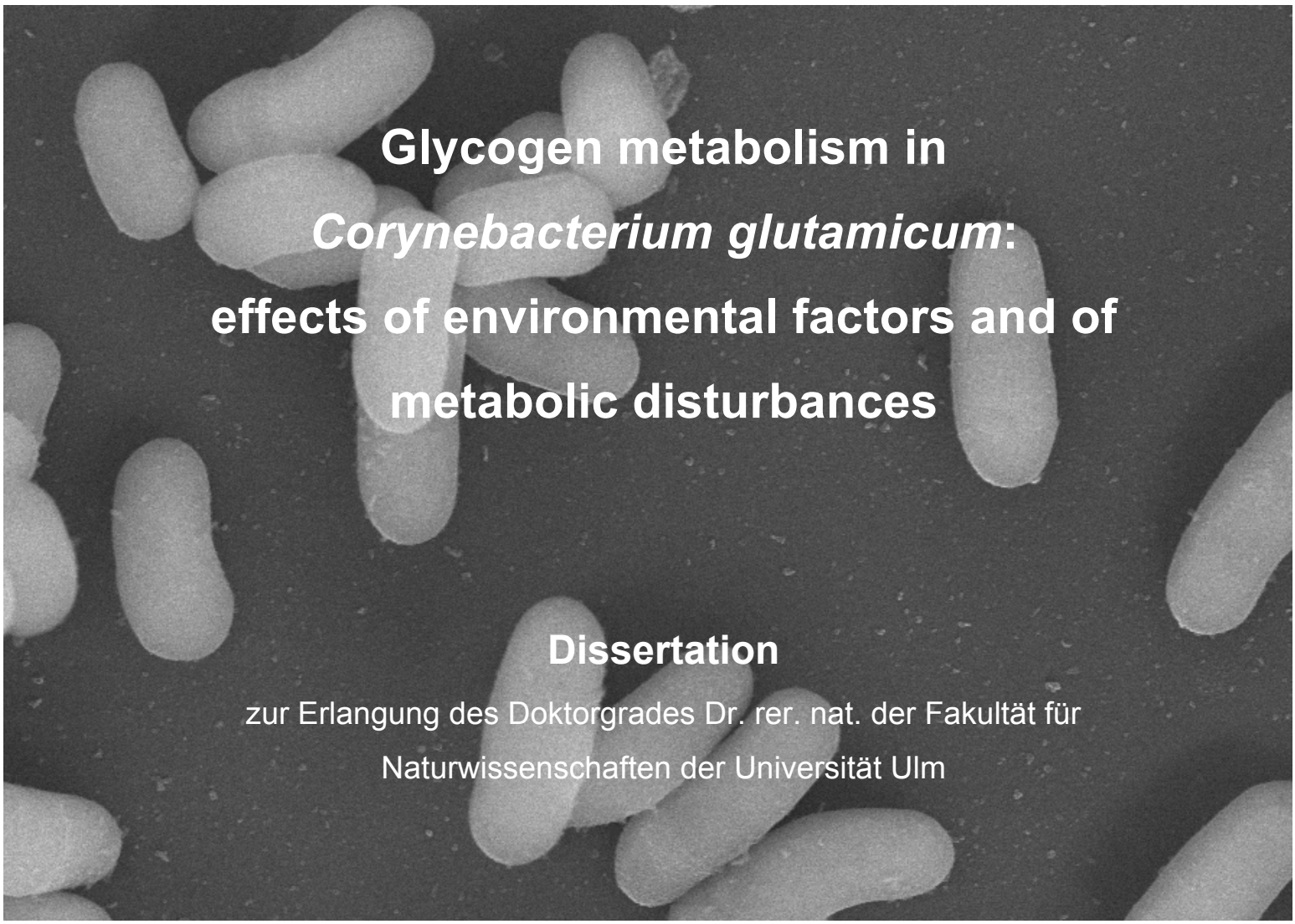
ulm university universität uulm



Glycogen metabolism in *Corynebacterium glutamicum*: effects of environmental factors and of metabolic disturbances

Dissertation

zur Erlangung des Doktorgrades Dr. rer. nat. der Fakultät für Naturwissenschaften der Universität Ulm

vorgelegt von

Philipp Rola Alexander von Zaluskowski

aus Gießen

2014

Die vorliegende Arbeit wurde am Institut für Mikrobiologie und Biotechnologie der Universität Ulm unter der Anleitung von Herrn Prof. Dr. Bernhard J. Eikmanns angefertigt.

Amtierender Dekan: Prof. Dr. Joachim Ankerhold

1. Gutachter: Prof. Dr. Bernhard J. Eikmanns
2. Gutachter: Prof. Dr. Peter Dürre

Tag der Promotion: 25.07.2014

Table of contents

ABSTRACT **1**

ZUSAMMENFASSUNG **3**

1 INTRODUCTION **5**

2 MATERIAL AND METHODS **11**

2.1 Bacterial strains, plasmids and oligonucleotides **11**

2.1.1 Bacterial strains 11

2.1.2 Plasmids 13

2.1.3 Oligonucleotides 14

2.2 Chemicals **17**

2.3 Instruments **19**

2.4 Enzymes and kits **21**

2.5 Growth media **22**

2.6 Cultivation conditions **23**

2.6.1 Aerobic cultivation of *Corynebacterium glutamicum* 23

2.6.2 Anaerobic incubation of *Corynebacterium glutamicum* 24

2.6.3 Cultivation of *Corynebacterium glutamicum* in a DASGIP parallel fermentation system 24

2.6.4 Cultivation of *Corynebacterium glutamicum* in survival experiments 25

2.7 Preparation and transformation of electro-competent *Escherichia coli* and *Corynebacterium glutamicum* cells **25**

2.7.1 Preparation and transformation of electro-competent *Escherichia coli* cells 25

2.7.2 Preparation and transformation of electro-competent *Corynebacterium glutamicum* cells 26

2.8 Chromosomal deletions in *Corynebacterium glutamicum* **26**

2.9 Construction of *Corynebacterium glutamicum* integration mutants **27**

2.10 Determination of luciferase activities **27**

2.11 DNA treatment **28**

2.11.1 Preparation of chromosomal DNA from *C. glutamicum* 28

2.11.2 Preparation of plasmid DNA 29

2.11.3 PCR 30

2.11.4 Agarose gel electrophoresis 32

2.11.5 Purification of DNA fragments 33

2.11.6 Enzymatic manipulation of DNA 33

2.11.7 Determination of DNA concentrations 33

2.12 RNA treatment **34**

2.12.1 Preparation of total RNA 34

2.12.2 Semi-quantitative real-time PCR 34

2.13 Analytics **35**

2.13.1 Determination of glycogen levels 35

2.13.2 Determination of sugars and organic acids in culture supernatants 36

2.13.3 Determination of intracellular metabolite concentrations 37
2.14 Determination of cell size 39
3 RESULTS 41
3.1 Characterisation of deletion mutants of genes involved in glycogen metabolism 41
3.1.1 Characterisation of single deletion mutants of genes involved in glycogen synthesis 41
3.1.2 Characterisation of single deletion mutants of genes involved in glycogen degradation 45
3.1.3 Characterisation of *C. glutamicum* strains with multiple deletions of genes involved in glycogen degradation 49
3.1.4 Comparison of constructed *Corynebacterium glutamicum* deletion strains 53
3.2 Influence of specific growth factors on glycogen levels in *C. glutamicum* 55
3.2.1 Influence of phosphate availability on glycogen levels 55
3.2.2 Influence of nitrogen availability on glycogen levels 56
3.2.3 Influence of pH on glycogen levels 58
3.2.4 Influence of anaerobic conditions on glycogen levels 60
3.3 Regulation of glycogen metabolism 67
3.3.1 Quantitative real-time transcription analysis of genes involved in glycogen metabolism during growth with reduced phosphate concentrations 67
3.3.2 *In vivo* and *real-time* transcription analysis of genes involved in glycogen metabolism using an integrated luciferase system 69
3.4 Effects of disturbed glycogen metabolism on production behaviour 74
3.4.1 Effects of disturbed glycogen metabolism on the production of organic acids 74
3.4.2 Glycogen levels and production pattern of the succinate producer *C. glutamicum ΔaceE Δpqo ΔldhA ΔC–T ilvN ΔalaT ΔavtA* (*C. glutamicum* ELB-P) with different carbon sources 76
3.5 Physiological role of glycogen metabolism in *C. glutamicum* 79
3.5.1 Effect of disturbed glycogen metabolism on energetic parameters in *C. glutamicum* 79
3.5.2 Effect of disturbed glycogen metabolism on survival of *C. glutamicum* 82
4 DISCUSSION 87
REFERENCES 106
ABBREVIATIONS 117
PUBLICATIONS 119
CONTRIBUTIONS OF OTHERS TO THIS WORK 121
DANKSAGUNG 123
***CURRICULUM VITAE* 125**
ERKLÄRUNG 127

Titelbild: Rasterelektronenmikroskopische Aufnahme von *Corynebacterium glutamicum* bei 10000-facher Vergrößerung. In Zusammenarbeit mit Prof. Dr. Paul Walther, Zentrale Einrichtung Elektronenmikroskopie, Universität Ulm.

