Glycogen Metabolism in *Corynebacterium glutamicum* ATCC 13032

Joy A Kensley

Thesis presented for the Degree of

DOCTOR OF PHILOSOPHY

In the Department of Molecular and Cell Biology

UNIVERSITY OF CAPE TOWN

October 2004

Acknowledgements

I would like to express my appreciation to my supervisor, Assoc. Prof. S. J. Reid, and the Equity Development Programme, PhD Scholarship. Much gratitide also goes to Herr Prof. Dr. B. J. Eikmanns for guidance and encouragement. Many thanks to my co-workers, in Cape Town and Ulm, for a good working atmosphere. Thank you to Stefan Dempf for assistance in some cloning and growth experiments. A special thanks to Ana for her friendship and Mark for his devotion to me. I especially wish to acknowledge my parents for their moral and spiritual support and continuous encouragement. This work is a testimony to their unwavering faith in God and me.

GENERAL CONTENTS

ABSTRACT

Corynebacterium glutamicum is a Gram-positive facultative aerobe particularly known for its industrial application in the synthesis of amino acids, such as L-glutamate and L-lysine. The central metabolic pathways of this organism has been an area of much research by many groups. Linked to glycolysis is the synthesis of glycogen, previously considered a storage molecule of excess glucose. No information concerning the role of glycogen or its metabolism in *C. glutamicum* was known, and the aim of this work was to elucidate glycogen metabolism in this industrially important organism.

C. glutamicum was unable to utilise glycogen as a sole carbon source, but possessed enzymes capable of degrading the polysaccharide intracellularly. Glycogen was accumulated mainly during logarithmic growth in minimal media and during stationary phase in complex media, both containing glucose. Glycogen accumulation was not necessarily proportional to the concentration of glucose in the media, accumulating a maximum of 81.8 mg glucose/g DW in a 5% glucose minimal medium compared to 53.4 mg glucose/g DW in a 10% glucose minimal medium. In maltose grown cultures, glycogen accumulation was the highest compared to glucose with 107.8 mg glucose/g DW. Growth on organic acids and sugars such as fructose and sucrose did not support high levels of glycogen accumulation indicating that low level accumulation of glycolytic intermediates were unable to support glycogen production in *C. glutamicum*.

Four genes involved in glycogen metabolism were identified by computer analysis of the genome sequence of *C. glutamicum*. These were: ADPglucose pyrophosphorylase (*glg*C), glycogen synthase (*glg*A), glycogen branching enzyme (*glg*B) and a glycogen debranching enzyme (*glg*E). The transcriptional arrangement of these genes was found to be unique in comparison to those of other published glycogen operons. Northern analysis confirmed the monocistronic transcription of *glg*A (~ 1230 nt) and *glg*C (~ 1250 nt) from a divergent, possible overlapping promoter region, while RT-PCR also confirmed the *glg*B and *glg*E messenger as polycistronic (~ 4000 nt). The regions upstream of each gene was analysed by promoter fusion constructs on different carbon sources. No significant regulation was found with the presence of glucose in complex media, however

reporter activity was down regulated 2.5-3.5 times with growth on organic acids. Reporter activity in maltose grown cultures were in contrast upregulated 1.2-1.6 times which supported the higher glycogen levels measured during growth on this substrate in comparison to glucose. The function of the genes, *glg*C and *glg*B, was determined by inactivation by plasmid insertion resulting in no glycogen accumulation by the organism. Growth comparisons of the wild type and mutant strains indicated that glycogen was not essential for growth, but could support a higher cell density on entry into stationary phase.

ABBREVIATIONS

aa	amino acid(s)
ADP	adenosine 5'-diphosphate
ADPGlc PPase	ATP:α-D-glucose-1-phosphate adenyltransferase
AMP	adenosine 5'-monophosphate
ATP	adenosine 5'-triphosphate
ATCC	American Type Culture Collection
bp	base pair(s)
C-	carboxy-(terminal)
CAT	chloramphenicol acetyltransferase
Cm	chloramphenicol
CoA	coenzyme A
CRP	cAMP receptor protein
CsrA	carbon storage regulator
*csr*B	small RNA associated with CsrA
*csr*C	small RNA associated with CsrA
d	distance
DIG	digoxygenin
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
DTNB	5,5'-dithiobis-2-nitrobenzoate
DW	dry weight
ExPASy	**Ex**pert **P**rotein **A**nalysis **Sy**stem
ΔE	extinction difference
g	gram
GD	glucose-6-phosphate dehydrogenase
h	hour
HK	hexokinase
IPTG	isopropyl-β-D-thiogalactopyranoside

kb	kilobase pairs
Km^R	kanamycin resistant
LB	Luria-Bertani medium
mg	milligrams
mRNA	messenger RNA
MW	molecular weight
N-	amino-(terminal)
NADH	nicotinamide adenine dinucleotide (reduced)
NADPH	nicotinamide adenine dinucleotide phosphate (reduced)
NCBI	National Centre For Biotechnology Information
nm	nanometers
nt	nucleotide(s)
OD_x	optical density at x nm
ORF	open reading frame
oriT	origin of transfer
oriV	origin of replication
p	plasmid
P	phosphate
P_i	inorganic phosphate
PCR	polymerase chain reaction
pgm	phosphoglucomutase
ppGpp	guanosine 3'-bisphosphate 5'-bisphosphate
PTS	phosphotransferase system
RBS	ribosomal binding site
RNase	ribonuclease
rpm	revolutions per minute
RT	room temperature
RT-PCR	reverse transcriptase polymerase chain reaction
s	seconds
scrK	sucrose kinase
SDS	sodium dodecyl sulphate

SIB	Swiss Institute of Bioinformatics
sRNA	small untranslated RNAs
TE	Tris EDTA
*tre*S	trehalose synthase
Tris	Tris(hydroxymethyl)aminomethane
V	volume
W	Watts
2xYT	yeast tryptone broth (two times)
α	alpha
β	beta
ε	molar extinctions coefficient (l x $mmol^{-1}$ cm^{-1})
λ	lambda
μ	micro

Amino Acids – Names and Abbreviations

Amino acid	Three –Letter Abbreviation	One-Letter Abbreviation
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamic acid	Glu	E
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

CHAPTER 1

GENERAL INTRODUCTION

CONTENTS

1.1 Introduction to *Corynebacterium glutamicum*

Physiological characteristics of *Corynebacterium* include that they are Gram-positive, facultatively anaerobic or aerobic, are unable to produce spores, are non-motile, and in the case of *C. glutamicum* are biotin auxotrophs (Collins and Cummins, 1986; Liebl, 2001). Generally the cells are rod-shaped but appearing club shaped at times, therefore the term "coryneform" was coined for this group of bacteria due its seemingly irregular morphology (Collins and Cummins, 1986). More than 50 species of *Corynebacterium* have been isolated and described and more than half of them in the 1990's (Liebl, 2001). The order, *Corynebacterium* includes both medically and industrially important species such as *C. diptheria* and *C. glutamicum*, respectively. Generally the genus is phenotypically very diverse and have been isolated from sources as different as soil, dairy products, animal skin, faeces and plant material (Liebl, 2001).

The organisation of *Corynebacterium* as a genus was first established by Lehmann and Neumann in 1896. Since then a more chemotaxonomic and molecular approach has been adopted to complement the morphological taxonomic classification of bacteria (Goodfellow and Minnikin 1981). Recent classification of bacteria using 16S rRNA/rDNA sequence data, organises the genus *Corynebacterium* under the class of Actinobacteria and within the family Corynebacteriaceae (Stackebrandt *et al.*, 1997). Another critical chemotaxonomic feature of *Corynebacterium* is the presence of mycolic acids (2-alkyl-branched-3-hydroxy-acids) in the cell wall (Collins *et al.*, 1982). Except for *C. amycolatum,* all Corynebacteria have the feature of mycolic acids directly crosslinking the peptidoglycan layer (Liebl, 2001). The cell wall of *C. glutamicum* is discussed in more detail relevant to the topic of this thesis in Section 1.9. Members of *Corynebacterium* have a typical G+C content of between 51-68% (Goodfellow and Minnikin, 1981) and with the recent sequencing of the *C. glutamicum* genome, the G+C content was found to be 53.8% (Kalinowski *et al.*, 2003).

The soil bacterium *C. glutamicum* was found to be a very efficient producer of L-glutamate by Kinoshito and co-workers in the 1950's when they were screening for organisms able to produce glutamate (Kinoshito *et al.,* 1957). The demand for L-

glutamate as a flavour enhancer was becoming increasingly high, and monosodium glutamate (MSG) was being extracted from plant species by chemical processes at that time (Hermann, 2003). With the discovery of a bacterium able to produce large quantities of amino acids, the fermentative production of amino acids boomed.

1.2 Amino acid production by coryneform bacteria

The industrial application of coryneform bacteria in amino acid and vitamin production has become very important where L-glutamate and L-lysine contribute the most in terms of tonnage and economical value. Other amino acids such as L-valine, L-isoleucine, L-threonine, L-aspartate, and L-alanine are also produced by *Corynebacteria* (Hermann, 2003; Liebl, 2001). Coryneform bacteria are responsible for the production of around 1,5 million tons of L-glutamic acid per year, while the world market for L-lysine was 550 000 tons in 2001 with a growth rate of 7% per annum (Hermann, 2003; Bott and Niebisch, 2003).

C. glutamicum was able to export glutamate without the necessity of creating mutant strains in order to excrete the amino acid. However, certain growth conditions such as biotin limitation were necessary (Clement and Lanéelle, 1986). It was first surmised that the membrane became leaky due to biotin limitation (Kimura, 1963; Shiio *et al.,* 1963) or, that glutamate excretion occurred via the reverse action of the glutamate uptake system (Clement and Lanéelle, 1986). Instead, the primary glutamate uptake system was found not to be reversible (Krämer, 1994a; Kronemeyer *et al.*, 1995), and neither was the membrane leaky (Hoischen and Krämer, 1990; Krämer, 1994a; Krämer, 1994b). Rather, an efficient energy-dependent efflux system for glutamate, against an existing chemical gradient played a major role in overproduction (Gutman *et al.*, 1992). The role of biotin limitation was still important and it was thought the condition altered the lipid content and composition of the cell membrane (Takinami *et al.*, 1968; Hoischen and Krämer, 1990). In industrial applications, *C. glutamicum* was not usually grown under biotin limitation since these strains are biotin auxotrophs. Therefore, other methods of compromising the cell wall were used, for example the addition of penicillin or Tween 60 during the logarithmic growth phase (Liebl, 2001; Eggeling *et al.*, 2001). The exact

mechanism of glutamate efflux is still not known, though altering the membrane fluidity did enhance glutamate efflux, and more recently, a mutation in oxoglutarate dehydrogenase led to high levels of glutamate excretion without biotin limitation (Kawahara *et al.*, 1997).

L-Lysine, although produced in lower quantities than L-glutamate, is considered the more economically important amino acid (Leuchtenberger, 1996). Lysine is an essential amino acid used to supplement animal feed which is largely deficient in this amino acid. Farmers prefer to use feed low in protein in order to reduce the amount of nitrogen excreted, therefore it would be more economical to add the amino acids specifically required by the animal (Hermann, 2003). L-Lysine forms part of the aspartate family of amino acids and is produced via a split pathway of DL-diaminopimelate, the precursor of L-lysine and cell wall peptidoglycan synthesis (Liebl, 2001). In the past the common practice of developing overproducing strains was by mutation and selection (Malumbres and Martin, 1996) but the precise physiological changes leading to increased overproduction were unknown (Kalinowski *et al.*, 2003). With the development of genetic tools (Katsumata *et al.*, 1984; Ozaki *et al.*, 1984; Santamaría *et al.*, 1984) for *C. glutamicum* and metabolic engineering by recombinant DNA technology (Bailey, 1991), various overproducing strains have been constructed. In contrast to glutamate overproduction which requires only adjusting culturing parameters, lysine overproduction needed to be induced by genetic modification of *C. glutamicum* (Liebl, 2001; Cremer *et al.*, 1988). One of the reasons it was quite easy to manipulate L-lysine overproduction, is the lack of regulation of the synthesis of lysine-specific enzymes in *C. glutamicum* (Yeh *et al.*, 1988). This is unlike the situation in *E. coli* where L-lysine production is very strictly regulated (de Graaf *et al.*, 2001). The export of L-lysine in *C. glutamicum* occurs actively via a specific lysine carrier which was found to be necessary to prevent the accumulation of intracellular lysine when grown on lysine-containing peptides (Vrljic *et al.*, 1996). Although the export of lysine is important (Bellman *et al.*, 2001), the relative ease with which the pathway toward L-lysine can be manipulated for example by deletion of homoserine dehydrogenase, or deregulation of aspartate kinase, has led to the

construction of very useful overproducing lysine strains (Sahm *et al.*, 2000; de Graaf *et al.*, 2001).

C. glutamicum hyperproducers of other amino acids have also been constructed for example for threonine, isoleucine, tyrosine, phenylalanine, or tryptophan (Liebl, 2001). These have been accomplished by both classical rounds of mutagenesis and screening as well as more specifically targeted applications of recombinant DNA technology. Manipulation of pathways via deregulation, overexpression or deletion have been very fruitful. With the availability of new techniques such as in-vivo-NMR or MALDI-TOF mass spectrometry, intracellular flux distributions can be studied and mathematical models developed (Vallino and Stephanopolous, 1993; Marx *et al.*, 1996; Park *et al.*, 1997; Wittmann and Heinzle, 2001). The DNA sequence of *C. glutamicum* further provides information concerning its central metabolism for example DNA chip technology (Loos *et al.*, 2001; Wendisch, 2003) or proteome analysis. A combination of these modern analytical approaches could lead to the design and development of further improved strains.

1.3 The central metabolism of *C. glutamicum*

The central metabolism of *C. glutamicum* has been, and still is an important area studied within this bacterium. Various groups have contributed to the current knowledge we have concerning the central metabolism of *C. glutamicum*, and a schematic of the main central pathway is dipicted in **figure 1.1**. There are two pathways via which the breakdown of six and five carbon sugars are oxidised in amino acid producing corynebacteria. These are, glycolysis and the pentose phosphate pathway. Building blocks for the synthesis of cellular components and energy is supplied via the breakdown of carbohydrates through these pathways, with the formation of phosphoenolpyruvate (PEP), pyruvate and acetyl-CoA (Gottschalk, 1986) . Further oxidation of these products occurs through the tricarboxylic acid (TCA) cycle generating ATP and NADPH. The TCA cycle has an additional function in anabolism, where intermediates of the cycle provide carbon precursors for biosynthetic processes. During growth, and particularly amino acid production, these precursors are removed from the cycle and anaplerotic

reactions come into play which are able to replenish the lost metabolites by generating oxaloacetate (Gerstmeir *et al.*, 2003). The production of aspartate family type amino acids in particular, leads to the depletion of oxaloacetate and is replenished by the carboxylation of either PEP or pyruvate. In *C. glutamicum* both carboxylation processes, via PEP carboxylase (PEPCx) and pyruvate carboxylase (PCx), are possible but it is PCx that is mainly responsible for anaplerosis during growth on glucose (Peters-Wendisch *et al.*, 1993, 1997, 1998). In fact, PCx was recently identified as the major bottle neck in glutamate and lysine production by *C. glutamicum* (Peters-Wendisch *et al.,* 2001) and PEPCx was previously found to be dispensible for growth and lysine production (Peters-Wendisch *et al.*, 1993).

Growth of *C. glutamicum* on certain substrates such as acetate stimulate gluconeogeneic reactions. PEP is an important precursor for gluconeogenesis and is provided mainly by the enzyme, PEP carboxykinase (PEPCk) (Riedel *et al.*, 2001). Two other enzymes might have a contribution toward gluconeogenesis in *C. glutamicum*, but their roles have not yet been clarified. Malic enzyme has been suggested as a decarboxylating enzyme converting malate to pyruvate (Cocaign-Bousquet and Lindley, 1995). The possible function of this reaction is to provide NADPH during growth on substrates where there is little or no flux through the penthose phosphate pathway (Gourdon *et al.*, 2000). *C. glutamicum* also does not possess transhydrogenases able to synthesise NADPH from NADH and the cycling of malate via pyruvate and oxaloacetate, although ATP-dependent, might supply NADPH without the loss of carbon (Cocaign-Bousquet and Lindley, 1995). The second unclarified decarboxylating enzyme found in *C. glutamicum* is oxaloacetate decarboxylase. Activity for this enzyme has been identified *in vitro* (Jetten and Sinskey, 1995) but no gene has yet been identified and no carbon flux has been shown to be directed from oxaloacetate to pyruvate *in vivo* (Petersen *et al.*, 2000). The generation of pyruvate by these two possible reactions would require the presence of a functional PEP synthetase but this was considered unlikely since a PCx mutant was unable to grow on gluconeogenic substrates (Peters-Wendisch *et al.*, 1998).

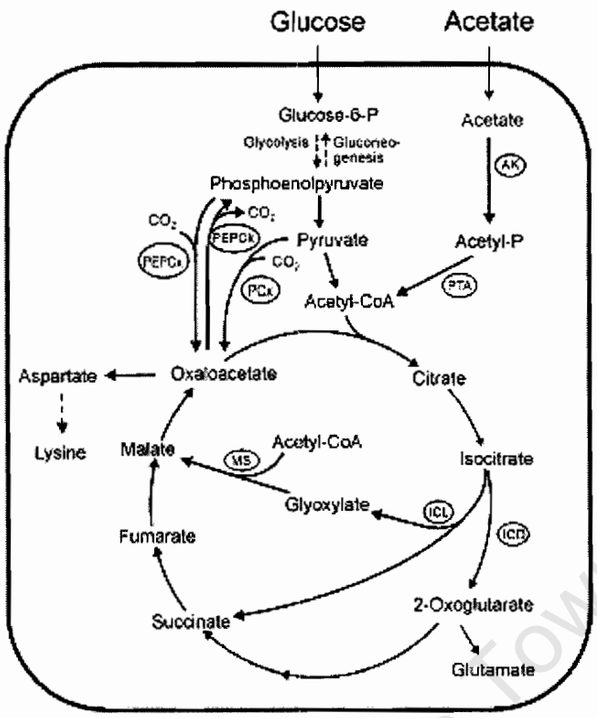

Figure 1.1 The central metabolism of *C. glutamicum* during growth on glucose and acetate. Dotted arrows represent pathways consisting of several reactions, uninterrupted arrows represent single reactions. AK, acetate kinase; PTA, phosphotransacetylase; ICD, isocitrate dehydrogenase; ICL, isocitrate lyase; MS, malate synthase; PEPCk, phosphoenolpyruvate carboxykinase; PCx, pyruvate carboxylase; PEPCx, phosphoenolpyruvate (Diagramme taken from Gerstmeir *et al.*, 2003)

Different carbon sources enter the central metabolism of *C. glutamicum* at different stages, and the flux distributions and amounts of energy derived are different for these carbon sources. During exponential growth on glucose it is believed that more than 50% of the carbon flux is distributed through the penthose phosphate pathway, in order to provide the reducing agent, NADPH (Cocaign-Bousquet *et al.*, 1996; Vallino and

Stephanopoulos, 1993; Dominguez *et al.*, 1998). Whereas growth on fructose leads to a major flux distribution of 80% toward glycolysis (Dominguez *et al.*, 1998; Shiio *et al.*, 1990), and NADPH is provided by pathways other than the pentose phosphate pathway. These have been estimated to include increased activity of the TCA cycle, but more importantly through the decarboxylation of malate, by malic enzyme, and the replenishing of oxaloacetate in the TCA cycle through the carboxylation of pyruvate by pyruvate carboxylase (PCx) (Dominguez *et al.*, 1998) as mentioned earlier. Acetyl-CoA enters the TCA cycle by its incorporation together with oxaloacetate into citrate. With growth on acetate, gluconeogenesis is required to supply the cells with 3-phosphoglycerate and with hexose and pentose sugars (Gerstmeir *et al.*, 2003). Another cycle called the glyoxylate cycle functions to provide C4 and C3 anabolic intermediates when cells grow on acetate as the sole carbon source. This anaplerotic pathway bypasses some steps of the TCA cycle and via isocitrate lyase (ICL) and malate synthase (MS) generates malate directly (Kornberg, 1966a,b) (**Fig. 1.1**). *C. glutamicum*, unlike *E. coli*, when grown on mixed substrates such as sugars, for example glucose, and organic acids such as lactate, and acetate shows no diauxic growth and is able to co-metabolise these substrates (Wendisch *et al.*, 2000). Thus so far there has been no concrete evidence for carbon catabolite repression in *C. glutamicum* (Gerstmeir *et al.*, 2003).

It had also been suggested that the uptake of carbon sources might be key rate-limiting reactions in growth and amino acid production (Linton 1990), and the uptake of different carbon sources in *C. glutamicum* has been investigated to some extent. The uptake of glucose is via two major transport systems (Gourdon *et al.*, 2003). One is via the mannose phosphotransferase system (PTS^{Man}) which has a high affinity for glucose (Kotrba *et al.*, 2001), and the other is a suggested permease type transporter (Dominguez and Lindley, 1996; Park *et al.*, 2000; Cocaign-Bousquet *et al.*, 1996). Fructose is transported into the cell through the fructose PTS (PTS^{Fru}) which also has a low affinity for glucose (Mori and Shiio, 1987). Sucrose uptake is also via a specific PTS and requires phosphorylation of the liberated fructose by fructose phosphotransferase, generating glucose and fructose-1-phosphate (Dominguez and Lindley, 1996). The uptake of maltose has been suggested to be via an ABC transport protein (Krings, 2003)

with possible intracellular cleavage by maltase similar to the related *Brevibacterium flavum* (Dominguez and Lindley 1996). This organism is included as part of the *C. glutamicum* species (Liebl, 2001). After the internalisation of acetate, it is converted to acetyl-CoA via the enzymes acetate kinase (AK) and phosphotransacetylase (PTA) (**Fig. 1.1**). Thus far an acetate uptake carrier has not been identified in the genome of *C. glutamicum* (Gerstmeir *et al.*, 2003). *C. glutamicum* can grow on DL-lactate as a sole carbon source using the enzymes D-and L-lactate dehydrogenases (Bott and Niebisch, 2003). So far, D-lactate dehydrogenase has been purified from a *C. glutamicum* strain and a possible gene identified in the genome sequence, however there is only a possible gene identification of a L-lactate dehydrogenase (Bott and Niebisch, 2003). Also there is no information on the uptake of DL-lactate in *C. glutamicum* although it is a substrate that promotes gluconeogenesis in the cell.

In general when glucose enters a bacterial cell and is phosphorylated, it is incorporated into glycolysis through the conversion of glucose-6-phosphate to fructose-1,6-bisphosphate by isomerisation and phosphorylation. The phosphorylation step is the commited step toward glycolysis with the final products PEP and pyruvate entering the TCA cycle for further oxidation. However, when glucose is in excess, glucose-6-phosphate can also be converted by the enzyme phosphoglucomutase to glucose-1-phosphate. Glucose-1-phosphate together with ATP are the building blocks for generating the storage compound glycogen.

1.4 The polysaccharide glycogen

Carbon is accumulated in many organisms in the form of glycogen as an energy reserve, and is utilised during temporary starvation conditions arising in the environment. Necleoside diphosphates were first discovered by Leloir and co-workers in the 1950s and they established the biosynthesis of glycogen via an activated form of glucose, either UDPGlc in mammals, fungi, and eukaryotic heterotrophic microorganisms or ADPGlc in bacteria and photosynthetic eukaryotes (Leloir *et al*, 1971). The glycogen molecule consists of 90-95% $\alpha(1\rightarrow4)$ linear glycosidic bonds and 5-10% $\alpha(1\rightarrow6)$ glycosidic bonded branch points every 8-12 linear glucose molecules, see **figure 1.2** (Preiss *et al.*,

1983; Voet and Voet, 1995). The one reduced molecule at the end of different branches can be cleaved to release glucose and subsequently made available for glycolysis. This can be very efficient for the rapid release of glucose (Voet and Voet 1995). With the many α-1,4- polyglucans in this high molecular weight, non-reducing, polysaccharide it has the advantage to minimally effect the internal osmotic pressure of the cell (Ballicora *et al.*, 2003). Glycogen can constitute 70% or more of the dry mass of a bacterial cell, and is stored in bacteria as either intracellular organic inclusion bodies or as dispersed cytoplasmic small granules (Matheron *et al.*, 1998). The function of glycogen in bacterial cells was largely thought to be a storage molecule able to prolong cell survival under starvation conditions (Preiss, 1984).

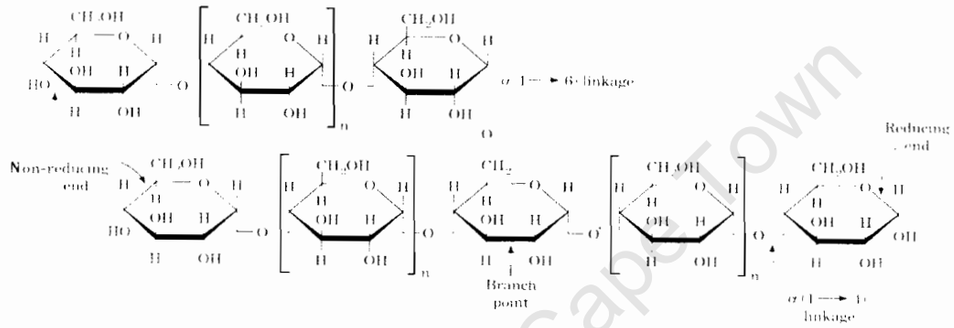

Figure 1.2. Glycogen chemical structure. The molecule has one reduced end at the end of each branch at which the addition or removal of one glucose molecule can occur. The red line indicates a branch point. (Adapted from Voet and Voet., 1995).

1.5 The role of glycogen in bacterial cells

The accumulation of glycogen in bacterial cells generally occur during stress conditions such as limiting nitrogen, phosphate or sulphur but with an excess of carbon available for growth, as was found in *E. coli* (Holme, 1957). In *E. coli* and *Aerobacter aerogenes*, it was shown that cells containing intracellular glycogen, but having no external carbon source in the media, were able to survive longer than cells that did not contain intracellular glycogen (Strange, 1968; Strange *et al*, 1961). This increased survival of

wild type strains compared to mutants in glycogen synthesis implied a function for glycogen as a storage compound. However, this role of the polysaccharide was not altogether clear since the organism, *Sarcina lutea,* showed no cell survival advantage under limited growth whether the cell accumulated intracellular glycogen or not (Burleigh and Dawes, 1967). Other proposed functions for glycogen have been suggested in sporulation (Rueda *et al.*, 2001; Kiel *et al.*, 1994), cell wall formation (Belanger and Hatfall., 1999) and maintaining turgour pressure (Bruton *et al.*, 1995). Glycogen has also been responsible for the development of caries in an overproducing mutant of *Streptococcus mutans* (Spatafora *et al.*, 1995). In *Mycobacterium smegmatis,* the accumulation and degradation of glycogen has also been proved to be essential for normal exponential growth in the organism (Belanger and Hatfull, 1999). Since the role of glycogen in bacteria is not clear a few examples of glycogen accumulation in different organisms are discussed in more detail.

1.5.1 Futile cycling and the carbon capacitor concept of glycogen in bacteria

Futile cycling of metabolites brings about the hydrolysis of ATP as a net reaction, and therefore would only preferably occur when ATP is in excess in the cell. Futile cycling of glycogen was identified in *Fibrobactor succinogenes*, where ^{1}H-NMR and ^{13}C-NMR spectra indicated the degradation and storage of glycogen at the same time (Gaudet *et al.*, 1992). This was confirmed later by Matheron *et al.*, (1998) where a further four strains of *Fibrobacte*r were shown to cycle glycogen. Glycogen normally accounts for approximately 70% of the dry mass of *F. succinogenes* and was found to be stored during all phases of growth, while even resting cells were able to accumulate and degrade glycogen (Gaudet *et al.*, 1992). By tracking labelled carbon and hydrogen it was found that between 12 and 16% of carbon entering glycolysis originated from prestored glycogen. (Matheron *et al.*, 1998). *M. smegmatis* was also shown to recycle glycogen during growth as mentioned earlier and these authors were the first to coin the phrase "carbon capacitor" as an explanation for the accumulation and breakdown of glycogen during normal growth of the cell (Belanger and Hatfull, 1999). Glycogen was proposed to serve as a temporary reservoir for glycolysis argueing the sequestering of free glucose into glycogen and the release of glucose only when it was required by the cell. Therefore

as the need for glycolytic breakdown of glucose fluctuated, so the rates of glycogen synthesis and degradation would adjust accordingly (Belanger and Hatfull, 1999). The above theory is oversimplified, but it was a reasonable attempt to explain the findings regarding cyclic glycogen metablism in *M. smegmatis*. Another example of glycogen accumulation during exponential growth and even under nutrient limitation was found in *Clostridium cellulolyticum* (Guedon *et al.*, 2000). This was an argument against futile cycling purely to consume excess ATP, but rather the controlled regulation of glycogen accumulation other than during limited growth conditions. Another important point demonstrated by this particular study was the control of the levels of glucose-6-phosphate and glucose-1-phosphate. In *C. cellulolyticum* a high carbon flux through glycolysis caused a decrease of the glucose-6-phosphate pool and an increase in the glucose-1-phosphate pool (Guedon *et al.*, 2000) indicating this branch point was able to control excess carbon and dissipate energy.

1.5.2 Accumulation of glycogen associated with sporulation, differentiation and virulence.

In the actinomycete, *Streptomyces coelicolor*, genes involved in glycogen metabolism have been identified and were found to be duplicated (Bruton *et al.*, 1995; Martin *et al.*, 1997). *S. coelicolor* undergoes complex morphological differentiation through the germination of spores to forming complex mycelium (Chater and Losick 1997). Glycogen accumulates differently during these developmental phases and different operons were found to be active at a different stage of development (Schneider *et al.*, 2000). This implied a role for glycogen during different developmental stages in *S. coelicolor* (Martin *et al.*, 1997; Schneider *et al.*, 2000). A proposed link between glycogen and trehalose has also been suggested because of the arrangement of *tre*S (gene converting maltose to trehalose) in the same operon as glycogen associated genes (Schneider *et al.*, 2000). Glycogen has also been associated with sporulation in *S. brasiliensis* where glycogen was accumulated in sporeforming hyphae and a physiological link with trehalose was also confirmed with the formation of trehalose from glycogen in a sporulation specific event (Rueda *et al.*, 2001). In *Bacillus,* glycogen was also been shown to provide energy for sporulation (Slock and Stahly, 1974). The operon

involved was isolated from *B. subtilis*, characterised and glycogen was found to be accumulated only on media that allowed efficient sporulation (Kiel *et al.*, 1994). As early as 1976, glycogen like molecules had been implicated as a significant factor in creating caries by *Streptococcus mutans* (Tanzer *et al.*, 1976). Later, a glycogen synthesis deficient mutant generated by mutating a regulator involved in accumulation, was found to be less cariogenic (Spatafora Harris *et al.*, 1992) confirming the importance of glycogen in bacterial virulance. Glycogen associated virulence was also indicated in *Salmonella entiridis* where the highest amounts of glycogen were accumulated in the more virulent strains which also produced large amounts of biofilm (Bonafonte *et al.*, 2000). In the above cases, the accumulation of glycogen was beneficial for the respective bacteria under certain growth or developmental stages. Yet glycogen could also be non-beneficial in bacteria, which was found in the symbiotic relationship between *Rhizobium tropici* and tropical plants (Marroqui *et al.*, 2001). This bacterium induces nitrogen fixing nodules in tropical plants (Martínez *et al.*, 1985) and a mutation in glycogen synthase enhanced respiration and symbiotic nitrogen fixation in *Phaseolus vulgaris* plants (Marroqui *et al.*, 2001). This was at least one case where the lack of glycogen accumulation in a bacterium was beneficial for its survival.

The classic function of glycogen as a storage molecule during limited growth conditions, with an excess of carbon available (Preiss, 1984; Preiss 1996a) is clearly not the only function of glycogen in bacterial cells. The examples above indicate a possible, more intricate role of the polysaccharide in the metabolism of different bacteria. The genes and their chromosomal arrangement associated with glycogen have been studied in some bacteria and particular attention has also been paid to the regulation of glycogen biosynthesis in *E. coli*. These will be discussed in the following pages.

1.6 The enzymatic reactions involved in glycogen metabolism

The metabolic processes involved in glycogen synthesis and degradation as well as the genes associated with these processes are discussed relative to bacteria. The reactions involved had been studied since 1964 (Preiss 1969) and an illustration of the general process is given in **figure 1.3**. The enzyme linking glycolysis to glycogen synthesis is

phosphoglucomutase (Lu and Kleckner, 1994) converting glucose between glucose-6-phosphate and glucose-1-phosphate. The first reaction in glycogen synthesis in bacteria is the priming of glucose to generate ADP-glucose, and this reaction is catalised by the enzyme ADPGlc pyrophosphorylase (ATP:α-D-glucose-1-phosphate adenyltransferase; EC 2.7.7.27; ADPGlc PPAse; GlgC) (Haugen *et al.*, 1976). The second reaction involves the transfer of the glucosyl unit to a glycogen primer by an ADPGlc-specific glycogen synthase (ADPglucose:1,4-α-D-glucan 4-α-glucosyltransferase; EC 2.4.1.21; GlgA) (Fox *et al.*, 1976). It was found recently that in *A. tumefaciens*, GlgA was not only transferred α(1,4) linked glucans, but also catalysed its own glucosylation (Ugalde *et al.*, 2003). The final reaction involved in synthesis, catalyses the formation of the branched α-1,6-glucosidic linkages in glycogen using a branching enzyme (α-1,4-glucan 6-glycosyltransferase; EC 2.4.1.18; GlgB) (Boyer and Preiss, 1977).

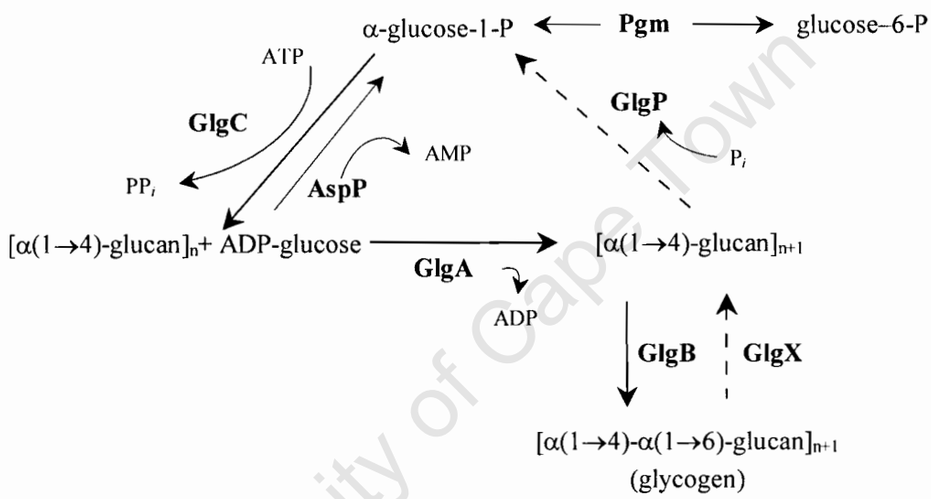

Figure 1.3 Representation of glycogen metabolism in bacteria. The reactions are described in the text. Abbreviations: PP_i : inorganic pyrophosphate; P_i : inorganic phosphate; P : phosphate. (The illustration was drawn according to the references in the text including Preiss and Romeo, 1989 and Preiss, 1996a).

