT with the promoter of the *malPQ* operon and clearly showed that the maltodextrin phosphorylase gene (*malP)* is up-regulated by MalT. This was one of the other genes also identified in the screen. MalP catalyses a very similar reaction to the previously discussed GlgP protein by either incorporating or liberating glucose-1-phosphate (Glc1P) from linear maltodextrins. The secondary protein structure of *E. coli* MalP is also similar to those of *E. coli* GlgP (Kumal, 1990) and this may indicate that they have similar functions *in vivo*. The main difference between MalP and GlgP, however, is the substrate they act on. GlgP has a high affinity for glycogen (Hu and Gold, 1975; Kasvinsky *et al*., 1978) while MalP has a high affinity for linear oligosaccharides and low affinity for glycogen (<1%) (Schwartz and Hofnung, 1967). Despite this, it can be speculated that MalP, in a similar manner to GlgP, could utilise glucose-1-6-bisphosphate as well as glucose 1-phosphate as substrates to incorporate phosphate into glycogen.

Given that both MalP and its regulator (MalT) was identified in this screen, it was decided to examine the amount of phosphate in glycogen from a *malp*$^{-}$ mutant. In addition, the glycogen content from mutants lacking araC-like transcriptional regulators, GadW and GadX, was also analysed. Mutants were obtained from the Yale *E. coli* stock centre and originated from the Keio collection (Baba *et al.*, 2006). They were grown in Kornberg medium and the glucose 6-phosphate content of the glycogen determined. Fig.2.4 shows that there was no difference in phosphate content in any of the mutants in comparison with the K-12 control strain, suggesting that none of the genes alone are involved in phosphorylating glycogen. In order to investigate this further, the relationship between MalP and GlgP, with the assumption that there might be functional redundancy between the two, was studied.

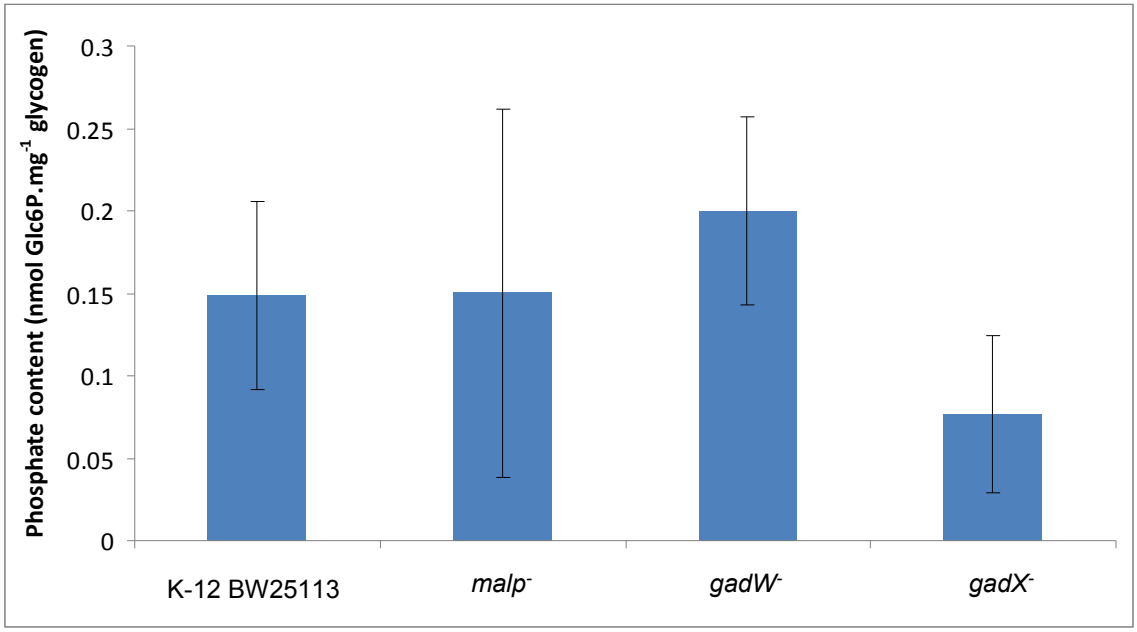

Figure 2.4 Glycogen phosphate content of three *E. coli* mutants (*malp*$^-$, *gadW*$^-$ *and gadX*$^-$) generated in a K-12 BW25113 background strain grown under high phosphate conditions. Values are presented as mean ± SE (n=3; K-12 BW25113 n=4) and no significant differences (P<0.05) was observed as determined by *Students t*-test from the K-12 control strain.