Abstract

In this work, several aspects of the glycogen metabolism in *C. glutamicum* were studied. First of all, the role of the *glgC*, *glgA*, *glgB*, *glgX*, *malP* and *glgP* genes in glycogen metabolism on glucose and maltose was investigated. Furthermore, the influence of phosphate, nitrogen and oxygen availability on glycogen levels was examined as well as the link between pH stress and glycogen metabolism. Additional effort was invested to elucidate the nature of glycogen metabolism regulation by the reduction of phosphate concentrations and to describe the transcriptional regulation of *glgC*, *glgA* and *glgX* during growth on glucose and maltose. The effect of disturbances in the glycogen metabolism on the production behaviour under anaerobic conditions was examined as well as the glycogen metabolism in the succinate-producing strain *C. glutamicum* Δ*aceE* Δ*pqo* Δ*ldhA* ΔC–T *ilvN* Δ*alaT* Δ*avtA*. Last, but not least, the influence of *glgC* (involved in glycogen synthesis) and *glgX* (involved in glycogen degradation) deletions on energetic parameters and survival of *C. glutamicum* was investigated.

Growth experiments with several deletion mutants on glucose corroborated that (i) the *glgC*, *glgA* and *glgB* genes are responsible for glycogen synthesis, (ii) the *glgX* gene is mainly responsible for glycogen degradation, (iii) the *malP* gene is also involved in glycogen degradation, but is less important, and (iv) the *glgP* gene played no role in glycogen metabolism under the tested conditions. Corresponding growth experiments with several deletion mutants on maltose as carbon source showed that (i) the *glgC* and *glgA* genes are not involved in glycogen synthesis, (ii) the *glgB* gene is not only involved in glycogen metabolism, but also in maltose utilization, (iii) the *glgX* and *malP* genes are both important for glycogen degradation with *malP* being also involved in maltose utilization, and (iv) *glgP* gene played no role under the tested conditions.

Another set of growth experiments with various levels of phosphate and nitrogen sources revealed that a decrease in nitrogen availability has no influence on glycogen levels, while decrease of phosphate concentrations led to an increase in glycogen levels. Additional semi-quantitative RT-PCR experiments confirmed that the higher glycogen levels due to phosphate reduction were caused at least partly by changes in the transcription profile of *glgC*, *glgA*, *glgB*, *glgX*, *malP* and *glgP* genes. By performing experiments under anaerobic conditions it was also shown that (i) *C. glutamicum* is able to accumulate glycogen under anaerobic conditions, (ii) this accumulation caused an increase of the OD_{600}, which was not a consequence of an increase in cell number, (iii) the production behaviour was not affected by disturbances in the glycogen metabolism, and (iv) changes in central metabolism, which led to accumulation of succinate, caused a different profile of glycogen accumulation compared to *C. glutamicum* WT, with glycogen levels being lower and

decreasing after reaching its maximum. Additionally, it was shown that the abolishment of glycogen synthesis improved growth under an extracellular pH of 6, whereas no such influence was observed under an extracellular pH of 7.

Transcriptional analysis with an integrated luciferase reporter system showed that there were no carbon source-dependent differences in the transcription of the *glgC*, *glgA* and *glgX* genes between cells growing on glucose or on maltose and that transcription levels of all three genes were the highest in the beginning of cultivation. Respective transcription levels of *glgC* and *glgA* were higher than the ones of *glgX*, but also dropped faster.

To investigate the role of glycogen metabolism in *C. glutamicum*, the influence of disturbances in the glycogen metabolism on energetic parameters was examined. This analysis revealed no effect of *glgC* or *glgX* deletion on the ATP, $NADH/H^+$ or $NADPH/H^+$ levels. Re-inoculation experiments after prolonged carbon starvation of *C. glutamicum* WT, *C. glutamicum* Δ*glgC* and *C. glutamicum* Δ*glgX* showed that glycogen metabolism is involved in the survival and that usable glycogen was beneficent for growth after re-inoculation.

Zusammenfassung

In dieser Arbeit wurden verschiedene Gesichtspunkte des Glycogenstoffwechsels in *Corynebacterium glutamicum* betrachtet. Zuerst wurde die Rolle der *glgC*-, *glgA*-, *glgB*-, *glgX*-, *malP*- und *glgP*-Gene im Glycogenstoffwechsel auf Glucose und auf Maltose untersucht. Weiterhin wurde der Einfluss der Verfügbarkeit von Phosphat, von Stickstoffquellen und von Sauerstoff auf die Glycogengehalte sowie die Verbindung zwischen pH-Stress und dem Glycogenstoffwechsel untersucht. Zusätzliche Bemühungen wurden unternommen, um die Art der Regulation des Glycogenstoffwechsels durch die Reduktion von Phosphatkonzentrationen zu beleuchten und um die transkriptionelle Regulation von *glgC*, *glgA* und *glgX* während des Wachstums auf Glucose und auf Maltose zu beschreiben. Die Auswirkung von Störungen des Glycogenstoffwechsels auf das Produktionsverhalten unter anaeroben Bedingungen wurde genauso untersucht wie der Glycogenstoffwechsel des Succinat-produzierenden Stammes *C. glutamicum* Δ*aceE* Δ*pqo* Δ*ldhA* ΔC–T *ilvN* Δ*alaT* Δ*avtA*. Als letztes wurde der Einfluss von Deletionen von *glgC* (an der Glycogensynthese beteiligt) und *glgX* (am Glycogenabbau beteiligt) auf energetische Parameter und auf das Überleben von *C. glutamicum* überprüft.

Wachstumsexperimente mit verschiedenen Deletionsmutanten auf Glucose zeigten, (i) dass die *glgC*-, *glgA*- und *glgB*-Gene für die Glycogensynthese verantwortlich sind, (ii) dass das *glgX*-Gen hauptverantwortlich für den Glycogenabbau ist, (iii) dass das *malP*-Gen auch am Glycogenabbau beteiligt, aber unwichtiger ist, und (iv) dass das *glgP*-Gen unter den getesteten Bedingungen keine Rolle im Glycogenstoffwechsel spielt. Ähnliche Wachstumsversuche mit verschiedenen Deletionsmutanten auf Maltose zeigten, (i) dass die *glgC*- und *glgA*-Gene nicht an der Glycogensynthese beteiligt sind, (ii) dass das *glgB*-Gen nicht nur am Glycogenstoffwechsel, sondern auch an der Maltose-Verstoffwechslung beteiligt ist, (iii) dass die *glgX*- und *malP*-Gene für den Glycogenabbau wichtig sind und dass das *malP*-Gen auch an der Maltose-Verstoffwechslung beteiligt ist, und (iv) dass das *glgP*-Gen unter den getesteten Bedingungen keine Rolle im Glycogenstoffwechsel spielt.

Experimente mit verschiedenen Konzentrationen an Phosphat und Stickstoffquellen eröffneten, dass die Verminderung der Stickstoffverfügbarkeit keinen Einfluss auf den Glycogengehalt hatte, während die Verminderung der Phosphatkonzentrationen zu einem Anstieg des Glycogengehalts führte. Zusätzliche semi-quantitative RT-PCR-Experimente bestätigten, dass der höhere Glycogengehalt durch Phosphatverminderung zumindest teilweise durch Veränderungen der Transkriptionsprofile der *glgC*-, *glgA*-, *glgB*-, *glgX*-, *malP*- und *glgP*-Gene verursacht wurde. Durch das Durchführen von Experimenten unter anaeroben Bedingungen wurde außerdem gezeigt, (i) dass *C. glutamicum* in der Lage ist, Glycogen unter anaeroben Bedingungen zu akkumulieren, (ii) dass diese Akkumulation

eine Erhöhung der OD_{600} verursachte, die nicht eine Folge der Erhöhung der Zellzahl war, (iii) dass das Produktionsverhalten nicht durch Störungen im Glycogenstoffwechsel beeinflusst wurde, und (iv) dass Veränderungen im Zentralstoffwechsel, welche zur Akkumulation von Succinat führten, ein anderes Profil der Glycogenakkumulation im Vergleich zu *C. glutamicum* WT verursachten, wobei der Glycogengehalt niedriger war und sich verminderte, nachdem das Maximum erreicht war. Zusätzlich wurde gezeigt, dass die Beseitigung der Glycogensynthese das Wachstum bei einem extrazellulären pH von 6 verbesserte, während ein solcher Einfluss bei einem extrazellulären pH von 7 nicht beobachtet wurde.