The glycogen molecule does not have many non-reduced ends and the breakdown of the molecule can occur at the few reduced ends with a glycogen specific phosphorylase (EC 2.4.1.1; GlgP) (Choi *et al.*, 1989; Yu *et al.*, 1988). This reaction releases α-glucose-1-phosphate and can only occur at greater than 5 or 6 residues from a reduced end, therefore a debranching enzyme (EC 3.2.1.*;GlgX) is required to hydrolyse the α(1→6) branch points and reform α(1→4) glycosyl links (Preiss, 1984). The core enzymes involved in synthesis and degradation were described but there is another enzyme that plays a regulatory role in the first step of glycogen synthesis. Adenosine diphosphate sugar pyrophosphatase (EC 3.6.1.21; ASPPase; AspP) hydrolises ADP-glucose generating the precursor molecule α-glucose-1-P and therefore is antagonistic to ADPGlc PPase (Moreno-Bruna *et al.*, 2001).

1.7 Glycogen metabolism in different organisms

The fundamental principle involved in glycogen synthesis and degradation in bacteria was described above and is generally the same in different bacteria (Preiss and Romeo 1994). However, a comparison is drawn between *E. coli* and other organisms where genes or enzymes involved in glycogen metabolism have been studied. Differences occur in terms of the control of synthesis, degradation and the function of the polysaccharide in the various organisms. The arrangements of the genes in the chromosome of these bacteria are also quite different to each other. A physical map illustration of the *E. coli* chromosomal gene arrangement of glycogen associated genes is shown in **figure 1.4** together with the genetic arrangements of other organisms which are discussed. The function of the enzymes associated with the various genes were discussed in the previous section, therefore reference to specific functions of genes will only be mentioned of those not discussed above (refer to **Fig. 1.3**).

1.7.1 Glycogen gene arrangement in E. coli

The genes involved in the synthesis and breakdown of glycogen are arranged together on the chromosome of *E. coli* on an approximate 10 kb stretch of DNA (Preiss and Romeo, 1994) (**Fig. 1.4**). The structural genes *glg*C, *glg*A and *glg*B were initially cloned on a single fragment and the arrangements of the genes identified by deletion mapping

experiments (Okita *et al.*, 1981; Okita *et al.*, 1982). Later two other genes were also identified, one ORF lies between *glg*B and *glg*C, and was designated *glg*X (Yang *et al.*, 1996) while the other ORF, *glg*P, was found downstream of *glg*A (Romeo *et al.*, 1988; Yu *et al.*, 1988). The coding regions of *glg*A and *glg*C are seperated by 2 bp and g*lg*B and *glg*X ORFs overlap by 1 bp. The coding region of *glg*P is seperated from *glg*A by 18 bp. The non-coding region seperating *glg*BX and *glg*CAP is involved in transcriptional regulation (Preiss, 1996a). The genes flanking the glycogen operon of *E. coli* are *asd* (aspartate semialdehyde dehydrogenase) and *glp*D (glycerol phosphate dehydrogenase) and are not involved in glycogen synthesis (Preiss and Romeo, 1994). Two operons, *glg*BX and *glg*CAP, are transcribed from their own promoters and in the same direction (Preiss and Romeo, 1994). Both operons each encode genes for synthesis and degradative enzymes, implying that the two functions are coordinately expressed and the clustering of the genes suggests translational coupling within the two operons (Romeo *et al.*, 1988).

1.7.2 Agrobacterium tumefaciens

Information concerning the entire *glg* operon in *A. tumefaciens* was completed in 1998 by Ugalde *et al.*, (1998). The region of *glg*CA and *pgm* were identified earlier by Uttaro and Ugalde, (1994) and transcriptional analysis was completed for the entire region by primer extension, RT-PCR, mutational analysis and fusion constructs. In this organism glycogen genes are clustered on a 9.1 kb DNA fragment. All five genes identified are transcribed in a single operon and essentially the arrangement was the same as in *E. coli*. However, the structural genes encoding *glg*B and *glg*C were not seperated by an ORF encoding *glg*X, and *glg*X was also not found within this cluster. Downstream of *glg*A, the gene, *pgm* (enzyme linking glycogen to glycolysis), was also transcribed as part of two mRNA transcripts. It forms part of a mRNA transcribed from the start of *glg*P and is also transcribed as a 71 amino acid shorter product by its own promoter. The monocistronic messenger starts 168 bp upstream of an internal ATG and this shorter *pgm* was able to complement an inactivated *pgm* (Ugalde *et al.*, 1998).

1.7.3 Rhodobactor sphaeroides 2.4.1 and R. capsulatus

An approximate 6 kb DNA region was isolated from a gene bank of *R. sphearoides* using a PCR generated probe for *glg*C (Igarashi and Meyer, 2000). The region contained the full sequence of *glg*C followed by the full sequence of *glg*A. The fragment was flanked by a truncated *glg*X on the 3' end, downstream of *glg*A, and flanked on the 5'end by *glg*P. In between *glg*P and *glg*C an apparent deletion of a *glg*B gene was found, however the authors were able to measure branching activity in the cells and concluded that *R. sphaeroides* coded for another full length *glg*B elsewhere on the chromosome (Igarashi and Meyer, 2000). *R. sphaeroides* also has two chromosomes increasing the likelyhood of having a full length *glg*B.

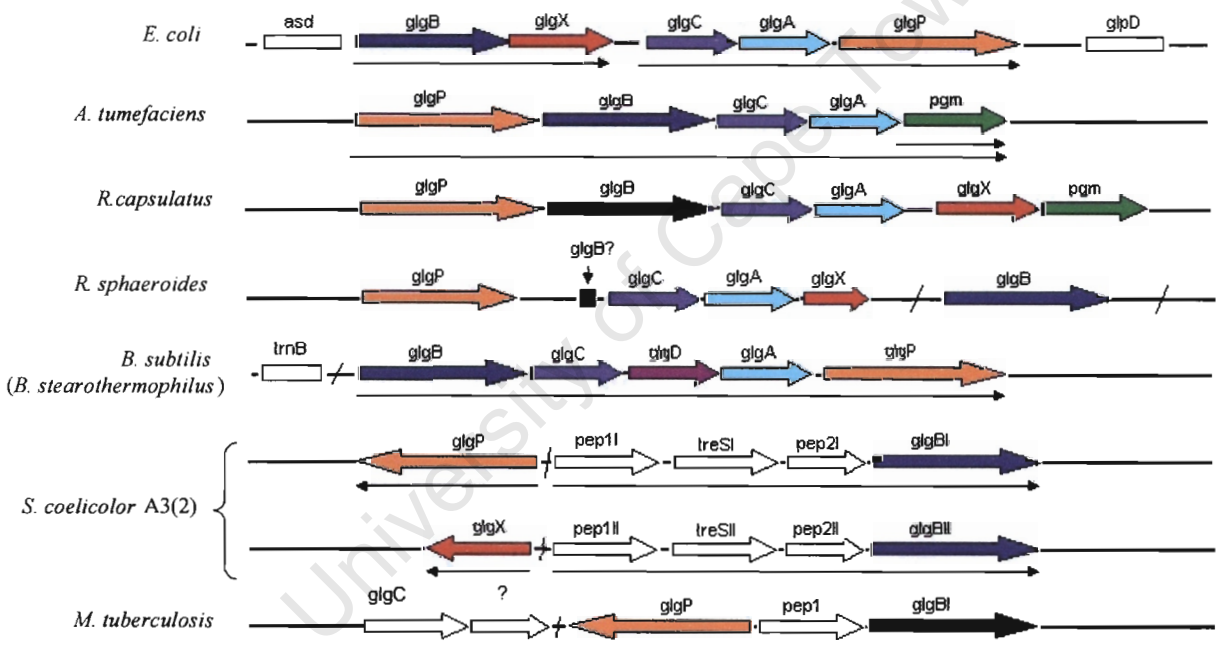

Figure 1.4. Comparison of the glycogen operons from different bacteria. Narrow black arrows indicate published mRNA transcripts. Organisms closely related to *C. glutamicum* are near the bottom. Diagramme was reconstructed from various sources discussed in the text. (Adapted from Halsey 2003)

In another Rhodobactor strain, *R. capsulatus*, the arrangment of the glycogen genes were similar, but encoded a full ORF for *glg*B between *glg*P and *glg*C (Haselkorn *et al.*, 1999). Also downstream of *glg*X was a *pgm* ORF similar to the situation in *A. tumefaciens* (Haselkorn *et al.*,1999) but it was not known if this gene was transcribed from its own promoter.

1.7.4 Bacillus subtilis and B. stearothermophilus

The DNA region required for glycogen biosynthesis in *B. subtilis* and in *B. stearothermophilus* was found to be the same and is depicted as one physical map (Kiel *et al.*, 1994; Takata *et al.*, 1997) in **figure 1.4**. The region includes five ORFs and is transcribed as a single mRNA. The important difference in this operon is the encoding of the structural gene, *glg*D, which lies between *glg*C and *glg*A. In both organisms this polypeptide has sequence similarity to *glg*C from other organisms and forms a unique heterotetrameric ADPGlc PPase (Takata *et al.*, 1997). Instead of allosteric regulation of homotetrameric ADPGlc PPase's as in other bacteria (discussed in Section 1.4.4.) it is implied that the GlgD subunit may control total ADPGlc PPase activity in *Bacillus* strains since the enzyme activity in this organism did not respond to usual allosteric regulators. An ORF for *glg*X was not found in this operon but it contains a gene encoding *glg*P downstream of *glg*A similar to *E. coli*.

1.7.5 Streptomyces coelicolor A3(2)

In *S. coelicolor* A3(2) it was found that this differentiating organism possesses two glycogen branching enzymes, which are required for the different phases of growth concerning forming aerial mycelia (phase I) and initiating sporulation by septation (phase II) (Bruton *et al.*, 1995). Subsequently, a gene encoding *glg*C responsible only for the formation of aerial mycelium was found in this organism, suggesting the probability of another *glg*C gene elsewhere on the chromosome (Cruz Martin *et al.*, 1997). In 2000, by sequencing and operon disruption, two duplicated clusters were found in the *S. coelicolor* A3(2) associated with either phase I or phase II of differential development (Schneider *et al.*, 2000). These operons were not very similar to the ones previously described, one important difference was the association of these operons with *tre*S (Schneider *et al.*,

2000). TreS is a gene product associated with the conversion of α-1,4 glucans (malto-oligosccharides or maltose) to α-1,1-glucans (trehalose). The ORF of *glg*B was duplicated and downstream of the phase I operon a structural gene of *glg*P was found. Downstream of the phase II operon a gene encoding *glg*X was found (see **Fig. 1.4**). The authors speculated as to the roles of the enyzmes encoded by the structural genes pep1 and pep2 suggesting both are associated with trehalose metabolism because of the association with *tre*S in the operon. No similarity had been found to Pep2 like proteins therefore its function was unknown. However, Pep1 was found to be a homologue of a glycanase enzyme designated GlgE in *M. smegmatis* (Belanger and Hatfull, 1999). This enzyme could be an intracellular glucanhydrolase or a maltosyltransferase converting glycogen into possible maltose units and could serve as a substrate for *tre*S. Insertional disruptions in *tre*SI and *tre*SII had a polar effect on the *glg*B genes suggesting the transcription of this operon as a single mRNA.

1.7.6 M. tuberculosis and M. smegmatis

The organisation of operons likely involved in glycogen metabolism of *M. tuberculosis* was drawn from the complete genome sequence (Cole *et al.*, 1998) and the revised annotation (Camus *et al.*, 2002). The structural genes of *glg*P and *glg*B should be transcribed divergently and are separated by a gene designated *pep*1. A gene designated *glg*E found in *M. smegmatis* was similar to the *pep*1 encoded in *S. coelicolor* as mentioned earlier (Belanger and Hatfull, 1999). These authors suggested that glycogen was recycled by GlgE (Pep1) during normal growth since a temperature sensitive mutant of *glg*E accumulated high concentrations of the polysaccharide. A different function was attributed to the PepI (GlgE) homologue in *S. coelicolor* where cycling of glycogen was thought to be with trehalose (Schneider *et al.*, 2000) and not linked directly to glycolysis as suggested in *M. smegmatis*. The gene homologue has also since been designated *pepI* in *M. tuberculosis*. The exact function of this enzyme in the two bacteria is not very clear. A gene encoding *glg*C was found associated with a ORF with homology to a putative glycosyl transferase but was not found clustered with *glg*P, *pep*1, and *glg*B.

From the diagramatic illustration in **figure 1.4**, the pattern of gene clustering was not identical in different bacteria, however, there is significant similarity between arrangements. In the cases of *E. coli*, *A. tumefaciens*, *B. subtilis* and *B. stearothermophilus,* transcription of the *glg* operons occur via one mRNA encoding both synthesis and degradative genes. Of interest would be the regulation of these genes. The clusters presented here are also associated with other genes in related pathways such as *pgm* in glycolysis and *tre*S in trehalose metabolism. The significance of the association of glycogen genes with those of trehalose is not clear although it has been alluded that the two carbohydrates interact within the cell or during different development stages (Rueda *et al.*, 2001; Zevenhuizen, 1992; Schneider *et al.*, 2000). Trehalose is a relatively inert molecule and a difussible carbon and like glycogen does not affect the osmostic pressure of the cell. It is thought glycogen and trehalose interconversion might participate in the spatial differentiation in *S. coelicolor* (Schneider *et al.*, 2000). In *S. brasiliensis,* it was also suggested from experimental data that the glycogen accumulated in hyphae was converted to trehalose during the final stages of sporulation (Rueda *et al.*, 2001). In some organisms the levels of trehalose remain constant while glycogen is accumulated and degraded (Zevenhuizen, 1992) while in others trehalose is cycled at the same time glycogen is accumulated and degraded (Noventa-Jordào *et al.*, 1996). A possible link also occurs between these carbohydrates in *C. glutamicum* in relation to the cell wall of the organism and is discussed in Section 1.9.

1.8 Regulation of glycogen metabolism in bacteria

Regulation of bacterial processes is through the control of the synthesis of enzymes or control of enzyme activity. The regulation of glycogen synthesis in bacteria has largely been studied in *E. coli* and control of this pathway in various degrees is by allosteric regulation of ADPGlc PPase (Ballicora *et al.*, 2003; Hengge-Aronis and Fischer 1992), phosphorylation of glycogen phosphorylase (Fletterick and Madsen, 1980) and transcriptional regulation of the structural genes (Preiss, 1996b; Preiss and Romeo, 1994).

1.8.1 Allosteric regulation of ADPGlc PPase

Glycogen synthesis in bacteria was found to be solely via the ADP-glucose pathway (Preiss, 1996b). The ADPGlc PPase from *E. coli* has been well characterised and excellent summaries are given in three reviews concerning glycogen biosynthesis in bacteria (Preiss and Romeo, 1994; Preiss, 1996a/b; Ballicora *et al.*, 2003.). *E. coli* and *S. typhimurium* mutants deficient in glycogen accumulation were found to have defective ADPGlc PPase and/or glycogen synthase activity (Krebs and Preiss, 1975; Preiss, 1984). Mutants of these organisms which were found to have glycogen in excess had overexpressing activities of ADPGlc PPase, glycogen synthase, and/or branching enzyme (Preiss 1983; Preiss, 1996a; Preiss and Romeo, 1994). Similarly those organisms accumulating less glycogen had lower enzyme activities of the enzymes mentioned. Many isolated ADPGlc PPases from plants and bacteria have also been subjected to site-directed mutagenesis of specific amino acids and many were found to be involved in allosteric regulation of the enzyme (Ballicora *et al.*, 2003). In most bacteria the enzyme is a homotetramer and is allosterically regulated by small effector molecules which form part of the major carbon assimilatory pathway of the organism (Ballicora *et al.*, 2003). The respective carbon utilisation pathways produce certain metabolites and these act as effector molecules in the regulation of ADPGlc PPase (Preiss, 1996b; Ballicora *et al.*, 2003). For the Embden-Meyerhof (glycolysis), Entner-Doudoroff pathways and the Krebbs cycle (TCA cycle), activators are fructose-1,6-bisphosphate, fructose-6-phosphate, and pyruvate while AMP, ADP and/or Pi are inhibitors (Ballicora *et al.*, 2003). ADPGlc PPase from various sources has since been grouped into nine classes (Ballicora *et al.*, 2003). These classes are largely classified according to the quaternary structure, respective activators and inhibitors, and the main carbon utilisation pathway employed. Glycolysis is the main carbon assimilation pathway in *E. coli* and the main activator of ADPGlc PPase is fructose 1,6-bisphosphate and its inhibitor, AMP. For some bacteria such as *B. stearothermophilus* and *B. subtilis,* ADPGlc PPase is not allosterically regulated, but rather the enzyme exists as a heterotetramer and is regulated by two GlgD polypeptides, which form a complex with two GlgC polypeptides (Takata *et al.*, 1997).

In addition to allosteric regulation of ADPGlc PPase, glycogen phosphorylase, *glg*P, is also similarly regulated, but the enzyme is also controlled by phosphorylation and dephosphorylation. The unphosphorylated (inactive) form of GlgP is phosphorylated (active form) via phosphorylase kinase with 2xATP's and the reverse reaction occurs via a phosphoprotein phosphatase and water (Fletterick and Madsen, 1980). Allosteric regulation of GlgP involves inhibition by ATP, glucose-6-phosphate and ADP-glucose and activation by AMP (Preiss and Romeo, 1994).

1.8.2 Transcriptional regulation of glycogen genes in E. coli

Regulation of the transcription of glycogen genes have been thoroughly studied only in *E. coli*. Glycogen is under the control of different regulation mechanisms in *E. coli,* but the exact mechanisms of the various components are not yet clear. However a picture can be drawn from what is known to date. For example, the committed enzyme of the pathway, ADPGlc PPase, is allosterically regulated by AMP and fructose-1,6-bisphosphate, which is likely the most important regulatory step in *E. coli* glycogen synthesis and was discussed above. The genetic expression of various glycogen genes are also positively and negatively regulated by various machanism.

1.8.2.1 Positive transcriptional regulation

The sigma factor associated with stationary phase growth, σ^S (*rpoS*), in *E. coli* has been identified as a positive regulator of glycogen synthesis. Together with cAMP, this sigma factor stimulates the synthesis of *glg*S, the protein product of which in turn is a positive regulator of glycogen synthesis (Hengge-Aronis and Fischer, 1992; Preiss and Romeo, 1994). The monocistronic gene, *glg*S, constitutes the third known glycogen operon in *E. coli*. GlgS stimulates glycogen synthesis, but its precise molecular function has not yet been established since it appears to have no direct effect on the *glg*CAP operon in *E. coli* (Hengge-Aronis and Fischer, 1992). In addition, a null mutant of *glg*S accumulated more glycogen than an *rpoS* mutant, suggesting the sigma factor had additional influence on glycogen synthesis (Hengge-Aronis and Fischer, 1992), although the mechanism has not yet been clarified (Preiss and Romeo, 1994). The expression of *glg*A and *glg*C was moderately enhanced by ppGpp which acted synergistically with cAMP (Romeo and

Preiss, 1989). Adenylate cyclase (*cya*) and CRP (cAMP receptor protein; *crp*) are also strong positive regulators of *glg*A and *glg*C glycogen synthesis (Urbanowski *et al.*, 1983). These components however, did not significantly effect the expression of *glg*B (Romeo *et al.*, 1990; Romeo and Preiss, 1989).

1.8.2.2 Negative transcriptional regulation of glycogen genes in E. coli

A novel regulator of carbon storage was identified which was able to exert a negative control on glycogen synthesis (Romeo and Gong, 1993; Romeo *et al.*, 1993) by destabilising mRNA (Baker *et al.*, 2002). The expression of *glg*B, *glg*CAP and *glg*S was shown to be post-transcriptionally regulated by this protein, called CsrA (Liu *et al.*, 1995; Yang *et al.*, 1996). This protein, although a negative regulator of glycogen and other genes expressed in stationary phase, can also stabilise mRNA expressed during exponential growth, for example transcripts involved in flagellum biosynthesis, motility and chemotaxis (Romeo, 1998; Gudapaty *et al.*, 2001; Wei *et al.*, 2001). In 1997, the CsrA protein was purified and found to be co-purified with a sRNA molecule designated *csr*B (Liu *et al.*, 1997). A second sRNA was recently isolated having the same function as *csr*B sRNA, designated *csr*C (Weilbacher *et al.*, 2003). Both sRNAs have the function of competing with cellular mRNA for binding to CsrA via the binding of the protein to imperfect repeat sequences. The sRNA *csr*B having 18 and *csr*C having 9 repeats (Liu *et al.*, 1997; Weilbacher *et al.*, 2003), act as antogonists to the function of CsrA. A model was proposed where sRNA controlled the activity of CsrA (Romeo,1998), but it was also found that CsrA could activate the expression of *csrB*, indicating that CsrA also regulated itself by regulating *csr*B. This activation of *csr*B transcription by CsrA was not expected to occur directly, since activation only occured *in vivo* and not *in vitro* implying CsrA either post-transcriptionally inhibited the expression of a transcriptional repressor or activated the expresssion of a transcriptional activator of *csr*B (Gudapaty *et al.*, 2001). The intermediate regulatory circuitry involved in the autoregulation of CsrA was recently reported to include the response regulator UvrY and the sensor kinase BarA (Suzuki *et al.*, 2002). This two component signal transduction system supported the activation of a transcriptional activator theory of the CsrA/CsrB and BarA/UvrY regulatory system (Suzuki *et al.*, 2002). The model of CsrA function has since been modified to incorporate

the latest findings and is presented in **figure 1.5**. The destablisation and degradation of RNA transcripts in the presence of CsrA was confirmed by Liu *et al.*, (1995), but it was also believed that in addition, CsrA might bind to the RBS and prevent translation (Romeo, 1998). This concept was further analysed on *glg*C mRNA, where they found that more than one molecule of CsrA could bind the mRNA, with binding of the protein to the leader sequence at the RBS and further upstream (Baker *et al.*, 2002). The sequence sites at which CsrA bound was similar to the repeated sequences found on the sRNAs *csr*B and *csr*C (Weilbacher *et al.*, 2003). More CsrA molecules were also predicted to bind other, already mRNA bound, CsrA molecules to stabilise the complex. This mechanism of preventing translation initiation was thought to be specific for *glg*CAP and *glg*B as previously it was established that the protein was not a general inhibitor of translation (Yang *et al.*, 1996; Liu and Romeo, 1997). However, just recently the translation of *cst*A, a gene induced by stationary phase sigma factors, was also found to be inhibited by the binding of CsrA to the RBS of its leader sequence (Dubey *et al.*, 2003). This confirmed the model presented by Baker *et al.*, (2002) and also included the existence of CsrA as a homodimer by cross-linking studies (Dubey *et al.*, 2003). It is also interesting to note that the debranching enzyme, *glg*X, is not regulated by CsrA while the branching enzyme, *glg*B, is (Yang *et al* ., 1996), confirming post transcriptional regulation of the *glg*BX operon in *E. coli.*

Another gene involved in the negative regulation of glycogen biosynthesis in *E. coli* is *glg*Q. Its gene product was able to decrease the expression of *glg*C, *glg*A and *glg*B and when it was mutated, glycogen was accumulated 5 to 10 times more than in the wild type controls (Romeo and Preiss, 1989; Dedhia *et al.*, 1994). GlgQ was not closely linked with the structural genes according to PI transduction whereas another negative regulator *glg*R was found closely linked to the structural genes (Romeo and Preiss, 1989). The gene product GlgR also decreased the synthesis of *glg*C and *glg*A, but had no affect on the synthesis of *glg*B in *E. coli* (Romeo and Preiss 1989). The last two mentioned regulators have to date not been well studied in *E. coli.*

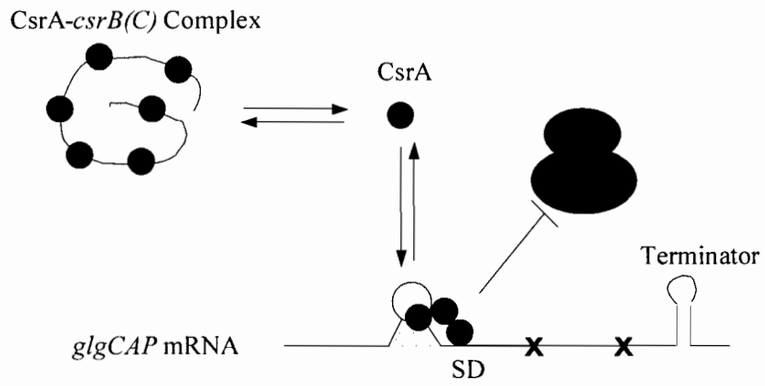

Figure 1.5 Model of CsrA-mediated regulation of *E. coli glg*C. sRNA, *csr*B forms a ribonucleoprotein complex with CsrA and acts as an antogonist of CsrA function. Translation of *glg*CAP is inhibited by the binding of CsrA to two positions in the leader sequence. The one site overlaps the Shine Delgarno (SD) sequence and the second binds within a short RNA hairpin. Binding in all cases is with ssRNA. Binding of CsrA to these sites facilitates the binding of more CsrA molecules generating protein-protein interaction and stabilising the complex. With the prevention of translation, endunucleolytic cleavage (X) results in rapid degradation of the message. (Illustration redrawn from Baker *et al.*, 2002).

An important concept to understand in the regulation of glycogen accumulation in *E. coli*, is that the genetic regulation is distinguished from the absolute level of glycogen. Glycogen genes are induced on entry into stationary phase, but if there is no excess carbon available glycogen is not accumulated. In contrast, glycogen is accumulated extensively when *E. coli* is grown on media containing excess glucose and nitrogen is limiting, even though there is low expression of glycogen genes under these conditions (Preiss and Romeo, 1994; Hengge-Aronis and Fischer, 1992). Allosteric regulation of ADPGlc PPase has been found to be the main means of regulating glycogen synthesis in *E. coli* (Ballicora *et al.*, 2003).

1.9 The cell wall of *C. glutamicum*

The cell wall of *C. glutamicum* is unusual for Gram-positive bacteria, and this separates it from other Gram-positives in that it contains a hydrophobic (lipid) bilayer (Minnikin & O'Donnell, 1984; Nikaido *et al.*, 1993). The organism shares this unusual cell envelope with a taxon of actinomycetes which include mycobacteriae, norcardiae, and rhodococci. In fact the function and structure of the Gram-positive corynebacteria cell wall resembles that of the Gram-negative cell envelope, which has an outer lipid bylayer composed of phospholipids and lypopolysaccharides (Liebl, 2001). *Corynebacterium* and their related organisms possess, in addition to the plasma membrane, a distinct outer barrier which forms an outer lipid layer, predominant constituents of which are the mycolic acid esters. This outer barrier lipid layer is composed of both covalently linked corynomycolates and non-covalently bound glycolipids (Puech *et al.*, 2001). The non-covalently bound glycolipids consist of trehalose dicorynemycolates and trehalose monocorynemycolates (Liebl, 2001). The mycolic acids play an important role in the cell wall permeability as mutants in mycoloyl transferases generated a decreased mycolate content and subsequent increased permeability (Jackson *et al.*, 1999; Puech *et al.*, 2000). Understanding the cell wall composition of *C. glutamicum* and its permeability aids in understanding the uptake of nutrients and also the excretion of certain molecules which is important for industrial strains. Trehalose is an important constituent of the non-covalently linked mycolates and a possible relationship between glycogen and trehalose in *C. glutamicum* is addressed in the next section.

1.9.1 The link between glycogen and trehalose in C. glutamicum

Tehalose forms part of two corynomycolic acid esters: trehalose monocorynomycolate (TMCM) and trehalose dicorynomycolate (TDCM). These two molecules have been shown to be the major free lipid (non-covalently bound lipid) fractions of the lipid bilayer in *C. glutamicum* (Puech *et al.*, 2000). The deletion of pathways involved in trehalose biosynthesis led to impaired growth of the cultures and altered cell wall lipid composition (Tzvetkov *et al.*, 2003). The two major pathways for trehalose synthesis is the OtsA-OtsB and the TreY-TreZ pathways utilising the substrates glucose-6-phosphate and UDP-glucose, and malto-oligosaccharides or α-1,4-glucan polysaccharide, respectively (Wolf

et al., 2003). A third pathway considered less significant in trehalose synthesis, converts maltose to trehalose via TreS (Wolf *et al.*, 2003). Glucose-6-phosphate could indirectly be one of the precursors for glycogen synthesis (**Fig. 1.3**), while glycogen also consists of α-1,4-glucan molecules (maltodextrins). Trehalose synthesis was thought to be linked to glycogen biosynthesis through the need of the TreYZ pathway for the substrate α-1,4-glucans (Tzvetkov *et al.*, 2003) (**Fig. 1.6**). The mutants, Δ*otsAB/glgA*::Km and Δ*otsAB/treS/glgA*::Km displayed the same growth conditions, and trehalose accumulation, as mutants in Δ*treZ*. Although the glycogen synthesis mutants may not affect cell wall composition of *C. glutamicum* excessively since the OtsA-OtsB pathway was energetically more favourable compared to the TreY-TreZ pathway. However, TreY-TreZ was able to support growth of *C. glutamicum* under high and low sugar conditions. Though the contribution of both pathways to trehalose biosynthesis still needs to be determined, a clear link between trehalose synthesis and glycogen was shown by a growth deficiency in mutants blocked in the OtsA-OtsB pathway and glycogen synthesis (Tzvetkov *et al.*, 2003).

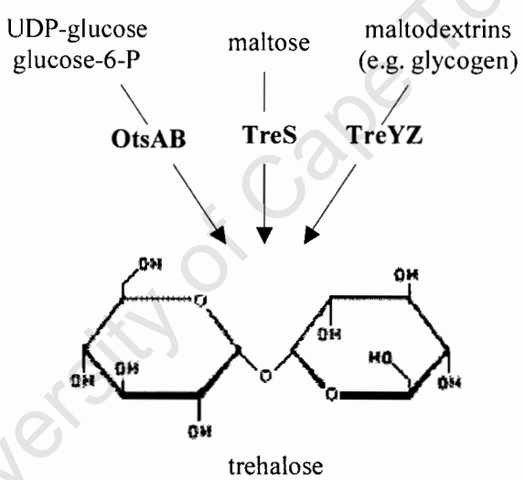

Figure 1.6 Known trehalose biosynthesis pathways in *C. glutamicum*.
Diagramme is redrawn from Wolf *et al.*, (2003).

Toward the end of this thesis, a part of the work presented in the this thesis was published by Tzvetkov *et al.*, (2003) where they identified the putative arrangement of *glg*A and *glg*C through scouring of the published genome and produced an integration mutant of *glg*A. This mutant was unable to accumulate glycogen in the presence of excess sucrose in the culture medium.

1.10 The aims of this study

The industrial significance of this organism is the reason that it has been so well studied (Hermann, 2003; Liebl, 2001) particularly with regard to amino acid production, precursor supply by central metabolic pathways and anaplerotic reactions, transport systems, and carbon and nitrogen fluxes (Bott and Niebisch, 2003). To this end, the genome of *Corynebacterium glutamicum* ATCC 13032 had been sequenced independently by various groups (Kalinowski *et al.*, 2003; Nakagawa, 2002). These have been made publicly available during the course of this work and can be accessed using the Genbank accession number NC_003450 (Nakagawa, 2002). In a review of the respiratory chain of *C. glutamicum*, Bott and Niebisch (2003) point to the importance of understanding the organism as a whole rather than simply parts thereof when considering constructing the 'optimal' production strain. Also, although the type strain *C. glutamicum* ATTC 13032 used throughout this study is non-pathogenic, information on *C. glutamicum* might serve as a model for pathogenic strains of *Corynebacteria* and the related *Mycobacteria.*

With this in mind, an introduction to glycogen metabolism in *C. glutamicum* ATCC 13032 was undertaken. The physiology of glycogen accumulation in *C. glutamicum* was studied under different culture conditions. The genome sequence was used to identify and isolate the genes involved in glycogen synthesis and degradation, and finally, regulation and genetic aspects were investigated to establish the importance and relevance of glycogen metabolism to this strain.

CHAPTER 2

PHYSIOLOGICAL STUDIES ON GLYCOGEN METABOLISM IN *C. glutamicum* ATCC 13032

CONTENTS

2.1 Summary

The breakdown and accumulation of the glycosidic polysaccharide, glycogen, was investigated in *Corynebacterium glutamicum* ATCC 13032. *C. glutamicum* was unable to grow on glycogen as a sole carbon source. It is very possible *C. glutamicum* does not have uptake mechanisms for the polysaccharide and neither is it likely to possess, and excrete, extracellular enzymes able to degrade glycogen. However, the intracellular extract was shown to possess enzymes able to degrade glycogen through the simple identification of a zone of clearance on a glycogen containing agar plate on which cell extract of *C. glutamicum* had been incubated. Some physiological aspects of glycogen accumulation in *C. glutamicum* were also investigated where the organism accumulated, up to 91 mg glucose/g DW of the polysaccharide during stationary phase on complex medium, with an excess of glucose. In contrast, maximum glycogen was detected during late logarithmic phase with growth of *C. glutamicum* on minimal medium supplemented with glucose. Interestingly a 5%, and not a 10%, glucose supplemented minimal media produced the most glycogen of 81,8 mg glucose/g DW, suggesting glycogen accumulation in *C. glutamicum* was not proportional to the concentration of glucose in the media. Maltose grown cells accumulated the highest levels of glycogen compared to glucose grown cells of 107.8 mg glucose/g DW. The sugars, fructose and sucrose did not support high levels of glycogen accumulation (< 40 mg glucose/g DW) and neither did growth on the organic acids, acetate and lactate, produce much glycogen (< 20 mg glucose/g DW). We suggest low level accumulation of glycolytic intermediates, under these conditions, were unable to stimulate glycogen production in *C. glutamicum*.

2.2 Introduction

The accumulation and break down of glycogen in *Corynebacterium glutamicum* has not been previously investigated, yet many organisms possess the enzymes required to accumulate and degrade this polysaccharide. Glycogen accumulates under stress conditions, for example when *E. coli* enters stationary phase growth due to reasons other than carbon limitation (Preiss 1984, Preiss and Romeo, 1994). It was not unreasonable to assume that the industrially important organism, *C. glutamicum*, also harboured enzymes involved in glycogen metabolism. In fact, the closely related *M. smegmatis* was previously shown to produce glycogen (Elbein and Mitchell, 1973).

C. glutamicum was grown on different concentrations of glucose and the intracellular glycogen content analysed to determine if the glucose concentration in the media was proportional to the amount of glycogen detected in the cells. The effect on glycogen accumulation when *C. glutamicum* was grown on different sugars as carbon sources were also investigated. *C. glutamicum* was also able to grow aerobically on the organic acids, acetate and lactate. Acetate would be incorporated into the citric acid cycle via acetyl-CoA (Gerstmeir *et al*., 2003) and lactate would be converted to pyruvate by quinone-dependent lactate dehydrogenases (Bott and Niebisch, 2003). Under these growth conditions, gluconeogenesis should be more active than glycolysis, resulting in the accumulation of fewer intermediates, which would usually stimulate ADPGlc PPase. There is evidence that in *E. coli,* a low concentration of glycolytic intermediates leads to the negative regulation of ADPGlc PPase (Preiss *et al*., 1983). If the same situation exists in *C. glutamicum,* one would expect less glycogen in cells grown on acetate or lactate. In the current chapter, a physiological investigation concerning glycogen accumulation in *C. glutamicum* was undertaken which might help build a platform for further genetic and regulatory characterisation.