2.3.4 Analysis of glycogen and maltodextrin phosphorylase activities in *glgp*$^-$ and *malp*$^-$ mutants

In order to examine the functional relationship between MalP and GlgP their activities were examined under growth conditions with different sugar substrates. MalP has been shown to be induced by maltose (Schwartz, 1965) and repressed by glucose (Chao and Weathersbee, 1973), but it isn't clear if the reverse is true for GlgP.

Fig. 2.5 shows the native glycogen phosphorylase activity gel of protein extracts from the *glgp*$^-$ and *malp*$^-$ mutants in the K-12 BW25113 (WT) strain. All strains were grown with either glucose or maltose in the medium. As can be observed, when the three strains were grown with glucose supplementation, GlgP activity was present in both the WT and MalP extracts, but absent in the *glgp*$^-$ mutant. When the strains were grown with maltose there was repression of GlgP activity in the WT. However, in the *malp*$^-$ mutant grown on maltose GlgP activity was also present suggesting that metabolic repression of *glgp* transcription by maltose is overcome in the *malp*$^-$ mutant.

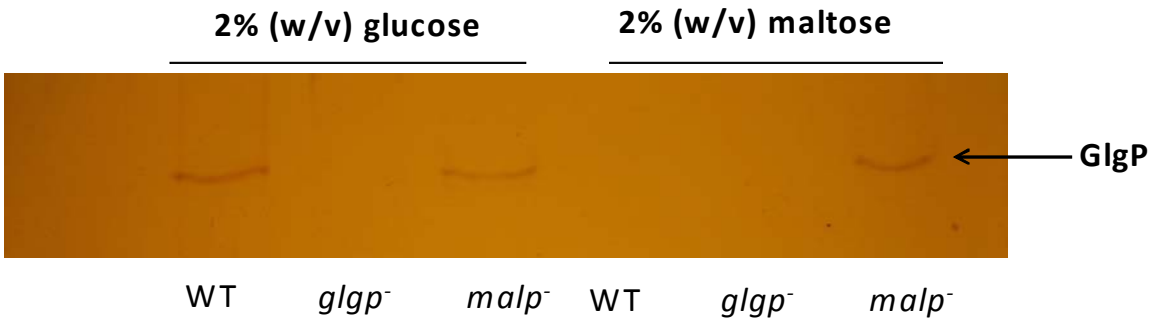

Figure 2.5. Non-denaturing activity gel of glycogen phosphorylase activities in K-12 BW25113 (WT) control, *glgp*$^-$ and *malp*$^-$ mutants grown in media supplemented with either 2% (w/v) glucose or 2% (w/v) maltose. Proteins extract were separated on 10% (w/v) non-denaturing PAGE containing 1% (w/v) glycogen. GlgP activities were assayed as described in Material and methods. The arrow indicates the expected band for the GlgP protein.

MalP activity in the protein extract from the same *E. coli* strains which were grown, as before, in either maltose or glucose was also determined (Fig.2.6). As expected, MalP activity was found in both the WT and *glgp*$^-$ mutant when grown on maltose. Also, when strains were grown in glucose there was a repression of MalP activity in both the WT and *glgp*$^-$ mutant strains. This demonstrates that MalP is induced specifically by maltose and that its activity is not up-regulated in the *glgp*$^-$ mutant when grown in glucose media. Taken together, the data on phosphorylase activities of GlgP and MalP suggests that GlgP might be able to compensate for a lack of MalP activity (as it is induced in the *malp*$^-$ mutant when grown in maltose), but that the opposite is not true (as MalP activity is not induced in the *glgp*$^-$ mutant when grown in glucose). It should, however, be pointed out that, since these activity measurements were only conducted at one time point, it is possible that MalP might still be present in the *glgp*$^-$ mutant grown with glucose at a different time point.