Transkriptionsanalysen mit einem integriertem Luziferase-Reportersystem zeigten, dass es keine Kohlenstoff-abhängigen Unterschiede in der Transkription der *glgC*-, *glgA*- und *glgX*-Gene zwischen Zellen, die auf Glucose oder auf Maltose gewachsen waren, gab, und dass das Transkriptionsniveau aller drei Gene am Anfang der Kultivierung am höchsten war. Die jeweiligen Transkriptionsniveaus von *glgC* und *glgA* waren zwar höher als die von *glgX*, aber sanken auch schneller ab.

Um die Rolle des Glycogenstoffwechsels in *C. glutamicum* zu untersuchen, wurde der Einfluss von Störungen im Glycogenstoffwechsel auf energetische Parameter überprüft. Diese Analyse ergab, dass die Deletion von *glgC* oder *glgX* keinen Einfluss auf die Konzentrationen von ATP, $NADH/H^+$ oder $NADPH/H^+$ hat. Wieder-Inokulationsexperimente nach verlängerter Kohlenstoffaushungerung mit *C. glutamicum* WT, *C. glutamicum* Δ*glgC* und *C. glutamicum* Δ*glgX* zeigten, dass der Glycogenstoffwechsel am Überleben beteiligt ist und dass verwertbares Glycogen nützlich für Wachstum nach Wieder-Inokulation ist.

1 Introduction

The Gram-positive soil bacterium *Corynebacterium glutamicum* was firstly isolated in the course of a screening for glutamate-excreting microorganisms (Kinoshita *et al.*, 1957). The organism was described as immobile, non-spore forming and facultative anaerobic bacterium, which is able to excrete glutamate under certain conditions, such as limitation of biotin or addition of detergents or penicillin (Abe *et al.*, 1967; Nishimura *et al.*, 2007; Takeno *et al.*, 2007; Shiio *et al.*, 1962; Takinami *et al.*, 1965; Nara *et al.*, 1964). *C. glutamicum* is a member of the suborder *Corynebacterineae*, which consists amongst others of the families *Corynebacteriaceae* (with *C. diphtheriae*) and *Mycobacteriaceae* (with *Mycobacterium tuberculosis* and *M. smegmatis*) (Stackebrandt *et al.*, 1997; Liebl, 2005). The presence of mycolic acids in the cell wall helps to form an additional barrier affecting the permeability (Minnikin *et al.*, 1978; reviewed in Bayan *et al.*, 2003; reviewed in Daffé, 2005). Unlike *C. diphteriae* and *M. tuberculosis*, *C. glutamicum* is a GRAS (Generally Regarded As Safe) organism, which makes it interesting as a model organism for these related pathogens (Funke *et al.*, 1997; Micklinghoff *et al.*, 2009).

The genome of *C. glutamicum*, which consists of 3.28 MBp and possesses a G+C content of 53.8 %, was sequenced in 2003 by two independent working groups (Ikeda and Nakagawa, 2003; Kalinowski *et al.*, 2003). Increasing availability of new molecular tools for deletion and overexpression of genes and the knowledge about the genome caused a dramatic increase in range of products produced by *C. glutamicum* (reviewed in Wendisch *et al.*, 2006ab). The important amino acids l-glutamate and l-lysine are produced with this organism in large scale (Leuchtenberger *et al.*, 2005). The worldwide demand in 2011 was estimated to be 2.60 million and 1.95 million tons (Ajinomoto, 2012; Ajinomoto, 2013). Besides l-glutamate and l-lysine, other amino acids, such as l-alanine, l-valine, l-isoleucine, l-histidine and l-arginine, and organic acids, such as succinate, lactate, pyruvate and acetate, are also interesting chemicals produced with *C. glutamicum* (Jojima *et al.*, 2010; Radmacher *et al.*, 2002; Sahm *et al.*, 1996; Kulis-Horn *et al.*, 2013; Schneider *et al.*, 2011; Okino *et al.*, 2008b, Litsanov *et al.*, 2012; Wieschalka *et al.*, 2013; Okino *et al.*, 2012a; Wieschalka *et al.*, 2012; Yasuda *et al.*, 2007). Furthermore, alcohols, such as isobutanol, ethanol and xylitol, and polyamides, such as putresine and cadaverine, are produced by metabolic engineered *C. glutamicum* strains (Smith *et al.*, 2010; Blombach *et al.*, 2011; Inui *et al.*, 2004a; Sasaki *et al.*, 2010; Schneider and Wendisch, 2010; Mimitsuka *et al.*, 2007).

One of the advantages of using *C. glutamicum* for production of chemicals is the ability to grow on mineral medium with a variety of substrates. *C. glutamicum* is able to use various sugars, such as glucose, sucrose, fructose and maltose, alcohols, such as ethanol and arabitol, and organic acids, such as acetate, pyruvate, propionate and lactate, for growth and production (Liebl *et al.*, 2005; Krause *et al.*, 2010; Seibold *et al.*, 2009; Arndt *et al.*, 2008; Arndt and Eikmanns, 2007; Laslo *et al.*, 2012; Blombach and Seibold, 2010). During growth on carbohydrates, *C. glutamicum* accumulates the polysaccharide glycogen in the early exponential growth phase (Seibold *et al.*, 2007; Seibold *et al.*, 2009; Seibold *et al.*, 2010). Glycogen consists of long α-1,4-glycosidic linked chains of glucose units, which possess about every twentieth glucose residue an additional α-1,6-glycosidic branch. In most bacteria, glycogen is regarded as carbon and energy reserve and is accumulated under limiting growth conditions (Preiss and Romeo, 1994; Wilson *et al.*, 2012; Wang and Wise, 2011). Glycogen is also involved in sporulation in *Bacillus subtilis* or in differentiation in *Streptomyces coelicolor* (Kiel *et al.*, 1994; Martin *et al.*, 1997). In *M. smegmatis*, a relative of *C. glutamicum*, glycogen can act as carbon capacitor to balance temporary disturbances in intracellular carbon homeostasis (Belanger and Hatfull, 1999). A similar role was also proposed for the glycogen metabolism of *C. glutamicum* (Seibold and Eikmanns, 2007; Seibold and Eikmanns; 2013). Other studies revealed that glycogen is connected to trehalose by different pathways (see Figure 1). Trehalose can be formed (i) from linear maltodextrins, which are precursors for glycogen and intermediates of degradation, via TreYZ (malto-oligosyltrehalose synthase and malto-oligosyltrehalose trehalohydrolase), (ii) from glucose 1-phosphate, which is also a precursor for glycogen, via GalU (UDP-glucose pyrophosphorylase) and OtsAB (trehalose 6-phosphate synthase and a trehalose 6-phosphate phosphatase), and (iii) from maltose via TreS (trehalose synthase) (Tzvetkov *et al.*, 2003; Wolf *et al.*, 2003; Padilla *et al.*, 2004ab).

Some bacteria, such as *Escherichia coli*, accumulate glycogen in the stationary phase, while others, such as *Streptococcus mitis*, accumulate it already in the exponential phase (Holme and Palmstierna, 1956a; Holme and Palmstierna, 1956b; Holme *et al.*, 1957; Gibbons and Kapsimalis, 1963). *C. glutamicum* differs from other bacteria, since glycogen is degraded already in the beginning of the late exponential phase (Seibold *et al.*, 2007). Based on the data from *E. coli* and *M. tuberculosis*, respective genes for enzymes involved in glycogen synthesis or degradation were identified in *C. glutamicum* (Kalinowski *et al.*, 2003; Seibold *et al.*, 2007): *cg1268* (*glgC*), *cg1268* (*glgA*), *cg1281* (*glgB*), *cg2310* (*glgX*), *cg1479* (*glgP1*) and *cg2289* (*glgP2*); additionally, *cg1382* (*glgE*) was identified as putative glycogen synthase. Several studies led to the present model of glycogen metabolism (and the connected trehalose and maltose metabolism in *C.*

glutamicum (Figure 1). Glucose is taken up and phosphorylated by the glucose-specific phosphotransferase system (Moon *et al.*, 2007). Glucose 6-phosphate can also be formed when glucose is transported into the cell by a permease and then is phosphorylated by an intracellular glucokinase (Lindner *et al.*, 2010; Park *et al.*, 2000). Glucose 6-phosphate then is converted to glucose 1-phosphate by the phosphoglucomutase PGM (Seibold *et al.*, 2009; Seibold and Eikmanns, 2013). The ADP-glucose pyrophosphorylase GlgC uses ATP and glucose 1-phosphate to form ADP-glucose (Seibold *et al.*, 2007). GlgC is essential for the formation of glycogen during growth on glucose (Seibold *et al.*, 2007).