2.3 Materials and Methods

2.3.1 Bacterial strains and culture conditions

Corynebacterium glutamicum was first isolated during screening for glutamate producers in 1957 according to Kinoshito *et al.* (1957). In this study *C. glutamicum* ATCC 13032 was routinely grown in complex medium, LB or 2xTY (Sambrook *et al.*, 2001), or modified CgC-minimal medium (CgC-MM; Appendix A; Kase *et al.*, 1972). All cultures were grown in fluted Erlenmyer flasks on a rotary shaker to facilitate aeration, at 120 rpm, and at a temperature of 30 ^{0}C. Carbon sources were filter sterilised and added to autoclaved media prior to inoculation.

2.3.2 Preparation of cell free extracts

2.3.2.1 Glycogen determination

For glycogen assays, cultures of *C. glutamicum* were grown on LB or CgC-MM broth (Appendix A) and 5 ml samples harvested during growth. Samples were washed once with 0.9% $NaCl_2$ and a second time with a resuspension buffer, 40 mM potassium acetate; pH 4.2. The cell pellet was reconstituted to a total volume of 1 ml with the resuspension buffer, and frozen at –20 ^{0}C till further analysis. The washing steps removed residual glucose (or other carbon sources) from the medium which might have interfered with the glycogen test described in section 2.3.4. The cells were disrupted in 2 ml screw-top eppendorf tubes filled with 250 mg acid washed glass beads (150-212 μm, Sigma-Aldrich Chemicals GmbH), using mechanical lysis of a Ryboliser (Hybaid RiboLyser Cell disruptor). The cell disruptor was used at a maximum speed of 6.5 for 45 s, and 3 times without cooling in between since glycogen rather than enzyme activity was to be measured. The cell debris and glass beads were cleared from the supernatant by centrifugation at 13,000 rpm, 20 min, and 4 ^{0}C.

2.3.2.2 Enzymatic analysis

For preparation of cell free extracts for enzymatic analysis, sampling was the same as above. Cells were disrupted in the appropriate reaction buffer at 4 ^{0}C in a similar

manner, with the addition of intermittent cooling between mechanical bursts of the Ryboliser.

2.3.3 Physiological glycogen plate assay

Cell free extracts of standardised protein concentration were placed on sterile nitrocellulose filters on CgC-MM plates containing 0.5 % glycogen. After incubation at 30 ^{0}C for 24 hours , the plates were stained with Lugol's iodine reagent (1.5 % KI; 0,15 % I). The $\alpha(1\rightarrow4)$ glucan chains in glycogen molecules form coils in which the iodine molecules fit and form glycogen iodine complexes. These complexes would have a blue-brown colour in comparison to the yellow-brown colour of the negative control.

2.3.4 Enzymatic glycogen assays

2.3.4.1 Degradation of glycogen to glucose

Cell free extracts for glycogen assays were obtained as described above in 2.3.2.1 and boiled for 5 min in order to inactivate glucose degrading enzymes. Each sample was divided into two 100 µl aliquots (reactions A and B), and 2 µl amyloglucosidase (10 mg/ml, Roche Diagnostics) was added to reaction A while reaction B remained as a reference. Both reactions A and B were incubated, with shaking, at 57 ^{0}C for 2 hours. With amyloglucosidase digestion, α-1,4- and α-1,6-glucosidic bonds were digested and glucose liberated. This enzyme was most active at 37 ^{0}C but also had the ability to release glucose from trehalose at 57 ^{0}C. Incubation of the sample with amyloglucosidase at 57 ^{0}C favoured the release of glucose from glycogen but prevented the breakdown of trehalose to glucose, as was shown by Parrou and Franscois, (1997). Since trehalose was not degraded to glucose, it would not interfere with the determination of glycogen through the measurement of glucose. Glucose concentration was then measured in both control (- amyloglucosidase) and experimental (+ amyloglucosidase) tubes, the control value was subtracted from the experimental value and the milligrams of glucose calculated, relative to the cell dry weight.

2.3.4.2 Enzymatic determination of glucose

After the digestion of glycogen by amyloglucosidase, the resulting glucose was measured enzymatically by the phosphorylation of glucose to glucose-6-phosphate by hexokinase and its subsequent oxidation with NADP by glucose 6-phosphate dehydrogenase. The following reactions being irreversible under these conditions:

$$\text{Glucose} + \text{ATP} \xrightarrow{\text{HK}} \text{Glucose-6-P} + \text{ADP}$$

$$\text{Glucose-6-P} + \text{NADP} \xrightarrow{\text{GD}} \text{6-phosphoglucan} + \text{NADPH}$$

HK: Hexokinase

GD: Glucose 6-phosphate dehydrogenase

The NADPH was measured spectrophometrically at room temperature at a wavelength of 340 nm and subsequently the amount of glucose could be calculated based on the extinction coefficient for NADPH as well as the molecular weight of glucose. The intracellular glucose resulting from the degradation of glycogen was determined by subtracting sample B, to which no amyloglucosidase had been added, from sample A, which had been incubated with amyloglucosidase as described in section 2.3.4.1. The measurements were calculated based on the volume of a 1 ml reaction volume using the following equation:

$$C_{glucose} = (V_{total} \times \Delta E) / (\varepsilon \times d \times V_{sample});\ \varepsilon_{340} = 6.3\ l.mmol^{-1} \times cm,\ d = 1cm;$$

Glycogen is reported as mg glucose/g dry cell weight (mg glucose/g DW). The dry cell weight was calculated according to the absorbance at OD_{600} where an OD_{600} of 1 correlated to 0.25 $g.l^{-1}$ for *C. glutamicum* (Börman *et al.*, 1992) In this study, the values of glycogen (mg glucose/g DW) are given as the average for at least 2 separate experiments where glycogen is measured each time in triplicate.

2.3.5 Protein determination

Protein concentration was determined using the BioRad dye reagent with bovine serum albumin as a standard with concentrations between 0.025 $mg.ml^{-1}$ to 0.1 $mg.ml^{-1}$ according to the principle of Bradford, (1976).

2.4 Results

2.4.1 Qualitative evidence for glycogen degrading enzymes in the cell extract of *C. glutamicum*.

The growth of *C. glutamicum* was analysed in minimal medium broth and solid media containing glycogen as the sole carbon source. The organism was unable to grow on this substrate but it may possess glycogen degrading enzymes in the cell extract. Cell extracts were prepared from *E. coli* DH5α and *C. glutamicum* as described in the Materials and Methods (2.3.2). The protein concentration of the cell free extracts were determined according to Bradford (1976) using bovine serum albumin as a standard, and the protein content of all samples was standardised for the experiment. Of each 10 mg/ml protein solution, 25 µl was pipetted onto a filter placed on a minimal medium plate containing glycogen and allowed to incubate at 30 ^{0}C overnight. A zone of clearance was visible upon staining with Lugol's iodine reagent, in the region of the filter, which implied enzymes capable of degrading glycogen were present in the cell extract of *C. glutamicum* (**Fig. 2.1**).

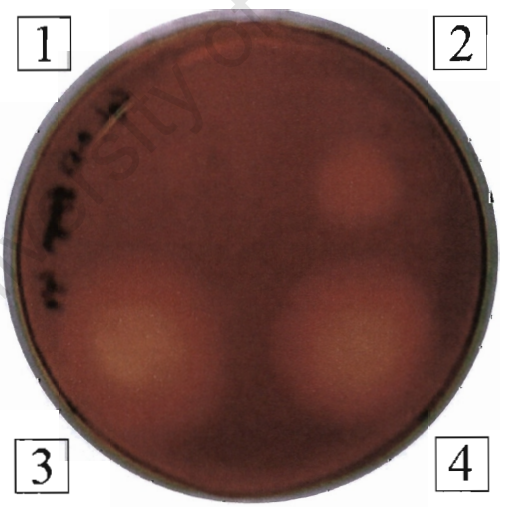

Figure 2.1 Glycogen degradation by enzymes in the cell extract of *C. glutamicum*.

1: Potassium acetate buffer only ; 2: *E. coli* DH5α cell extract*; 3 and 4: *C. glutamicum* cell extract*. *extracts were of a standard protein solution of 10 mg/ml and 25 µl loaded per sample.

2.4.2 Development of an assay to detect intracellular glycogen

An enzymatic glycogen test for *C. glutamicum* was developed to measure the intracellular levels of glycogen (Materials and Methods 2.3.4). The test was based on the degradation of glycogen to glucose by amyloglucosidase at a temperature of 57 ^{0}C, which prevented the breakdown of trehalose to glucose at this temperature (Parrou *et al.*, 1997). Different concentrations of glucose were analysed to optimise glycogen detection, and to see if these concentrations were proportional to the amount of glycogen accumulated in the cells.

Table 2.1 Glycogen content, measured as mg glucose/g DW, in *C. glutamicum* cells grown in minimal medium with 2% glucose.

Time [h]	Glucose [mg/g DW]
0	44.2 ± 0.03
4	50.2 ± 0.53
7	65.8 ± 0.29
12	32.7 ± 1.59
24	17.9 ± 0.15
72	4.9 ± 0

An example of the glycogen content and standard deviations calculated for different time points during growth of *C. glutamicum* on minimal media supplemented with 2% glucose are given in Table 2.1.

2.4.3 Accumulation of glycogen in *C. glutamicum* grown on complex media with 4% glucose

Initial quantitative detection of glycogen in *C. glutamicum* was performed on complex media with an excess of glucose, where intracellular glycogen was measured during growth. Cells grown on complex media without glucose, accumulated no glycogen after 16 hours, therefore complex media precultures for this experiment contained no glucose. During exponential growth of *C. glutamicum* on complex media with 4% glucose, glycogen was accumulated between 30 and 60 mg glucose/g DW. During stationary phase, inhibitory metabolites or products are accumulated by the cells and oxygen becomes limited because of an increase in cell density. However, the culture was able to maintain growth to OD_{600} 47 after 24 h with a growth rate (μ) of 0.55 h^{-1} and a doubling time of 1.3 h. (**Fig. 2.2 A**). Higher levels of glycogen were measurable during stationary phase than during logarithmic phase, with a maximum accumulation of 91 mg glucose/g DW at 24 hours (**Fig. 2.2 B**).

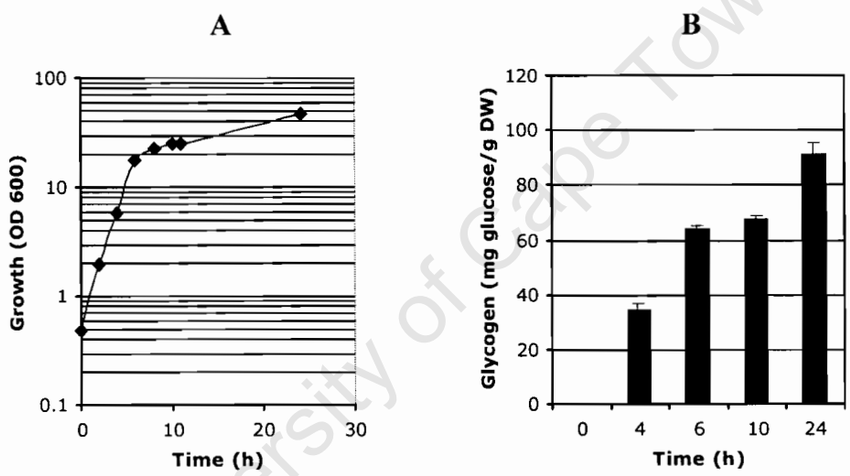

Figure 2.2. Accumulation of glycogen on complex media containing excess glucose.
A.: *C. glutamicum* grown in LB + 4% glucose. **B:** Accumulation of glycogen during growth of *C. glutamicum.*

2.4.4 Growth of *C. glutamicum* and the glycogen accumulation profile on different concentrations of glucose

The effect of different concentrations of glucose in minimal medium broth on the accumulation of glycogen in *C. glutamicum* was investigated. Three concentrations of the carbon source were tested, 2%, 5%, and 10% glucose. The profiles of these experiments are depicted in **figure 2.3 A** and **B**, where glycogen was measurable at T=0, because the minimal medium preculture contained an initial 1% glucose concentration. Minimal medium plus glucose was used as a preculture in order to prevent the carry over of other nutrients from a complex media preculture, and to allow the cells to adapt to the medium in which they were to be tested. The growth rate on minimal media for both 2% and 10% glucose was 2.6 h^{-1}, while on 5% glucose it was 2.7 h^{-1}. This was half the rate determined on complex media supplemented with 4% glucose ($\mu = 0.55\ h^{-1}$). The highest growth density was determined with cells grown on 5% glucose supplemented minimal media, reaching a maximum OD_{600} of 72 at 24 hours and a final OD_{600} of 54 after 72 hours. Minimal media containing an initial 2% glucose concentration grew to a maximum OD_{600} 40.5 with a final OD_{600} 24.7 reached after 72 hours. Surprisingly the medium supplemented with 10% glucose did not support the highest growth rate or reach the highest growth density. However, between 24 hours and 72 hours this culture was able to maintain its maximum OD_{600} of 56.

The accumulation of glycogen in *C. glutamicum,* grown on glucose supplemented minimal medium, did not follow the same profile as growth on complex media, where maximum glycogen was accumulated in stationary phase (**Fig. 2.2 B**). The maximum glycogen concentration detected, of 81.8 mg glucose/g DW, was determined on 5% glucose during late logarithmic growth, or entry into stationary phase ie at T=7 hours (**Fig. 2.3 A** and **B**). The 2% and 10% supplemented cultures, produced less glycogen in comparison after 7 hours, but these were also the highest levels for these cultures during their growth profile. After 24-hour growth, the 10% supplemented culture maintained glycogen levels of 50 mg glucose/g DW and the 2% and 5% glucose supplemented culture produced less than 20 mg glucose/g DW. After 72 hours growth, glycogen was still produced up to 25.6 mg glucose/g DW in the cultures containing the initial 10%

glucose concentration. This culture did not support the highest glycogen accumulation but contributed to measurable glycogen over a longer culturing time. The 2% and 5% glucose containing cultures exhausted their supply of glucose after 10 and 14 hours, respectively, while after 72 hours cultures containing initially 10% glucose still had 0.5% glucose in the medium.

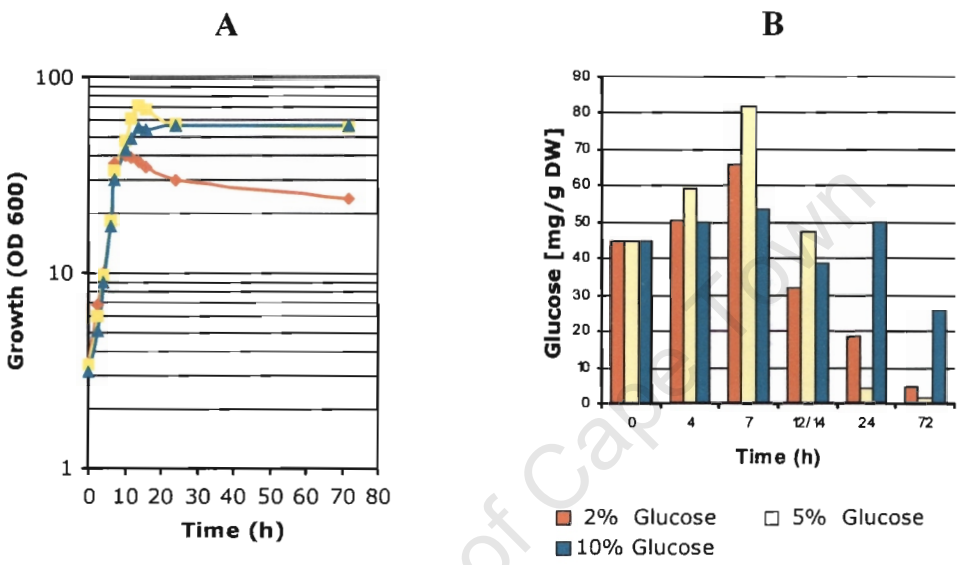

Figure 2.3. Glycogen accumulation in minimal medium with 2%, 5% and 10% glucose. **A.:** Growth of *C. glutamicum* in 2%, 5% and 10% glucose. **B:** The accumulation of glycogen during growth on different glucose concentrations. Where 12/14 h is the measurement of glycogen at 12 h for the 2% glucose CgC-MM culture and the measurement at 14 h for the 5% and 10% glucose CgC-MM culture. The preculture for each experiment contained glucose which explains the initial 44.5 mg glucose/g DW measured at 0 h. Standard deviations for these experiments were less than 20%.

2.4.5 Growth of *C. glutamicum* and accumulation of glycogen on different carbohydrate substrates

The effect of carbohydrates, other than glucose, on glycogen accumulation was investigated. *C. glutamicum* was cultured as described in the materials and methods with

glucose, maltose, fructose and sucrose at a final substrate concentration of 2% in minimal medium broth. The minimal media precultures contained the relevant carbohydrates at an initial concentration of 1%. Cell extracts from the cultures were harvested at 0 hours, 4 hours, 7/10 hours, 12/14, hours and 24 hours. Samples taken at 7/10 hours and 12/14 hours indicate the sampling of glucose and maltose grown cells at 7 and 12 hours, and the sampling of fructose and sucrose grown cells at 10 and 14 hours. Glycogen was subsequently assayed to determine the glycogen accumulation profile of *C. glutamicum* during growth on the different carbohydrates. The growth pattern of *C. glutamicum* on glucose, maltose, fructose and sucrose are shown in **figure 2.4 A**. On media supplemented with glucose, the cells grew at a rate of $\mu = 0.26\ h^{-1}$, which was maximal for the different sugars tested, while fructose grown cells displayed the slowest growth rate of $0.19\ h^{-1}$. In a different study by Dominguez *et al.*, 1998, the growth rate of *C. glutamicum* ATCC 17965 on fructose was also found to be less in comparison to growth on glucose. The cultures grown on the four different sugars all reached an approximate final OD_{600} of 30 after 24 hours.

Glycogen accumulation during the growth of *C. glutamicum* on the various sugars is depicted in **figure 2.4 B**. Maltose grown cells accumulated maximum glycogen levels, during practically the entire growth phase, reaching a maximum accumulation of 107.8 mg glucose/g DW after 4 hours of growth. Glucose grown cells showed a maximum level of glycogen of 65.8 mg glucose/g DW, measured after 7 hours growth. After 4 hours of growth on maltose and glucose, maltose grown cells had more than twice the amount of glycogen. However, it must be noted that the enzyme amyloglucosidase, used to degrade glycogen in the enzyme assay (see Materials Methods Section 2.3.4), has both α-1,4 and α-1,6 glucosidase activity, and maltodextrins (formed from maltose) consist of α-1,4 bonded glucose molecules. Degradation of intracellular maltodextrins by amyloglucosidase may give artificially high levels of glycogen measured as glucose. This might be particularly true under growth conditions such as minimal medium containing maltose. However, in the next chapter it was established that the promoter regions upstream of these structural genes, were also upregulated when grown on maltose in comparison to glucose grown cells.

C. glutamicum cultured on the sugars, fructose and sucrose, showed low levels of glycogen accumulation with the maximum reached after 4 hours with the values 21.9 and 42 mg glucose/g DW, respectively (**Fig. 2.4 B**). Glucose, fructose, and sucrose are assimilated via specific phosphoenolpyruvate phosphotransferase systems (PTS) and therefore would be found in the cytoplasm in a phosphorylated form. Dominguez and co-workers established that two fructose uptake mechanisms function in *C. glutamicum* ATCC 17965, with the main uptake via PTS^{Fru} generating fructose-1-phosphate, while the rest was taken up by the lower affinity PTS^{Man}, forming fructose-6-phosphate (Dominguez *et al.*, 1998). We found that cells grown in fructose media had the slowest growth rate, and accumulated the lowest levels of glycogen.

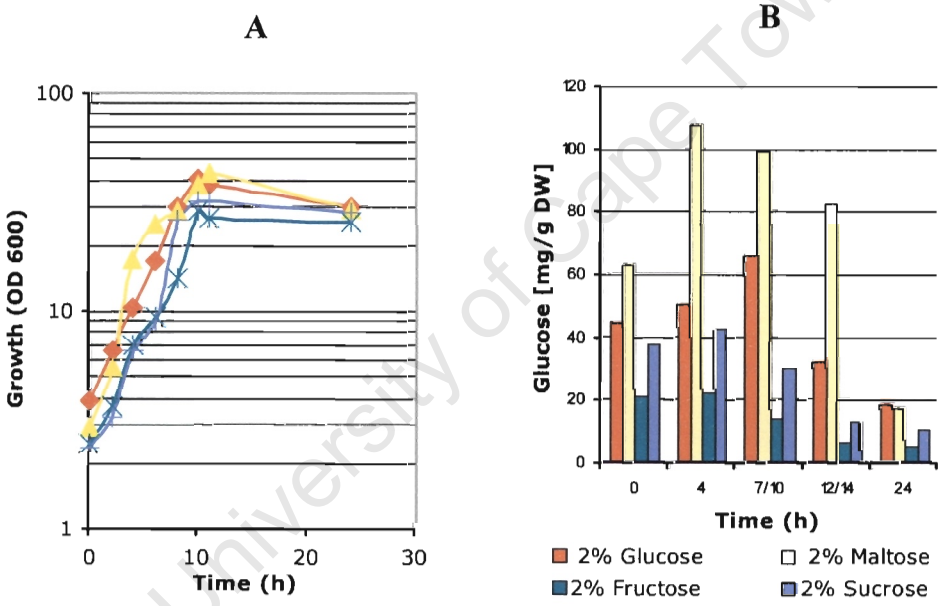

Figure 2.4. Glycogen accumulation on different carbon sources. A: Growth pattern of *C. glutamicum* in CgC-MM broth with the carbohydrate sources glucose, maltose, fructose and sucrose at a final concentration of 2%. **B:** Glycogen accumulation during the growth of *C. glutamicum* on different carbohydrates. Standard deviations for glycogen assays were less than 15%.

2.4.6 Intracellular glycogen content of *C. glutamicum* during growth on acetate and lactate

In an attempt to determine the accumulation of glycogen during growth on organic acids which enter the TCA cycle via acetyl-CoA and pyruvate, *C. glutamicum* was grown on minimal media supplemented with either 1% acetate or lactate, and 1% glucose was used in the control culture. *C. glutamicum* grew on all three substrates, and samples were taken for glycogen assays during the logarithmic growth phase 4 hours after inoculation at OD_{600} 1 in minimal medium broth. The organic acids supported growth to a final OD_{600} of approximately 8.8 while 1% glucose supported growth for *C. glutamicum* to a higher OD_{600} of 17.9 after 24 hours. The amount of glycogen measured after growth on acetate and lactate was considerably less, at 16.5 and 9.2 mg glucose/g DW respectively, compared to 49 mg glucose/g DW glycogen for the glucose containing culture (**Fig. 2.5**).

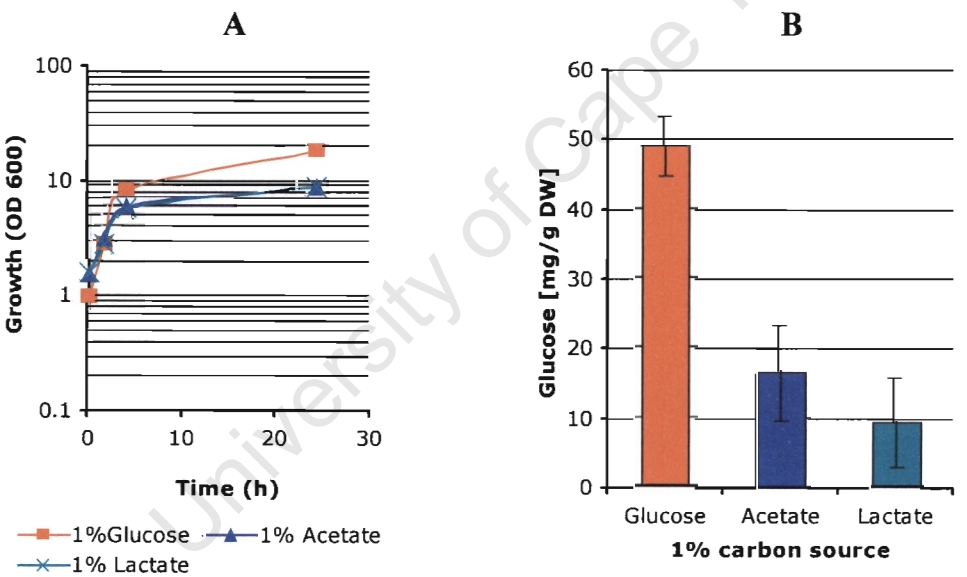

Figure 2.5. Accumulation of glycogen with growth on organic acids A: The growth of *C glutamicum* on the organic acids, acetate and lactate, in comparison to glucose. **B:** The measured accumulation of glycogen on the carbon sources glucose, acetate and lactate taken during the logarithmic growth phase of the WT. Glycogen was measured after 4 h. The precultures of complex medium contained no carbon source and the cultures were allowed to adapt to the carbon source during a 16 h overnight culture with the appropriate carbon source.

2.5 Discussion

Since *C. glutamicum* is an important amino acid producing organism, its central metabolism and the metabolic fluxes prevalent in the cell are of importance and glycogen accumulation may at first glance be of secondary interest. Conditions that might affect the flow of carbon to the TCA cycle may have an effect on the availability of intermediates for amino acid and vitamin production. An investigation of glycogen accumulation was therefore undertaken with respect to conditions such as excess glucose as well as glycogen accumulation during growth on different carbon sources. As a first introduction to glycogen metabolism in *C. glutamicum*, we attempted to grow the organism on solid medium and in liquid medium with glycogen as the sole carbon source. As expected, the organism was unable to degrade the complex substrate, presumably because extracellular glycogen degrading enzymes are not produced by this organism. *C. glutamicum* did however, have the ability to degrade glycogen intracellularly.

Glycogen levels were highest in samples taken from stationary phase cultures grown in complex media with 4% glucose (**Fig. 2.2**). After 24 hours, 91 mg glucose/g DW was accumulated under these conditions. In contrast, glycogen was highest when measured during the late exponential growth phase regardless of the concentration of glucose when grown on minimal medium (**Fig. 2.3**). The availability of other nutrients, such as peptides in complex media would result in glucose being metabolised, but more importantly the synthesis of metabolites from glucose would be blocked. Glucose not required immediately for growth could have been incorporated into glycogen resulting in higher glycogen levels in stationary phase on complex media. The higher concentrations of glucose in minimal media supported the accumulation of glycogen over a longer growth period. In both the 5% and 10% glucose containing cultures, the maximum OD_{600} of 56.2 was maintained from approximately 12 hours to 72 hours (**Fig. 2.3**). However it was the 5% glucose containing media that accumulated the most glycogen, indicating the accumulation of glycogen was not proportional to the concentration of glucose in the media. This in turn suggests the uptake and distribution of glucose (into glycolysis or glycogen) in *C. glutamicum* could be regulated. In *E. coli,* glycogen is mostly accumulated in the stationary phase (Preiss, 1984), however in *M. smegmatis,* glycogen is

constantly accumulated and degraded during growth (Ballinger and Hatfull, 1999). Cycling of glycogen was also established in *Clostridium* and in *Fibrobacter* strains implying glycogen cycling could be prominent in all bacteria and not only Gram-positive bacteria (Guedon *et al.*, 2000; Matheron *et al.*, 1998). To more fully understand the accumulation and degradation of glycogen in *C. glutamicum*, mutants within the glycogen biosynthesis pathway would have to be generated and investigated for growth characteristics.

When considering the uptake of sugars from the medium, *C. glutamicum* utilises the phosphotransferase system (PTS) for the assimilation of sugars such as glucose, sucrose and fructose (Dominguez and Lindley, 1996; Parche *et al.*, 2001), with specific enzyme II complexes. The PTS components in the related *C. diphtheriae* have been previously described (Parche *et al.*, 2001), and the presence of these components in *C. glutamicum* have recently also been identified in its genome sequence (Kalinowski *et al.*, 2003). However, in the case of glucose, a suspected permease transporter might also be present, since there was still residual growth on glucose when the two glucose PTS's, PTS^{Man} and PTS^{Fru}, were deleted (Park *et al.*, 2000; Dominguez and Lindley 1996). A schematic of the flow of carbon from these sugars toward glycolysis or glycogen is given in **figure 2.6**. Growth of *C. glutamicum* on different sugars would lead to the accumulation of different levels of glycolytic intermediates, one of which would be fructose-1,6-bisphosphate, the stategic branch point of glycolysis and glycogen formation. Fructose-1,6-bisphosphate is a known regulator of ADPGlc PPAse in *E. coli* (Preiss and Romeo 1994) and therefore different levels of this intermediate could regulate glycogen formation. In the case of fructose grown cells, an excess of fructose-1,6-bisphosphate could result leading to the activation of ADPGlc PPase. The reaction leading to glycolysis from fructose-1,6-bisphosphate requires ATP, but the precursor for glycogen, glucose-1-phosphate, could also be formed via the enzymes fructose-1,6-bisphosphatase, glucose-6-phosphate isomerase and phosphoglucomutase. The activation of ADPGlc PPase correlates with the levels of glycogen measured with growth of *C. glutamicum* on fructose which were the lowest (**Fig. 2.4**) in comparison to the other sugars. With the assimilation of sucrose, once phosphorylated, it would be degraded into one glucose-6-phosphate molecule and

one fructose molecule. Even though fructose-1,6-bisphosphate is an activator of glycogen synthesis, only half the number of glucose molecules would be available to enter glycolysis, therefore the accumulation of glycogen would likely not occur. We found growth of *C. glutamicum* on sucrose led to less glycogen accumulation in comparison to glucose but more in comparison to fructose (**Fig. 2.4**). In another physiological aspect, the growth of *C. glutamicum* on inorganic acids led to even less glycogen accumulation of < 20 mg glucose/g DW relative to > 20 mg glucose/g DW measured on fructose. The carbon source, acetate, is incorporated in the TCA via acetyl-CoA while lactate is first converted to pyruvate via lactate dehydrogenase. In the above cases, the glycolytic pathway is not being used so that gluconeogenesis would be required in order to generate the glycolytic intermediates that would normally stimulate glycogen production. We found the highest accumulation of glycogen when *C. glutamicum* grew on the substrate maltose (**Fig. 2.4**). Via synthesising enzymes, maltose is either incorporated into growing maltodextrin chains or can be phosphorylated and subsequently degraded to yield glucose-1-phosphate (the precursor toward glycogen) (**Fig. 2.6**). The maltodextrin chains form a pool of maltodextrins of various lengths, and due to the nature of the enzymatic glycogen assay it is possible this pool contributed to the higher intracellular levels of 'glycogen' measured with growth on maltose. Since maltodextrins are $\alpha(1\rightarrow4)$ linked glucose molecules these are also degraded by amyloglucosidase, the enzyme used to degrade glycogen, however the contribution of the carbon source was excluded from the test by several washing steps before lysing the cells (Materials and Methods, Section 2.3.4).

In summary, we have established that *C. glutamicum* ATCC 13032 has the ability to synthesise and degrade glycogen. The accumulation of glycogen was highest during logarithmic growth on 1% or 2% glucose but was also maximally accumulated during stationary phase when there was an excess of glucose (**Fig. 2.2**). A further objective would be to identify the genes involved in glycogen metabolism, which would facilitate the analysis of regulatory mechanisms in glycogen metabolism. Understanding the regulation and function of glycogen accumulation may help lead to a better understanding of the contribution of this polysaccharide to the central metabolism of *C. glutamicum*.

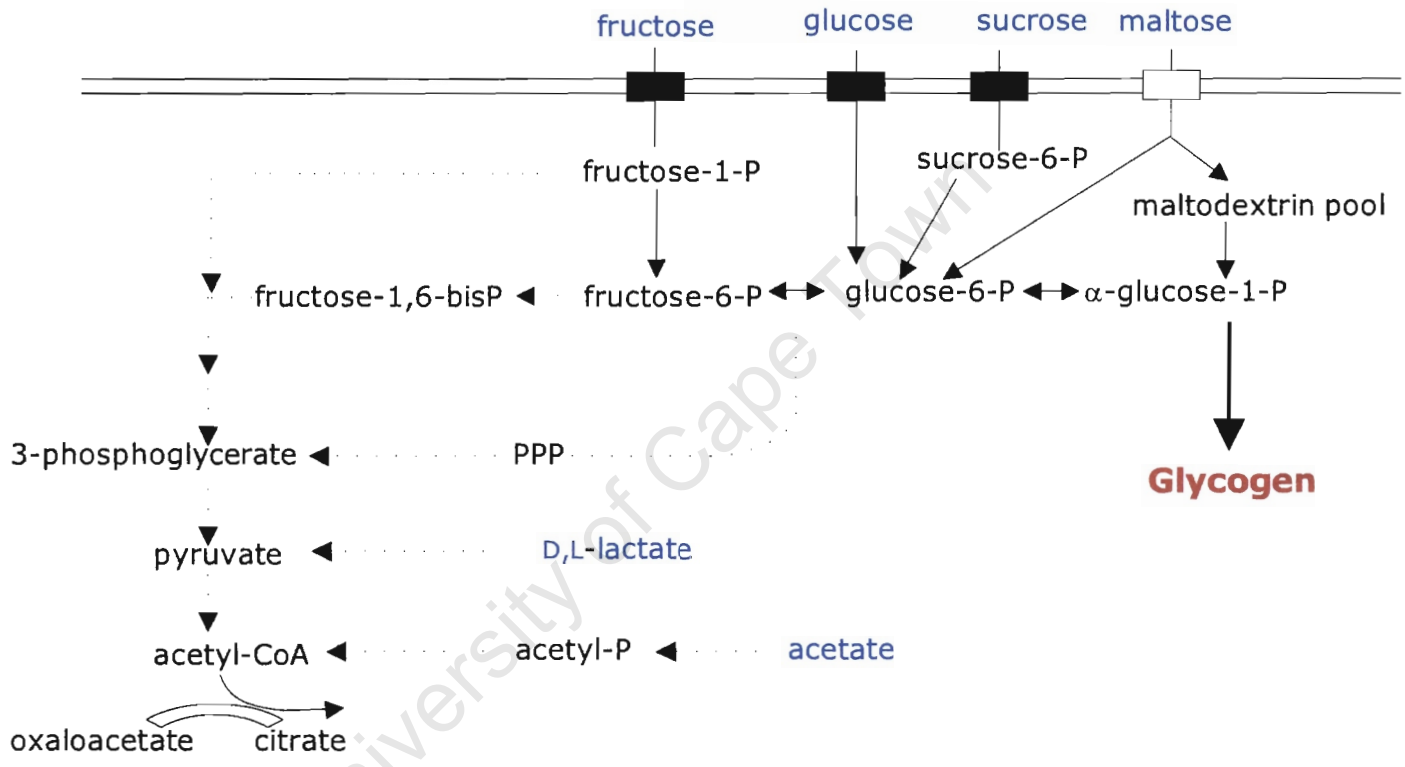

Figure 2.6 A schematic representation of the flow of different carbon sources used to analyse glycogen accumulation in *C. glutamicum*. Carbon sources are in blue. Bold solid line represent reactions toward glycogen synthesis. Dotted lines represent the pathways toward the TCA. PPP : Pentose Phosphate Pathway. Black boxes represent specific PTS, white box represents putative ABC transport process.