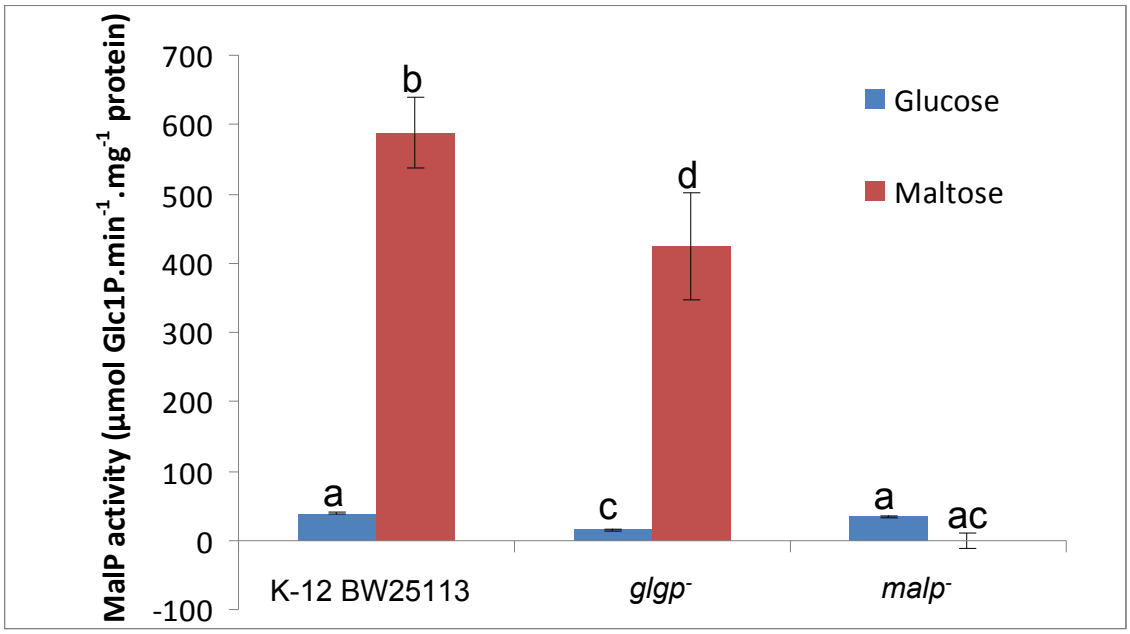

Figure 2.6. Maltodextrin phosphorylase activity measured in protein extracts from K-12 BW25113 (WT) control, *glgp*$^-$ and *malp*$^-$ mutants. *E. coli* were grown supplementated either 2% (w/v) glucose (blue bar) or maltose (magenta bar). One unit enzyme activity is defined as 1.0µmol of glucose-1-phosphate formed per minute. Values are presented as mean ± SE (n=4) and different letter denomination represents values that were determined by ANOVA to be significantly different (P<0.05) from each other.

2.3.5 Production of a *malp*$^-$/*glgp*$^-$ double mutant

In order to examine if *E. coli* glycogen is phosphorylated by the combination of MalP and GlgP, it was decided to construct a *malp*$^-$/*glgp*$^-$ double mutant. There are several ways to induce mutations in bacteria. One of the easiest is by means of recombination using a red recombinase method developed by Datsenko and Wanner (2000). *E. coli* are not readily transformed by linear DNA because of the presence of the intracellular exonuclease *Rec*BCD that degrades linear DNA (Benzinger *et al*., 1975). The red system include three genes λ, β,and *exo* from bacteriophage λ carried on a plasmid. These genes produce the proteins Gam, Bet and Exo, respectively (Murphy *et al*., 2000). The Gam protein inhibits the *Rec*BCD nuclease from attacking linear DNA while Bet and Exo are involved in creating and protecting single stranded overhangs from the linear DNA for recombination into the bacterial genome (Murphy *et al.,* 2000). The strategy involves three basic steps. Firstly, the synthesis of oligo-nucleotides and preparation of the cassette. Secondly, electroporation of the cassette into the cells carrying the target gene and thirdly, recombination. The synthesis of

oligo-nucleotides is normally achieved by chemical synthesis with 30-50nt homology to the target sequence. The linear DNA is prepared by amplifying an antibiotic resistance gene (in this case the chloramphenicol resistance gene). This is electroporated into the cells to be mutated which contain the pKD46 plasmid (Datsenko and Wanner, 2000) that allows expression of the λ, β,and *exo* genes required for recombination. Expression of the genes on pKD46 are driven by the arabinose promoter and the plasmid contains a temperature sensitive replicon to allow it to be easily cured from the cells. The linear DNA then replaces the targeted gene by homologous recombination and transformed cells can be selected based on acquired resistance to the antibiotic (Fig.2.7).