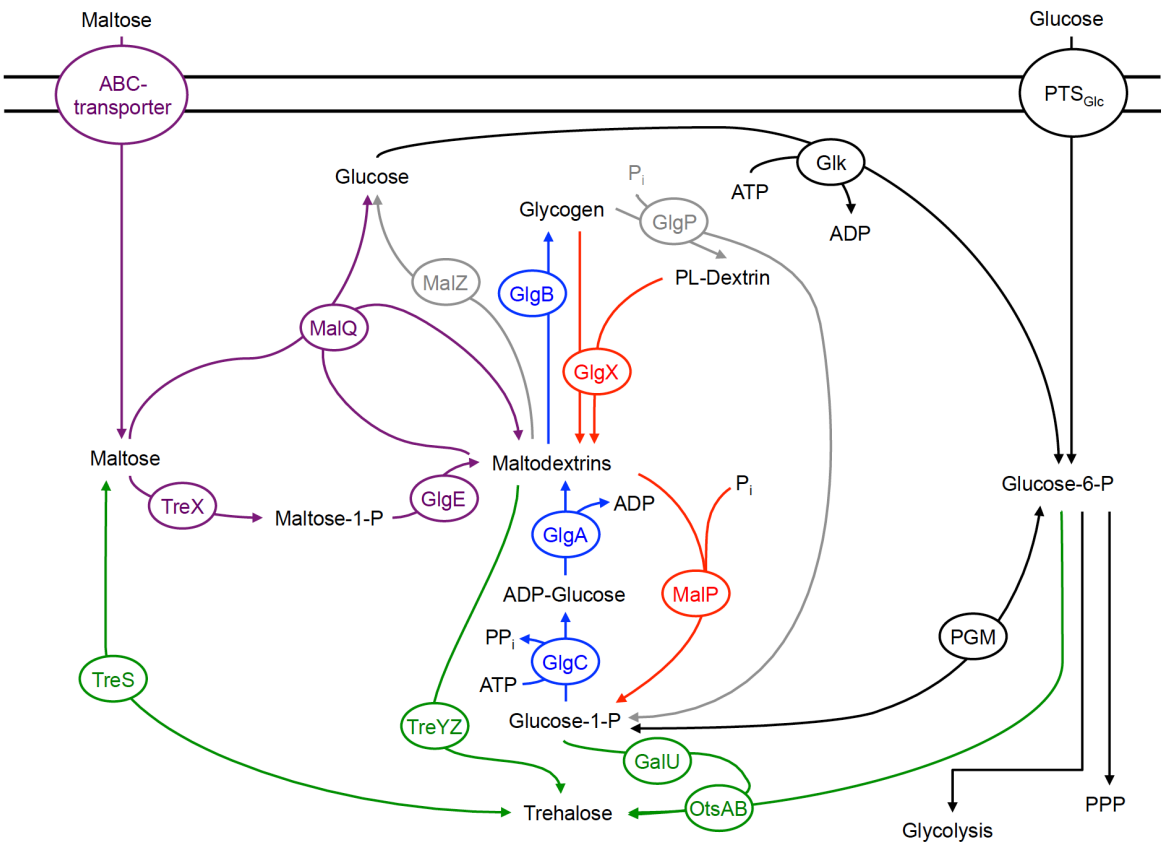


Figure 1: Model of glycogen metabolism and connected trehalose and maltose metabolism in *C. glutamicum* (based on genomic data from *C. glutamicum* and data from *E. coli* and *M. tuberculosis*). Glycogen synthesis is shown in blue, glycogen degradation in red, maltose related reactions in purple, trehalose related reactions in green, glucose related reactions in black, reactions without candidate genes in grey; ABC-transporter: ATP-binding cassette transporter for maltose; ADP adenosine diphosphate; ATP: adenosine triphosphate; GalU: UDP-glucose pyrophosphorylase; GlgA: glycogen synthase; GlgB: glycogen branching enzyme; GlgC: ADP-glucose pyrophosphorylase; GlgE: maltosyl transferase; GlgP: glycogen phosphorylase; GlgX: glycogen debranching enzyme; Glk: glucokinase; MalP: maltodextrin phosphorylase; MalQ: 4-alpha-glucanotransferase; MalZ: maltodextrin glucosidase; OtsA: trehalose 6-phosphate synthase OtsB: trehalose 6-phosphate phosphatase; PGM: phosphoglucomutase; PP_i/P_i: inorganic pyrophosphate/phosphate; PPP: pentose phosphate pathway; PTS_{Glc}: glucose-specific phosphotransferase system; TreS: trehalose synthase; TreX: maltokinase; TreY: malto-oligosyltrehalose synthase; TreZ: malto-oligosyltrehalose trehalohydrolase;;

Glycogen synthase (GlgA) uses ADP-glucose to elongate already existing linear maltodextrins by addition of one glucose residue and is also required for glycogen synthesis with glucose as carbon source (Tzvetkov *et al.*, 2003). α-1,6-glycosidic linkages are introduced by the glycogen branching enzyme (GlgB) (Seibold *et al.*, 2011). It was found that the glycogen debranching enzyme (GlgX) is the key player in glycogen degradation and deletion of *glgX* leads to problems in the response to osmotic shifts due to abolished trehalose formation, indicating its involvement in trehalose metabolism (Seibold and Eikmanns, 2007). Both glycogen and maltodextrin phosphorylase activity were detected in *C. glutamicum*. A deletion of *cg1479* (*glgP1*) led to a loss of maltodextrin phosphorylase activity (Seibold *et al.*, 2009). Hence, it was assumed that *cg2289* (now *glgP*) encodes a glycogen phosphorylase and that *cg1479* (now *malP*) encodes a maltodextrin phosphorylase (Seibold *et al.*, 2009). Glycogen phosphorylase activity was observed, but it was not confirmed that it is encoded by *glgP*. Glycogen phosphorylases are able to cleave glucose residues at the non-reducing ends of α-1,4-glycosidic linked glucose polymers, forming glucose 1-phosphate (Alonso-Casajús *et al.*, 2006). By action of phosphoglucomutase (PGM), glucose 1-phosphate is converted to glucose 6-phosphate, which can be fed into central metabolism (Seibold *et al.*, 2009; Seibold *et al.*, 2013). Glycogen debranching enzyme makes linear maltodextrins, which are a substrate for the maltodextrin phosphorylase (MalP) (Seibold *et al.*, 2009). Similarly to the glycogen phosphorylase, the maltodextrin phosphorylase can cleave glucose residues from the linear maltodextrins and form glucose 1-phosphate. Glucose 1-phosphate can be fed again into the central metabolism by action of the PGM. Besides maltodextrin phosphorylase activity, the activities of maltodextrin glucosidase MalZ and of 4-alpha-glucanotransferase MalQ, which linked glycogen metabolism to maltose metabolism, were detected in cell extracts of *C. glutamicum* (Seibold *et al.*, 2009). Maltodextrin glucosidase forms glucose, which is phosphorylated by an intracellular glucokinase Glk to glucose 6-phosphate (Lindner *et al.*, 2010; Park *et al.*, 2000). A gene coding for MalQ was identified (*cg2523*), whereas a candidate for MalZ is still missing (Seibold *et al.*, 2009).

Maltose metabolism of *C. glutamicum* is connected to glycogen metabolism. Maltose is taken up by the ABC transporter system MusEFGK$_2$I, one glucose residue of maltose is transferred to linear maltodextrins by the 4-alpha-glucanotransferase MalQ and the other glucose residue set free is phosphorylated to glucose 6-phosphate, which enters the central metabolism (Henrich *et al.*, 2013; Seibold *et al.*, 2009; Lindner *et al.*, 2010). Glycogen branching enzyme then introduces α-1,6-glycosidic linkages in the linear maltodextrins. The α-1,4-glycosidic linked maltodextrins are substrate for the maltodextrin phosphorylase, which cleaves glucose residues from the non-reducing ends (Seibold *et*

al., 2009). Resulting glucose 1-phosphate is converted to glucose 6-phosphate and fed into central metabolism (Seibold *et al.*, 2009; Seibold and Eikmanns, 2013). Additionally, in *M. tuberculosis* and *M. smegmatis* glycogen can be formed by a maltokinase, a maltosyl transferase and a glycogen branching enzyme (Elbein *et al.*, 2010; Kalscheuer *et al.*, 2010). Genomic analysis revealed the candidate genes for a maltokinase (*treX*) and maltosyl transferase (*glgE*) (Kalinowski *et al.*, 2003; Henrich, 2011). *GlgE* forms an operon with *glgB*, indicating the involvement in glycogen metabolism (Seibold *et al.*, 2011). Trehalose metabolism is also connected to the glycogen metabolism in *C. glutamicum* via three pathways: (i) The trehalose synthase TreS converts maltose (precursor for glycogen) to trehalose, (ii) the maltooligosyl trehalose synthase TreY and the malto-oligosyltrehalose trehalohydrolase TreZ use linear maltodextrins for trehalose formation, and (iii) the UDP-glucose pyrophosphorylase GalU, trehalose 6-phosphate synthase OtsA and the trehalose 6-phosphate phosphatase OtsB form trehalose from glucose 1-phosphate (Tzvetkov *et al.*, 2003; Wolf *et al.*, 2003; Padilla *et al.*, 2004ab; Carpinelli *et al.*, 2006).