CHAPTER 3

IDENTIFICATION OF THE GENES INVOLVED IN GLYCOGEN METABOLISM IN *C. glutamicum*: TRANSCRIPTIONAL ANALYSIS AND REGULATION

CONTENTS

3.1 Summary

C. glutamicum has been shown to accumulate and degrade glycogen. Subsequent bioinformatic analysis of the then unannotated genome of *C. glutamicum* and Blast searches with known and putative glycogen genes from other bacteria, led to the identification of four genes possibly involved in glycogen metabolism. Genes coding for the ADPGlc pyrophosphorylase (*glg*C), a glycogen synthase (*glg*A), a branching enzyme (*glg*B) and a debranching enzyme (*glg*E) were identified according to their arrangement and amino acid sequence similarity of the respective deduced proteins to other (homologous) enzymes. Transcription analysis of *C. glutamicum glg*A, *glg*C, *glg*B and *glg*E revealed the monocistronic transcription of *glg*A (~ 1230 nt) and *glg*C (~ 1250 nt), while the transcription of *glg*B (~ 2500 nt) and *glg*E (~ 2000 nt), as a single mRNA transcript was confirmed by RT-PCR as well as Northern blots. The genes *glg*A and *glg*C were transcribed divergently by possible overlapping promoter regions which was confirmed by reporter gene analysis of the promoter region. Transcriptional regulation of these genes was analysed during growth on different carbon sources by measuring the activity of promoter regions cloned ahead of a promoterless chloramphenicol acetyl transferase reporter gene. These assays were performed in *C. glutamicum* and it was found that all promoters were down regulated 2.5-3.5 times when grown on acetate and lactate in comparison to glucose, while in maltose grown cells the promoters where upregulated 1.5 times relative to glucose. These results indicate regulation at the transcriptional level of the genes *glg*A, *glg*C and *glg*EB in *C. glutamicum.*

3.2 Introduction

Before the current annotated genome sequence of *C. glutamicum* (Nakagawa, 2002; Kalinowski *et al.*, 2003) was made publicly available, the identification of the relevant genes for glycogen synthesis and degradation were identified by comparison of previously published enzymes against the unannotated genome sequence available. Multiple alignments of published sequences with that of the recently annotated *C. glutamicum* genome sequence using BLAST (Altschul *et al.*, 1997), allowed a more thorough investigation of the genes involved in glycogen metabolism in *C. glutamicum.* The arrangement of genes involved in glycogen metabolism in many organisms are clustered and computer analysis also allowed us to indentify the genetic arrangement of glycogen genes in *C. glutamicum.* In *E. coli,* the genes coding for glycogen synthesis and degradation are coordinately expressed in two operons (Romeo *et al.*, 1988). In *S. coelicolor* glycogen related genes are not only found in an operon but are also duplicated and the different operons are expressed at different stages of development in the bacterium (Schneider *et al.*, 2000). In Chapter 2, we established that glycogen accumulation in *C. glutamicum* was dependent on the carbon source, whether it was carbohydrates or organic acids, suggesting some sort of regulation of these genes. Glycogen accmulation also varied during the growth phase of the bacterium. In this chapter, we were able to identify and analyse four genes involved in glycogen metabolism in *C. glutamicum.* Regions of DNA upstream of the translational starts of these genes were also analysed for an indication of transcriptional control of gene expression under different conditions.

3.3 Materials and Methods

3.3.1 Computer analysis of genes involved in glycogen metabolism

Computation was performed at the SIB (Swiss Institute for Bioinfomatics; http://www.isb-sib.ch) and NCBI using the BLAST network services (Altschul *et al.*, 1997). The protein and DNA sequences were analysed using the online ExPASy proteomics server (Gasteiger *et al.*, 2003) and ClustalW (Higgins *et al.*, 1994), Clone Manager version 5 and Genedoc software packages. The SWISSPROT accession number for *C. glutamicum* GlgC is Q8NRD4. Additional sequences used for comparison with this polypeptide and their respective Genbank accession numbers are as follows: *M. tuberculosis glg*C (O05314), *S. coelicolor glg*C (P72394), *E. coli glg*C (P00584). The Genbank accession number for *C. glutamicum glg*A is AP005277; protein ID BAB98510.1 and additional sequences used for comparison are *M. tuberculosis* CDC1551 (AE007001; protein ID AAK45507.1), *S. coelicolor* (AJ243803; protein ID CAB50741.1) and *E. coli* (BAB37697). For multiple alignment analysis of various GlgB proteins the following sequences were used: *C. glutamicum glg*B (BX927151; protein ID CAF19928.1); *M. tuberculosis glg*B (BX842576, protein ID CAA98090.1); *S. coelicolor glg*BI (X83397); *S. coelicolor glg*BII (X73903); *E. coli glg*B (M13751). The putative α-amylase (GlgE) of *C. glutamicum* (BX927151; protein ID CAF19929) was aligned with *M. smegmatis* putative glucanase GlgE (AF172946; protein ID AAF07898.1) and two putative glucanohydrolases from *S. coelicolor* designated Pep1 I (AJ001205; protein ID CAA04600.1) and Pep1 II (AJ001206; protein ID CAA04606.1).

3.3.2 Bacterial strains, growth conditions and plasmids

C. glutamicum was routinely grown aerobically in 500 ml fluted Erlenmyer flasks at 120 rpm to facililate aeration. The medium was either complex medium with or without a carbon source, or CgC-MM supplemented with different carbon sources (Appendix A). Cloning and subcloning were conducted with the *E. coli* strain DH5α, which was grown in 2xTY broth or agar (Sambrook *et al.*, 2001). Kanamycin was added to the growth medium at 50 $\mu g.ml^{-1}$ to select for transformants. Bacterial strains, plasmids and oligonucleotides used in this chapter are described in the tables below.

Table 3.1. Bacterial strains

Strain	Relevant characteristics	Reference
E. coli DH5α	*supE44, hsdR17, recA1, thi-1, endA1, lacZα, gyrA96, relA1*	Hanahan, 1985
C. glutamicum ATCC 13032	Wild type	Abe *et al.*, 1967

Table 3.2 Plasmids

Plasmid	Relevant characteristics	Reference
pEKEx2	Km^R expression vector carrying *lacI*^{q}, the *tac* promoter and the pUC18 mutiple cloning site	Eikmanns *et al.*, 1994
pEKglgA	pEKEx2 derivative, 1.3 kb fragment of *glg*A ORF including RBS	this work
pEKglgE	pEKEx-2 derivative, 2.1 kb fragment of *glg*E ORF including RBS	this work
pK19*mobsacB*	Km^R, non-replicating in *C. glutamicum*, oriT (mobilizable), oriV	Schäfer *et al.*, 1994
pK19IMA	pK19*mobsacB* derivative, 550 bp internal region of *glg*A, cloned via PCR generated *Sal*I-*Bam*HI restriction sites	this work
pK19IMC	pK19*mobsacB* derivative, 371 bp internal region of *glg*C, cloned via PCR generated *Bam*HI and internal *Sal*I restrictions sites	this work
pET2	Km^R, *cat,* ori pBL1, ori colE1	Vasicova *et al.*, 1998
pETPA1	pET2 derivative, 437 bp *glg*A promoter fragment	this work
pETPC4	pET2 derivative, 437 bp *glg*C promoter fragment	this work
pETPEB1	pET2 derivative, 556 bp *glg*E promoter fragment	this work

Table 3.3 Oligonucleotide primers*

Primer	Sequence (5'-3')	Restriction sites
DMglgE-rev4	GGAATTCGGTAGGTCTTGATTTCGC	*Eco*RI
PR-glg*A*C-for	CGGGATCCTGCACCCATGCAGTGAAC	*Bam*HI
PRAC-2-rev	ACGCGTCGACTCCGGAGTTCACCA	*Sal*I
PRAC-2-for	ACGCGTCGACTGCACCCATGCAGTGAAC	*Sal*I
PR-glg*A*C-rev	CGGGATCCGAATCCGGAGTTCACCAG	*Bam*HI
PR-glgEB-for	CCGCTCGAGGATTCGGTTGCCTAATGC	*Xho*I
PR-glgEB-rev	CGGGATCCTTCCATCCAAAATGCGGG	*Bam*HI
DM-glgE-for3	CGACATTTTGCATGCGTC	-
RT-revB2	ATCAGCCTTGACCTGCGG	-
DM-glgE-for1	ACGCGTCGACCGGTATCGATGATGTTCG	*Sal*I
DM-glgE-rev2	GGAATGGAGTATGGAAGTTGG	-
IM-glgB-for	CCCAAGCTTATCCGATGCGTTCTATGG	*Hind*III
IM-glgB-rev	CGGGATCCTCAGAGAATGCGTACACC	*Bam*HI

*oligonucleotide primers were synthesised by MWG-Biotech, GmBh

3.3.3. General recombinant DNA procedures

All DNA modifications and manipulations were performed according to standard procedures (Sambrook *et al.,* 2001). *C. glutamicum* chromosomal DNA was isolated according to Eikmanns *et al.,* (1994). Plasmid DNA from *E. coli* was isolated using a modified method of alkali lysis according to Birnboim (1983) or with the GFX-kit for plasmid isolation (Amersham).

3.3.4. Isolation of chromosomal DNA from *C. glutamicum*

Chromosomal DNA was isolated from a 5 ml overnight culture (2 x TY with 0.5% glucose) according to the method described by Eikmanns *et al.,* (1994). The culture was centrifuged (5000 rpm; 5 min), washed twice with TE buffer and resuspended in 1 ml TE containing 15 $mg.ml^{-1}$ lysozyme. After 3 hours incubation at 37 ^0C, 3 ml lysis buffer (10 mM Tris-HCl, 400 mM $NaCl_2$, 2 mM EDTA, pH 8.2), 220 µl SDS (10%), and 150 µl Proteinase K (20 $mg.ml^{-1}$) was added and the solution incubated at 37 ^0C overnight.

Proteins were precipitated by addition of 2 ml salt saturated NaCl and the solution centrifuged (5000 rpm, 15 min, RT) to recover the supernatant. The chromosomal DNA was precipitated with addition of ice cold 100% ethanol and spooling with a pasteur pipette, washed in 70 % ethanol, dried and allowed to resuspend overnight in 100 – 200 µl TE at 4 ^{0}C (or 10 mM Tris pH 7.6).

3.3.5. RNA extractions

RNA was harvested from mid-exponential phase *C. glutamicum* cultured in 60 ml CgC-MM, containing 1% or 2% glucose as the carbon source (Appendix A). An equal volume of killing buffer (20 mM Tris-HCl pH 8.2; 20 mM NaN_3; 5 mM $MgCl_2$) was added to 20 ml of chilled culture, and centrifuged for 10 min at 5000 rpm and 4 ^{0}C to pellet the cells. Pelleted cells were flash frozen in liquid nitrogen and stored at -70 ^{0}C or RNA was extracted immediately. The cell pellet was defrosted on ice and the cells added to 2 ml screw top eppendorfs previously filled with 0.25 ml glassbeads (150-212 µm, Sigma-Aldrich Chemicals GmbH); 0.5 ml acidic phenol; and 0.5 ml RLT buffer (RNeasy® Mini Kit, Qiagen GmbH, Hilden). These were lyzed with a Ryboliser (Hybaid RiboLyser Cell disruptor) 3 times for 45 sec at a setting of 6.5 and without cooling on ice except at the end. The cell free extract was added to 15 ml falcon tubes containing 1.5 ml acidic phenol; 1.5 ml chloroform isoamyl alcohol; and 2.5 ml AE Buffer (40 mM sodium acetate; 1 mM EDTA), which had been heated to 60 ^{0}C in a water bath. The aqueous phase was extracted by centrifugation and treated to three more subsequent phenol extractions. The aqueous phase was finally extracted with chloroform isoamyl alcohol (24:1) and precipitated with 0.1 volume 3M sodium acetate and 2.5 volume absolute ethanol at -20 ^{0}C for 2 h to overnight. The RNA was washed with 70% ethanol and resuspended in RNAse-free H_20. For Northern blots and RT-PCR, the RNA was treated with DNase I digestion according to standard protocols (Sambrook *et al.*, 2001).

3.3.6 Reverse Transcription PCR (RT-PCR)

RT-PCR was performed using the primers RT-revB and DM-glgE-for3 (**Table 3.3**), with RNA extracted from exponential phase *C. glutamicum* cultures grown in CgC-MM + 1% glucose or CgC-MM + 1% acetate (Appendix A). In order to determine a single mRNA

transcript for *glg*E and *glg*B, RNA was extracted as described above and treated with a DNase-1 digestion according to standard protocols. The reactions were performed according to the manufacturers specifications for the Ready to Go RT-PCR Beads, Amersham Biosciences. Contaminating DNA was excluded in the experiment by performing a control PCR with heat-inactivation of the reverse transcriptase at 42 ^{0}C for 20 min. Two positive control RT-PCR reactions were included to confirm the presence of each gene. A 540 bp region on the 5' end of *glg*E was amplified using RT-PCR with the primers DM-glgE-for1 and DM-glgE-rev2. The second control was the amplication of a 1.01 kb internal region of *glg*B with the primers, IM-glgB-for and IM-glgB-rev.

3.3.7 Northern hybridisation

Total RNA was isolated from mid-exponential phase cultures of *C. glutamicum* strains as described above (Section 3.3.4). RNA was seperated by electrophoresis in 1.0% denaturing formaldehyde gels according to standard RNA protocols. Two concentrations of RNA; 4 µg and 6 µg were loaded per lane. Except for positive controls, where 1 µg of RNA was loaded on the RNA agarose gel. The positive controls were RNA extracted from *C. glutamicum* containing the plasmids pEKglgA and pEKglgE. These plasmids have the complete ORF, including a sufficient region upstream to contain possible ribosomal binding sites of *glg*A and *glg*B (**Table 3.2**). RNA was transferred by vacuum extraction onto a nitrocellulose membrane and the RNA fixed according the manufacturers instructions. Hybridisation with a P^{32} labeled specific probe was allowed overnight at 60 ^{0}C, and after the washing steps the blots were exposed to X-ray film and developed. The RNA molecular marker (0.24-9.5kb), was obtained from Invitrogen Life Technologies.

3.3.8 Construction of transcriptional fusions

PCR (Appendix B) was used to amplify fragments upstream of *glg*A, *glg*C, and *glg*EB using *C. glutamicum* chromosomal DNA as a template. The primers shown in **Table 3.3** were tagged with appropriate restriction enzyme sites to facilitate cloning into the promoter-reporter vector pET-2 using the reporter gene, chloramphenicol acetyl transferase (CAT). The promoter region of *glg*A was constructed using the primers, PR-

glgAC-for and PRAC-2-rev which generated a 437 bp fragment and was cloned into pET2 with the restriction sites *Sal*I and *Bam*HI. The promoter region of *glg*C was the same fragment but amplified with the primers PRAC-2-for and PR-glgAC-rev, which had the restriction sites reversed, and the fragment was cloned in the pET2 vector. The promoter probe vector pETPEB1 was constructed using the primers PR-glgEB-for and PR-glgEB-rev and the 556 bp fragment cloned using the PCR generated restriction sites *Xho*I and *Bam*HI. The promoter DNA fragments for analysis were sequenced from the vector by the company MWG Biotech, Germany. The sequencing results were analysed using NCBI Blast searches and the DNA software programme Clone Manager, version 5. All plasmid constructs were introduced into *C. glutamicum* using the high efficiency electroporation protocol of van der Rest *et al.*, 1999.

3.3.9 Preparation of cell free extracts

For the chloramphenicol acetyl transferase (CAT) assays, *C. glutamicum* cultures were grown overnight (16h) in complex media and inoculated into fresh complex media with and without glucose to a starting OD_{600} of 0.3. Cells were harvested at various growth stages by centrifugation (5000 g for 5 min at 4 ^{0}C), and resuspended in 500 µl of 50 mM Tris-HCl (pH 7.6). Cells were disrupted either by sonication at 95 W using 30 s bursts for 6 min (Virsonic Digital 475 Cell Disruptor, Virtis) or by mechanical lysis with a Ryboliser (Hybaid RiboLyser Cell disruptor) using the maximum setting, for 45 s bursts, 3 times, and with cooling inbetween. The samples were centrifuged at 14000 g for 20 min at 4 ^{0}C, and the cell-free extracts (CFE) obtained used for CAT assays. For CAT activity from samples grown in CgC-MM + carbon source*, *C. glutamicum* was grown in complex medium for 6 hours, inoculated into fresh CgC-MM + carbon source* and incubated overnight. This preculture was inoculated into a main culture of CgC-MM + carbon source* and the cells harvested during the exponential growth phase. Cell extracts were prepared as described for complex medium.

*Carbon sources were 1% glucose, 1% maltose,1% acetate, or 1% lactate

3.3.10 Protein determination of cell free extracts

Protein concentrations of the cell free extracts were determined using the BioRad dye reagent with bovine serum albumin as a standard (concentrations of 0.025 $mg.ml^{-1}$ to 0.1 $mg.ml^{-1}$) according to the principle of Bradford (1976).

3.3.11 Determination of CAT activities

Chloramphenicol acetyl transferase catalyses the following reaction:

Chloramphenicol + 2 Acetyl-CoA → Chloramphenicol-1,3-diacetate + 2 CoA

The reduced CoA is catalyzed further in a second reaction with 5,5'-dithiobis-2-nitrobenzoate (DTNB):

CoA + DTNB → Dithionitrobenzene-CoA + 5'-thio-2-nitrobenzoate

5-Thio-2-nitrobenzoate has an extinction coefficient of 13.6 $mM^{-1}cm^{-1}$ at 412 nm. CAT assays were performed according to a modified method of Shaw (1979). Where 10 µl cell extract was added to 950 µl of reaction mixture (0.4 g DTNB/l, 100 mM Tris-HCl, pH 7.8, 0.1 mM acetyl-CoA) and incubated at 37 ^{0}C for 2 min. The reaction was started with the addition of 50 µl 5 mM chloramphenicol and followed photometrically where 1 Unit of CAT activity is defined as 1 µmol of chloramphenicol acetylated per minute. Specific CAT activity in the samples were calculated as U CAT/mg protein.

3.4 Results

3.4.1 Identification of GlgC and GlgA by computer analysis

The first two genes identified by computer analysis was an ADPGlc pyrophosphorylase (*glg*C) and a glycogen synthase (*glg*A). Both genes are involved in anabolic reactions in the metabolism of glycogen. These genes have been previously identified and studied in *E. coli* (Leung *et al.*, 1986; Preiss, 1996a) and have also been identified in organisms such as *A. tumefaciens* (Ugalde *et al.*, 1998) which was summarised in Chapter 1.

3.4.1.1 Analysis of the C. glutamicum GlgC subunit by multiple alignment

ADPGlc pyrophosphorylase (ATP:α-D-glucose-1-phosphate adenyltransferase; EC 2.7.7.27; ADPGlc PPase) consists of four subunits encoded by the same genetic sequence. The amino acid sequence for one subunit was analysed (**Fig. 3.1**). Conserved regions in bacterial and plant ADPGlc PPase subunits have been annotated according to the computer programme, PROSITE, where three regions were identified as signature patterns for this enzyme. The first two were N-terminal and most likely regions involved in substrate binding and/or allosteric regulation, while the third motif was a conserved region within the central part of the enzyme. These ADPGlc PPase signatures are as follows: signature 1:- [AG]-G-G-x-G-[STK]-x-L-x(2)-L-[TA]-x(3)-A-x-P-A-[LV] ; signature 2:- W-[FY]-x-G-[ST]-A-[DNSH]-[AS]-[LIVMFYW]; and signature 3:- [APV]-[GS]-M-G-[LIVMN]-Y-[IVC]-[LIVMFY]-x(2)-[DENPHK] (Preiss *et al.*, 1991; Nakata, *et al.*, 1991). An alignment of the putative *C. glutamicum* GlgC subunit was made with similar subunits in related Gram-positive organisms such as *M. tuberculosis* and *S. coelicolor,* where the polypeptides showed 60% and 55% identity, respectively. A comparison was also drawn to the not so closely related Gram-negative organism *E. coli,* where the polypeptides had 36% identity. The well characterised *E. coli* GlgC has been discussed earlier in Chapter 1 (Preiss and Romeo, 1994), and the same functional domains such as, the three signatures for ADP-glucose pyrophosphorylase, were present in all four organisms and are underlined in the alignment shown in **figure 3.1**. The absolutely conserved amino acid in bacteria and plants, denoted in *E. coli* as Lys-195 (Preiss and Romeo, 1994; Ballicora *et al.*, 2003), binds the phosphate moiety of the

substrate and was conserved in the *C. glutamicum* GlgC polypeptide (Lys-180) as well as in the *M. tuberculosis* and *S. coelicolor* polypeptides (black highlighted column in **Fig. 3.1**). Another amino acid important for catalysis recently identified in *E. coli* (Frueauf *et al.*, 2001), was also conserved in *C. glutamicum* GlgC at Asp-126. The domain important for nucelotidyltransferase activity was found to be N- terminal, between amino acids 8-279 in *C. glutamicum,* and includes the three conserved motifs for this type of enzyme.

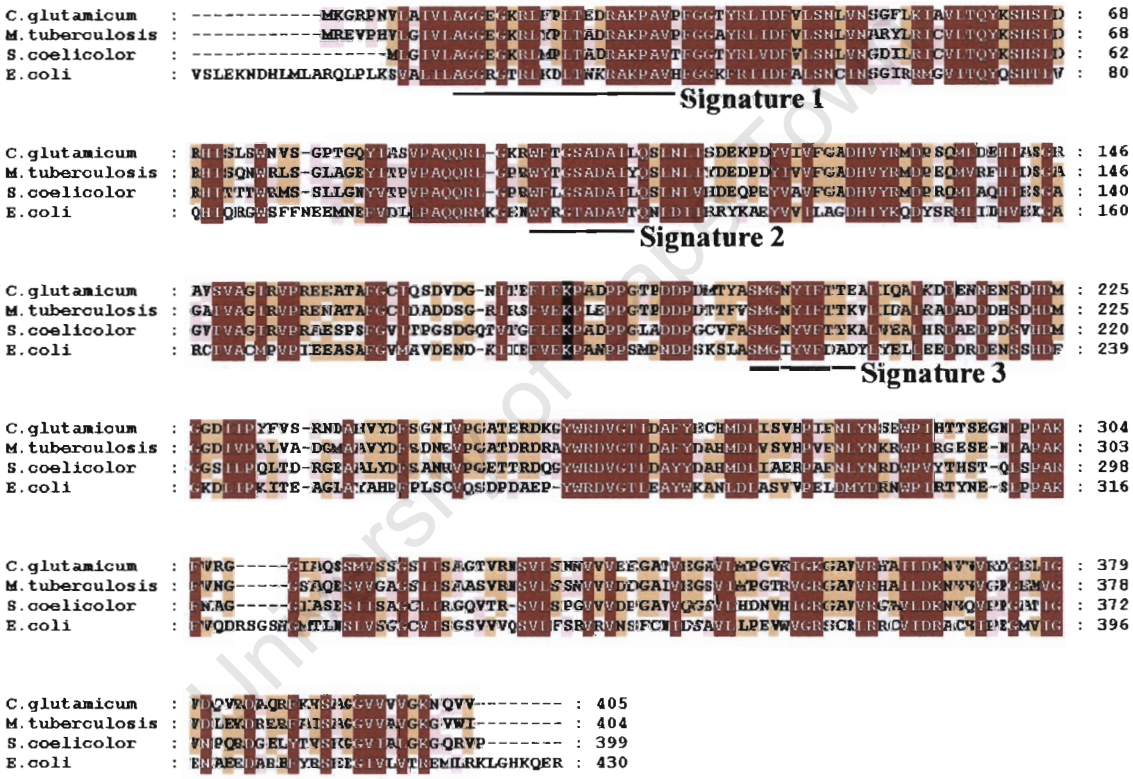

Figure 3.1. Multiple amino acid alignment of *C. glutamicum* GlgC polypeptide with that of other GlgC bacterial polypeptides. The ADP-glucose pyrophosphorylase signatures 1, 2 and 3 considered important in allosteric regulation and substrate binding, are underlined. The amino acid, *E. coli* Lys-195, conserved in both bacteria and plants, is highlighted in black.

3.4.1.2 Alignment of C. glutamicum GlgA amino acid sequence

The *C. glutamicum glg*A gene encodes a 409 aa predicted glycosyltransferase with the given IUP enzyme nomencleture, EC 2.4.1.21 (Nakagawa, 2002). An alignment of the GlgA polypeptide was computed with putative glycosyl transferases from *M. tuberculosis* (59%) and *S. coelicolor* (49%) and with the previously characterised *glg*A from *E. coli* (24%) (Kumar *et al.*, 1986). The *C. glutamicum glg*A polypeptide had a very low identity to the polypeptide from *E. coli* yet the protein family domain was confirmed to belong to glycosyl transferases using nucleotide diphospho-sugar, nucleotide monophospho-sugar and sugar phosphates [EC: 2.4.1.-] to form glycosidic bonds with specific acceptor molecules. The classification was according to Campbell *et al.* (1997), where they classified these glycosyltransferases based on amino acid sequence similarity. In the *C. glutamicum* GlgA polypeptide, this region lies between amino acids 209-387 and corresponds to the same C-terminal regions of the polypeptides of the other two actinomycetes (domain underlined in black in **Fig. 3.2**) and of *E. coli*. GlgA polypeptides characterised in *E. coli* (Kumar *et al.*, 1986), *Rb. Sphaeroides* (Igarashi and Meyer, 2000), and *R. tropici* (Marroqui *et al.*, 2001) were also classed in the glycosyl transferase family 1, even though the percentage identity they had with GlgA from *C. glutamicum* was less than 25% on the protein level.

The gene from *C. glutamicum,* according to sequence analysis, has two possible translational start sites seperated by 60bp (starred amino acids in **Fig. 3.2**). A putative ribosomal binding site was not identifiable near either translational start. The alignment with similar but putative polypeptides from *M. tuberculosis* and *S. coelicolor* speculates the translational start to be the second methionine, with a polypeptide size of 390 bp. According to the complete genome sequence annotation by Kalinowski *et al.* (2003) this shorter polypeptide (Accession number BX927151; Protein ID CAF19823.1) had been designated as the putative *glg*A gene. The annotation according to Kalinowski and co-workers was the most recently released (27th July 2004), yet no EC number has been given to this 390 aa polypeptide. The *C. glutamicum* GlgA has been very recently confirmed to be involved in glycogen synthesis (Tzvetkov *et al.*, 2003). Nevertheless, the

gene, *glg*A, had been disrupted (Tzvetkov *et al.*, 2003) and no glycogen was measurable confirming its function in glycogen synthesis.

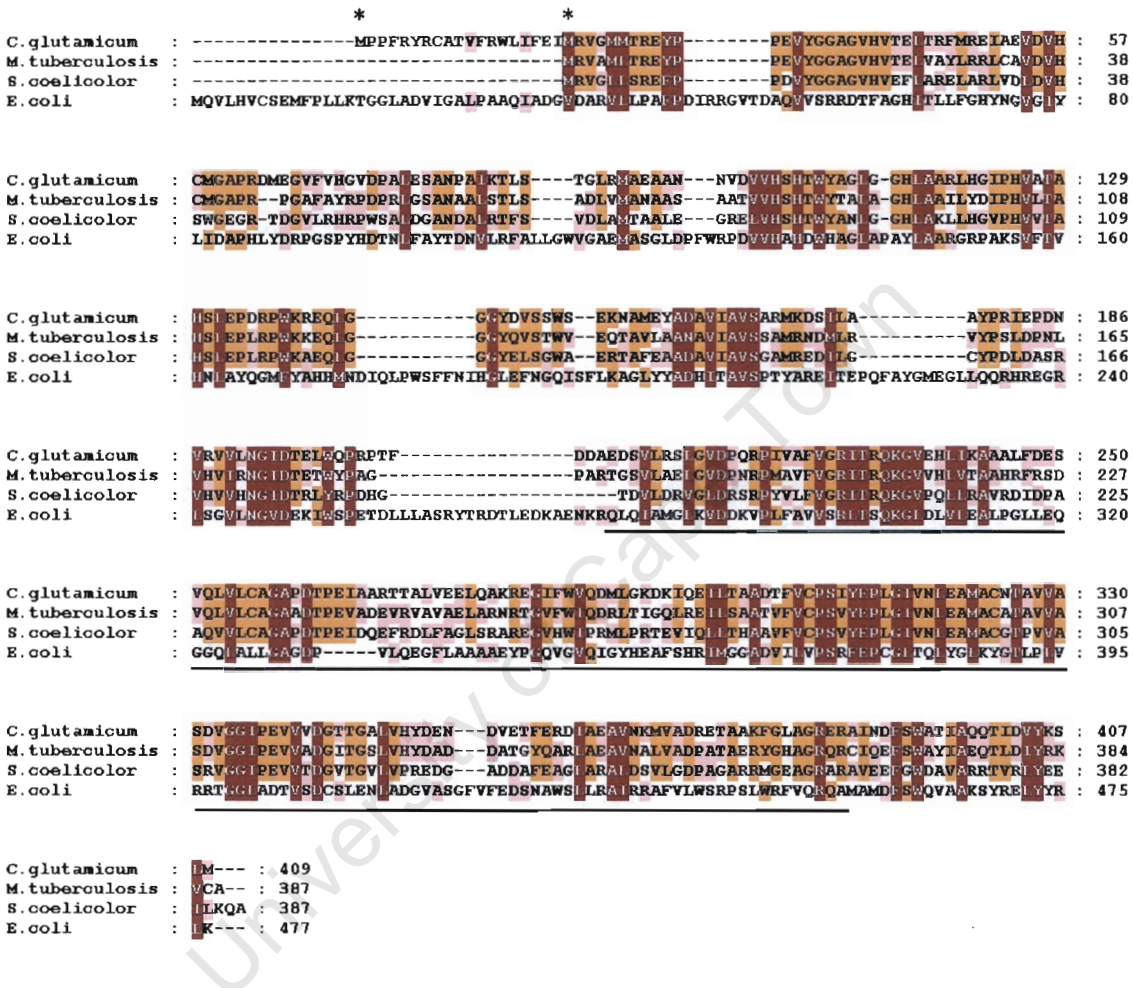

Figure 3.2. Amino acid multiple alignment of the *C. glutamicum glg*A gene product with that of two *glg*A polypeptides of related species. The domain involved in glycosyl transferase activity is underlined in black and the two putative translational starts are starred for *C. glutamicum.*

3.4.2 Chromosomal arrangement of *glg*A and *glg*C in *C. glutamicum* and transcriptional analysis

With the recent availability of the genome sequence of *C. glutamicum* (GenBank NC_00345) the genes surrounding *glg*A and *glg*C could be analysed. The chromosomal arrangement of these genes are depicted in **figure 3.3 A**. The genes encoding glycogen synthase and ADPGlc PPase are shown to be transcribed divergently from each other. The ORF for *glg*A encodes a DNA sequence size of 1170 bp (390 aa) and *glg*C has a DNA sequence size of 1217 bp. The region between the two genes possibly encodes a divergent promoter region able to control the expression of both genes. Possible ribosomal binding sites for both genes were not easily identified from sequence analysis of this region. This chromosomal region had been described in a recent publication (Tzvetkov *et al.*, 2003), where the researches suggested the start codons of *glg*A and *glg*C were separated by 51 bp, but we propose the separation to be at least 97 bp based on the amino acids sequence of 409 aa (Nakagawa, 2000). In addition, the GlgA protein has also been predicted to encode a 390 aa polypeptide from the genome sequence by Kalinowski *et al.* (2003). The intergenic distance between the genes would then be increased by 20 amino acids, to 157 bp. In comparison with the other two related actinomycetes, *M. tuberculosis* and *S. coelicolor*, by alignment (**Fig. 3.2**), we propose the intergenic region between *glg*A and *glg*C to be at least more than 51 bp as previously published. Mapping the transcriptional start would help define the promoter region and the true translational start for these genes. The gene directly upstream of *glg*A is transcribed in the same direction and has been annotated as a putative sucrose hydrolase. The region between the glycogen synthase and putative sucrose hydrolase is 147 bp. The gene immediately upstream of *glg*C has been annotated as a methyl transferase (NCgl1074) and is transcribed in the opposite direction to *glg*C. A gene further downstream of *glg*C is transcribed in the same direction and encodes a putative RNA polymerase sigma factor (NCgl1075).

To confirm the sizes of the transcripts from the *glg*A and *glg*C genes, Northern hybridisation was performed. Total RNA was obtained from *C. glutamicum* cells grown on minimal media containing 1% glucose. An internal 550 bp *glg*A specific probe (**Fig.**

3.3 A) was made from the vector pK19IMA (**Table 3.2**), and hybridised strongly with RNA isolated from *C. glutamicum* wild type (WT) (**Fig. 3.3 B**, 6 µg RNA, lane 3). A positive transcript was included in the experiment and took the form of mRNA extracted from *C. glutamicum* harbouring pEKglgA. This strain overexpressed the full sequence, of *glg*A on a plasmid which was induced with addition of IPTG to the growth media. The probe hybridised with a *glgA* mRNA transcript (lane1) synthesised from the inducible overexpression vector generating a band of ~ 1200 nt. The size of the band generated with RNA from *C. glutamicum* WT was slightly larger and the native transcript size was estimated at ~ 1230 nt. The difference in mobility of these bands may be due to different RNA concentrations of specific RNA but still confirms the monocistronic transcription of *glgA* mRNA in *C. glutamicum,* and agrees well with the predicted size of the gene which is 1170 bp.

The *glg*C mRNA transcript was similarly analysed and an internal specific probe, 371 bp *Sal*I-*Bam*HI fragment (**Fig. 3.3 A**) from pK19IMC (**Table 3.2**) hybridised with RNA isolated from *C. glutamicum* WT. Two concentrations of RNA were loaded on the gel and hybridised with the probe. A band was visible with 4 µg of RNA in lane 2 and 6 µg of RNA in lane 3 (**Fig. 3.3 C**). The size of a transcript of ~ 1250 nt was confirmed against a RNA marker, confirming the predicted sizes for the gene of 1210 bp. The *glg*C transcript was therefore also transcribed monocistronically, which seemed likely since the gene directly downstream of *glg*C would be transcribed in the opposite direction (**Fig. 3.3 A**).