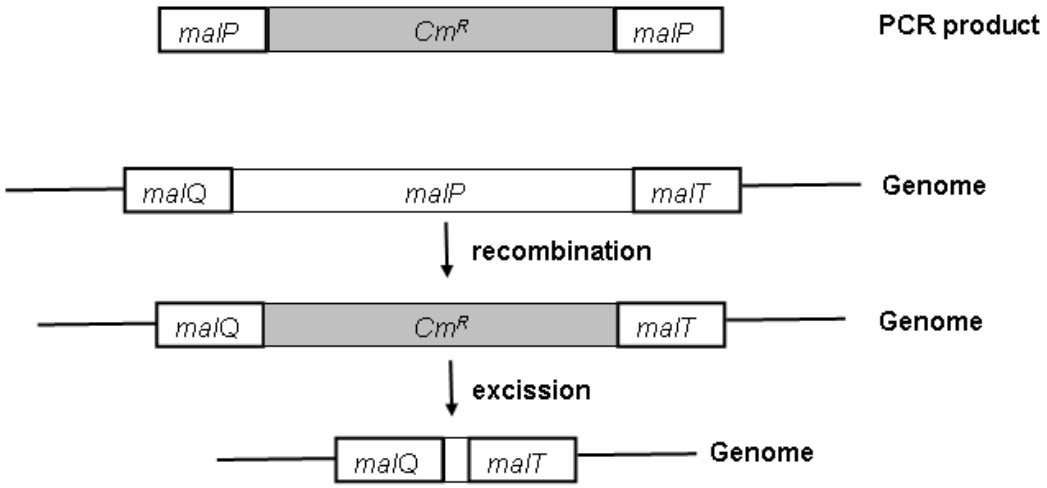

Figure 2.7 Strategy for replacing the *malp* gene in the *E. coli* genome based on the method of Datsenko and Wanner (2000). *malp* is replaced with a chloramphenicol resistance (Cm^R) gene in a $glgp^-$ mutant background in order to produce a $malp^-/glgp^-$ double mutant.

Using this method, Datsenko and Wanner (2000) were able to disrupt more than 40 genes on the *E. coli* chromosome without a single failure, and it has been used to knock out all non-lethal genes in *E. coli* as part of the Keio collection initiative (Baba *et al*., 2006), demonstrating the reliability of the method. However, one of the pitfalls in this method involves the formation of many false positives from the plasmid due to aberrant PCR errors. To circumvent this, Datsenko and Wanner (2000) constructed plasmids that were unable to replicate in *E. coli* strains. Because I did not have access to these, the plasmid pACYC184 was used as a template for the PCR with

the expectation to also find a significant amount of false positives. The resulting PCR product showed a band of approximately 700bp which should contain the *chloramphenicol acetyltransferase* gene flanked by nucleotides homologous to either end of *malp* (Fig.2.8).

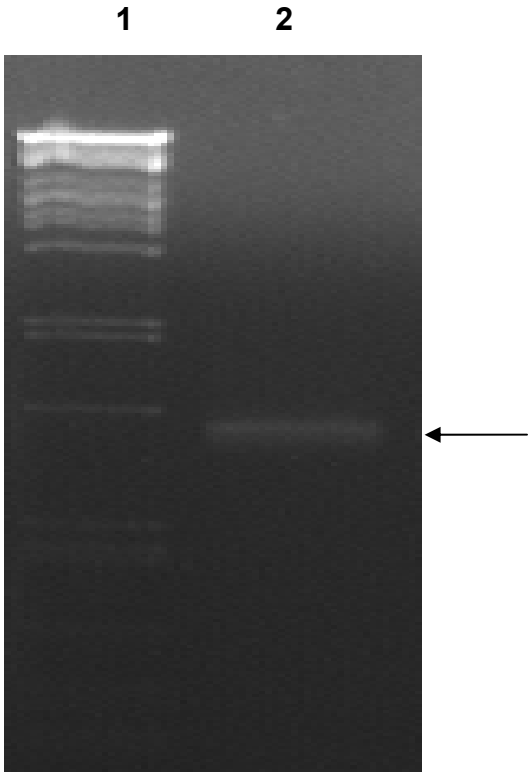

Figure 2.8 Agarose gel showing a PCR product designed to produce an insertion mutation in the *malP* gene through homologous recombination. Lane 1: Lambda DNA marker, Lane2: PCR product. The arrow indicates the PCR product with an approximate size of 700bp.

The excised band was transformed into *glgp*$^{-}$::pKD46 cells and colonies were isolated that were resistant to both kanamycin and chloramphenicol. A screening of twenty of these colonies for insertion of the *Cm*^{R} gene into *malp* yielded no positives. The occurrence of these false positives are probably due to aberrant PCR as was found previously (Datsenko and Wanner, 2000). Unfortunately, due to time constraints I was unable to repeat this experiment; however, it will be repeated in future in order to test the hypothesis that MalP and GlgP in combination can lead to phosphate incorporation into glycogen.