In *C. glutamicum*, the regulation of the glycogen metabolism has been scarcely investigated. Except the involvement of the global regulators RamA and RamB in the transcription of *glgC* and *glgA*, no other regulators were reported to be connected to glycogen metabolism control. Since the reduction of phosphate concentrations led to an increase of glycogen levels, it is very likely that other regulators can influence the glycogen metabolism (Woo *et al.*, 2011).

One aim of the present study was the construction of single and multiple deletion mutants of *C. glutamicum* of genes coding for enzyme (putatively) involved in glycogen metabolism. By analysing growth and glycogen levels with the carbon sources glucose or maltose, the role of the respective genes in the glycogen metabolism was investigated. Defined deletion mutants with clear phenotype (abolishment of glycogen synthesis and strong impairment of glycogen degradation) were used for further studies.
Additionally, the influence of several environmental conditions on the glycogen levels of *C. glutamicum* was tested to identify possible regulatory stimuli of the glycogen metabolism. To investigate this, growth and glycogen levels in *C. glutamicum* were determined under decreasing concentrations of phosphate or of nitrogen sources. Following the predictions of a dynamic flux model, it was tested, whether *C. glutamicum* is able to form glycogen under anaerobic conditions.
A further goal was to investigate the regulation of the glycogen metabolism. This was done by the promoter analysis of genes (*glgC*, *glgA* and *glgX*) coding for key enzymes of

the glycogen metabolism. Additional insight should be gained by expression analysis of genes involved in glycogen metabolism under newly identified conditions, which led to altered glycogen levels.

Another important point was the investigation of advantages of a functional glycogen metabolism in *C. glutamicum*. For this, the effects of disturbed glycogen metabolism on growth under slightly acidic extracellular pH and on the production behaviour under anaerobic conditions were analysed. Additional, the glycogen levels of the succinate-producer *C. glutamicum* Δ*aceE* Δ*pqo* Δ*ldhA* ΔC-T *ilvN* Δ*alaT* Δ*avtA* were also examined (Wieschalka *et al.*, 2012; Wieschalka *et al.*, 2013).

Last, but not least, the role of the glycogen metabolism in *C. glutamicum* was subject of other experiments. One approach was to analyse intracellular levels of energetic parameters (AMP, ADP, ATP, NAD^+, $NADH/H^+$, $NADP^+$, $NADPH/H^+$) in *C. glutamicum* WT and *C. glutamicum* strains with disturbances in glycogen metabolism (abolishment of glycogen synthesis or glycogen degradation) during growth with glucose. Additionally, the influence of disturbances in glycogen metabolism (abolishment of glycogen synthesis or glycogen degradation) on survival and growth behaviour after prolonged carbon starvation was investigated.

2 Material and methods

2.1 Bacterial strains, plasmids and oligonucleotides

2.1.1 Bacterial strains

All strains used in this work are listed in table 1.

Table 1: Strains used in this work

Strain	Relevant characteristics	Reference or source
E. coli DH5α	*supE44 hsdR17 recA1 endA1 gyrA96 thi-1 relA1 gyrA96 ΔlacU169 (φ80lacZΔM15)*	Hanahan, 1985
C. glutamicum WT	Wild-type strain ATCC 13032, biotin-auxotrophic	Abe *et al.*, 1967
C. glutamicum RES167	Restriction deficient derivative of *C. glutamicum* ATCC13032	Tauch *et al.*, 2002
C. glutamicum Δ*glgC*	*C. glutamicum* WT with deletion of *glgC* coding for ADP-glucose pyrophosphorylase	This work, in cooperation with Katrin Breitinger
C. glutamicum Δ*glgA*	*C. glutamicum* WT with deletion of *glgA* coding for glycogen synthase	This work, in cooperation with Alissa Grässer
C. glutamicum Δ*glgB*	*C. glutamicum* WT with deletion of *glgB* coding for glycogen branching enzyme	Gerd Seibold, unpublished
C. glutamicum Δ*glgX*	*C. glutamicum* WT with deletion of *glgX* coding for glycogen debranching enzyme	This work, in cooperation with Katrin Breitinger
C. glutamicum Δ*glgP*	*C. glutamicum* WT with deletion of *glgP2* coding for a putative glycogen phosphorylase	This work, in cooperation with Katrin Breitinger
C. glutamicum Δ*malP*	*C. glutamicum* WT with deletion of *glgP1* (*malP*) coding for maltodextrin phosphorylase	Seibold *et al.*, 2009
C. glutamicum Δ*glgX*Δ*glgP*	*C. glutamicum* WT with deletions of *glgX* and *glgP2*	This work
C. glutamicum Δ*glgX*Δ*malP*	*C. glutamicum* WT with deletions of *glgX* and *glgP1* (*malP*)	This work
C. glutamicum Δ*glgP*Δ*malP*	*C. glutamicum* WT with deletions of *glgP2* and *glgP1* (*malP*)	This work
C. glutamicum Δ*glgX*Δ*glgP* Δ*malP*	*C. glutamicum* WT with deletions of *glgX*, *glgP2* and *glgP1* (*malP*)	This work

C. glutamicum RESluxIN	Restriction deficient derivative of *C. glutamicum* ATCC13032 with *luxAB* coding for two subunits of the luciferase from *Photorhabdus luminescens* and *fre* coding for a flavin mononucleotide reductase from *Escherichia coli* integrated into copy B of 16S rDNA	Laslo, 2013
C. glutamicum RESluxIN_p_{glgX1}	*C. glutamicum* RESluxIN with promoter fragment p_{glgX1} integrated into copy B of 16S rDNA	This work, in cooperation with Leonie Schnell
C. glutamicum RESluxIN_p_{glgX2}	*C. glutamicum* RESluxIN with promoter fragment p_{glgX2} integrated into copy B of 16S rDNA	This work, in cooperation with Leonie Schnell
C. glutamicum RESluxIN_$p_{glgC(ATG)}$	*C. glutamicum* RESluxIN with promoter fragment $p_{glgC(ATG)}$ integrated into copy B of 16S rDNA	This work, in cooperation with Leonie Schnell
C. glutamicum RESluxIN_$p_{glgC(GTG)}$	*C. glutamicum* RESluxIN with promoter fragment $p_{glgC(GTG)}$ integrated into copy B of 16S rDNA	This work, in cooperation with Leonie Schnell
C. glutamicum RESluxIN_p_{glgA}	*C. glutamicum* RESluxIN with promoter fragment p_{glgA} integrated into copy B of 16S rDNA	This work, in cooperation with Leonie Schnell
C. glutamicum Δ*aceE* Δ*pqo* Δ*ldhA* ΔC–T *ilvN* Δ*alaT* Δ*avtA* (*C. glutamicum* ELB-P)	*C. glutamicum* WT with deletions of *aceE* coding for E1p subunit of PDHC, of *pqo* coding for pyruvate:quinone oxidoreductase, of *ldhA* coding for L-lactic acid dehydrogenase, of the last 249 bp of C-terminal domain of *ilvN* coding for the small subunit of acetohydroxyacid synthase, of *alaT* coding for alanine aminotransferase, and of *avtA* coding for valine:pyruvate aminotransferase	Wieschalka *et al.*, 2012

2.1.2 Plasmids

All plasmids used in this work are listed in table 2.

Table 2: Plasmids used in this work

Plasmid	Characteristics	Reference or source
pK19*mobsacB*	Km^r; mobilizable (*oriT*); *oriV*	Schäfer *et al.*, 1994
pK19*mobsacB*_Δ*glgC*	pK19*mobsacB* with a truncated *glgC* gene	This work, in cooperation with Katrin Breitinger
pK19*mobsacB*_Δ*glgA*	pK19*mobsacB* with a truncated *glgA* gene	This work, in cooperation with Alissa Grässer
pK19*mobsacB*_Δ*glgB*	pK19*mobsacB* with a truncated *glgB* gene	Gerd Seibold, unpublished
pK19*mobsacB*_Δ*glgX*	pK19*mobsacB* with a truncated *glgX* gene	This work, in cooperation with Katrin Breitinger
pK19*mobsacB*_Δ*glgP*	pK19*mobsacB* with a truncated *glgP* gene	This work, in cooperation with Katrin Breitinger
pK19*mobsacB*_Δ*malP*	pK19*mobsacB* with a truncated *malP* gene	Seibold *et al.*, 2009

2.1.3 Oligonucleotides

All primers in this work are listed in table 3.