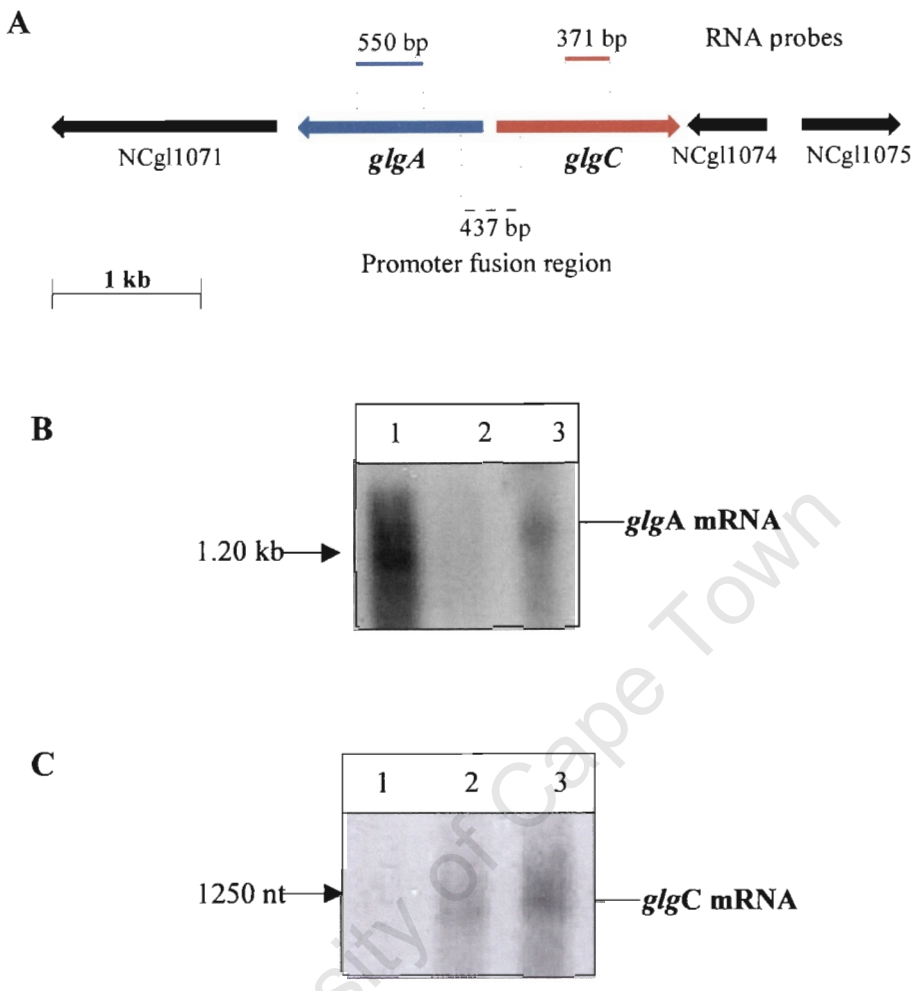

Figure 3.3 The gene arrangement of *glg*A and *glg*C on the chromosome of *C. glutamicum* and Northern blots. A: Chromosomal arrangement. Annotation of the genes are as follows: NCgl1071: putative sucrose hydrolase (*csc*A); NCgl1074: putative methyl transferase; NCgl1075: putative RNA polymerase sigma factor, ECF family. **B: Northern blot analysis of *glg*A expression.** Hybridised with a specific *glg*A internal probe (550 bp). Lane 1: positive control: 1 µg RNA extracted from *C. glutamicum* overexpressing *glg*A on a plasmid. Lane 2 and 3 are WT RNA: 4 µg RNA in lane 2; 6 µg RNA in lane 3. **C: Northern blot analysis of *glg*C expression.** RNA extracted from *C. glutamicum* was hybridised with a *glg*C specific probe (371 bp). Lanes 2 and 3 represent 4 and 6 µg, respectively. Sizes corresponding to a RNA reference marker are indicated on the left of the Northern blots. The intergenic region cloned for promoter analysis is shown by the dashed line (437 bp).

3.4.3 Multiple alignments of GlgB and GlgE and transcriptional analysis

Two further enzymes involved in synthesis and degradation of glycogen were analysed. These enzymes have opposite functions and are responsible for generating branching points α(1→6) (*glg*B) and for removing branches (*glg*E) from the α(1→4) linked glycogen chains.

3.4.3.1 Analysis of the deduced GlgB amino acid sequence

A comparison of the *C. glutamicum* glycogen branching enzyme (GlgB) amino acid sequence with others contained in the genetic databases indicated that it shares a significant identity with known GlgB branching enzymes (**Fig. 3.4**). Glycogen branching enzyme, [EC 2.4.1.18], belongs to the α-amylase family of enzymes (Romeo *et al.*, 1988) and three domains have been identified by secondary structure analysis (Matsuura *et al.*, 1984); Buisson *et al.*, 1987). An amino-terminal domain involved in the size of the glucan chain transferred; a carboxy-terminal domain involved in substrate preference; and a central (α/β) barrel involved in catalysis. The α-amylase catalytic domain in the *C. glutamicum* branching enzyme is underlined in black in the **figure 3.4**, betweeen amino acids 256 and 596 (Falquet *et al.*, 2002). Amino acids in the catalytic centre of branching enzyme, shown to be conserved for various organisms (Abad *et al.*, 2002), lie at positions Tyr^{303}, Asp^{338}; His^{343}; Arg^{406}; Asp^{408}; Glu^{461}; Glu^{462}; His^{528}; Asp^{529}, in the *C. glutamicum* branching enzyme. The recent crystallisation of a truncated, but active, *E. coli* branching enzyme revealed the first three-dimensional structure of this enzyme and more information concerning functions of the three domains are now available (Abad *et al.*, 2002). Analysis of the overall amino acid sequence identity revealed the highest similarities with the Gram-positive actinomycetes, *M. tuberculosis* (57% identity) and *S. coelicolor* (56% identitiy) in comparison to the well described enteric *E. coli* GlgB enzyme (45% identity). Two GlgB proteins are described in *S. coelicolor* (Bruton *et al.*, 1995) and both have a 56% similarity to the *C. glutamicum* GlgB. The predicted branching enzyme of *C. glutamicum* shares much amino acid similarity to the *E .coli* known branching enzyme and therefore most likely shares the role of creating α(1→6) branch points in α(1→4) glucan linked molecules.

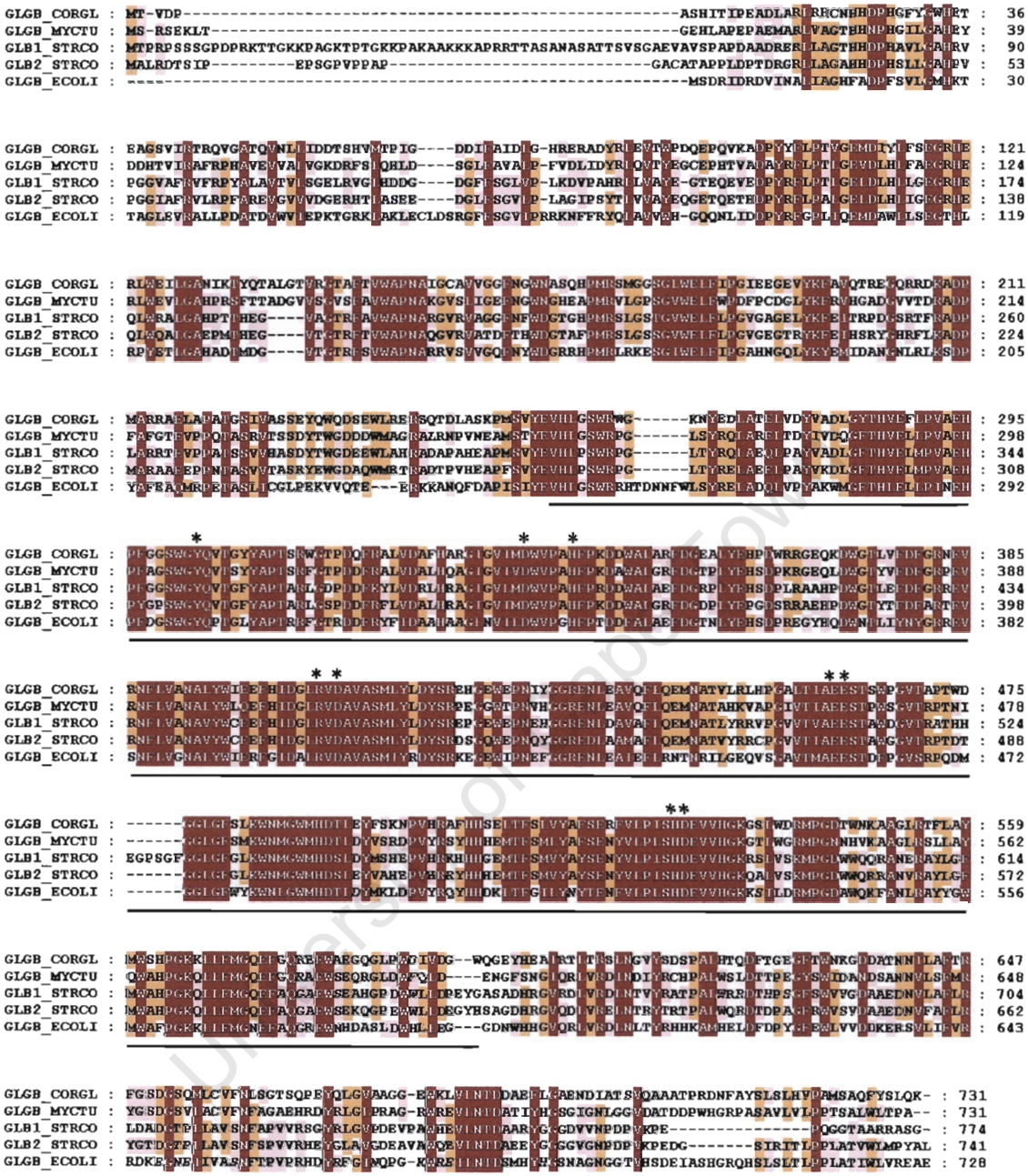

Figure 3.4. Amino acid similarities of *C. glutamicum* GlgB with that of other GlgB enzymes. The origin of these proteins are as follows: GLGB_CORGL: *C. glutamicum glg*B; GLGB_MYCTU: *M. tuberculosis glg*B; GLB1_STRCO: *S. coelicolor glg*B1; GLB2_STRCO: *S. coelicolor glg*B2; GLGB_ECOLI: *E. coli glg*B. The catalytic domain of *C. glutamicum* is underlined in black and the amino acids involved in catalytic activity are starred.

3.4.3.2 Analysis of the putative GlgE amino acid sequence

The amino acid sequences of GlgE polypeptides with which the putative *C. glutamicum glg*E gene product were aligned have been characterised at the functional level in *M. smegmatis* (Belanger and Hatfull, 1999) and at the genetic level in *S. coelicolor* (Schneider *et al.*, 2000) (**Fig. 3.5**). The *pep*1 gene is duplicated in *S. coelicolor* (Schneider *et al.*, 2000) and designated *pep*1 I and *pep*1 II (see Chapter 1, figure 1.4). No significant similarity was found between the *C. glutamicum* GlgE and the functional equivalent *E. coli* GlgX enzyme (results not shown), but according to Pujadas and Palau, (2001) it is not uncommon for members of the GH-13 (glucoside hydrolase 13) family to have common enzyme activity but unsimilar sequences. However, *C. glutamicum* GlgE has 60% identity to *M. smegmatis* GlgE enzyme, the function of which has been previously reported (Belanger and Hatfull, 1999). The GlgE polypeptide also had 51 and 50% identity to the Pep1 I and Pep1 II polypeptides from *S. coelicolor*, respectively. Another argument in favour of its involvement in glycogen degradation would be the chromosomal arrangement it has with *glg*B. In *E. coli*, the branching and debranching enzymes, *glg*BX, are transcribed on a single mRNA transcript (Romeo and Preiss, 1988) and so it is possible that *C. glutamicum glg*E, which lies downstream of *C. glutamicum glg*B, also forms an operon with *glg*E. In fact, the genes *glg*E and *glg*B in *M. smegmatis* also lie in tandem, although it is not yet known whether they are transcribed as a single transcript (Belanger and Hatfull, 1999). The *C. glutamicum* GlgE reported in this study was based on that annotated by Kalinowski *et al.* (2003), with the predicted polypeptide size of 675 aa which stands in better agreement than the GlgE polypeptide predicted by Nakagawa (2002) of 498 aa.

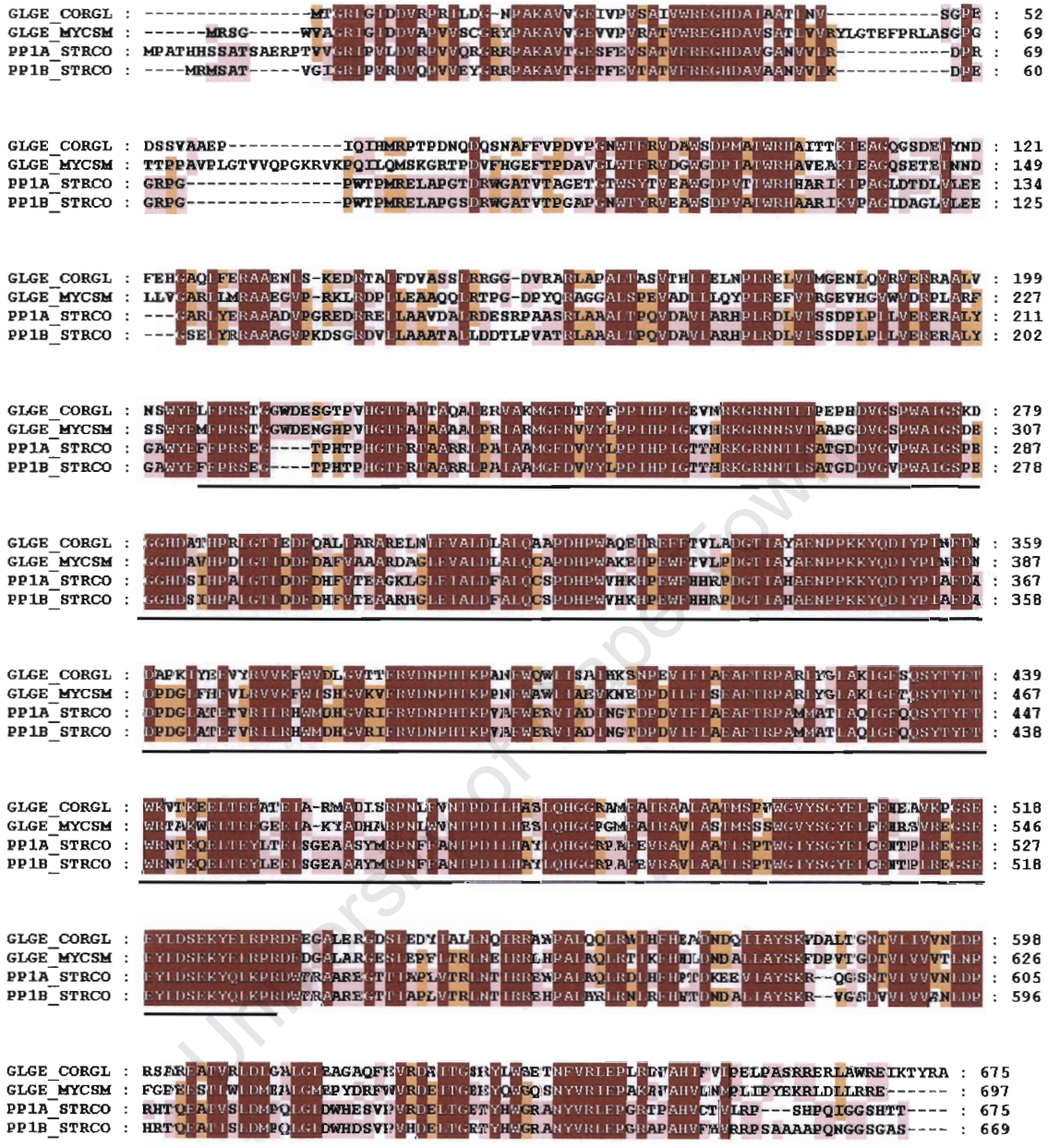

Figure 3.5. Amino acid sequence multiple alignment of the *C. glutamicum glg*E gene product (GLGE_CORGL) with that of published and related glucan hydrolases from *M. smegmatis* (GLGE_MYCSM), *S. coelicolor pepI*A (PP1A_STRCO) and *S. coelicolor pep*1B (PP1B_STRCO). The region identified as belonging to the alpha_amylase protein family (pfam) is underlined in black.

3.4.4 Chromosomal arrangement of *glg*E and *glg*B and transcriptional analysis in *C. glutamicum*

The structural genes of *glg*E and *glg*B where also identified in the genome of *C. glutamicum* by BLAST searches and the flanking region analysed. The flanking region is set out in the **figure 3.6 A**. Two genes downstream of *glg*B have homologies to sulphonate binding and export in *Pseudomonas putida* and are called *ssu*A and *ssu*B, respectively. Both genes are transcribed in the opposite direction to *glg*E and *glg*B. The neighbouring gene to *glg*B is also transcribed in the opposite direction and has a strong similarity to a ferric enterobactin transport protein from *E. coli.* The region between the translational end of *glg*E and the translational start of *glg*B consists of only 37 bp, and is most likely too short to contain a promoter region able to drive the transcription of *glg*B. Although it can also be argued that even though the length of the non-coding region between *glgE* and *glgB* is only 37 bp, there might be a specific promoter of *glgB* present, as intragenic promoters within operons were found in *C. glutamicum* even in gene clusters where much shorter intergenic regions were present (e.g. *hom-thrB, trp* operon) (Mateos *et al.*, 1994;O'Gara and Dunican, 1994). In order to test whether *glgE* and *glgB* are cotranscribed, Northern blot analysis was done to confirm the transcriptional mRNA size of the two genes, and the intergenic region between was also amplified by RT-PCR. For Northern analysis, RNA was also extracted from *C. glutamicum* WT and *C. glutamicum* harbouring pEKglgE (see Table 3.2 in the Materials and Methods for a description of the plasmid). A *Pst*I specific probe (486 bp **Fig. 3.6 A**) was generated from the plasmid pEKglgE (Section 3.3.9) and hybridised with the RNA. This probe hybridised strongly to a *glgE* mRNA transcript synthesised from a plasmid under IPTG induction and a transcript size of 2.03 kb was detected as seen in lane 1 of **figure 3.6 B**. Relative to the band in lane 1, a transcript of ~ 4 kb was visible in lane 3, suggesting the expression of *glg*E and *glg*B as a single mRNA transcript.

To confirm a single mRNA transcript for *glg*E and *glg*B, RNA was isolated from *C. glutamicum* cultures grown on minimal media containing 2% glucose and reverse transcriptase experiments performed. The intergenic region was amplified by synthesising cDNA from the RNA template and subsequent amplification of the region

with specific primers. The primers, RT-revB2 and DM-glgE-for3 (**Table 3.3**), amplified a 940 bp product across the intergenic region, (**Fig. 3.6 C,** lane 4). To exclude the possibility of contaminating DNA in the RNA sample, a negative control was included where the reverse transcriptase had been heat inactivated and the reaction generated no product (lane 1). Thus the PCR products seen in lane 2 - 4 were due to the amplification of cDNA transcribed from RNA. Lane 2 and lane 3 were positive controls of *glg*E and *glg*B, respectively, where an internal region for each gene was amplified to prove functionality of the reaction. Although equal volumes of all RT-PCR reactions were loaded on the agarose gel, it appeared *glg*E expression might be higher than *glg*B expression due to the brighter RT-PCR band seen in lane 2. However, the RT-PCR was not quantitative and the intensity of the bands varied in repeated experiments. The same pattern, seen in **figure 3.6 C**, was found with RT-PCR experiments on minimal media containing acetate as the sole carbon source and on medium containing a mixed glucose/acetate carbon source (results not shown).

From the experiments described above, we concluded that *glg*E and *glg*B are transcribed as a single operon. The glycogen genes analysed in *C. glutamicum* are therefore found in two clusters unlike the arrangement of these genes in *E. coli* (Preiss, 1996a). These clusters were not found in the near vicinity of each other and except for the sucrose hydrolase downstream of *glg*A, no other neighbouring genes are involved in carbohydrate metabolism. Another gene that is important for glycogen degradation is a glycogen phosphorylase which removes single glucose molecules from the reduced end of the polymer. This gene was not found clustered with the other glycogen metabolic genes described here.

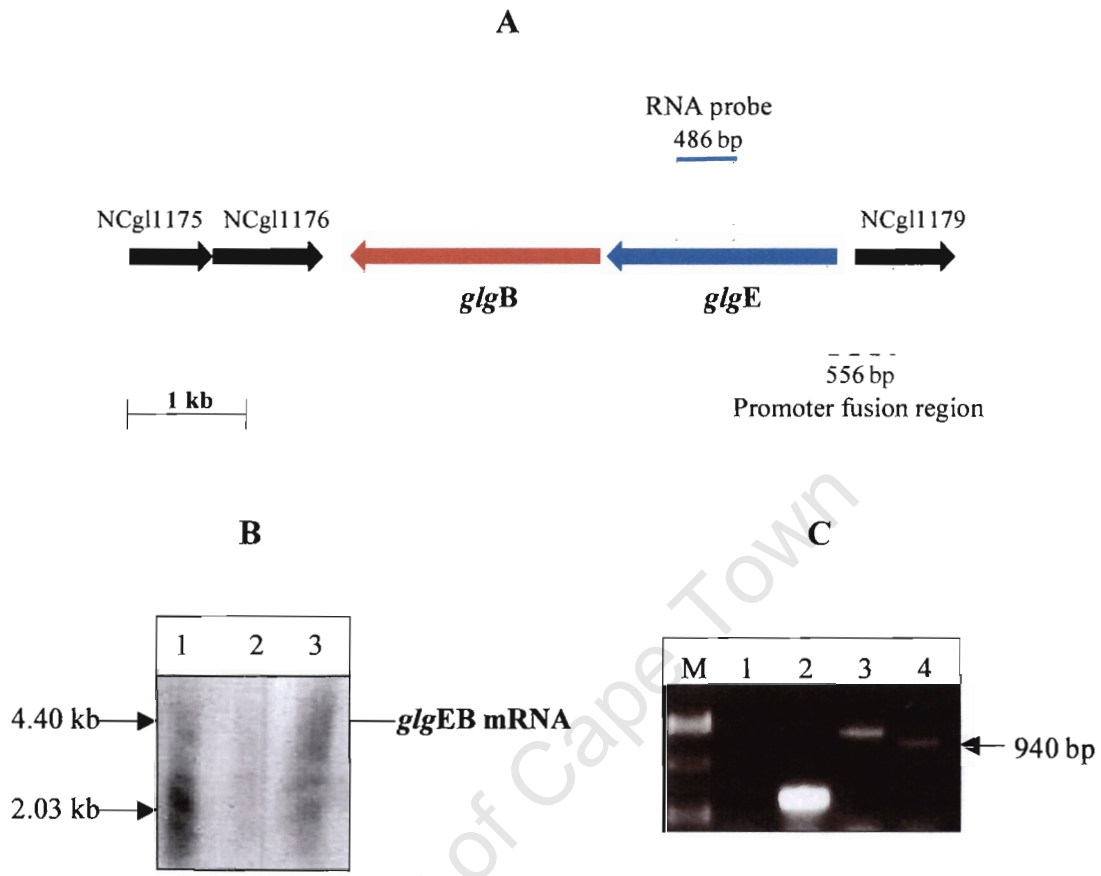

Figure 3.6. Organisation of *glg*B and *glg*E on the *C. glutamicum* chromosome, Northern analysis and reverse transcriptase reactions. A: Chromosomal arrangement. The *glg*B and *glg*E are flanked by the following genes: NCgl1175: a putative *ssu*B; NCgl1176: a putative *ssu*A; NCgl1179: a putative *fep*C. For description of the genes see text. **B: Northern blot.** Lane 1: 1 µg of RNA extracted from *C. glutamicum* overexpressing *glg*E (positive control). Lanes 2 and 3: 4 and 6 µg of *C. glutamicum* RNA, respectively. The probe hybridised to a band at ~ 4 kb in lane 3. Sizes according to a RNA marker are shown on the left of the Northern blot. **C: A 0.8% agarose gel of RT-PCR.** Lane 4: RT-PCR of the 940 bp intergenic region between *glg*E and *glg*B amplified with the primers DM-glgE-for3 and RT-rev2 from RNA extracted from *C. glutamicum* exponential phase grown cells. Lane 1: negative control of heat inactivated reverse transcriptase. Lanes 2 and 3: positive controls, where internal 540 bp and 1.01 kb products were amplified within *glg*E and *glg*B, respectively. Lane M: *Pst*I digested λ-DNA-size marker. The region cloned to test promoter activity is represented by the dash line (556 bp).

3.4.5 Reporter gene analysis of the promoter regions upstream of *glg*A, *glg*C and *glg*EB

The promoter regions of *glg*A, *glg*C and *glg*EB were tested for activity during different growth stages of *C. glutamicum* on complex media with and without glucose, as well as on minimal media with different carbon sources. Transcriptional fusions were constructed with a promoterless chloramphenicol acetyl transferase reporter gene as described in the Materials and Methods (Section 3.3.8) and the resulting plasmids were transformed into *C. glutamicum* by electroporation. Reporter gene activity was quantified in cell free extracts as a measure of choramphenicol acetyl transferase gene expression under the control of the different promoters. The promoter fusion construct, pETPA, contained a 437 bp fragment of *glg*A, that extended 255 bp upstream and 182 bp downstream of the putative translational start (this region also contained the second putative translational start discussed in 3.4.1). While the promoter fusion construct, pETPC, contained the same 437 bp fragment, cloned in the opposite direction (for a diagramatic representation see **Fig. 3.3 A** dashed line). The analysed promoter region of *glg*C extended 279 bp upstream and 158 bp downstream of the putative translational start. The third promoter fusion construct, pETPEB, contained a 556 bp fragment that extended 508 bp upstream and 49 bp downstream of the putative translational start and is represented by the dashed line in **figure 3.6 A**. A negative control of the vector pET2 without an insert was also electroporated into *C. glutamicum* and CAT activity measured in the cell extract.

3.4.5.1 Promoter activity on complex media with and without glucose

All three promoters were active during early and late exponential growth phase as well as stationary phase for cultures grown on complex medium and complex medium + 2% glucose (**Fig. 3.7**). The control plasmid, without insert, showed specific CAT activity of < 0.01 U.mg^{-1} protein indicating the expression of chloramphenicol acetyl transferase measured in the other plasmids was due to the activity of the regions cloned upstream of the CAT gene. Maximal gene expression was measured for all three promoters at 24 hours. The promoter activity on media containing glucose was approximately 1.5 times less than on media without glucose. This small difference in expression was not

considered significant, since glycogen was not accumulated in cells grown on complex medium without a carbon source as discussed in Chapter 2. Rather, promoter activity from these promoter regions, indicated they were constitutively expressed in the cell regardless of the availability of glucose. The promoter regions upstream of *glg*A, *glg*C and *glg*EB also showed little regulation during the growth phase of *C. glutamicum* and promoter activity remained relatively low under the conditions tested.

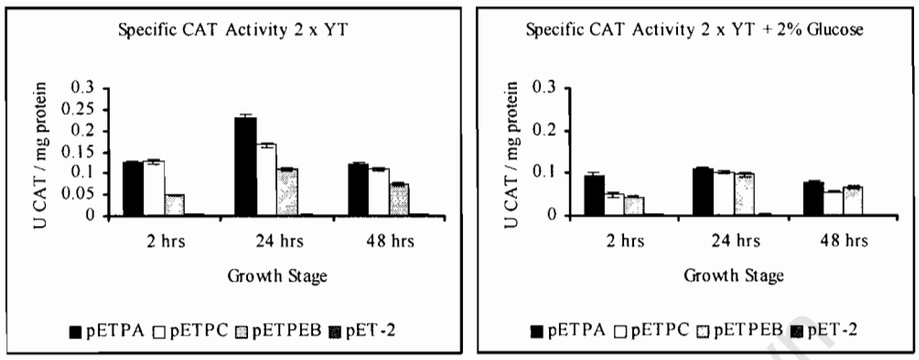

Figure 3.7 Chloramphenicol acetyl transferase activity on complex media, with and without glucose, of the three promoter constructs pETPA, pETPC, and pETPEB. Samples were harvested and analysed during the exponential, stationary and late stationary growth phase of *C. glutamicum*. The control plasmid, pET-2, contained no additional DNA and the read through activity measured was always <0.01 U CAT.mg^{-1} protein for all time points.

3.4.5.2 Promoter activity on different carbon sources

Promoter gene expression of the three constructs were also compared on different carbon sources. Samples were harvested in mid-exponential phase from *C. glutamicum* cultures grown on minimal medium batch culture containing either glucose, maltose, acetate or lactate with an initial concentration of 1% in the culture medium. All promoters showed high specific CAT activity of approximately 0.420 U.mg^{-1} protein when grown on minimal media with glucose (**Table 3.4**). Acetate and lactate grown cells had 2.5-3.5 times lower promoter gene expression compared to glucose. Interestingly promoter activity of all the genes was approximately 1.5 times higher in maltose grown cells than

glucose grown cells. Glycogen was also accumulated 1.5 times more in the cell extract of *C. glutamicum* when grown on maltose compared to glucose. Reporter gene activity measured in the cell extract containing the vector without insert was always less than 0.005 U CAT/mg protein.

Table 3.4 Specific chloramphenicol acetyl transferase (CAT) activity from cell extracts of the *C. glutamicum* strains, 13032 (pETPA), 13032 (pETPC), and 13032 (pETEB) taken during the logarithmic growth phase of respective cultures. Standard deviations were ± 0.015 for plasmids containing inserts.

***C. glutamicum* strain**	**Carbon source**	**Specific CAT activity ($U.mg^{-1}$ protein)**
	Glucose	0.395
13032 (pETPA)	Maltose	0.506
	Acetate	0.113
	Lactate	0.160
	Glucose	0.446
13032 (pETPC)	Maltose	0.701
	Acetate	0.162
	Lactate	0.158
	Glucose	0.467
13032 (pETPEB)	Maltose	0.625
	Acetate	0.083
	Lactate	0.117
	Glucose	<0.005
13032 (pET-2)	Maltose	<0.005
	Acetate	<0.005
	Lactate	<0.005

A shortened promoter region (355 bp) upstream of *glg*EB was cloned by using an internal *Sal*I site within the upstream region. *Sal*I –*Bam*HI region extending 304 bp upstream and 49 bp downstream of the putative translation start was also constructed and this clone showed the same promoter activity as pETPEB1 (results not shown). However it can be assumed the transcriptional start of *glg*EB must lie within this 304 bp region upstream of the translational start.

3.5 Discussion

Subsequent to the physiological studies presented, four genes were identified to be involved in glycogen metabolism in *C. glutamicum*. These genes encoded an ADPGlc PPase, glycogen synthase, glycogen branching enzyme and a glycogen debranching enzyme. These proteins or polypeptides were aligned with other known enzymes of the same function. In three cases, these genes or enzymes had been characterised in *E. coli,* these were ADPGlc PPase (GlgC), glycogen synthase (GlgA) and glycogen branching enzyme (GlgB). The glycogen debranching enzyme (GlgE) was most similar to the *M. smegmatis* GlgE which had been named the functional, but not the genetic equivalent of GlgX in *E. coli* (Ballinger and Hatfull, 1999). GlgC from *C. glutamicum* encoded a 405-aa polypeptide with a predicted M_r of 43.86 kDa, and was in agreement with the GlgC polypeptide encoded by *E. coli* of approximately 50 kDa (Salamone *et al.*, 2002). The amino acid sequence shared only 36% identity to the well characterised *E. coli* polypeptide, but had in common the three signatures of a ADPGlc PPase as well as critical conserved amino acids for substrate binding and allosteric regulation (**Fig. 3.1**). The predicted glycosyl transferase [EC 2.4.1.21] from *C. glutamicum* was denoted to belong to the glycosyl transferase family 1. The glycogen synthase from *C. glutamicum* had very little amino acid similarity to the GlgA of *E. coli* (24%) and the predicted size of the polypeptide was also in question. However, it has been argued that amino acid polypeptides with more than 20% or more identity have common ancestory (Doolittle, 1981). During the course of this work, the function of *glg*A had been confirmed by Tzvetkov *et al.*, (2003) in relation to the link between trehalose and glycogen. An integration mutant of *glg*A produced less trehalose and glycogen in comparison to the wild type when grown on sucrose (Tzvetkov *et al.*, 2003). The ORFs encoding *glg*A and

*glg*C were clustered together but transcribed divergently by possible overlapping promoters (**Fig. 3.2**). The transcript for *glg*C hybridised at approximately 1250 nt. Both *glg*A and *glg*C were transcribed monocistronically. The promoter region between the two genes was cloned in both directions into the chloramphenicol acetyltransferase expression vector pET2 and tested for activity (section 3.4.3). Both orientations of the promoter drove transcription of the reporter gene, therefore the region between *glg*A and *glg*B encoded functional promoters in both directions. Divergent and overlapping promoters are not uncommon in *C. glutamicum* (Pátek *et al.*, 2003) and the genes transcribed are usually involved in the same pathway one of the more recent examples being P-*ace*A and P-*ace*B (Gerstmeir *et al.*, 2003) involved in the glyoxylate cycle. We were not able to determine whether this 157 bp region encodes overlapping promoters. Mutation, studies and primer extension would help define this region further.

Unlike the clustering of glycogen encoding genes in the same region in other bacteria (Igarashi and Meyer, 2000), the ORFs encoding *glg*E and *glg*B were not found clustered with *glg*A and *glg*C. In fact except for the sucrose hydrolase directly downstream of *glg*A (**Fig. 3.3 A**), no other genes involved in glycogen or carbohydrate metabolism were identified near these clusters. Glycogen branching enzyme (*glg*B) from *C. glutamicum* was compared to the glycogen branching enzyme from *E. coli* where they shared 45% identity. The *E. coli* branching enzyme had been recently well characterised through the obtaining of a partial crystal structure (Abad *et al.*, 2002). We suggest therefore the encoding of this 731 aa protein with a molecular weight of 82.59 kDa as the *C. glutamicum* branching enzyme. The predicted glycogen debranching enzyme of *C. glutamicum* had no genetic equivalent in *E. coli*. However, *glg*E had been previously characterised in *M. smegmatis* (Belanger and Hatfull, 1999) which had 60% amino acid identity to the *C. glutamicum glg*E. The GlgE from *M. tuberculosis* has been subsequently renamed as a Pep1 polypeptide, because of its high similarity to the duplicated Pep1 enzymes in *S. coelicolor* (Schneider *et al.*, 2000). The *S. coelicolor* Pep1 enzymes had also about 50% identity to that of the polypeptide designated GlgE in *C. glutamicum*. The difference in nomencleture is likely due to the slightly different function given to GlgE and Pep1 enzymes. In *Mycobacterium* the authors eluded to the

degradation of glycogen by GlgE, and the release of glucose available for glycolysis (Belanger and Hatfull, 1999), whereas in *Streptomyces* the breakdown of glycogen was instead associated with the subsequent conversion of maltose units to trehalose by TreS (Schneider *et al.*, 2000). The intergenic region between *glg*B and *glg*E was estimated at only 37 bp, and the probability that these two genes were transcribed as a single operon was investigated. Northern analysis and RT-PCR confirmed this, while primer extension analysis was unsuccessful and the promoter region could not be defined closer than 304 bp upstream of the *glg*B translational start. The co-ordinated expression of the ~ 2.2 kb *glg*B, branching, and ~ 2.02 kb *glg*E, debranching genes from one promoter confirmed the synchronised expression of synthetic and degradative enzymes. The expression of both a degrative and synthetic gene on one transcript indicates that these proetins are likely post-transcriptionally regulated. In fact, in *E. coli* the *glg*BX messenger transcript has been shown to be post-transcriptionally regulated by the protein CsrA (Liu *et al.*, 1995).