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Chapter 3: Investigation of the effect of Sex4, Lsf1, Lsf2, Lafora and GWD proteins on glycogen metabolism in *Saccharomyces cerevisiae.*

3.1 Introduction

Phosphatases (EC 3.1.3) are enzymes which are able to remove phosphate groups from a substrate. They are an important group of enzymes and can be subdivided into several classes. One of these are the protein phosphatases which are able to remove phosphate groups from amino acids within proteins which have been phosphorylated by protein kinases (Hanks *et al.,* 1988). Different classes of protein phosphatases exist, such as tyrosine phosphatases (EC 3.1.3.48) and serine/threonine phosphatases (EC 3.1.3.16). Both of these remove phosphate from, as the name suggests, one amino acid from a specified protein. However, another class of protein phosphatases, known as dual specificity phosphatases (EC 3.1.3.38), have the ability to dephosphorylate two amino acids of the same protein (Denu and Dixon, 1995).

Although dual specificity phosphatases are generally described as being protein phosphatases, some are able to dephosphorylate polyglucans. The best known examples of these include the Laforin protein of animals (Worby *et al*., 2006) and the SEX4 protein in plants (Edner *et al.,* 2007; Kötting *et al.,* 2009*)*. Both of these proteins are probably involved in dephosphorylating either glycogen or starch prior to its degradation, and their role was reviewed in detail in the introductory chapter (section 1.2.2 and section 1.2.3). The question arises whether this process of glycogen dephosphorylation (and phosphorylation) occurs in other species. One way to examine this is to examine the role of dual-specificity phosphatases that are present in other organisms. Baker's yeast (*Saccharomyces cerevisiae*) contains several genes coding for putative dual-specificity phosphatases (Table 3.1)

Table 3.1 Dual-specificity phosphatase identified in the *Saccharomyces cerevisiae* genome

Locus	Reference
YVH1/YIR026C	Beeser and Cooper, 2000
PPS1/YBR276C	Ernsting *et al* ., 1997
CDC14/YFR028C	Culotti and Hartwell, 1971
TEP1/YNL128W	Heymont *et al.* , 2000
MSG5/YNL053W	Doi *et al* ., 1994
SDP1/YIL113W	Collister *et al* ., 2002

One of these dual-specificity phosphatases (Yvh1p) has been demonstrated to be involved in glycogen accumulation (Beeser and Cooper, 2000) Yvh1p is induced by nitrogen starvation and cold temperatures (Beeser and Cooper, 2000), and required for cell growth, effective sporulation (Park *et al*., 1996), vegetative growth (Sakumoto *et al.,* 1999, 2001) and, interestingly, glycogen metabolism (Beeser and Cooper, 2000). When it is eliminated by mutation the yeast cells cannot accumulate glycogen when grown on plates lacking nitrogen (Guan *et al*., 1992; Beeser and Cooper, 2000). The glycogen-less phenotype noted here in *yvh1p*$^{-}$ mutant cells is different to what would be expected if it acts in a similar way to the SEX4 or Laforin proteins. Mutations in the genes coding for these lead to an accumulation of starch or glycogen and so it would be expected that if Yvh1p acts in a similar manner it would also lead to a glycogen excess phenotype. Although this is not the case it is interesting to speculate that this yeast protein may influence glycogen metabolism in some way similar to SEX4 and Laforin, yet have the opposite effect. Because Yvh1p is the only DSP in the yeast genome known to be involved in glycogen metabolism it was decided to examine if it could be complemented by human and plant DSP's. In addition the effect of DSP's and the GWD protein on glycogen metabolism in both wild type and *yvh1*p mutant yeast cells will be assessed. It is hoped that this will provide evidence whether or not yeast glycogen is phosphorylated.

3.2 Materials and methods

3.2.1 Chemicals

PEG100 was purchased from Roth (Karlsruhe, Germany). All other chemicals used in enzymes essays were purchased from Roche Biochemicals (Mannheim, Germany)

and Sigma Aldrich Fluka (SAF) chemical company (St. Louis, MO, USA) unless otherwise stated. All reagents and chemicals were of analytical grade.

3.2.2 Genes, vector and strains used in this study

A *SEX4* EST (At3g52180) was obtained from the Ohio state DNA stock center, while cDNA's of *Lsf1* (At3g01510) and *Lsf2* (At3g10940). were kind gifts from Samuel Zeeman (ETH Zurich, Switzerland). A cDNA coding for Laforin (IMAGE ID≠: 824559) was obtained from the IMAGE collection while one coding for potato GWD: pPVD1construct was from James Lloyd (Institute for Plant Biotechnology (IPB, Stellenbosch University, South Africa). The yeast shuttle vector pPVD1, was obtained from the Institute of Wine Biotechnology (IWBT, Stellenbosch University, South Africa).