Table 3: Oligonucleotides used in this work. Restriction sites are solid underlined, homologous regions are dashed underlined.

Oligonucleotide	Sequence	Purpose
glgCDel-For1	5'-CGGGATCCCGGTGTCTTAATC GCAGGGTTC-3'	Generation of 5'-end fragment of *glgC* for deletion; proof of deletion; *Bam*HI restriction site
glgCDel-Rev1	5'-ATGTACTGCCCCGTTGGACCA GACACGTTCCACGACAATG-3'	Generation of 5'-end fragment of *glgC* for deletion
glgCDel-For2	5'-GGTCCAACGGGGCAGTACATA CTCCGTGCTGTCCAACAAC-3'	Generation of 3'-end fragment of *glgC* for deletion
glgCDel-Rev2	5'-CCCAAGCTTGGGTTGTCTTCG GCCAAGTCTCC-3'	Generation of 3'-end fragment of *glgC* for deletion; proof of deletion; *Hind*III
glgPDel-For1	5'-CGGGATCCCGCTTGGGTCTTG CTGATGTAG-3'	Generation of 5'-end fragment of *glgP* for deletion; *Bam*HI restriction site
glgPDel-Rev1	5'-TTCTCGCCCGCAAGCACACCA AGTCCGCCAGAGTAGATTG-3'	Generation of 5'-end fragment of *glgP* for deletion
glgPDel-For2	5'-GGTGTGCTTGCGGGCGAGAA CAGCGTTCGCGTTGATTCCG-3'	Generation of 3'-end fragment of *glgP* for deletion
glgPDel-Rev2	5'-CCCAAGCTTGGGGAACGTCAG CCACGCAATCC-3'	Generation of 3'-end fragment of *glgP* for deletion; *Hind*III restriction site
GlgPupstr	5'-AGCCCGTCAATTCTATGTGG-3'	Proof of deletion of *glgP*
GlgPdwnstr	5'-GTGTCATGCACGGTAACTGG-3'	Proof of deletion of *glgP*
glgXDel-For1SalI	5'-ACGCGTCGACGTCGCCAAAGA TGGTGCGGAAGTC-3'	Generation of 5'-end fragment of *glgX* for deletion; proof of deletion; *Sal*I restriction site

glgXDel-Rev1	5'-TCAAAATCTCCATCGAAAGCAC GAGCATAGGGATCAAC-3'	Generation of 5'-end fragment of *glgX* for deletion
glgXDel-For2	5'-TTTCGATGGAGATTTTGAGGCA GAACGTGCTTCGACTC-3'	Generation of 3'-end fragment of *glgX* for deletion
glgXDel-Rev2	5'-CCCAAGCTTGGGCGTCGCTATC CACTCCGATG-3'	Generation of 3'-end fragment of *glgX* for deletion; proof of deletion; *Hind*III restriction site
GlgX-upstr	5'-GAGCAGCGAGCAAGCATT-3'	Proof of deletion of *glgX*
GlgX-dwnstr	5'-TGAGGACCCGCAGGTAAGAT-3'	Proof of deletion of *glgX*
Del_glgA.for1 *Hind*III	5'-ATCCATAAGCTTGGCCGTGATCT GGTGAGG-3'	Generation of 5'-end fragment of *glgA* for deletion; *Hind*III restriction site
Del_glgA.rev1	5'-ATCCGACGTTGGAGGCATCCCT GAGGTTGTTGTCGACGGCA-3´	Generation of 5'-end fragment of *glgA* for deletion
Del_glgA.for2	5'-GGATGCCTCCAACGTCGGATGC AATGCGTAAACCGGTGGAC-3'	Generation of 3'-end fragment of *glgA* for deletion
Del_glgA.rev2 *Xba*I	5'-CGAGGTTCTAGAAGTCGATCAA TCTGTAAG-3'	Generation of 5'-end fragment of *glgA* for deletion; *Xba*I restriction site
Del_glgA-ctrl.for	5´-ACTGGATAGTGGAAGTGGG-3'	Proof of deletion of *glgA*
Del_glgA-ctrl.rev	5'-GCGCAGGAACAGAAGCAATG-3'	Proof of deletion of *glgA*
DelGlgB-For 1	5'- GCTCTAGATTGCAGCAACTAC GCAACATCC-3'	Proof of deletion of *glgB*; (Generation of 5'-end fragment of *glgB* for deletion; *Xba*I restriction site)
DelGlgB-Rev 2	5'-CCCAAGCTTTGAATGGGCTGAA GACAACAC-3'	Proof of deletion of *glgB*; (Generation of 3'-end fragment of *glgB* for deletion; *Hind*III restriction
MalP_ctrl-ext2.for	5'-GGCGTCCAATTTTGCGGCGT-3'	Proof of deletion of *malP*
MalP_ctrl-ext2.rev	5'-TGGTACGTCACCGGGGCTCT-3'	Proof of deletion of *malP*

qRT_glgX_for	5’-CTTCGACTGGGGCAACGATC-3’	qRT-PCR for *glgX*
qRT_glgX_rev	5’-GGCAGTCACTCCCAGATCAC-3’	qRT-PCR for *glgX*
qRT_glgC_for	5’-CATGGACCCAAGCCAGATGC-3’	qRT-PCR for *glgC*
qRT_glgC_rev	5’-AGGATCATCAGGGGTTCCCG-3’	qRT-PCR for *glgC*
qRT_glgB_for	5’-GACCTACCAAACTGCGCTCG-3’	qRT-PCR for *glgB*
qRT_glgB_rev	5’-TCGCCTTCCTCTATGCCTGG-3’	qRT-PCR for *glgB*
qRT_glgA_for	5’-TACGACGTGTCCTCCTGGTC-3’	qRT-PCR for *glgA*
qRT_glgA_rev	5’-GTCATCGAAAGTCGGGCGAG-3’	qRT-PCR for *glgA*
qRT_malP_for	5’-ACCCAGGACTACCCCGTTAC-3’	qRT-PCR for *malP*
qRT_malP_rev	5’-GCGCGGGTTTTCATATCGTC-3’	qRT-PCR for *malP*
qRT_glgP_for	5’-GCGGGCGAGAACATGAAGTC-3’	qRT-PCR for *glgP*
qRT_glgP_rev	5’-GACCTGCTCGCCGTTCTTATC-3’	qRT-PCR for *glgP*

2.2 Chemicals

Chemical	Manufacture
α-D(+)-glucose monohydrate	Carl Roth GmbH, Karlsruhe, Germany
Agarose NEEO Ultra-Qualität	Carl Roth GmbH, Karlsruhe, Germany
Ammonium sulphate	Sigma Aldrich Chemie GmbH, Steinheim, Germany
Adenosine-5'-triphosphate (ATP)	Roche Diagnostics GmbH, Mannheim, Germany
BactoTM agar	Becton Dickinson GmbH, Heidelberg, Germany
BactoTM tryptone	Becton Dickinson GmbH, Heidelberg, Germany
BactoTM yeast extract	Becton Dickinson GmbH, Heidelberg, Germany
Biotin	Sigma Aldrich Chemie GmbH, Steinheim, Germany
Brain heart infusion (BHI)	Becton Dickinson GmbH, Heidelberg, Germany
Calcium chloride dihydrate	Merck KGaA, Darmstadt, Germany
Chloroform	Carl Roth GmbH, Karlsruhe, Germany
Chromanorm (H_2O for HPLC)	VWR International GmbH, Darmstadt, Germany
Copper(II) sulphate pentahydrate	Merck KGaA, Darmstadt, Germany
Deoxyribonucleotide triphosphates (dNTPs)	Fermentas GmbH, St. Leon-Rot, Germany
Dimethylsulfoxide (DMSO)	Merck KGaA, Darmstadt, Germany
Dipotassium hydrogen phosphate	Sigma Aldrich Chemie GmbH, Steinheim, Germany
DNA Loading Dye (6x)	Fermentas GmbH, St. Leon-Rot, Germany
Ethanol	VWR International GmbH, Darmstadt, Germany
Ethidium bromide	Carl Roth GmbH, Karlsruhe, Germany
Ethylenediaminetetraacetic acid (EDTA)	AppliChem GmbH, Darmstadt, Germany
GeneRuler™ 1 kb DNA Ladder	Fermentas GmbH, St. Leon-Rot, Germany
Glycerol	Sigma Aldrich Chemie GmbH, Steinheim, Germany
Hydrogen chloride (HCl)	Merck KGaA, Darmstadt, Germany
Isoamyl alcohol	Carl Roth GmbH, Karlsruhe, Germany
Isopropanol	Merck KGaA, Darmstadt, Germany
Ferrous(II) sulphate heptahydrate	Merck KGaA, Darmstadt, Germany
Kanamycin	Carl Roth GmbH, Karlsruhe, Germany