Promoter activity was measured by cloning the region upstream of each gene in front of a reporter gene. No transcriptional regulation of these genes in *C. glutamicum* was evident when cells where grown on complex media with or without glucose. A similar situation was observed in *E. coli* where expression of glycogen genes on complex media or complex media with glucose was similar on entry into stationary phase (Hengge and Fischer, 1992). However, the promoter activities measured with cells grown on acetate and lactate were all reduced between 2.5-3.5 times in comparison to those grown on glucose. Growth on maltose, in contrast, caused the upregulation of all three constructs by between 1.2-1.6 times. Glycogen content in these cells were also decreased or increased by the same amounts according to the substrate in the growth medium. This implies transcriptional regulation of these genes in terms of glycolytic or gluconeogenic carbon sources in *C. glutamicum*. In *E. coli* glycogen accumulation is under positive transcriptional regulation by GlgS, RpoS, cAMP, CRP, adenylate cyclase, and ppGpp (Preiss and Romeo, 1994; Preiss, 1996a) as discussed in Chapter 1, under Section 1.8. There is also strong negative post-transcriptional control by the carbon storage regulator (CsrA) protein (Preiss and Romeo, 1994; Preiss, 1996a). Database searches using this

CsrA protein against the *C. glutamicum* genome gave no clear indication that this same carbon storage regulator was also found in *C. glutamicum*. In fact, no carbon catabolite repression has yet been identified in *C. glutamicum* (Gerstmeir *et al.*, 2003).

CHAPTER 4

CONSTRUCTION OF MUTANTS IN *glg*B AND *glg*C AND PHYSIOLOGICAL STUDIES

CONTENTS

4.1 Summary

Two mutants in glycogen biosynthesis were constructed in the wild type strain *C. glutamicum* ATCC 13032, generating *C. glutamicum* CgIMB and *C. glutamicum* CgIMC where the *glg*B and the *glg*C genes, respectively were disrupted by insertion of a plasmid through sequence specific homologous recombination. The disruption of each gene was confirmed by Southern hybridisation and physiological studies. The mutants were analysed for growth variation as well as the ability to produce glycogen. Both strains showed a slight deviation in growth rate on minimal media supplemented with glucose but were able to reach the same final cell density as the wild type strain. However, on complex media containing an excess of glucose, the maximum cell density of the WT was approximately 1.5 times higher than both mutants yet the mutants showed no deviation in growth rate on this medium. In the wild type, maximum glycogen concentrations as high as 90 mg glucose/g DW were measured. Both mutant strains were unable to produce glycogen on minimal media containing 1% glucose, and produced significantly less glycogen (maximum ~ 10 mg glucose/g DW) on complex media containing glucose in excess. The genes designated *glg*B and *glg*C therefore have been successfully inactivated by insertion of a non-replicating plasmid and the function of the genes in the synthesis of glycogen in *C. glutamicum* has been confirmed.

4.2 Introduction

In Chapter 2, physiological aspects of glycogen biosynthesis were described and in Chapter 3, four genes from *C. glutamicum* were identified by data base searches and alignments of the relevant amino acid sequences. Inactivation of these genes by gene disruption or gene replacement would help prove their functionality and their role in glycogen metabolism.

The importance of *C. glutamicum* in fermentative production of economically important amino acids had established a need for the development of genetic tools. In the mid 1980's, the first molecular genetic engineering in *C. glutamicum* was reported (Katsumata *et al.*, 1984; Ozaki *et al.*, 1984; Santamaría *et al.*, 1985 and 1987). Transformation techniques, and the construction of plasmid vectors for *C. glutamicum* have since developed into a wide range of available genetic techniques for this organism. Recently the published literature on the tools for genetic engineering in *C. glutamicum* was summarised by Kirchner and Tauch, (2003). Information concerning transformation techniques, cloning of genes for overxpression or promoter regions for analysis as well as the generation of deletions was expounded in the publication. In this Chapter, the inactivation of genes involved in the glycogen biosynthesis pathway in *C. glutamicum* was undertaken. The method of inactivating the two genes, *glg*B and *glg*C, was via the integration of a plasmid within the gene. The principle of this method entailed the cloning of an internal region of the gene into a suitable vector. The vectors, in this case, did not possess an origin of replication for *C. glutamicum* but were able to propagate in *E. coli*. In fact, in this study, the use of a commercial *E. coli* T-tailed cloning vector was also suitable for inactivation of a gene in *C. glutamicum*. Preferred methods of inactivation of a gene would be the deletion of an internal region within the gene or generation of point mutations. We decided to disrupt the target genes by insertion of a non-replicating plasmid. Downstream genes would not be affected by the insertion of a plasmid since neither *glg*B nor *glg*C form an operon with other genes (The genetic arrangements of the glycogen genes in *C. glutamicum* were discussed in Chapter 3 but are given again in the Materials and Methods of this Chapter). Although different vectors were used to inactivate each gene, the principle of generating an integration mutant was

mutant was the same. Both vectors were unable to replicate in *C. glutamicum* and carried a region of DNA homologous to an internal region within the gene that was to be inactivated. A forced integration by homologous recombination would be expected after introduction of the recombinant plasmid *C. glutamicum* and cells harbouring the integrated plasmid were selected for with an appropriate low concentration of antibiotic. The effect of these mutants on growth and glycogen accumulation was of interest and the strains where thus characterised.

Glycogen synthesising genes have been disrupted in other organisms. The insertional inactivation of *glg*C, from the related actinomycete, *S. coelicolor,* led to the accumulation of less glycogen (Cruz Martin *et al.*, 1997) during a specific period of growth. An *E. coli* strain, deficient in glycogen branching enzyme (*glg*B) and unable to form branched forms of glycogen, was used to express branching enzymes from maize where different branching enzymes were found to play an important part in the structure of the polysaccharide formed (Guan *et al.*, 1995). The inactivation of *glg*C and *glg*B in this work would only be the second time the synthesis pathway of glycogen metabolism in *C. glutamicum* would have been disrupted. During this work, glycogen synthase (*glg*A) in *C. glutamicum* was disrupted by Tzvetkov *et al.*, (2003) and they were unable to measure glycogen on sucrose grown cells.

4.3 Materials and Methods

4.3.1 Bacterial strains, plasmids and culturing conditions

The strains and plasmids used in this study are given in Table 4.1.

Table 4.1 Bacterial strains and plasmids

Strain/Plasmid	Relevant characteristics	Reference
Strains		
E. coli DH5α	*supE44, hsdR17, recA1, thi-1, endA1, lacZα, gyrA96, relA1*	Hanahan, 1985
C. glutamicum 13032	Wild type	Abe *et al.*, 1967
CglMB	*C. glutamicum glg*B integration mutant	this work
CglMC	*C. glutamicum glg*C integration mutant	this work
Plasmids		
pDrive	Km^R, non-replicating in *C. glutamicum*	Qiagen
pK19*mobsacB*	Km^R, non-replicating in *C. glutamicum*	Schäfer *et al.*, 1994
pDrlMB	pDrive derivative, 1.043 kb internal fragment of glg*B*, Km^R, oriV	this work
pK19lMC	pK19*mobsacB* derivative, 371 bp internal region of *glg*C, Km^R, oriT (mobilizable), oriV	this work

Cultures of *C. glutamicum* were routinely grown in fluted Ehrlenmeyer flasks for complex media and minimal media containing glucose (Appendix A). Cultures were grown on a rotary shaker at 120 rpm at 30 ^{0}C. The *E. coli* strain, DH5α, was used for cloning, subcloning and propagation of plasmids and was grown in 2xTY broth or agar (Sambrook *et al.*, 2001). Two plasmids, pDrive and pK19*mobsacB*, were used for cloning of PCR generated fragments and transformed into *E. coli* via electroporation. For *E. coli*, kanamycin was added to the growth medium at 50 μg.ml^{-1} to select for plasmids. While mutant strains of *C. glutamicum* were selected on 25 μg.ml^{-1} kanamycin in order to maintain the single copy of the plasmid borne Km^R gene integrated into the chromosome.

4.3.2 General recombinant DNA procedures

All standard DNA modifications and manipulations were performed according to Sambrook *et al.,* (2001). While plasmid DNA, from *E. coli*, was isolated using a modified method of alkali lysis according to Birnboim (1983) or with the GFX-kit for plasmid isolation (Amersham).

4.3.3 Construction of the vectors, pDrIMB and pK19IMC for mutation by integration

The primers used to generate PCR (Appendix B) fragments for cloning are found in Table 4.2.

Table 4.2 Oligonucleotide primers

Primer	Sequence (5'-3')	Restriction site
IM-*glgB*-for	CCCAAGCTTATCCGATGCGTTCTATGG	*Hind*III
IM-*glgB*-rev	CGGGATCCTCAGAGAATGCGTACACC	*Bam*HI
IM-*glgC*-for	ACGCGTCGACACCACGTGTATCGCATGG	*Sal*I
IM-*glgC*-rev	CGGGATCCTGTGGATTGGCCACTCAG	*Bam*HI

A plasmid for integration into *glg*B was generated by amplification of a 1,043 kb internal fragment (**Fig. 4.1**) and cloned directly into the *E. coli* T–tailed cloning vector pDrive from Qiagen according to the manufacturers instructions. The primers for amplification were, IM-glgB-for and IM-glgB-rev, and are listed in **Table 4.2**. The generated plasmid, pDrIMB, was propogated in *E. coli* and subsequently used to generate mutants in *C. glutamicum*.

For construction of the plasmid, pK19IMC, a 500 bp internal fragment of *glgC* was amplified by PCR (**Fig. 4.2**) with the primers IM-glgC-for and IM-glgC-rev, listed in **Table 4.2**. An internal 3' *Sal*I restriction site and the PCR generated 5' *Bam*HI restriction site was used to clone a 371 bp fragment into a pK19*mobsacB* vector, which had been modified with the same enzymes and desphosphorylated according to standard procedures. Both plasmids, pDrIMB and pK19IMC, were sequenced to verify the

cloning of the correct fragments, before construction of the respective mutants. Below, the cloning strategies for generating the plasmids for integration into the genes *glg*B and *glg*C of *C. glutamicum* are depicted.

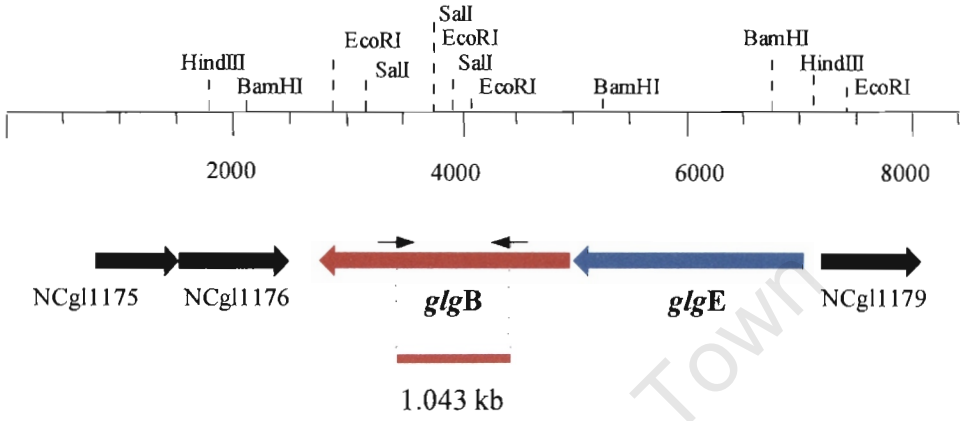

Figure 4.1. The genetic arrangement of *glg*B and *glg*E*. The PCR amplified 1.043 kb internal region of *glg*B was cloned into pDrive to generate pDrIMB, described in section 4.3.3.

*previously shown in results section of Chapter 3 as figure 3.3 A.

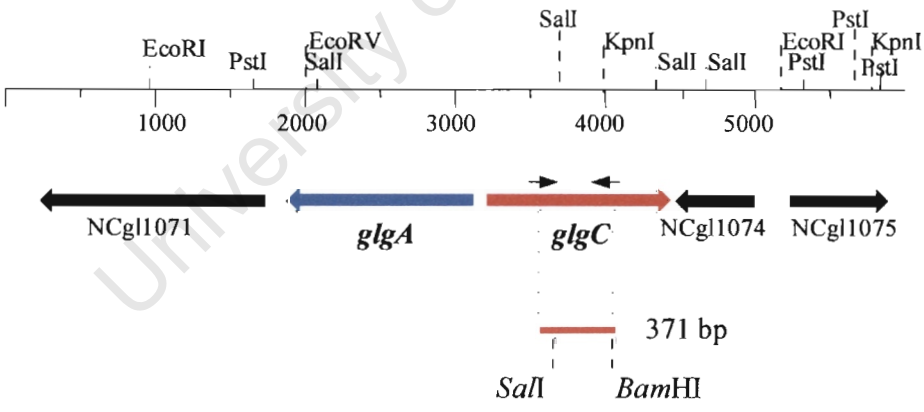

Figure 4.2. The chromosomal arrangement of *glg*C and *glg*A*. The internal region of *glg*C, (500 bp) amplified by PCR. The restricted *Sal*I - *Bam*HI (371 bp) region of *glg*C was cloned into the vector pK19*mobsacB* to generate pK19IMC, described in section 4.3.3.

* previously depicted in Chapter 3 as figure 3.6 A.

4.3.4 Generation of the integration mutant strains in *C. glutamicum*

The plasmids, pDrlMB and pK19lMC, were extracted from *E. coli* DH5α using a modified alkaline lysis method according to Birnhom (1983). The final DNA preparation was precipitated with 100% butanol in order to remove salts that could interfere with the electroporation of the plasmid into *C. glutamicum*. Electroporation followed the protocol of van der Rest *et al.*, (1999). Mutants were selected on LBHIS containing 25 $\mu g.ml^{-1}$ kanamycin (Appendix A) and confirmed by Southern hybridisation with the appropriate probes. Both vectors do not possess an origin of replication for *C. glutamicum* and therefore cells that were able to grow on kanamycin must have integrated the vector into the chromosome through homologous recombination. A diagramatic representation of the construction of the mutants is depicted in **figure 4.3**.

4.3.5 Extraction of chromosomal DNA from *C. glutamicum*

Chromosomal DNA was isolated from a 5 ml overnight culture (2 x TY with 0.5% glucose) according to the method described by Eikmanns *et al.*, (1994). The culture was centrifuged (5000 rpm; 5 min), washed twice with TE buffer and resuspended in 1 ml TE containing 15 $mg.ml^{-1}$ lysozyme. After 3 hours incubation at 37 ^{0}C, 3 ml lysis buffer (10 mM Tris-HCl, 400 mM $NaCl_2$, 2 mM EDTA, pH 8.2), 220 µl SDS (10%), and 150 µl Proteinase K (20 $mg.ml^{-1}$) was added and the solution incubated at 37 ^{0}C overnight. Proteins were precipitated by addition of 2 ml salt saturated NaCl and the solution centrifuged (5000 rpm, 15 min, RT) to recover the supernatant. The chromosomal DNA was precipitated with addition of ice cold 100% ethanol and spooling with a pasteur pipette, washed in 70% ethanol, dried and allowed to resuspend overnight in 100 – 200 µl TE at 4 ^{0}C (or 10 mM Tris pH 7.6).

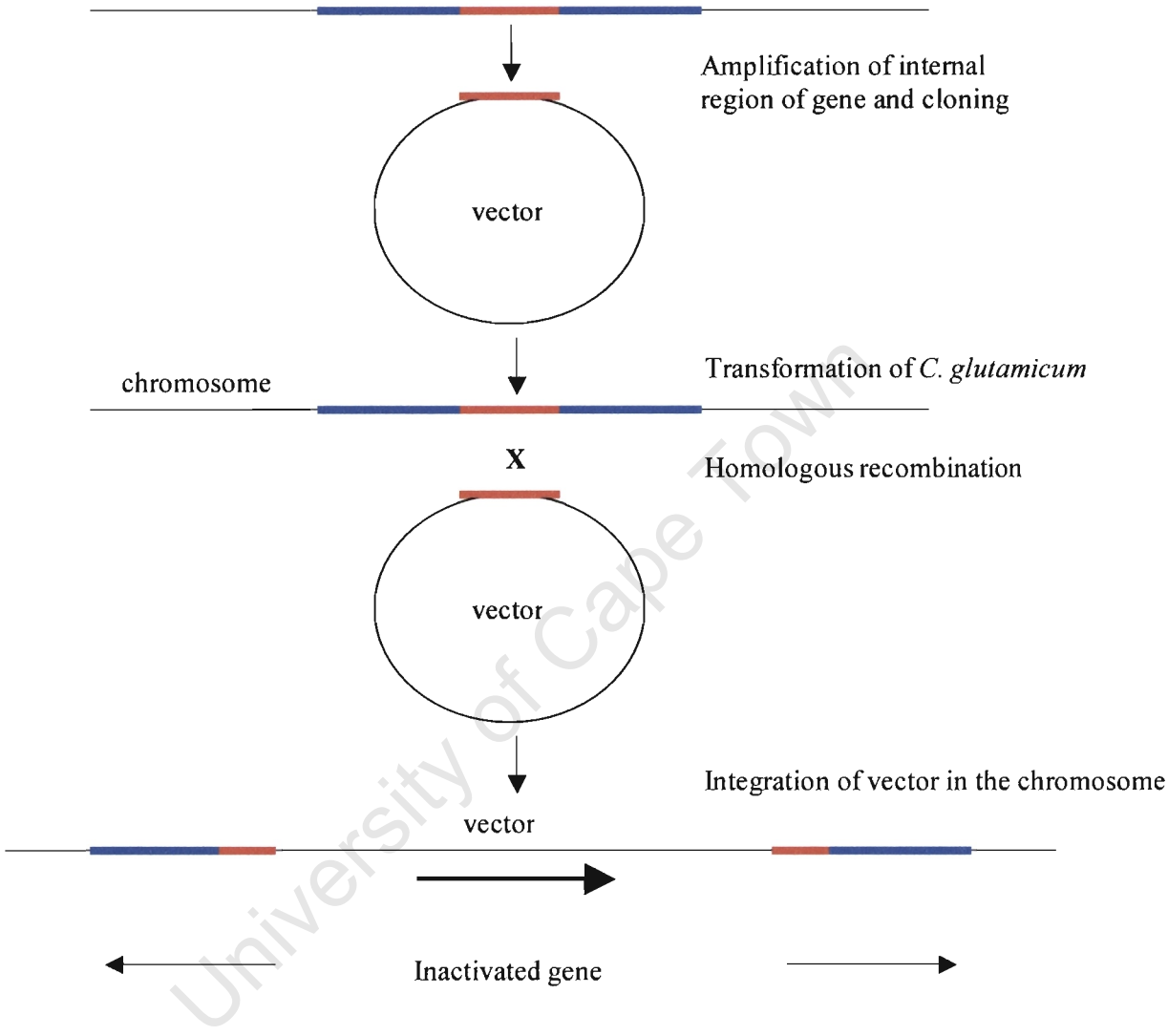

Figure 4.3. A diagramatic scheme of generating an integration mutant in *C. glutamicum* by using the vector pK19*mobsacB*. An internal region of the gene to be inactivated was cloned into the MCS of the vector and transformed into the WT by electroporation. The cloned region and the vector integrated into the chromosome by homologous recombination. Schäfer *et al.*, (1994).

4.3.6 Southern blot analysis

The integration of the vector pDrIMB into the chromosome of *C. glutamicum* was confirmed by Southern hybridisation. *Stu*I restricted chromosomal DNA, extracted from *C. glutamicum* WT and a putative *glg*B integration mutant, CgIMB, were electrophoresed according to standard protocols (Sambrook *et al.*, 2001) on a 0.8 % agarose gel, transferred to a nylon membrane with a vacuum pump and hybridised with a DIG-labelled internal 1.042 kb *glg*B probe prepared from the plasmid pDrIMB. Hybridisation was performed according to the Roche Biochemicals Manufacturers' Manual for DIG-oxygenin labelling and detection of the bands was with CSPD® (Roche). Confirmation of the integration of the plasmid pK19IMC, followed the same procedure as described above for pDrIMB, except the chromosomal DNA of the WT and corresponding mutant were restricted with the enzyme *Bam*HI and hybridised with a 371 bp *glg*C specific DIG-oxygenin labelled probe generated from pK19IMC.

4.3.7 Preparation of cell free extracts for glycogen determination

Cell free extracts of *C. glutamicum* WT and mutant strains, CgIMB and CgIMC, were prepared as previously described in Chapter 2, Section 2.3.3.

4.3.8 Glycogen determination in CgIMB and CgIMC

Levels of glycogen, during growth, was determined with cell free extracts from the *C. glutamicum* WT strain as well as the mutant strains, as previously described in Chapter 2, Section 2.3.5.

4.4 Results

4.4.1 Construction and confirmation of a *glgB* integration mutant

In order to disrupt the *glg*B gene in *C. glutamicum*, an internal region was amplified from the chromosome, and cloned directly using a TA-based cloning kit (pDrive, Qiagen). The construct was sequenced to confirm its origin and further propagated in *E. coli* DH5α in order to generate enough plasmid for transformation into *C. glutamicum*. The recombinant plasmid named, pDrIMB, was not able to replicate in *C. glutamicum* because it has an origin of replication for *E. coli* and not for *C. glutamicum*. The plasmid, pDrIMB, was transformed via electroporation into *C. glutamicum* according to the protocol of van der Rest *et al.*, (1999) and mutant colonies were selected on LBHIS containing 25 $\mu g.ml^{-1}$ kanamycin. The integrated vector was maintained in the chromosome by selective pressure of antibiotic resistance carried on the vector. To confirm integration of the vector, chromosomal DNA was extracted from the potential sequence specific integration mutant and from the WT, restricted with *Stu*I, and probed with a sequence specific probe for *glg*B. Southern hybridisation confirmed the construction of the mutant as shown in **figure 4.4**. The restriction enzyme *Stu*I did not digest the vector, pDrive, nor the cloned internal region of *glg*B, therefore a single band was expected for both strains, but the expected band of the mutant would be larger by 4.9 kb, ie the size of the intergrated vector. The WT hybridised specifically with the internal probe and generated a band of 5.6 kb (**Fig. 4.4**, lanes 2 and 3), while the band generated in lane 1, was larger by 4.9 kb and gave the expected size of 10.5 kb. The *C. glutamicum* integration mutant of *glg*B was designated CgIMB.

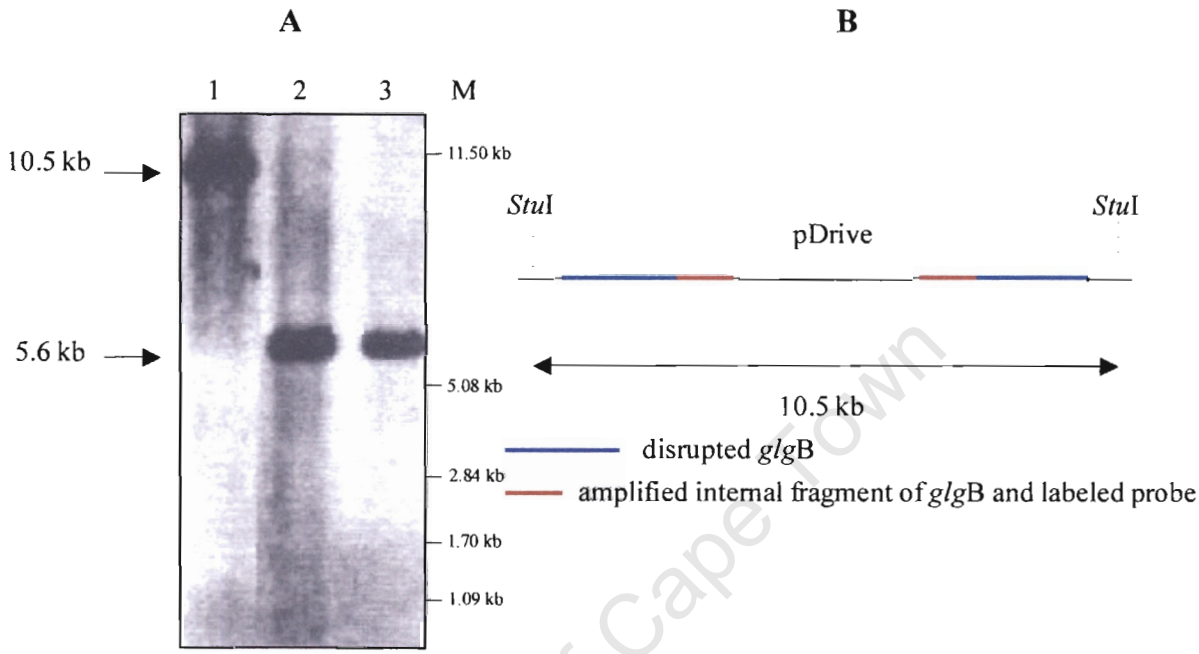

Figure 4.4. Southern hybridisation to confirm inactivation of the *C. glutamicum glg*B gene. A: Autoradiograph of fragments generated from *Stu*I digested chromosomal DNA after probing with a sequence specific, *glg*B, probe. Lane 1: chromosomal DNA from putative *glg*B mutant; lanes 2 and 3: chromosomal DNA from *C. glutamicum* WT. **B:** Schematic representation of the expected autoradiograph signal from the *C. glutamicum glg*B insertion mutant (CgIMB). Lane M indicates the size markers for *Pst*I digested λ DNA.

4.4.2 Construction and confirmation of *glg*C integration mutants

The sequence directed insertion mutant of *glgC* was constructed using the vector pK19*mobsacB*. This vector was constructed with part of the transfer machinery of RP4 (the *mob* gene), and is a pBR322 derivative. The other determinants necessary for conjugation are carried on the chromosome of the *E. coli* strain S17-1 and the vector is able to mobilise to both Gram-negative and Gram-positive bacteria. The vector also has the ability to replicate in *E. coli* and closely related species but is unable to replicate in *C. glutamicum* (Schäfer *et al.*, 1994). An internal region of *glgC* was amplified by PCR as described in the Materials and Methods (Section 4.3.3) and the product was digested with *Sal*I and *Bam*HI and cloned into pK19*mobsacB*. The vector pK19*mobsacB* would preferably be used to generate deletions within a specific gene, since it was constructed with a modified *Bacillus sac*B gene (Schäfer *et al.*, 1994), the expression of which confers sucrose sensitivity to *C. glutamicum* (Jäger *et al.*, 1992). Subsequent growth of integrants, on sucrose would lead to excision of the plasmid from the chromosome resulting in either the WT phenotype or a specific deletion within the target gene. The technique requires two cross over events by homologous recombination, and for *glg*C the region cloned was likely too small (371 bp) to allow two such events. However, an integration mutant of *glg*C would be sufficient to describe the function of the gene, since downstream genes would not be affected by the insertion of the non-replicating plasmid (see **figure 4.3**). The recombinant plasmid, pK19IMC, could be mobilised by conjugation between *E. coli* and *C. glutamicum*, however the DNA transfer was done by electrotransformation (van der Rest *et al.*, 1999), since a higher DNA transfer efficiency (or higher integration frequency) was achieved via this method.

Three potential mutants were selected on rich media containing sorbitol, which supported growth and protected the cells, after they had been electroporated (Materials and Methods 4.3.4). To confirm the disruption of *glg*C, chromosomal DNA was extracted from the putative mutants and the WT, and digested with *Bam*HI. The different samples were probed with a sequence specific *glg*C probe by Southern hybridisation. The multiple cloning site of pK19*mobsacB* could be digested with *Bam*HI, but no internal *Bam*HI site existed within the plasmid or within the cloned region of *glg*C. Therefore, 2 signals were

expected for the integrants and one for the WT. The corresponding fragments in the putative mutants generated were 9.2 kb and 7.1 kb while the WT gave a signal of 10.2 kb, shown in **figure 4.5**. The band visible above 10.2 kb in lanes 3 and 4 was undigested DNA, as this band was not found in lane 5 (where the chromosomal DNA had been digested to completion) and was the sum of the two expected fragments from the mutant strain. These integration mutants of *C. glutamicum* where designated CgIMC.

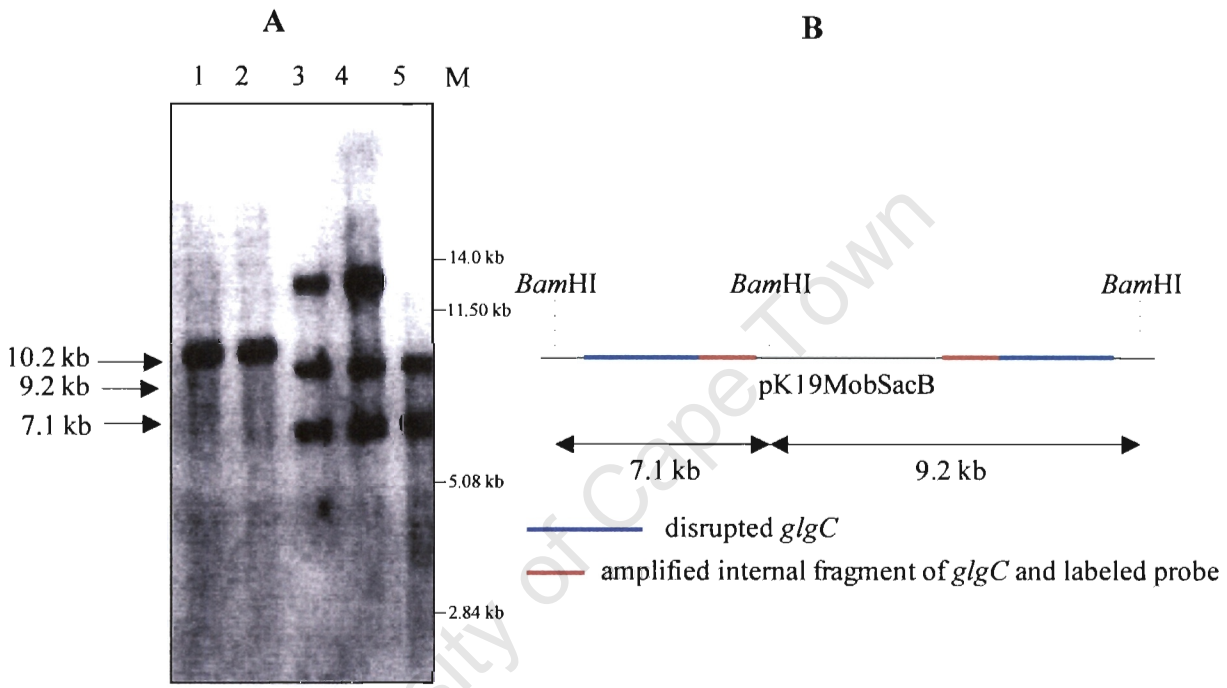

Figure 4.5. Southern hybridisation to confirm the inactivation of the *C. glutamicum glg*C gene. **A:** Autoradiograph of *C. glutamicum*, WT and CgIMC chromosomal DNA digested with *Bam*HI and probed with a sequence specific *glg*C probe. Lanes 1 and 2: Chromosomal DNA from *C. glutamicum* WT; lanes 3-5: chromosomal DNA from *C. glutamicum* CgIMC, where in lanes 3 and 4 the chromosomal DNA was not completely digested by *Bam*HI. **B** Diagramatic representation of the expected autoradiograph signals for the integration mutant *C. glutamicum* CgIMC. Lane M represents size markers according to *Pst*I digested λ DNA.

4.4.3 Glycogen production of the *glg*B and *glg*C mutants in comparison to the WT during growth on minimal medium containing glucose

The constructed integration mutants were tested for variability in growth and for their ability to produce glycogen. Both integration mutants generated were within biosynthetic genes, the ADP-glucose pyrophosphorylase (*glg*C) and the glycogen branching enzyme (*glg*B). The integrated plasmid was maintained in the chromosome by selective pressure, but for the following physiological experiments the main culture was always cultured without antibiotic in order to allow comparitive studies with the WT strain. Loss of the integrated plasmids was not found when cultured for 24 hours without antibiotic selection (results not shown). The two mutants, CglMB and CglMC, and the WT were cultured on minimal media containing 1% glucose and the glycogen levels were measured during growth. Both integrants grew at similar rates ($\mu = 0.36\ h^{-1}$), yet the WT grew at a slightly faster rate of $0.45\ h^{-1}$ (**Fig. 4.6 A**). The maximum OD_{600} reached in all three cultures was approximately the same, with the mutants showing only a minor difference of 1.5 OD_{600} units less (WT had a maximum OD_{600} 20.6). The final optical density of the three cultures were: WT = OD_{600} 15.6; CglMB = OD_{600} 13.4; and CglMC = OD_{600} 14.6.

Glycogen levels were analysed during the growth phases of the different strains, the profile of which is shown in **figure 4.6 B**. The glycogen branching enzyme was no longer active in CglMB, since no intracellular glycogen was measured, except after 4 hours of growth where low levels of 4.5 mg glucose/g DW were measured. This low level of glycogen in a mutant lacking GlgB, a branching enzyme, could be due to unbranched 'glycogen' being present in the cells during the early logarithmic phase. For CglMC, inactivation of the glucose priming enzyme completely inhibited glycogen accumulation during the entire growth profile. In contrast, glycogen levels were measured in the WT strain in logarithmic and stationary phase, reaching a maximum of 51.4 mg glucose/g DW after 6 hours. After 24 hours the levels of glycogen in the WT was below 20 mg glucose/g DW. From the above information, it is evident that the genes, *glg*B and *glg*C, from *C. glutamicum* are involved in glycogen metabolism to the extent that they are essential for glycogen production. No clear phenotypic growth effect was found under the conditions of growth of the mutants on minimal media containing

glucose. Whether complex media containing an excess of glucose has an effect on the growth of the mutants in comparison to the WT, was subsequently tested.