Table 3.2. Yeast strains used in this study.

Strain name	Genotype	Reference
BY4742	*MAT* α; *his3Δ1; leu2Δ0; lys2Δ0; ura3Δ0*	Brachmann *et al* ., 1998
Y15714	Matα; his3Δ1; leu2Δ0; lys2Δ0; ura3Δ0; YAL064c-a::kanMX4	Brachmann et al., 1998

3.2.3 Construct preparation

All cDNA's were present in pBluescript SK(+) (Stratagene, La Jolla, California). Laforin was digested from pBluescript with *Not*I and *Sal*I and ligated into the same restriction sites of pPVD1. SEX4 and LSF2 were both excised from pBluescript with *EcoR*I and *Sal*I and ligated into pPVD1 in the same restriction sites. Lsf1 was digested from pBluescript with *EcoR*I and *Xba*I and ligated into the same restriction sites of pPVD1.

3.2.4 Preparation of yeast cells

Yeast cells for transformation were prepared according to Dohemen *et al.* (1991) with few modifications. The cells were grown in 5mL Yeast Peptone Dextrose (YPD) (5g.L^{-1} yeast extract, 10g.L^{-1} peptone, 10g.L^{-1}glucose) media overnight. The culture was transferred to 150mL of the same media the following day and grown to an OD_{600}. The cells were centrifuged at 4000**g** for 5min and washed in a 50mL solution of 1.0M sorbitol, 10mM Lysine-NaOH (pH 8.35), 3% (v/v) ethylene glycol, 5% (v/v)

DMSO. This was followed by centrifugation at 4000**g** for 5min. The pellet was resuspended in 2mL of the same solution.

3.2.5 Yeast transformation

To transform the constructs into the WT and *yvh1p*$^-$ mutant, a 20µL mixture of 5-7µg plasmid DNA, 10 mg.mL^{-1} single stranded carrier DNA (herring sperm DNA) was added to 20µL of freshly prepared *yvh1p*$^-$ mutant or WT cells. The solution was mixed by inverting the tube several times before adding 1.4mL of 40% (v/v) PEG100, 0.2M Lysine-NaOH (pH 8.35) and mixed by vortexing for 1min. The solution was incubated at 30°C for 1hr, sedimented by centrifugation at 3000**g** for 5sec and resuspended in 1.0mL of 0.15 M NaCl, 10 mM Lysine-NaOH (pH 8.35). The cells were spread on selective media containing appropriate amino acid according to Brachmann *et al.* (1998) and incubated at 28°C.

3.2.6 Determination of glycogen content in yeast cells

To measure glycogen, a 10mL culture was inoculated with a colony and grown at 28°C for 72hrs before being centrifuged at 4000**g** for 3min at 4°C. The media was discarded and the pellets were suspended in 10mL of ice cold water and centrifuged again for 3min at the same speed. Pellets were frozen in liquid nitrogen and stored at -80°C until further use. Glycogen was measured with the method adapted from Becker (1978) and Lillie and Pringle (1980). 500µL of 0.25M Na_2CO_3 at 60°C was added to the frozen cells and the samples placed in boiling water for 2hrs. A 450µL sample was taken and acidified with addition of 67µL of 6M acetic acid. 200µL of this solution was mixed with 800µL of NaOAc (pH 4.8) and incubated with 20µL amyloglucosidase (10 mg.mL^{-1}) at 37°C for 12 hrs. The solution was neutralised with 10µL of NaOH and centrifuged for 10min at 20 000**g** at room temperature. Glucose was determined by combining 290µL of assay buffer (300mM Tris, pH 6.8, 10mM $MgCl_2$, 1mM NAD, 1mM ATP) and 10µL of the digest. Determination was done by adding 0.34U/0.17 U of glucose-6-phosphate dehydrogenase/hexokinase from yeast to the wells and the increase in absorbance was followed at 340nm.

3.3 Results and discussion

3.3.1 Preparation of constructs for investigation of glycogen metabolism in yeast cells

Yvh1p has been previously identified as a dual-specificity phosphatase which repression leads to significant decreases in glycogen (Beeser and Cooper, 2000). In order to identify whether plant and human dual-specificity phosphatases could complement Yvh1p, constructs carrying *sex4, Lsf1, Lsf2* and *Laforin*, as well as the starch phosphorylating enzyme *GWD* were ligated in sense orientation with respect to the strong constitutive PGK promoter in the pPVD1 shuttle plasmid. These were then transformed into wild type and *yvh1p*$^{-}$ mutant yeast cells and positive transformants identified based on amino acid selection media.