Magnesium chloride hexahydrate	Merck KGaA, Darmstadt, Germany
Magnesium sulphate heptahydrate	Sigma Aldrich Chemie GmbH, Steinheim, Germany
Manganese(II) sulphate monohydrate	Merck KGaA, Darmstadt, Germany
Methanol	VWR International GmbH, Darmstadt, Germany
3-(N-morpholino)propanesulfonic acid (MOPS)	Carl Roth GmbH, Karlsruhe, Germany
Nickel(II) chloride hexahydrate	Merck KGaA, Darmstadt, Germany
β-Nicotinamide adenine dinucleotide disodium salt, oxidized (NAD)	GERBU Biotechnik GmbH, Wieblingen, Germany
β-Nicotinamide adenine dinucleotide disodium salt, reduced (NADH/H+)	Roche Diagnostics GmbH, Mannheim, Germany
β-Nicotinamide adenine dinucleotide phosphate disodium salt, oxidized (NADP)	GERBU Biotechnik GmbH, Wieblingen, Germany
β-Nicotinamide adenine dinucleotide phosphate disodium salt, reduced (NADPH/H+)	GERBU Biotechnik GmbH, Wieblingen, Germany
L-Ornithine	Merck KGaA, Darmstadt, Germany
Ortho-phthaldialdehyde (OPA)	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
Phosphoenolpyruvate (PEP)	Roche Diagnostics GmbH, Mannheim, Germany
Poly(deoxyinosinic-deoxycytidylic) (Poly(d[I;C]))	Roche Diagnostics GmbH, Mannheim, Germany
Potassium acetate	Merck KGaA, Darmstadt, Germany
Potassium dihydrogen phosphate (KH_2PO_4)	Merck KGaA, Darmstadt, Germany
Potassium hydroxide (KOH)	Merck KGaA, Darmstadt, Germany
Potassium phosphate dibasic (K_2HPO_4)	Sigma Aldrich Chemie GmbH, Steinheim, Germany
Roti®-phenol/chloroform/isoamyl alcohol	Carl Roth GmbH, Karlsruhe, Germany
D-Sucrose	Carl Roth GmbH, Karlsruhe, Germany
Sodium acetate	Merck KGaA, Darmstadt, Germany
Sodium chloride (NaCl)	Sigma Aldrich Chemie GmbH, Steinheim, Germany

Spectinomycin dihydrochloride pentahydrate	Sigma Aldrich Chemie GmbH, Steinheim, Germany
D-Sorbitol	Sigma Aldrich Chemie GmbH, Steinheim, Germany
Struktol 674 antifoam	Schiller und Seilacher, Hamburg, Germany
Sulphuric acid, 95 – 97 % (H_2SO_4)	Merck KGaA, Darmstadt, Germany
Tris(hydroxymethyl)aminomethane (Tris)	Sigma Aldrich Chemie GmbH, Steinheim, Germany
Urea	Carl Roth GmbH, Karlsruhe, Germany
Zinc sulphate heptahydrate	Merck KGaA, Darmstadt, Germany

2.3 Instruments

Anthos ht III microplate-reader	Anthos Mikrosysteme GmbH, Krefeld, Germany
Biofuge pico Heraeus #3325B	DJB Labcare Ltd, Buckinghamshire, Great Britain
Centrifuge 5804 R	Eppendorf AG, Hamburg, Germany
Rotor A-4-44	Eppendorf AG, Hamburg, Germany
Rotor F-45-30-11	Eppendorf AG, Hamburg, Germany
Cuvettes . Micro, 1,6 mL	SARSTEDT AG & Co., Nümbrecht, Germany
DASGIP parallel fermentation system	DASGIP Information and Process Technology GmbH, Jülich, Germany
Electrophoresis Power Supply EPS 601	Amersham Pharmacia Biotech GmbH, Freiburg, Germany
Electroporation cuvettes, 2 mm electrode gap	Peqlab Biotechnologie GmbH, Erlangen, Germany
Gene Pulser	Bio-Rad Laboratories GmbH, Munich, Germany
Glass beads, diameter 0.1 mm	Sigma Aldrich Chemie GmbH, Steinheim, Germany
LC 1100/1200 system with reflective index detector (G1321A) and variable wavelength detector (G1314B)	Agilent Technologies, Böblingen, Germany
precolumn (Organic Acid, 40 x 8 mm)	CS-Chromatographie Service GmbH, Langerwehe, Germany
main column (Organic Acid, 300 x 8 mm)	CS-Chromatographie Service GmbH, Langerwehe, Germany

precolumn (C18-Multohyp, ODS-5µm, 40 x 4 mm)	CS-Chromatographie Service GmbH, Langerwehe, Germany
main column (C18-Multohyp, ODS-5µm, 125 x 4 mm)	CS-Chromatographie Service GmbH, Langerwehe, Germany
Vials for HPLC (R1 klar/ 6mm)	CS-Chromatographie Service GmbH, Langerwehe, Germany
LightCycler	Roche Diagnostics GmbH, Mannheim, Germany
LumiNuncTM 96-well plate	Thermo Fisher Scientific GmbH, Ulm, Germany
micro scales	Mettler-Toledo GmbH, Gießen, Germany
NanoDrop 2000 Spectrophotometer	Thermo Fisher Scientific GmbH, Ulm, Germany
pH-Meter WTW pH521	Wissenschaftlich-Technische Werkstätten, Weilheim, Germany
Photographic documentation	Decon Science Tec GmbH, Mühlbachwiesen, Germany
UV-Screen TFP-M/WL, 312 nm	Bachhofer, Reutlingen, Germany
Photometer Ultrospec 2100 pro	Amersham Pharmacia Biotech GmbH, Freiburg, Germany
Photometer Ultrospec 3000	Amersham Pharmacia Biotech GmbH, Freiburg, Germany
Pipettes	
PIPETMAN classic	Abimed GmbH, Langenfeld, Germany
DISCOVERY Comfort DV10C	Abimed GmbH, Langenfeld, Germany
PS-Microplate 96 well, flat bottom	Greiner Bio-One GmbH, Frickenhausen, Germany
Hybaid RiboLyser	Thermo Fisher Scientific, Dreieich, Germany
Rotary Shaker Certomat SII	B. Braun Biotech International GmbH, Melsungen, Germany
Sartorius BP 2100	Sartorius AG, Göppingen, Germany
Sartorius BP 8199	Sartorius AG, Göppingen, Germany
Sterile filters Filtropur S	SARSTEDT AG & Co., Nümbrecht, Germany
Tecan Infinite M200	Tecan Group Ltd., Männedorf, Switzerland
Thermo-cycler	Biometra GmbH, Göttingen, Germany
Ultracentrifuge L8-60M	Beckman Coulter, Inc., Fullerton, USA
Rotor TFT65.13	
Water bath	Köttermann GmbH & Co KG, Uetze/Hänigsen, Germany

2.4 Enzymes and kits

DNase I	Fermentas GmbH, St. Leon-Rot, Germany
E.Z.N.A.®Plasmid Mini Kit I	Omega Bio-tek, Inc., Norcross, USA
FastAP™ thermosensitive alkaline phosphatase	Fermentas GmbH, St. Leon-Rot, Germany
HotStar HiFidelity Polymerase Kit	Qiagen GmbH, Hilden, Germany
Lactate dehydrogenase	Roche Diagnostics GmbH, Mannheim, Germany
M-MuLV reverse transcriptase	Fermentas GmbH, St. Leon-Rot, Germany
NucleoSpin® Gel and PCR Clean-up	Macherey-Nagel GmbH & Co. KG, Düren, Germany
Perfectprep Plasmid Mini	Eppendorf AG, Hamburg, Germany
Phusion® polymerase	New England Biolabs, Ipswich, MA, USA
Pierce* BCA* Protein Assay	Thermo Fisher Scientific GmbH, Ulm, Germany
Pyruvate kinase	Roche Diagnostics GmbH, Mannheim, Germany
Qiagen RNeasy Mini Kit	Qiagen GmbH, Hilden, Germany
Qiagen RNeasy Midi Kit	Qiagen GmbH, Hilden, Germany
5'/3' RACE Kit 2nd Generation Kit	Roche Diagnostics GmbH, Mannheim, Germany
Restriction endonucleases	Fermentas GmbH, St. Leon-Rot, Germany
SensiMix SYBR One-Step RT-PCR Kit	Bioline GmbH, Luckenwalde, Germany
T4 DNA ligase	Fermentas GmbH, St. Leon-Rot, Germany
Taq DNA polymerase S	Genaxxon Bioscience GmbH, Ulm, Germany
Terminal deoxynucleotidyl transferase (TdT)	Fermentas GmbH, St. Leon-Rot, Germany

2.5 Growth media

For cultivation of *Corynebacterium glutamicum* and *Escherichia coli*, the following nutrients were used. For production of agar plates, 17 g agar/l was added to the respective media. For selection, kanamycin or spectinomycin were added in concentrations of up to 50 µg/mL and 100 µg/ml, respectively. For selection during generation of deletion mutants, 100 g sucrose/l and 17 g agar/l were added to 2x TY medium.