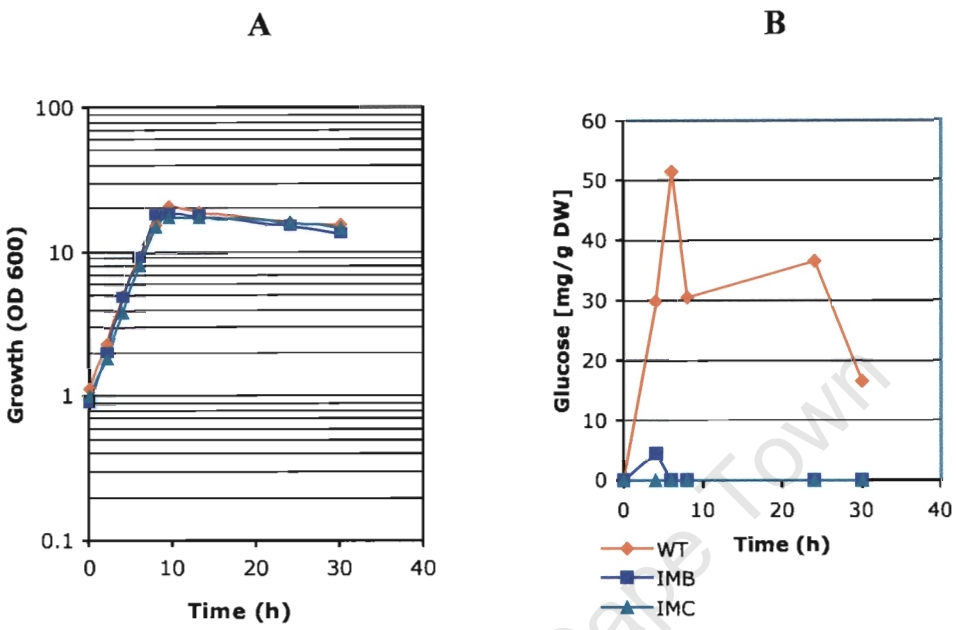

Figure 4.6. Glycogen content of the *C. glutamicum* mutants CgIMB and CgIMC in comparison to the WT. **A:** Growth of *C. glutamicum* WT (WT), CgIMB (IMB), and CgIMC (IMC) on CgC-MM with 1% glucose as the carbon source. **B:** Glycogen accumulation of the three strains during growth.

*standard deviations were less than 20%

4.4.4 Characterisation of the integration mutants on complex media containing glucose

The effect on the growth of the integration mutants, CgIMB and CgIMC, was examined in complex media containing 2% and 4% glucose. The growth rate of the WT and the mutant strains in this case was similar. On complex media containing 2% glucose, the rate was ~ 0.35 h^{-1} (**Fig. 4.7 A1**), while growth of the three strains on media containing 4% glucose had a higher rate of 0.55 h^{-1} (**Fig. 4.7 B1**). This was different relative to growth on minimal media where the growth rate was slightly less in the mutant strains compared to the WT strain. However, with growth on complex media supplemented with

2% and 4% glucose, the maximum OD_{600} reached for the different strains was significantly different. Both glucose concentrations supported a higher cell density in the WT than the integration mutants. The maximum OD_{600} reached in the WT culture on 2% glucose was OD_{600} 35.4 and on 4% glucose was OD_{600} 47, while both mutants only reached a maximum of OD_{600} ~ 25 and OD_{600} ~ 28 in the 2% and 4% glucose cultures, respectively. Growth of *C. glutamicum* in 4% glucose medium sustained slower growth but for longer periods than the 2% glucose medium.

The intracellular glycogen levels were also measured in these cultures, where high levels of glycogen was found in WT cells harvested in late logarithmic phase from the 2% glucose culture (67.3 mg glucose/g DW). However, the highest glycogen levels were obtained in stationary phase cells from the 4% glucose culture (91 mg glucose/g DW) (**Fig. 4.7 A2** and **B2**). In these cultures, a steady state increase in growth could be observed which is not evident in the 2% glucose medium. This suggests that the higher glycogen concentrations observed in 4% glucose medium is being utilised to sustain slow growth. Some glycogen was found in the cell extracts from the mutants grown on this media, but it was between 6 and 9 times less than the levels of glycogen in the WT (~ 10 mg glucose/g DW) through the entire growth phase. The difference in glycogen concentration between the mutants was not significant with the standard deviation of the glycogen assay at +/- 3 mg glucose/g DW. Although on minimal media (1% glucose) no glycogen was measured for CgIMC, on complex media (4% glucose) a maximum glycogen content of ~ 15 mg glucose/g DW was measured in cells harvested after 6 and 10 hours of growth. Glycogen content in the mutants was nevertheless significantly reduced when grown on complex media with 2% or 4% glucose.

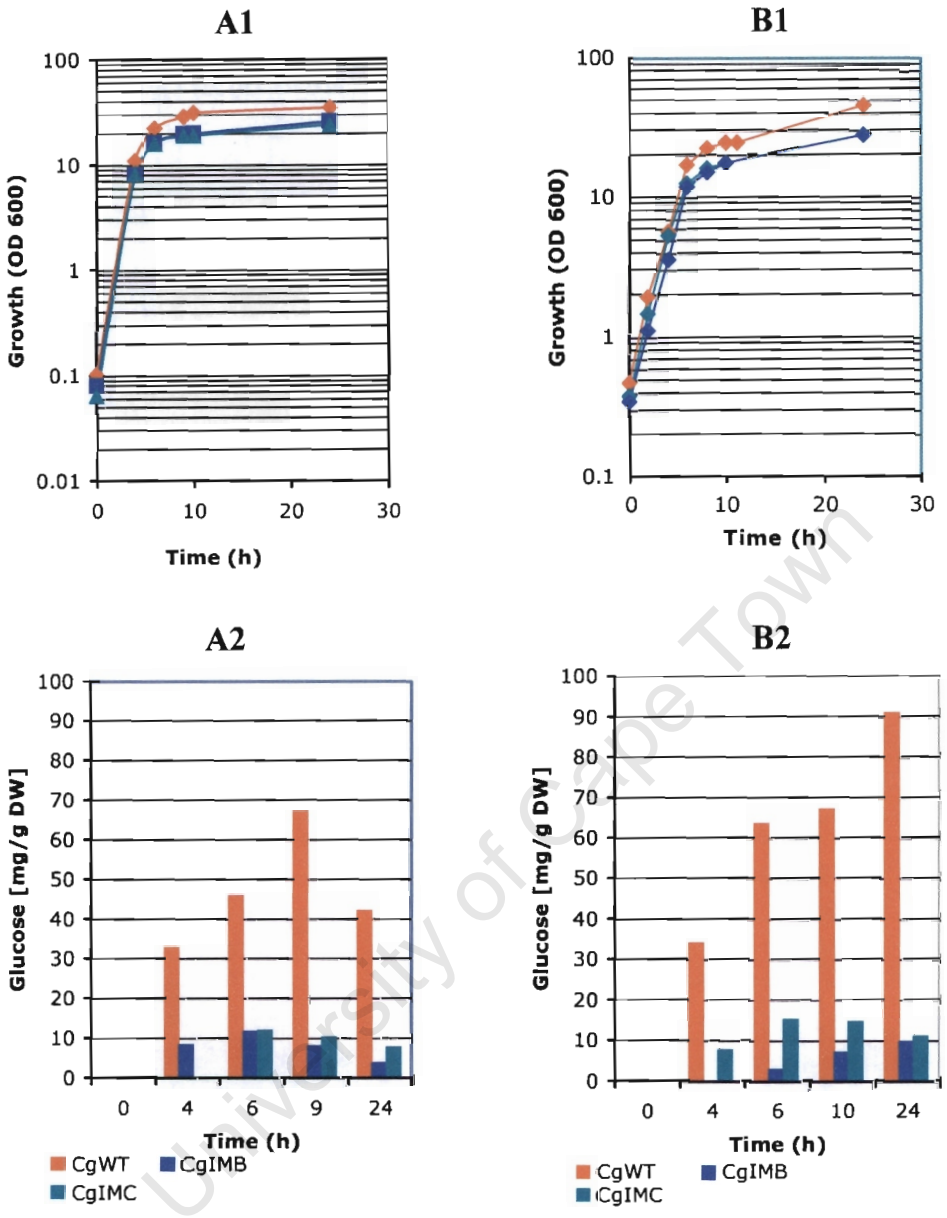

Figure 4.7. Characterisation of CgIMB and CgIMC on complex media with 2% (A) and 4% (B) glucose. **A1:** Growth curve of *C. glutamicum* WT, *C. glutamicum* CgIMB and *C. glutamicum* CgIMC on complex media + 2% glucose. **A2:** Glycogen accumulation profile of the three strains on complex media + 2% glucose. **B1:** *C. glutamicum* growth curve of WT, CgIMB and CgIMC on complex media + 4% glucose. **B2:** Accumulation of glycogen during growth of the WT and mutants on complex media + 4% glucose.

In all cases standard deviations were less than 20%

4.5 Discussion

Two genes essential for the synthesis of glycogen in *C. glutamicum* were inactivated by homologous integration. The glucose priming enzyme, ADPGlc PPase, is considered the critical step in glycogen production, and inactivation of this gene prevented accumulation of glycogen on 1% glucose minimal media. Another important enzyme for synthesising glycogen is the branching enzyme which breaks $\alpha(1\rightarrow4)$ glucose bonds and reforms $\alpha(1\rightarrow6)$ glycosidic bonds generating the branch points in glycogen molecules. In early logarithmic growth, CgIMB (*glg*B mutants) some glucose was measured, but this could be due to the breakdown of unbranched 'glycogen' formed during the early growth phase by an active *glg*C (**Fig. 4.6 B**). Glycogen accumulation of the mutants on complex media with an excess of glucose of 2 and 4% was reduced approximately 10-fold (**Fig. 4.7 A2**; **B2**). Residual but significant levels of glycogen were found in both mutant strains when grown on minimal medium or complex medium containing glucose as seen in **figure 4.7, A2 and B2.** The glycogen test was designed to exclude the measurement of free glucose in the medium as described under the Materials Methods section 2.3.2 and 2.3.4. The residual glucose measured therefore did not likely stem from glycogen but rather from other oligosaccharides such as maltooligosaccharides which also have $\alpha(1\rightarrow4)$ glycosidic bonds. The pathways synthesising maltooligosaccharides were not deleted in the mutants strains presented in this work, leading to the possibility that these complex carbohydrates were degraded by the enzyme, amyloglucosidase, used to degrade glycogen *in vitro*. However, despite the non-specific nature of the glycogen test, which results in the residual levels detected in the mutant, glycogen levels were significantly reduced by the deletion of *glgC* and *glgB,* confirming the role of these genes in glycogen synthesis.

The growth of the mutant strains on complex media with an excess of glucose supported similar growth rates to the WT strains, but interestingly were unable to grow to the same or similar cell density as the WT (**Fig. 4.7 A1; B1**). Cells entered stationary phase under these media conditions not because of a lack of carbon but rather because other factors such as oxygen or nitrogen had become limiting. The limitation of oxygen leads to the accumulation of NADH through a remaining active TCA cycle. The accumulation of

NADH has the effect of inhibiting enzymes such as pyruvate dehydrogenase and glyceraldehydephosphate dehydrogenase (Dominguez *et al.*, 1998; Garrigues *et al.*, 1997). Pyruvate dehydrogenase would normally produce acetyl CoA from pyruvate. The inhibition of glyceraldehydephosphate dehydrogenase causes an accumulation of dihydroxyacetone phosphate which eventually would lead to the breakdown of glycolysis. Essentially, a breakdown in glycolysis and the TCA cycle occurs with entry into stationary phase. In cells that are able to produce glycogen, some of the excess glucose can be converted into glycogen, and thus delay the breakdown of glycolysis and the TCA cycle by the accumulation of NADH. At least the storage of glucose in the form of glycogen may help control the flux of glucose into glycolysis. Support for this hypothesis is provided by the results obtained with growth of *C. glutamicum* in 4% glucose medium. Under these conditions, higher levels of glycogen was demonstrated and slow growth was maintained between 10 - 24 hours, presumably due to the utilisation of glycogen stores. Competition studies, involving the inoculation of one of the mutants and the WT into the same flask at equal concentrations and allowing growth, could indicate if glycogen would be beneficial in the WT under normal culturing conditions. The implication is that glycogen, although not essential for growth, might provide *C. glutamicum* WT with the advantage of growing to a higher OD_{600} when the cells enter stationary phase growth.

CHAPTER 5

GENERAL CONCLUSIONS

The physiology, genetics and regulation of glycogen metabolism in *C. glutamicum* has not been investigated to any real extent. To date, the exact function of the polysaccharide is not known in bacteria although it can serve as an energy reserve compound (Preiss and Romeo 1994). Glycogen is linked to the central metabolic pathway of bacteria by the incorporation of glucose into either glycolysis or into glycogen (Gottschalk, 1985). The distribution of glucose toward glycogen could be considered industrially inefficient for actively growing cells during amino acid production in *C. glutamicum*. We established the intracellular degradation of glycogen by *C. glutamicum* which was also found to synthesise the complex polysaccharide. In the present study four enzymes directly involved in the synthesis and degradation of glycogen were identified. These are: glucose-1-phosphate adenyltransferase (*glg*C), glycogen synthase (*glg*A), glycogen branching enzyme (*glg*B) and glycogen debranching enzyme (*glg*E).

The dependence of glycogen accumulation on glycolytic intermediates in *C. glutamicum* was found when the organism was grown on different carbohydrates and organic acids. Compounds which would normally stimulate gluconeogenesis, such as acetate and lactate, accumulated low levels of glycogen under normal culturing conditions. Even carbohydrates such as fructose and sucrose, the components of which enter the committed pathway to glycolysis at fructose-1,6-bisphosphate, did not stimulate glycogen accumulation. In contrast, growth of *C. glutamicum* in glucose and maltose media stimulated higher levels of glycogen accumulation. Fructose would normally be directed into glycolysis through the phosphorylation of fructose to fructose-1,6-bisphosphate, and from this point the sugar is committed to glycolysis. Even though in *E. coli* fructose-1,6-bisphosphate is a positive regulator of ADPGlc PPase (Preiss, 1992), at least one of the substrates for ADPGlc PPase, ie glucose-1-phosphate would not be available in order to synthesise glycogen. Glucose-1-phosphate would have to be generated from pyruvate via gluconeogenesis. The requirement for gluconeogenesis to generate glucose-1-phosphate

might have inhibited glycogen production and therefore low levels of glycogen were measured when *C. glutamicum* was grown on fructose. In *E. coli* the phosphorylated form of sucrose can be hydrolysed to generate the monosaccharides α-D-glucose-6-phosphate and β-D-fructose. Fructose is then phosphorylated to fructose-6-phosphate by *scrK* (sucrose kinase) and glucose is converted to fructose-6-phosphate via glucose-6-phosphate isomerase (Sauter and Gilles, 2004). With the further phosphorylation of fructose-6-phosphate to fructose-1,6-bisphosphate, the sugar is unable to take any other path besides that of glycolysis. However, glucose-6-phosphate can also be converted to glucose-1-phosphate by the reversible reaction of phosphoglucomutase. If approximately half the number of molecules of glucose were available for glycogen synthesis in *C. glutamicum* when cells were grown on sucrose, it would explain the higher glycogen accumulation in sucrose grown cells in comparison to fructose grown cells.

The low levels of glycogen measured under conditions requiring gluconeogenesis suggests that *C. glutamicum* ADPGlc PPase may be subject to negative allosteric regulation. In addition, the accumulation of glycolytic intermediates with growth on glucose and maltose could possibly stimulate the ADPGlc PPase of *C. glutamicum*. In this study the gene, *glg*C, encoding the enzyme ADPGlc PPase, was inactivated and its function in glycogen synthesis confirmed in *C. glutamicum*. The probability of allosteric regulation of the *C. glutamicum* ADPGlc PPase was strengthened by the presence of the three signatures of the ADPGlc PPase of *E. coli* which were also found in this polypeptide (**Fig 3.1**). The two signatures nearest the N-terminal, are regions suggested to be involved in allosteric regulation in *E. coli* (Preiss *et al.*, 1991). Even the critical conserved amino acids in *E. coli* involved with substrate binding and allosteric regulation are present in the amino acid sequence of the *C. glutamicum* polypeptide. We predict the GlgC of *C. glutamicum* forms a homotetramer (M_r of 175,44 kDa) as is the case for most bacterial ADPGlc PPases as reviewed by Ballicora *et al.*, (2003). Glycolytic intermediates play an important role in the regulation of ADPGlc pyrophosphorylase (Ballicora *et al.*, 2003) and both glycolytic and pentose phosphate pathways are active in

C. glutamicum, therefore activators and inhibitors of this enzyme could be metabolites from both these pathways.

In association with *glg*C, another gene, *glg*A was found transcribed in the opposite direction (**Fig. 3.3 A**). The genes identified immediately upstream and downstream of *glg*A and *glg*C were not involved in glycogen or carbohydrate metabolism, except for a putative sucrose hydrolase directly downstream of *glg*C. The GlgA polypeptide had a very low similarity to that of *E. coli,* but its function was confirmed by Tzvetkov *et al.*, (2003) when they inactivated this gene by integration and showed that very little glycogen was subsequently synthesised. The genetic arrangement *glg*C and *glg*A in *C. glutamicum* was unique to that of other bacteria where each gene was transcribed monocistronically and not within a polycistronic operon. The intergenic region between the two genes was found to include two overlapping promoters. The promoter regions were however not significantly regulated in the presence or absence of glucose, but showed regulation on acetate and lactate as well as maltose. It was not clear in this work what regulatory mechanisms are involved under these conditions.

A second cluster of glycogen associated genes were identified in *C. glutamicum* but it was not found in the near vicinity of the *glg*A and *glg*C diverging operons. The transcription of *glg*E and *glg*B as a single mRNA transcript was proven by Northern analysis and RT-PCR (**Fig. 3.6**). GlgB has the predicted function of breaking $\alpha(1\rightarrow4)$ glucose bonds and introducing $\alpha(1\rightarrow6)$ glycosidic bonds generating the branch points in glycogen molecules. The gene associated with this enzyme was inactivated in the chromosome of *C. glutamicum* resulting in the inability of the organism to accumulate glycogen under the conditions tested. Although the GlgE polypeptide from *C. glutamicum* had very little identity to the debranching enzyme in *E. coli,* its involvement in glycogen breakdown was compared to the GlgE polypeptide from *M. smegmatis* and the PepI polypeptides in *S. coelicolor* (Belanger and Hatfull, 1999; Schneider *et al.*, 2000). In these organisms, the polypeptides mentioned have a function in degrading glycogen (Benlanger and Hatfull, 1999; Schneider *et al.*, 2000). In *E. coli*, *A. tumefaciens*, *B. subtilis, B. stearothermophilus* and *S. coelicolor*, the genes associated

with synthesis and degradation have been shown to be transcribed together (Preiss and Romeo, 1994; Ugalde *et al.*, 1998; Takata *et al.*, 1997; Schneider *et al.*, 2000). The significance of two genes of opposite function forming an operon suggests their regulation is most likely not at the transcriptional level but rather at the post-transcriptional or translational level. Post-transcriptional regulation has been previously proved for the glycogen operons in *E. coli*, and is mediated by the global regulatory system CsrA/CsrB (Liu *et al.*, 1995).

Transcriptional regulation is quite intricate in *E. coli* involving induction by stationary phase sigma factors, cAMP/CRP and *glg*S (Hengge-Aronis and Fischer, 1992) and negative post-transcriptional regulation by the CsrA/CsrB system (Preiss, 1994). However, it was the allosteric regulation of ADPGlc by metabolic intermediates which had the strongest effect on the accumulation of glycogen in *E. coli* (Hengge–Aronis and Fischer, 1992). From the work presented here, the expression levels of glycogen genes in *C. glutamicum* showed no significant regulation when grown on complex media or complex media containing glucose, neither did expression levels change significantly during growth. However, *C. glutamicum* accumulated high levels of glycogen during stationary phase growth on complex media with excess glucose, indicating that an excess of fructose-1,6-bisphosphate may regulate glycogen synthesis by activating ADPGlc PPase. Indications are that glycogen synthesis in *C. glutamicum* may be strongly metabolically regulated. However, not much is known concerning carbohydrate or carbon regulation in *C. glutamicum*. The further identification of mechanisms involved in regulation of glycogen synthesis and degradation would provide a better understanding of the roles of the enzymes associated with glycogen metabolism in *C. glutamicum*.

The "carbon capacitor" concept of glycogen might be supported in *C. glutamicum* in part by an active promoter of *glg*EB during the exponential phase. Also the concerted transcription of the branching and debranching enzymes indicates either post-transcriptional regulation of the enzymes, or that glycogen might be synthesised and degraded during growth. However, deletions in *glg*E might confirm whether glycogen is necessary for growth as is the case in *M. smegmatis* where the glucose incorporated into

glycolysis also originated from glycogen stores (Belanger and Hatfull, 1999). Similar cycling of glucose through glycogen was found in other bacteria such as *F. succinogenes* (Gaudet *et al.*, 1992) and *C. cellulolyticum* (Guedon *et al.*, 2000). Growth experiments of the mutants did give some hint to a possible function of glycogen in *C. glutamicum*, since wild type strains were able to grow to higher optical densities than the mutants which did not accumulate glycogen on complex media plus glucose. Glycogen was therefore not essential for growth of the organism but on entry into stationary phase was able to support growth to a higher cell density, promoting some advantage for having accumulated the polysaccharide.

The work presented in this thesis provides an introduction to the synthesis and breakdown of glycogen in *C. glutamicum* and an insight into its regulation and possible function, which may serve as the basis for future studies. The mechanisms controlling the flow of carbon is important in *C. glutamicum* as efficient amino acid and vitamin production is based on the central metabolism of this organism. Four enzymes central to glycogen synthesis and degradation were identified and the functions of two of these genes were confirmed in this study by mutational analysis. The arrangement of these genes on the chromosome of *C. glutamicum* was different from other glycogen encoding genes in bacteria known thus far. However, additional studies involving the construction and analysis of a *C. glutamicum* mutant impaired in GlgE activity is required. The characterisation of a mutant in GlgE activity might help clarify its function in *C. glutamicum* and would also perhaps clarify the function in *Mycobacteria* and *Streptomyces* strains which encode similar enzymes. The four genes represented in this thesis do not constitute a complete picture of glycogen metabolism in *C. glutamicum*, as there are other enzymes such as glycogen phosphorylase, phosphoglucomutase and adenosine diphosphate sugar pyrophosphatase (see Chapter 1, figure 1.3) which would be involved in glycogen accumulation. Analysis of these other components would result in a more complete model for glycogen metabolism and would form the basis for future work on the mechanisms involved in regulation of glycogen metabolism.

APPENDIX A

Culture Media

Complex media 2xTY (Sambrook *et al.*, 2001)

Tryptone	16	g/l
Yeast Extract	10	g/l
NaCl	5	g/l

Complex media LB (modified from Sambrook *et al.*, 2001)

Tryptone	10	g/l
Yeast Extract	5	g/l
NaCl	5	g/l

Minimal media CgC (CgC-MM) (modified from Kase *et al.*, 1972)

$(NH_4)_2 SO_4$	5	g/l
Urea	5	g/l
MOPS	21	g/l
K_2HPO_4	0.5	g/l
KH_2PO_4	0.5	g/l
$MgSO_4$	0.25	g/l
$CaCl_2$	0.01	g/l
Trace elements solution	1	ml/l
Biotin (200 mg/l)	1	ml/l

The pH was adjusted with 1N NaOH to 6.8 or 6.3, depending on the carbon source, glucose or acetate respectively, and demin. H_2O added to 90% of the final volume. The carbon source, biotin and trace elements, were added after autoclaving.

Trace Elements Solution

$FeSO_2$ x 7 H_2O	10	g/l
$MnSO_4$ x H_2O	10	g/l
$CuSO_4$	0.2	g/l
$ZnSO_4$ x 7 H_2O	1	g/l
$NiCl_2$	0.02	g/l

The 100 X trace elements solution was filter sterilized, stored at 4 ^{0}C and added aseptically to the sterilized medium.

Carbon Source

Glucose	20	g/l
Maltose	10	g/l
Fructose	10	g/l
Sucrose	10	g/l
Acetate	10	g/l
Lactate	10	g/l

A 10 X carbon stock solution was filter sterilized and in the case of acetate and lactate neutralized before filter sterilising, and added to the autoclaved medium at the required concentration. To prepare solid media 15 g/l agar was added to the liquid medium before autoclaving

BHIS (Brain-Heart-Infusion-Sorbitol) (Liebl, *et al.*, 1989)

Brain-Heart-Infusion	18.5	g/l
Sorbitol	91.0	g/l

The brain-heart-infusion and sorbitol were autoclaved separately, and mixed before inoculation.

For LBHIS plates the following substances were added:

Tryptone	5.0	g/l
Yeast Extract	2.5	g/l
NaCl	5.0	g/l
Agar	18.0	g/l

To prepare the solid media BHI, tryptone, yeast extract and NaCl were autoclaved separately from the sorbitol, and combined before pouring the plates. Antibiotics were added as required when the media had cooled to 60 ^{0}C.

All media required in this work was constituted with demin. H_20

Antibiotics

Stock solutions were made of required antibiotics; filter sterilised and added to the medium at a temperature of approximately 60 ^{0}C.

Kanamycin:	Stock solution: 50 mg/ml	Concentration in media: 50 ug/ml
Ampicillin:	Stock Solution: 100 mg/ml	Concentration in media: 100 ug/ml
Chloramphenicol	Stock solution: 50 mg/ml in 70% ethanol	Concentration in media: 50 ug/ml
Nalidixic acid	Stock solution: 30 mg/ml on 1N NaOH	Concentration in media: 30 ug/ml

APPENDIX B

Polymerase chain reaction

PCR was routinely performed to isolate various regions or genes for cloning, to generate probes for Northern and Southern blots as well as for general quantification. The general reaction:

Template	10 ng
Primer 1	0.2 pmol
Primer 2	0.2 pmol
dNTPs	4 mM
$MgCl_2$	2.5 mM* * this can vary
DMSO	Not more than 10% of the reaction max 5 µl in 50 µl total volume
DNA polymerase	1 U
H_2O	x µl

The general PCR was performed with an initial denaturation of 95 ^{0}C for 2 min. Then 30 cycles of denaturation at 95 ^{0}C for 30 sec, annealing at the desired temperature for 30 sec and an elongation at 72 ^{0}C with the required length of time according to the product being amplified. Then a final elongation step of 72 ^{0}C for 10 min to ensure the products were synthesized to completion and a final cooling to 8 ^{0}C. All PCR's were performed with the Biometra. PCR reactions were purified with the Microcon YM100 column.

LITERATURE CITED

Abad, M. C., Binderup, K., Rios-Steiner, J., Arni, R. K., Preiss, J., and Geiger, J. H. (2002). The X-ray crystallographic structure of *Escherichia coli* branching enzyme. *The J. Biol. Chem.* **277:**42164-42170.

Abe, S., Takayama, K., and Kinoshita, S. (1967). Taxonomical studies on glutamic acid producing organisms. *J. Gen. Appl. Microbiol. (Tokyo)* **13:**279-301.

Altschul, S. P., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D. J. (1997). Gapped BLAST and PSI- BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* **25:**3389-3402.

Bailey, J. E. (1991). Toward a science of metabolic engineering. *Science* **252:**1668-1675.

Baker , C. S., Morozov, I., Suzuki, K., Romeo, T., and Babitzke, P., (2002). CsrA regulates glycogen biosnthesis by preventing translation of *glgC* in *Escherichia coli. Mol. Microbiol.* **44:**1599-1610.

Ballicora, M. A., Iglesias, A. A., and Preiss, J. (2003). ADP-glucose pyrophosphorylase, a regulatory enzyme for bacterial glycogen synthesis. *Microbiol. and Mol. Biol. Rev.* **67:**213-225.

Belanger, A. E., and Hatfull, G. (1999). Exponential-phase glycogen recycling is essential for growth of *Mycobacterium smegmatis. J. Bacteriol.* **181:**6670-6678.

Bellman, A., Vrljic, M., Patek, M., Sahm, H., Krämer, R., and Eggeling, L. (2001). Expression, control and specificity of the amino acid exporter LysE of *Corynebacterium glutamicum. Microbiol* **47:**1765-1774.

Birnboim, H. C. (1983). A rapid alkaline extraction method for the isolation of plasmid DNA. *Methods Enzymol.* **100:**243-255.

Bonafonte, M. A., Solana, C., Sesma, B., Alvarez, M., Montuenga, L., Garcia-Ross, D., and Gamazo, C. (2000). The relationship between glycogen synthesis, biofilm formation and virulence in *Salmonella enteritidis. FEMS Microbiol. Letters* **191:**31-36.

Börmann, E. R., Eikmanns, B. J., and Sahm, H. (1992). Molecular analysis of the *Corynebacterium glutamicum gdh* gene encoding glutamate dehydrogenase. *Mol Microbio.* **6:**317-326.

Bott, M., and Niebisch, A. (2003). The respiratory chain of *Corynebacterium glutamicum. J. Biol. Chem.* **104:**129-153.

Boyer, C. and Preiss, J. (1977). Biosynthesis of bacterial glycogen. Purification and properties of the *Escherichia coli* B α-1,4-glucan:α-1,4-glucan 6-glycosyltransferase. *Biochemistry* **16:**3693-3699.

Bradford, M. M. (1976). A rapid and sensitive method for quantification of microgram quantities of protein utilising the principle of protein-dye binding. *Analytical Biochemistry.* **72:**248-254.

Bruton, C. J., Plaskitt, K. A., and Chater K. F. (1995). Tissue-specific glycogen branching isoenzymes in a multicellular prokaryote, *Streptomyces coelicolor* A3(2). *Mol. Microbiol.* **18:**89-99.

Buisson, G., Duee, E., Haser, R., and Payan, F. (1987). Three dimensional structure of porcine pancreatic alpha-amylase at 2.9 A resolution. Role of calcium in structure and activity. *EMBO J.* **6:**3909-3916

Campbell, J. A., Davies, G. J., Bulone, V., Henrissat, B. (1997). A classification of nucleotide-diphospho-sugar glycosyltransferases based on amino acid sequence similarities. *Biochem. J.* **326:**929-939.

Camus, J.-C., Pryor, M. J., Médigue, C., and Cole, S. T. (2002). Re-annotation of the genome sequence of *Mycobacterium tuberculosis* H37Rv. *Microbiology* **148:**2967-2973.

Chater , K. F., and Losick, R. (1997). The mycelial life-style of *Streptomyces coelicolor* A3(2) and its relatives. In: Shapiro J. H., Dworkin, M (eds) Bacteria as multicellular organisms. Oxford University Press, New york, pp 149-182.

Choi, Y. L., Kawamukai, M., Utsumi, R., Sakai, H., and Komano, T. (1989). Molecular cloning and sequencing of the glycogen phosphorylase gene from *Escherichia coli. FEBS Letters* **243:**193-198.

Clement, Y., and Lanéelle, G. (1986). Glutamate excretion mechanism in *Corynebacterium glutamicum*: Triggering by biotin starvation or by surfactant addition. *J. Gen. Microbiol.* **132:**2589-2594.

Cocaign-Bousquet, M., Guyonvarch, A., and Lindley, N. D. (1996). Growth rate dependent modulation of carbon flux through central metabolism and the kinetic consequences for glucose-limited chemostat cultures of *Coryenbacterium glutamicum. Appl. Env. Microbiol.* **62:**429-436.

Cocaign-Bousquet, M., and Lindley, N. D. (1995). Pyruvate overflow and carbon flux within the central metabolic pathways of *Corynebacterium glutamicum* during growth on lactate. *Enz. Microbial. Technol.* **17:**260-267.

Cole, S. T., Brosch, R., Parkhill, J., Garnier, T., Churcher, C., Harris, D., Gordon, S. V., Eiglmeier, K., Gas, S., Barry III, C. E., Tekaia, F., Badcock, K., Basham, D., Brown, D., Chillingworth, T., Connor, R., Davies, R., Devlin, K., Feltwell, T., Gentles, S., Hamlin, N., Holroyd, S., Hornsby, T., Jagels, K., Krogh, A., Mclean, J., Moule, S., Murphy, L., Oliver, K., Osborne, J., Quail, M. A., Rajandream, M.-A., Rogers, J., Rutter, S., Seeger, K., Skelton, J., Squares, R., Squares, S., Sulston, J. E., Taylor, K., Whitehead, S., and Barrel, B. G. (1998). Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. *Nature* **393:**537-544.

Collins, M. D., and Cummins, C. S. (1986). Genus *Corynebacterium* Lehmann and Neumann 1896 In: P. H. A. Sneath,N. S. Mair, M. E. Sharp, and J. G. Holt (Eds) *Bergey's Manual of Systematic Bacteriology* Williams and Wilkins Baltimore **2:**1266-1276.

Collins, M. D., Goodfellow, M., and Minnikin, D. E. (1982). A survey of the structures of mycolic acids in *Corynebacterium* and related taxa. *J Gen Microbiol.* **128:**129-49.

Cremer, J., Treptow, C., Eggeling, L., and Sahm, H. (1988). Regulation of enzymes of lysine biosynthesis in *Corynebacterium glutamicum. J. Gen. Microbiol.* **134:**3221-3229.

Cruz Martin, M., Schneider, D., Bruton, C. J., Chater, K., and Hardisson, C. (1997). A *glgC* gene essential only for the first of two spatially distinct phases of glycogen synthesis in *Streptomyces coelicolor* A3(2). *J. Bacteriol.* **179:**7784-7789.

Dedhia, N. N., Hottiger, T., and Bailey, J. E. (1994). Overproduction of glycogen in *Escherichia coli* blocked in the acetate pathway improves cell growth. *Biotechnol. Bioeng.* **44:**132-139.

Dedhia, N., Chen, W., and Bailey, J. E. (1996). Design of expression systems for metabolic engineering: Coordinated synthesis and degradation of glycogen. *Biotechnol. Bioeng.* **55:**419-426.

de Graaf, A. A., Eggeling, L., and Sahm, H. (2001). Metabolic engineering of L-lysine production by *Corynebacterium glutamicum. Adv. Biochem. Eng. Biotechnol.* **73:**9-29.

Dominguez, H., Lindley N. D. (1996). Complete sucrose metabolism requires fructose phosphotransferase activity in *Corynebacterium glutamicum* to ensure phosphorylation of liberated fructose. *Appl. Environ. Microbiol.* **62:**3878-3880.

Dominguez, H., Rollin, C., Guyonvarch, A., Guerquin-Kern, A., Cocaign-Bousquet, M., and Lindley, N. D. (1998). Carbon-flux distribution in the central metabolic pathways of *Corynebacterium glutamicum* during growth on fructose. *Eur. J. Biochem.* **254:**96-102.

Doolittle, R. F. (1981). Similar amino acid sequences: chance or common ancestry? *Science* **214:**149-159.

Dubey, A. K., Baker, C. S., Suzuki, K., Jones, A. D., Pandit, P., Romeo, T., and Babitzke, P. (2003). CsrA regulates translation of the *Escherichia coli* carbon starvation gene, *cstA*, by blocking ribosome access to the *cstA* transcript. *J. Bacteriol.* **185:**4450-4460.

Eggeling, L. Krumbach, K., and Sahm, H. (2001). L-glutamate efflux with *Corynebacterium glutamicum*: why is penicilin treatment or Tween addition doing the same? *J. Mol. Microbiol. Biotechnol.* **3:**67-68.

Eggeling, L. and Sahm, H. (2003). New ubiquitous translocators: amino acid export by *Corynebacterium glutamicum* and *Escherichia coli. Arch Microbiol* **180:**155-160

Eikmanns, B. J., Kleinertz, E., Liebl, W., and Sahm, H. (1991). A family of *Corynebacterium glutamicum/Escherichia coli* shuttle vectors for cloning, controlled gene expression, and promoter probing. *Gene* **102:**93-98.

Eikmanns, B. J., Eggeling, L., and Sahm, H. (1993). Molecular aspects of lysine, threonine, and isoleucine biosynthesis in *Corynebacterium glutamicum. Antonie van Leeuwenhoek* **64:**145-163.