3.3.2 Measurement of glycogen content in the yeast

Following the selection of colonies, the glycogen content in the different constructs was measured at a stationary growth phase (Fig.3.1). The results demonstrated that there was no significant difference between the glycogen in the wild type and the *yvh1p*$^{-}$ mutants containing the empty plasmid (pPVD1). This is surprising since results obtained by Guan *et al.* (1992) and Beeser and Cooper (2000) indicated that the *yvh1p*$^{-}$ mutant is unable to accumulate glycogen at the stationary phase. One plausible explanation for this discrepancy could be due to strain differences. The strain (By4742) used in this experiment is haploid, in comparison with the diploid HPy120 strain used by Guan *et al*. (1992) and Beeser and Cooper (2000).

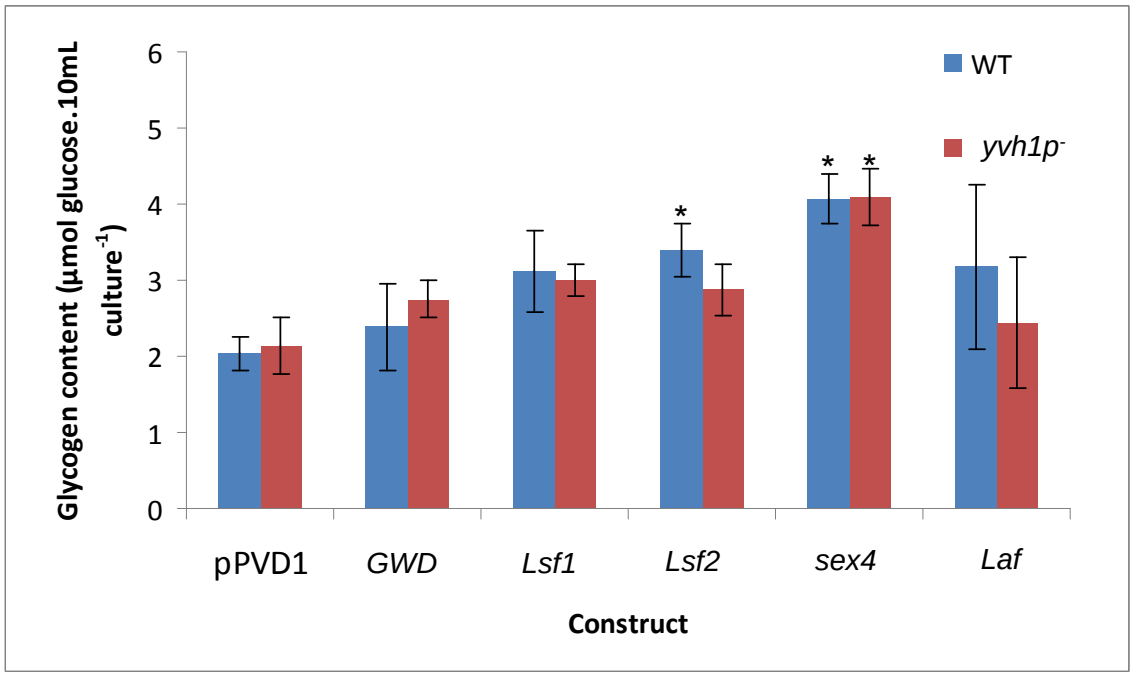

Figure 3.1 Glycogen content of different yeast constructs after it reached stationary phase at 72hrs. Potato glucan water dikinase (GWD), dual-specificity phosphatases (or phosphatases-like) genes *Lsf1, Lsf2* and *sex4*, as well as the human dual-specificity phosphatase Laforin (*Laf*) was transformed into either wild type (WT; blue bar) or *yvh1p*$^{-}$ (magenta bar) yeast mutant backgrounds (strain By4742). An empty vector control (pPVD1) was also included. Values are presented as mean ± SE (n=4) and an asterisk (*) indicates a value that were determined by *Students t*-test to be significantly different (P<0.05) from the empty vector control in the respective background strains.