Eikmanns, B. J., Thum-Schmitz, N., Eggeling, L., Luedtke, K., and Sahm, H. (1994). Nucleotide sequence, expression and transcriptional analysis of the *Corynebacterium glutamicum gltA* gene encoding citrate synthase. *Microbiology* **140:**1817-1828.

Elbein, A. D., and Mitchell, M. (1973). Levels of glycogen and trehalose in *Mycobacterium smegmatis* and the purification and propterties of the glycogen synthetase. *J. Bacteriol.* **113:**863-873.

Falquet, L., Pagni, M., Bucher, P., Hulo, N., Sigrist, C. J., Hofmann, K., and Bairoch, A. (2002). The PROSITE database, its status in 2002. *Nucleic Acids Res.* **30:**235-238.

Fletterick, R. J. and madsen N. B. (1980). The structure and related functions of phosphorylase *a*. *Ann. Rev. Biochem.* **49:**31-61.

Fox, J., Kawaguchi, K., Greenberg, E., and Preiss, J. (1976). Biosynthesis of bacterial glycogen. Purification and properties of the *Escherichia coli* B ADPglucose:1,4-α-glucosyltransferase. *Biochemistry* **15:**849-857.

Frueauf, J. B., Ballicora, M.,A., Preiss, J. (2001). Aspartate residue 142 is important for catalysis by ADP-glucose pyrophosphorylase from *Escherichia coli. J Biol Chem.* **276:**46319-25.

Garrigues, C., Loubiere, P., Lindley, N. D., Cocaign-Bousquet, M. (1997). Control of the shift from homolactic acid to mixed-acid fermentation in *Lactococcus lactis*: predominant role of the NADH/NAD+ ratio. *J. Bacteriol.* **179:**5282-7.

Gasteiger E., Gattiker A., Hoogland C., Ivanyi I., Appel R.D., Bairoch A. (2003). ExPASy: the proteomics server for in-depth protein knowledge and analysis. *Nucleic Acids Res.* **31**:3784-3788.

Gaudet, G., Forano, E., Dauphin, G. & A. Delport. (1992). Futile cycling of glycogen in *Fibrobacter succinogenes* as shown by in situ ^{1}H-NMR and ^{13}C-NMR investigation. *Eur. J. Biochem.* **207**: 155-162.

Gerstmeir, R., Wendisch, V. F., Schnicke, Ruan, H., Farwick, M., Reinscheid, D., and Eikmanns B. J. (2003). Acetate metabolism and ist regulation in *Corynebacterium glutamicum. J. Biotechnol* **104:**99-122.

Goodfellow, M., and Minnikin, D.E. (1981). Introduction to the coryneform bacteria, pp. 1811-1826. In: Starr, M.P., Stolp, H., Trueper, H.G., Balows, A., and Schlegel, H.G. (Eds.), The Prokaryotes, Vol. 2. Springer Verlag, Berlin, Germany.

Gottschalk, G. (1986). Bacterial metabolism second edition. Springer-Verlag New York Inc.

Gourdon, P., Baucher, M. F., Lindley, N.D., and Guyonvarch, A. (2000). Cloning of the malic enzyme gene from *Corynebacterium glutamicum* and role of the enzyme in lactate metabolism. *Appl. Environ. Microbiol.* **66:**2981-2987.

Gourdon, P., Raherimandimby, M., Dominguez, H., Cocaign-Bousquet, M., and Lindley N. D. (2003). Osmotic stress, glucose transport capacity and consequences for glutamate overproduction in *Corynebacterium glutamicum. J. Biotechnol* **104:**77-85.

Guan, P., Kuriki, T., Sivak, M., and Preiss, J. (1995). Maize branching enzyme catalyzes synthesis of glycogen-like polysaccharide in *glgB*-deficient *Escherichia coli. Proc. Natl. Acad. Sci. USA.* **92:**964-967

Gudapaty, S., Suzuki, K., Wang, X., Babitzke, P., and Romeo, T. (2001). Regulatory interactions of csr components: the RNA binding protein CsrA activiates *csrB* transcription in *Escherichia coli. J. Bacteriol.* **183:**6017-6127.

Guedon, E., Desvaux, M., and Petitdemange, H. (2000). Kinetic analysis of *Clostridium celluolyticum* carbohydrate metabolism: Importance of Glucose 1-phosphate and glucose 6-phosphate branch points for distribution of carbon fluxes inside and outside cells as revealed by steady-state continuous culture. *J. Bacteriol.* **182:**2010-2017.

Gutman, M.C., Hoischen, C., and Krämer, R. (1992). Carrier-mediated glutamate excretion by *Corynebacterium glutamicum* under biotin limitation. *Biochem. Biophys. Acta.* **1112:**115-123.

Halsey, R. (2003). Personal communication.

Hanahan, D. (1985). Techiniques for transformation of *E. coli.* In: DNA cloning. A practical approach. Voll (Glover D. M.; Hrsg.), IRL-Press, Oxford, Washington DC, USA: S. 109-135

Haselkorn, R., Fonstein, M., Kogan, Y., Kumar, V., Maltsev, N., Milgram, J. J., Paces, V., and Vlcek, C. (1999) in The Phototrophic Prokaryotes (Peschek, G. A., Loffelhardt, W., and Schmetterer, G., Eds.) Chapters 49, pp. 421-430, Kluwer Academic/Plenum, New York.

Haugen, T. H., Ishaque, A., and Preiss, J. (1976). Biosynthesis of bacterial glycogen. *J. of Biological Chemistry* **251:**7880-7885.

Hengge-Aronis, R., and Fischer, D. (1992). Identification and molecular analysis of *glgS*, a novel growth-phase-regulated and *rpoS*-dependent gene involved in glycogen synthesis in *Escherichia coli. Molecular Microbiology* **6:**1877-1886.

Hermann, T. (2003). Industrial production of amino acids by coryneform bacteria. *J. Biotechnol.* **104:**155-172.

Higgins, D., Thompson, J., Gibson, T., Thompson J. D., Higgins, D.G., and Gibson T.J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* **22:**4673-4680.

Hoischen, C., and Kramer, R. (1990). Membrane alteration is necessary but not sufficient for effective glutamate secretion in *Corynebacterium glutamicum. J. Bacteriol.* **172:**3409-3416.

Holme, T. (1957). Continuous culture studies on glycogen synthesis in *Escherichia coli* B. *Acta. Chem. Scand.* **11:**763-75.

Igarashi, R. Y., and Meyer C. R. (2000). Cloning and sequencing of glycogen metabolism genes from *Rhodobacter sphaeroides* 2.4.1. Expression and characterization of recombinant ADP-glucose pyrophosphorylase. *Archives of Biochemistry and Biophysics* **376:**47-58.

Jackson, M., Raynaud, C., Laneele, M.-A-, Guilhot, C., Laurent-Winter, C., Enserguix, D., Gicquel, B., and Daffe, M. (1999). Inactivation of the antigen 85C gene profoundly affects the mycolate content and alters the permeability of the *Mycobacterium tuberculosis* cell envelope. *Mol. Microbiol.* **31:**1573-1587.

Jetten, M. S., and Sinskey, A. J. (1995). Purification and properties of oxaloacetate decarboxylase from *Corynebacterium glutamicum. Ant. Van Leeuwenhoek* **67:**221-227.

Kalinowski, J., Bathe, B., Bartels, D., Bischoff, N., Bott, M., Burkovski, A., Dusch, N., Eggeling, L., Eikmanns, B. J., Gaigalat, L., Goesmann, A., Hartmann, M., Huthmacher, K., Kraemer, R., Linke, B., McHardy, A. C., Meyer, F., Moeckel, B., Pfefferle, W., Puehler, A., Rey, D. A., Rueckert, C., Rupp, O., Sahm, H., Wendisch, V. F., Wiegraebe, I., and Tauch A. (2003). The complete *Corynebacterium glutamicum* ATTC 13032 genome sequence and its impact on the production of L-aspartate-derived aminoacids and vitamins. *J. Biotechnol.* **104:**5-25.

Kase, H., and Nakayama, K. (1972). Production of L-threonine by analogue-resistant mutants. *Agric. Biol. Chem.* **36:**1611-1621.

Katsumata, M., Ozaki, A., Oka, T., and Furuya, A. (1984). Protoplast transformation of glutamate-producing bacteria with plasmid DNA. *J. Bacteriol.* **159:**306-311.

Kawahara, Y., Takahasi-Fuke, K., Shimizu, E., Nakamatsu, T., and Nakamori, S. (1997). Relationship between the glutamate production and actitivty of the 2-oxoglutarate dehydrogenase in *Brevibacterium lactofermentum. Biosc. Biotechnol. Biochem.* **61:**1109-1112.

Kiel, J. A., Boels, J. M., Beldman, G., and Venema, G. (1994). Glycogen in *Bacillus subtilis*: molecular characterisation of an operon encoding enzymes involved in glycogen biosynthesis and degradation. *Mol. Microbiol.* **11:**203-218.

Kimura, M. (1963). The effect of biotin on the amino acid biosynthesis by *Micrococcus glutamicus. J. Gen. Appl. Microbiol.* **9:** 205-212.

Kinoshito, S., Udaka, L., Shimono, M. (1957). Studies of the amino acid fermentation. I. Production of L-glutamic acid by various organisms. *J. Gen. Appl. Microbiol.* **3:**193-205.

Kirchner, O., and Tauch, A. (2003). Tools for genetic engineering in the amino acid-producing bacterium *Corynebacterium glutamicum. J. Biotechnol.* **104:**287-299.

Kornberg, H. L. (1966a). The role and control of the glyoxylate cycle in *Escherichia coli. Biochem. J.* **99:**1-11.

Kornberg, H. L. (1966b). Anaplerotic sequences and their role in metabolism. In: Campbell, P. N., Greville, G. P. (Eds.), Essays in Biochemistry, vol. 2. Academic Press, New York, pp. 1-31

Kotrba, P., Inui, M., Yukawa, H. (2001). The ptsI gene encoding enzyme I of the phosphotransferase system of *Corynebacterium glutamicum. Biochem. Res. Commun.* **289:**1307-1313.

Krämer, R. (1994a). Secretion of amino acids by bacteria: physiology and mechanism. *FEMS. Microbiol. Rev.* **13:** 75-94.

Krämer, R. (1994b). Systems and mechanisms of amino acid uptake and excretion in prokaryotes. *Arch. Microbiol.* **162:**1-13.

Krebs, E. G. and Preiss, J. (1975). Regulatory mechanisms in glycogen metabolism, p. 337-389. *In* W. J. Whelan (ed.), *International Review of Science, Biochemistry of Carbohydrates.* University Park Press, Baltimore.

Kronemeyer, W., Peekhaus, N., Krämer, R., Sahm, H., and Eggeling, L. (1995). Structure of the *gluABCD* cluster encoding the glutamate uptake system of *Corynebacterium glutamicum. J. Bacteriol.* **177:**1152-1158.

Kumar, A., Larson, C. E., and Preiss J. (1986). Biosynthesis of bacterial glycogen. Primary structure of *Escherichia coli* ADP-glucose:alpha-1,4-glucan, 4-glucosyl transferase as deduced from the nucleotide sequence of the *glg*A gene. *J. Biol. Chem.* **261:**16256-16259.

Lehmann, K. B., and Neumann, R. (1896). Atlas und Grundriβ der Bakteriologie und Lehrbuch der speziellen bakteriologischen Diagnostik, 1st ed., Lehmann, Munchen, Germany.

Leuchtenberger, W. (1996). Amino Acids – technical production and use, pp. 465-502. In: H.-J. Reed, A. Pühler, and P. Stadler (Eds.). Biotechnology, 2nd ed. Vol. 6, Wiley/VCH, Weinheim, Germany.

Leung, P., Lee, Y. M., Greenberg, E., Esch, K., Boylan, S & J. Priess. (1986). Cloning and expression of the *Escherichia coli glgC* gene from a mutant containing an ADPglucose pyrophosphorylase with altered allosteric properties. *J. Bacteriol.* **167:** 82-88.

Liebl, W. (2001). Corynebacterium-nonmedical. In: Balows, A., Trüper, H.G., Dworkin, M., Harder, W.,Schleifer, K.H. (Eds.), The Procaryotes, 3rd Edition: Electronic release 3.7 Springer, New York.

Linton, J. D. (1990). The relationship between metabolite production and growth efficiency of the producing organims. *FEMS Microbiol. Rev.* **75:**1-18.

Liu, M. Y., Gui, G., Wei, B., Preston, J. F., Oakford, L., Yuksel, U., Giedroc, D. P., and Romeo, T. (1997). The RNA molecule csrB binds to the global regulatory protein csrA and antagonises its activity in *Escherichia coli. J. Biological Chem.* **272:**17502-17510.

Liu, M. Y., and Romeo, T. (1997). The global regulator CsrA of *Escherichia coli* is a specific mRNA-binding protein. *J. Bacteriol.* **179:**4639-4642.

Liu, M. Y., Yang, H., and Romeo, T. (1995). The product of the pleiotropic *Escherichia coli* gene *csrA* modulates glycogen biosynthesis via effects on mRNA stability. *J. Bacteriol.* **177:**2663-2672.

Loos, A., Glanemann, C., Willis, L. B., O'Brien, X. M., Lessard, P. A., Gerstmeir, R., Guillouet, S., and Sinskey, A. J. (2001). Development and validation of *Corynebacterium glutamicum* DNA microarrays. *Appl. Envrion. Microbiol.* **67:**2310-2318.

Lu, M., and Kleckner, N. (1994). Molecular cloning and characterisation of the *pgm* gene encoding phosphoglucomutase of *Escherichia coli. J. Bacteriol.* **176:**5847-5851.

Malumbres, M. and Martin, J. F. (1996). Molecular control mechanisms of lysine and threonine biosynthesis in amino acid-producing corynebacteria: redirecting carbon flow. *FEMS Microbiol Lett.* **143:**103-114.

Marroqui, S., Zorreguietta, A., Santamaria, C., Temprano, F., Soberon, M., Megias, M., and Downie, J. A. (2001). Enhanced symbiotic performance by *Rhizobium tropici* glycogen synthase mutants. *J. Bacteriol.* **183:**854-864.

Martín, M. C., Schneider, D., Bruton, C. J., Chater, K. F., and Hardisson, C. (1997). A *glgC* gene essential only for the first two spatially distinct phases of glycogen synthesis in *Streptomyces coelicolor* A3(2). *J. Bacteriol* **179:**7784-7789.

Martínez, E., Pardo, M. A., Palacios, R., and Cevallos, M. A. (1985). Reiteration of nitrogen fixation gene sequences and specificity of *Rhizobium* in nodulation and nitrogen fixation in *Phaseolus vulgaris. J. Gen. Microbiol.* **131:**1779-1786.

Marx, A., de Graaf, A. A., Wiechert, W., Eggeling, L., Sahm, H. (1996). Determination of fluxes in the central metabolism of *Corynebacterium glutamicum* by nuclear magnetic resonance spectroscopy combined with metabolite balancing. *Biotechnol. Bioeng.* **49:**111-129.

Mateos, L. M., Pisabarro, A., Patek, m., Malumbres, M., Guerrero, C.,Eikmanns, B. J., Sahm, H., and Martin, J. F. (1994). Transcriptional analysis and Regulatory signals of the *hom-thrB* cluster of *Brevibacterium lactofermentum. J. Bacteriol.* **176:**7362-7371.

Matheron, C., Delort, A., Gaudet, G., Forano, E., and Liptaj, T. (1998). ^{13}C and ^{11}H Nuclear magnetic resonance study of glycogen futile cycling in strains of the genus *Fibrobacter. Appl. Environ. Microbiol.* **64:**74-81.

Matsuura, Y., Kusunoki, M., Harada, W., and Kakudo, M. (1984). Structure and possible catalytic residues of Taka-amylase. *J. Biochem. (Tokyo)* **95:**697-702

Minnikin, D. E., and O'Donnel, A. G. (1984). *Actinomycete* envelope lipid and peptidoglycan composition In: M. Goodfellow, M. Mordarski, and S. T. Williams (Eds.) The Biology of *Actinomycetes.* Academic Press London, UK. Pp337-388.

Minnikin, D. E., Goodfellow, M., and Collins, M. D. (1978). Lipid composition in the classification and identification of coryneform and related taxa In: I. J. Bousfeld and A. G. Callely (Eds.) Coryneform Bacteria. Academic Press London, UK. Pp.85-160.

Moreno-Bruna, B., Baroja-Fernandez, E., Munoz, F. J., Bastarrica-Berasategui, A., Zandueta-Criado, A., Rodriguez-Lopez, M., Lasa, I., Akazawa, T., and Pozueta-Romero, J. (2001). Adenosine diphosphate suagr pyrophosphatae prevents glycogen biosynthesis in *Escherichia coli. PNAS* **98:**8128-8132.

Mori, M., and Shiio, I. (1987). Pyruvate formation and sugar metabolism in an amino acid-producing bacterium, *Brevibacterium flavum. Agric. Biol. Chem.* **51:**129-138.

Nakagawa, S. (2002). Complete genomic sequence of *Corynebacterium glutamicum* ATCC 13032. *Submission to EMBL/Genbank/DDBJ databases.*

Nakayama, A., Yamamoto, K., and Tabat, S. (2001). Identification of the catalytic residues of bifunctional glycogen debranching enzyme. *J. Biol. Chem.* **276:**28824-28828.

Noventa-Jordao, M. A., de Lourdes, M., Polizeli, T. M., Bonini, B. M., Jorge, J. A., and Terenzi, H. F. (1996). Effects of temperature shifts on the activities of *Neurospora crassa* glycogen synthase, glycogen phosporylase and trehalose-6-phosphate synthase. *FEBS Letters* **378:**32-36.

O'Gara, J. P. and Dunnican L.K. (1994). Direct evidence for a constitutive internal promoter in the tryptophan operon of *Corynebacterium glutamicum. Biochem Biophys Res Commun.* **203:**820-827.

Okita, T. W., Rodriguez, R. L., and Preiss, J. (1981). Isolation of *Escherichia coli* structural genes coding for the glycogen biosynthetic enzymes. *J. Biol. Chem.* **256:**6944-6952.

Okita, T. W., Rodriguez, R. L., and Preiss, J. (1982). Biosynthesis og bacterila glycogen: Cloning of the glycogen biosynthetic enzyme structural genes of *Escherichia coli. Methods Enzymol* **83:**549-556.

Ozaki , A., Katsumata, R., Oka, T., and Furuya, A. (1984). Fucntional expression of the genes of *Escherichia coli* in Gram-positive *Corynebacterium glutamicum. Mol. Gen. Gen.* **196:**175-178.

Parche, S., Burkovski, A., Sprenger, G. A., Weil, B., Krämer, R., and Titgemeyer, F. (2001). *Corynebacterium glutamicum*: a dissection of the PTS. *J. Mol. Microbiol. Biotechnol.* **3(3):**423-428.

Parrou, J. L., Francois, J. (1997). A simplified procedure for a rapid and reliable assay of both glycogen and trehalose in whole yeast cells. *Anal. Biochem.* **248**:186-188.

Park, S. Y., Kim, H. K., Yoo, S. K., Oh, T. K., and Lee, J. K. (2000). Characterisation of *glk*, a gene coding for glucose kinase of *Corynebacterium glutamicum. FEMS Microbiol. Lett.* **188:**209-215.

Park, S. M., Shaw-Reid, C., Sinskey, A. J., and Stephanopoulus, G. (1997). Elucidation of anaplerotic pathways in *Corynebacterium glutamicum* via 13C-NMR spectroscpoy and GC-MS. *Appl. Microbiol. Biotechnol.* **47:**430-440

Patek, M., Nesvera, J., Guyonvarch, A., Reyes, O., and Leblon, G. (2003). Promoters of *Corynebacterium glutamicum. J. Biotechnol* **104:**311-323.

Petersen S., de Graaf, A. A., Eggeling, L., Möllney, M., Wiechert, W., and Sahm, H. (2000). In vivo quantification of parallel and bidirectional fluxes in the anaplerosis of *Corynebacterium glutamicum. J. Biol. Chem.* **275:**35932-35941.

Peters-Wendisch, P.G., Eikmanns, B. J., Thierbach, G., Bachmann, B., and Sahm, H. (1993). Phosphoenolpyruvate carboxylase in *Corynebacterium glutamicum* is dispensable for growth and lysine production. *FEMS Microbiol. Lett.* **112:**269-274.

Peters-Wendisch, P. G., Kreutzer, C., Kalinowski, J., Patek, M., Sahm, H., and Eikmanns, B. J. (1998). Pyruvate carboxylase from *Corynebacterium glutamicum*: characterisation, expression and inactivation of the *pyc* gene. *Microbiol.* **144:**915-927.

Peters-Wendsich, P. G., Schiel, B., Wendisch, V. F., Katsoulidis, E., Mockel B., Sahm, H., and Eikmanns, B. J. (2001). Pyruvate carboxylase is a major bottleneck for glutamate and lysine production by *Corynebacterium glutamicum. Appl. Environ. Microbiol.* **68:**5422-5428.

Peters- Wendisch, P. G., Wendisch, V. F., Paul, S., Eikmanns, B. J., and Sahm, H. (1997). Pyruvate carboxylase as anaplerotic enzyme in *Corynebacterium glutamicum. Microbiol.* **143:**1095-1103.

Preiss, J. (1969). The regulation of the biosynthesis of α-1,4 glucans in bacteria and plants. *Curr. Top. Cell. Regul.* **1:**125-160.

Preiss, J. (1984). Bacterial glycogen synthesis and its regulation. *Annu. Rev. Microbiol.* **38:**419-458.

Priess, J. (1996a). Regulation of glycogen synthesis,. *In* F. C. Niedhart, R CurtissIII, J. L. Ingraham, E. C. C. Lin, K. B. Low, B. Magasnik, W. S. Reznikoff, M. Riley, M. Schaechter & H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella*: cellular and molecular biology, 2nd ed., vol.1. American Society for Microbiology, Washington D.C. **p** 1015-1024

Preiss, J. (1996b). ADPglucose pyrophophorylase: basic science and applications in biotechnology. *Biotechnol. Annu. Rev.* **2:**259-279.

Preiss, J., Ball, K., Hutney, J., Smith-White, B. J., Li, L., Okitsa, T. W. (1991). Regulatory mechanisms involved in the biosynthesis of starch. *Pure Appl. Chem.* **63:**535-544.

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

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

Preiss, J., Yung, S. G., and Baecker, P. A. (1983). Regulation of bacterial glycogen synthesis. *Mol. Cell Biochem.* **57:**61-80.

Puech, V., Bayan, N., Salim, K., Leblon, G., and Daffe, M. (2000). Characterisation of the in vivo acceptors of the mycolyl residues transferred by the corynebacterial PS1 and the related mycobacterial antigens 85. *Mol. Microbiol.* **35:**1026-1041.

Puech, V., Chami, M., Lemassu, A., Laneele, M.-A., Schiffler, B., Gouon, P., Bayan, N., Benz, R., and Daffe, M. (2001). Structure of the cell envelope of *corynebacteria*: Importance of the non-covalently bound lipids in the formation of the cell wall permeability barrier and fracture plane. *Microbiol.* **147:**1365-1382.

Pujadas, G., and Palau, J. (2001). Evolution of α-amylases: Architectural features and key residues in the stabilisation of the $(\beta/\alpha)_8$ scaffold. *Mol. Biol. Evol.* **18:**38-54.

Riedel, C., Rittman, D., Dangel, P., Mockel, B., Petersen, S., Sahm, H., and Eikmanns, B. J. (2001). Characterisation of the phosphoenolpyruvate carboxykinase gene from *Corynebacterium glutamicum* and significance of the enzyme for growth and amino acid production. *J. Mol. Biotechnol.* **3:**573-583.

Romeo, T. (1998). Global regulation by the small RNA-binding protein CsrA and the non-coding RNA molecule CsrB. *Mol. Microbiol.* **29:**1321-1330.

Romeo, T., Black, J., and Preiss, J. (1990). Genetic regulation of glycogen synthesis in *Escherichia coli*: In vivo effects of the catabolite repression and stringent response systems in *glg* gene expression. *Curr. Microbiol.* **21:**131-137.

Romeo, T. and Gong, M. (1993). Genetic and physical mapping of the regulatory gene *csr*A on the *Escherichia coli* K-12 chromosome. *J. Bacteriol.* **175:**131-137.

Romeo, T., Gong, M., Liu, M. Y., and Brun-Zinkernagel, A. (1993). Identification and molecular characterisation of *csrA,* a pleiotropic gene from *Escherichia coli* that affects glycogen biosynthesis, gluconeogenesis , cell size and surface properties. *J. Bacteriol* **175:**4744-4755.

Romeo, T., Kumar, A., and Preiss, J. (1988). Analysis of the *Escherichia coli* glycogen gene cluster suggests that catabolic enzymes are encoded among the biosynthetic genes. *Gene* **70:**363-376.

Romeo, T., and Preiss, J. (1989). Genetic regulation of glycogen biosynthesis in *Escherichia coli*: in vivo effects of cyclic AMP and guanosine 5'-diphosphate and analysis of in vivo transcripts. *J. Bacteriol.* **171:**2773-2782.

Rueda, B., Miguelez, E. M., Hardisson, C., and Manzanal, M. B. (2001). Changes in glycogen and trehalose content of *Streptomyces brasiliensis* hyphae during growth in liquid cultures under sporulating and non-sporulating conditions. *FEMS Microbiol.* **194:**181-185.

Sahm, H., Eggeling, L., and de Graaf, A. A. (2000). Pathway analysis and metabolic engineering in *Corynebacterium glutamicum. Biol Chem.* **381:**899-910.

Salamone, P. R., Kavakli, I.H., Slattery, C.J., and Okita, T. W. (2002). Directed molecular evolution of ADP-glucose pyrophosphorylase. *PNAS* **99:**1070-1075.

Sambrook J., Fritsch, E. F., and Maniatis, T. (2001). Molecular cloning: a laboratory manual. Cold Spring Harbor; NY

Santamaria, R., Gil, J. A., Mesas, J. M., and Martin, J. F. (1984). Characterisation of an endogenous plasmid and development of cloning vectors and a transformation system in *Brevibacterium lactofermentum. J. Gen. Microbiol.* **130:**2237-2246.

Sauter, T. and Gilles E. D. (2004). Modelling and experimental validation of the signal transduction via the *Escherichia coli* sucrose phospho-transferase system. *J. Biotechnol.* **110(2):**181-199.

Schäfer, A. A., Tauch, A., Jäger, W., Kalinowski, J., Thierbach, G., and Pühler, A. (1994). Small mobilizable multi-pirpose cloning vectors derived from the *E. coli* plasmids pK18 and pK19: selection of defined deletions in the chromosome of *Corynebacterium glutamicum. Gene* **145:**69-73.

Shaw, W. V. (1975). Chloramphenicol acetyltransferase from chloranphenicol-resistant bacteria. *Methods Enzymol.* **43:**737-755.

Schneider, D., Bruton, C. J., and Chater, K. F. (2000). Duplicated gene clusters suggest an interplay of glycogen and trehalose metabolism during sequential stages of aerial mycelium development in *Streptomyces coelicolor* A3(2). *Mol. Gen. Genet* **263:**543-553.

Shiio, I., Otsuka, S.I., and Katsuya, N. (1963). Cellular permeability and extracellular formation of glutamic acid formation in *Brevibacterium flavum*. *J. Biochem.* **53**:333-340.

Shiio, I., Sugimoto, S. I., and Kawamura, K. (1990). Effects of carbon source sugars on yield of amino acid production and sucrose metabolism in *Brevibacterium flavum. Agric. Biol. Chem.* **54:**1513-1519.

Slock, J. A. and Stahly, D. P. (1974). Polysaccharide that may serve as a carbon and energy storage compound for sporulation in *Bacillus cereus*. *J. Bacteriol.* **120:**399-406.

Spatafora Harris, G., Michalek, S. M., and Curtiss III, Roy. (1992). Cloning of a locus involved in *Stretococcis mutans* intracellular polysaccharide accumulation and virulence testing of an intracellular polysaccharide-deficient mutant. *Infection and Immunity* **60:**3175-3185.

Spatafora, G., Rohrer, K., Barnard, D., and Michalek, S. (1995). A *Streptococcus mutans* mutant that synthesises elevated levels of intracellular polysaccharide is hypercariogenic *in vivo. Infection and Immunity* **63:**2556-2563.

Stackebrandt, E., Rainey, F. A., and Ward-Rainey, N. L. (1997). Proposal for a new hierarchal classification system, Actinobacteria classis nov. *Int. J. Syst. Bacteriol.* **47:** 479-491.

Strange, R.E. (1968). Bacterial glycogen and survival. *Nature*. **220**:606-607.

Strange, R.E., Dark, F.A., Ness, A.G. (1961). The survival of stationary phase *Aerobacter aerogenes* stored in aqueous suspension. *J. Gen. Microbiol.* **25**:61-76.

Suzuki, K., Wang, X., Weilbacher, T., Pernestig, A.-K., Melefors, O., Georgellis, D., Babitzke, P., and Romeo, T. (2002). Regulatory circuitry of the CsrA/CsrB and BarA/UvrY systems of *Escherichia coli. J. Bacteriol.* **184:**5130-5140.

Tanzer, J. M., Freedman, M. L., Woodiel, F. N., Eifert, R. L., and Rinehimer, L. A. (1976). Association of the *Streptococcus mutans* virulence with the synthesis of intracellular polysaccharide, p. 597-616. In H. M. Stiles, W. J. Loesche, and T. L. O'Brien (ed.), Proceedings in microbiology. Aspects of dental caries. Special supplement in Microbiology abtracts, vol3. Information Retrieval, Inc., London.

Takata, H., Takaha, T., Okada, S., Takagi, M., and Imanaka, T. (1997). Characterisation of a gene cluster for glycogen biosynthesis and a heterotetrametric ADP-glucose pyrophosphorylase from *Bacillus stearothermophilus*. *J. Bacteriol.* **179:**4689-4698.

Takinami, K., Yoshii, H., Yamada, Y., Okada, H., and Kinoshita, K. (1968). Control of L-glutamic acid fermentation by biotin and fatty acid. *Amino Acid Nucl. Acid* **18:**120-160.

Tzvetkov, M., Klopprogge C., Zelder O., and Lieble W. (2003). Genetic dissection of trehalose biosynthesis in *Corynebacterium glutamicum*: inactivation of trehalose production leads to impaired growth and an altered cell wall lipid composition. *Microbiol.* **149:**1659-1673.

Urbanowski, J., Leung, P., Weissbach, H., and Preiss, J. (1983). The *in vitro* expression of the gene for *Escherichia coli* ADP glucose pyrophosphorylase is stimulated by cyclic-AMP and cyclic-AMP receptor protein. *J. Biol. Chem.* **258:**2782-2784.

Ugalde, J. E., Parodi, A. J., and Ugalde, R. A. (2003). *De novo* synthesis of bacterial glycogen: *Agrobacterium tumefaciens* glycogen synthase is involved in glucan initiation and elongation. *PNAS* **100:**10659-10663.

Ugalde, J. E., Lepek, V., Uttaro, A., Estrella, J., Iglesias, A., and Ugalde, R. A. (1998). Gene organization and transcription analysis of the *Agrobacterium tumefaciens* glycogen (glg) operon: Two transcripts for the single phosphoglucomutase gene. *J. Bacteriol* **180:**6557-6564.

Uttaro, A., and Ugalde, R. A. (1994). A chromosomal cluster of genes encoding ADP-glucose synthetase, glycogen synthase and phosphoglucomutase in *Agrobacterium tumefaciens*. *Gene* **150:**117-122.

Van der Rest, M. E., Lange, C., and Molenaar, D. (1999). A heat shock following electroporation induces highly efficient transformation of *Corynebacterium glutamicum* with xenogenic plasmid DNA. *Appl Micrbiol. Biotechnol.* **52:**541-545.

Vallino, J. J., and Stephanopoulos, G. (1993). Metabolic flux distributions in *Corynebacterium glutamicum* during growth and lysine overproduction. *Biotechnol. Bioeng.* **41:**633-646.

Vasicova, P., Abrhamova, Z., Nesvera, J., Patek, M., Sahm, H., and Eikmanns, B. J. (1998). Integrative and autonomously replicating vectors for analysis of promoters in *Corynebacterium glutamicum*. *Biotechnol. Techniques.* **12:**743-746.

Vrljic, M., Eggeling, L., and Sahm, H. (1996). A new type of transporter with a new type of cellular function: L-lysine export from *Corynebacterium glutamicum*. *Mol. Microbiol.* **22:**815-826.

Voet, D.,and Voet, J. G. (1995) Biochemistry, 2nd ed, John Wiley and Sons Inc., New York. p 258-264, 484-504.

Wei, B. I., Brun-Zinkernagel, A.-M., Simecka, J. W., Prü, B. M., Babitzke, P., and Romeo, T. (2001). Positive regulation of motility and *flhDC* expression by the RNA-binding protein CsrA of *Escherichia coli. Mol. Microbiol.* **40:**245-256.

Weilbacher, T., Suzuki, K., Dubey, A. K., Wang, X., Gudapaty S., Morozov, I., Baker, C. S., Georgellis, D., Babitzke, P., and Romeo, T. A Novel sRNA component of the carbon storage regulatory system of *Escherichia coli. Mol. Microbiol.* **48:**657-670.

Wendisch, V. F. (2003). Genome-wide expression analysis in *Corynebacterium glutamicum* using DNA microarrays. *J. Biotechnol.* **104:**273-285.

Wendisch, V. F., De Graaf, A. A., Sahm, H., and Eikmanns, B. J. (2000). Quantitative determination of metabolic fluxes during co-utilization of two carbon sources: Comparative analyses with *Corynebacterium glutamicum* during growth on acetate and/or glucose. *J.Bacteriol.* **182:**3088-3096.

White, D., Hart, M. E., and Romeo, T. (1996). Phylogenetic distribution of the global regulatory gene *csrA* among eubacteria. *Gene* **182:**221-223.

Wittman, C., and Heinzle, E. (2001). Application of MALDI-TOF MS to lysine-producing *Corynebacterium glutamicum*. A novel approach for metabolic flux analysis. *Eur. J. Biochem.* **268:**2441-2455.

Wolf, A., Krämer, R., and Morbach, S. (2003). Three pathways for trehalose metabolism in *Corynebacterium glutamicum* ATCC 13032 and their significance in response to osmotic stress. *Mol. Microbiol.* **49:**1119-1134.

Yang, H., Liu, M. Y., and Romeo, T. (1996). Coordinate genetic regulation of glycogen catabolism and biosynthesis in *Escherichia coli* via the CsrA gene product. *J. Bacteriol.* **178:**1012-1017.

Yeh, P., Sicard, A. M., and Sinskey, A. J. (1988). General organization of the genes specifically involved in the diaminopimelate-lysine biosynthetic pathway of *Corynebacterium glutamicum. Mol Gen Genet.* **212:**105-111.

Yu, F., Jen, Y., Takeuchi, E., Inouye, M., Nakayama, H., Tagaya, M., and Fukui, T. (1988). α-glucan phosphorylase from *Escherichia coli* cloning of the gene, and purification and characterisation of the protein. *J. Biol. Chem.* **263:**13706-13711.

Zevenhuizen, L.P.T.M. (1992). Levels of trehalose and glycogen in *Arthrobacter globiformis* under conditions of nutrient starvation and osmotic stress. *Antonie van Leeuwenhoek* **66:**61-68.