Despite the fact that the there was no significant difference in glycogen content between the wild type and mutant cells, the effect of plant and human genes involved in polyglucan metabolism was further evaluated in the two yeast background strains (WT and *yvh1p*$^{-}$). This indicated that the expression of GWD in yeast did not cause an increase in yeast glycogen content (Fig.3.1) as has been reported when it is expressed in *E. coli* (Viksø-Nielsen *et al.,* 2002). Also, the Lsf1 did not lead to a decrease in glycogen in yeast as observed in *E. coli* (Scheidig, 2006). While Lsf2 did show a significant increase in glycogen content, this was only evident in the WT background (Fig.3.1). Studies within the IPB have demonstrated that the expression of *AtSEX4* in *E.coli* leads to bacteria that are unable to accumulate glycogen (James Lloyd, *unpublished data*). Surprisingly, in yeast, expression of SEX4 led to more glycogen than the empty vector control of the corresponding background strains

although there was no difference between the amount of glycogen between the WT and yvh1p$^-$ mutant strains (Fig.3.1). This suggests that this gene does not influence glycogen in the same way as in *E. coli*. There was also no significant difference noted in glycogen accumulation between the empty vector control and the *Laf* construct (Fig.3.1).

There might be plausible explanation to explain the results observed. Despite the fact that an increase in glycogen has been previously observed in *yvh1p*$^-$ mutants (Beeser and Cooper, 2000), yeast cells increase their glycogen content as a carbon reserve during nutrient limitations (as previously discussed). Recent studies have shown that Yvh1 is an essential component for ribosomal biogenesis in yeasts (Lo *et al.,* 2009; Kemmler *et al.,* 2009), and that abolishment of the C-terminal (and not the N-terminal where the phosphatase domain is situated) is important in this regard (Lo *et al.,* 2009). It is therefore likely that increases in glycogen accumulation in *yvh1p*$^-$ mutants are independent from its phosphatase activity, and rather due to a restriction in cell growth due to an impaired ribosomal mechanism.

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Chapter 4: General conclusion

In this study we confirmed previous reports of covalently bound phosphate in *E. coli* glycogen (Lorberth *et al.,* 1998; Viksø-Nielsen *et al.,* 2002). This was done by digestion of glycogen in *E. coli* pellets and determination of the amount of glucose 6-phosphate in comparison with glucose in the different *E.coli* strains. The glucose 6-phosphate content ranged from 0.3-0.8 nmol.mg^{-1} glycogen, similar to those reported in previous studies. The exception to this was in a glycogen branching enzyme mutant strain (KV832) which accumulated up to 2.3 nmol glucose-6-phosphate.mg^{-1} glycogen. If this is true it would be the first report of linear polyglucans containing covalently bound phosphate as in starch it is only the branched amylopectin fraction that is phosphorylated (Posternak 1951; Hizukuri *et al*., 1970; Takeda and Hizukuri, 1982; Blennow *et al*., 2002).

We also tried to isolate the gene responsible for the incorporation of phosphate using an *E.coli* gDNA library in a functional expression system. This was due to an observation that a *glgb*$^{-}$ *E. coli* mutant accumulates more glucans when it expresses the plant GWD protein than when it doesn't. We therefore transformed the genomic library into the *glgb*$^{-}$ mutants cells, screened and isolated colonies that accumulated increased amounts of glucans. Sequencing of the inserts from the plasmids in the colonies identified several genes coding for putative phosphorylating proteins, including some obvious false positives. One gene, interestingly, encoded the MalP protein. This might be able to incorporate phosphate into glycogen utilising glucose-1,6-bisphospate as a substrate. Determination of glycogen phosphate in a *malp*$^{-}$ mutant, however indicated that there might be another protein working together with MalP. It could be, however, that the method which was used to measure the phosphate in *E. coli* still needs to be fully optimised. Because of the similarities between MalP and GlgP it would be interesting to investigate the phosphorylation of *E. coli* by the combination of the two genes coding for these protein by producing a *glgp*$^{-}$*/malp*$^{-}$ double mutant. We failed to produce such a mutant, but this will be repeated in the near future.

Lastly, the effect of plant and animal genes involved in polyglucan phosphate metabolism in yeast glycogen was also investigated. Preliminary results from this indicated that GWD, Lsf1, Lsf2 and Laforin have little or no effect on yeast glycogen

metabolism while there appears to be an increase in glycogen content in *yvh1p*$^-$ yeast mutant cells complemented with SEX4. While the reason behind this remains unclear, it provides an exciting topic for future research. If expression of SEX4 truly increases yeast glycogen accumulation it would imply that there is indeed phosphate present there. It would be of great interest, therefore, to determine if this is the case as a first step to try and explain the phenotype. After the confirmation of phosphate in yeast glycogen the next step would be to construct a yeast cDNA library in order to screen for the gene that is incorporating the phosphate. This could be done by transforming the library into the *glgb*$^-$ *E. coli* mutant and screen for accumulation of glycogen by exposing the plate to solid iodine as was described in Chapter 2.

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