2x TY complex medium (Green and Sambrook, 2012)

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

BHI complex medium (Liebl *et al.*, 1989)

Brain heart infusion powder	37	g/l

BHIS complex medium (Tauch *et al.*, 2002)

Brain heart infusion powder	37	g/l
Sorbitol	91	g/l

Both ingredients were prepared separately and combined after autoclaving to reach the final concentrations.

CgC minimal medium (modified after Eikmanns *et al.*, 1991)

$(NH_4)_2SO_4$	5	g/l
Urea	5	g/l
3-(N-morpholino)propanesulfonic acid (MOPS)	21	g/l
K_2HPO_4	1	g/l
KH_2PO_4	1	g/l
$MgSO_4$ x 7 H_2O	0.25	g/l
$CaCl_2$	0.01	g/l

$MgSO_4$ and $CaCl_2$ were prepared separately as 1000 x stock solutions and autoclaved. Appropriate volumes were added to CgC minimal medium. KOH was used to adjust the pH of the minimal medium to 6.8 (for glucose and maltose as carbon sources) or 6.3 (for

acetate as carbon source), respectively. After autoclaving 1 ml/l of a micronutrient stock solution (1000 x) and a biotin stock solution (200 µg/ml) were added to the minimal medium. The carbon sources D-glucose, D-maltose or potassium acetate were added to the CgC minimal medium to obtain final concentrations up to 2 % (w/v).

Micronutrient stock solution (1000 x)

$FeSO_4$ x 7 H_2O	16.4	g/l
$MnSO_4$ x H_2O	10.0	g/l
$CuSO_4$ x 5 H_2O	0.2	g/l
$ZnSO_4$ x 7 H_2O	1.0	g/l
$NiCl_2$ x 6 H_2O	0.02	g/l

The salts were solved in water by adding concentrated HCl. The solution was sterile filtered and stored in a screw cap bottle under nitrogen atmosphere.

2.6 Cultivation conditions

Bacterial strains were stored as glycerol cultures [30 % (v/v) glycerol] in 2 mL screw cap tubes at - 80 °C and used for streaking out on 2x TY agar plate with a sterile inoculation loop. Plates with respective strains were incubated for 24 h at 37 °C (*E. coli*) or for 48 h at 28 °C (*C. glutamicum*), respectively. Single colonies on these plates were used to inoculate respective precultures.

2.6.1 Aerobic cultivation of *Corynebacterium glutamicum*

For cultivation of *C. glutamicum*, a single colony of the respective strain was used to inoculate 5 mL of 2x TY complex medium. After incubation for 8 h at 28 °C on a rotary shaker at 120 rpm, this preculture was used to inoculate a second preculture of 50 mL 2x TY medium in a 500 mL baffled Erlenmeyer flask, which was incubated over night at 28 °C and 120 rpm on a rotary shaker. The second preculture was centrifuged (4,500 x g, 8 min, 4 °C) and washed twice with ice-cold 0.9 % (w/v) NaCl solution. Cells were suspended in a final volume 2 mL 0.9 % (w/v) NaCl solution and used to inoculate the CgC minimal medium to an optical density (OD_{600}) of around 1 at a wavelength of 600 nm. Growth was followed by periodical measurements of the OD_{600}. For determination of glycogen levels, substrate consumption, and production behaviour, samples were taken at different time points and prepared according to the specific use. For experiments with reduced concentrations of phosphate or nitrogen sources, the concentrations of both phosphate sources (dipotassium hydrogen phosphate and potassium dihydrogen

phosphate) or of both nitrogen sources (ammonium sulphate and urea) were reduced equally according to the desired final concentrations of phosphate and nitrogen sources.

2.6.2 Anaerobic incubation of *Corynebacterium glutamicum*

For anaerobic incubation of *C. glutamicum*, cells of the respective strains were treated as for aerobic cultivation, but the second preculture was used to inoculate 50 mL of CgC in a 100 mL Schott glass bottle with rubber plug and screw cap to an OD_{600} of about 10 to 15. The cultures were incubated at 28 °C on a rotary shaker at 120 rpm. Samples for determination of OD_{600} and analysis of glycogen levels, substrate consumption, and production behaviour were taken at different time points with sterile syringes and needles. The gas phase in the bottles was replaced by nitrogen before autoclaving.

2.6.3 Cultivation of *Corynebacterium glutamicum* in a DASGIP parallel fermentation system

For cultivation of *C. glutamicum* in a DASGIP parallel fermentation system, the precultures of the respective strains were treated as for aerobic cultivation in Erlenmeyer flask. Cells from a 50 mL 2x TY preculture were washed twice with cold 0.9 % (w/v) NaCl solution and used to inoculate CgC minimal medium in a 400 mL bioreactor to an OD_{600} of around 10 and a final volume of 250 ml. Inoculum was added as well as glucose, biotin and micronutrients through a rubber septum, using sterile syringes and needles. The cultivation temperature was held constant at 30 °C by placing the four bioreactors in a tempered water bath. The pH was monitored by using pH electrodes and adjusted automatically by the “DASGIP Control” software by adding either 4 M KOH or 4 M H_2SO_4. During fermentations, the aeration rate with air was set to “3”, which corresponds to a rate of 0.75 vol air per volume medium and minute (vvm) (Blombach *et al.*, 2011). The amount of dissolved oxygen (DO) – in these experiments it was set to 30 % saturation – was adjusted and monitored with pO_2 electrodes. The regulation of DO took place by adjustment of the stirring frequency. The formation of foam was inhibited by manual addition of diluted solutions of the anti-foam agent “Struktol 674 antifoam“, when needed. The “DASGIP Control” software was used for adjustment of the respective pH values and % DO, calibration of the respective electrodes, and monitoring the whole fermentation process. Samples for determination of OD_{600} and analysis of glycogen levels, substrate consumption, and production behaviour were taken at different time points with sterile syringes and needles.

2.6.4 Cultivation of *Corynebacterium glutamicum* in survival experiments

For survival experiments, *C. glutamicum* was treated as described above to inoculate a first culture of CgC minimal medium with 2 % glucose or 1 % acetate, respectively, as carbon source. These main cultures served as inocula for second cultures. After 2, 3, and 5 days in the first culture with glucose as carbon source, cells were washed once with 0.9 % NaCl solution and used to inoculate a second culture with fresh CgC minimal medium and 2 % glucose. When acetate was used as carbon source in the first culture, cells were harvested after 1, 2, and 3 days, washed once with 0.9 % NaCl solution and used to inoculate a second culture with fresh CgC minimal medium and 2 % glucose. Growth was monitored by periodical determination of OD_{600}. Samples for glycogen levels in the first cultures were taken at the time points when cells of the first culture served as inoculum for the second cultures.

2.7 Preparation and transformation of electro-competent *Escherichia coli* and *Corynebacterium glutamicum* cells

2.7.1 Preparation and transformation of electro-competent *Escherichia coli* cells

For preparation of electro-competent *E. coli* DH5α cells, a method modified according to Green and Sambrook (2012) was used. 500 µL of an overnight culture (5 mL of 2x TY) served as inoculum for 250 mL 2x TY medium in a 1000 mL baffled Erlenmeyer flask. This main culture was cultivated at 37 °C on a rotary shaker at 120 rpm until it reached an OD_{600} of around 0.3. After incubation of the cells in precooled 50 mL Falcon tubes on ice for around 15 minutes, cells were harvested by centrifugation (4,500 x g, 12 min, 4 °C). The supernatant was discarded and cells were washed twice with 20 mL of ice-cold H_2O (4,500 x g, 12 min, 4 °C). After two additional washing steps with 20 mL of ice-cold glycerol solution [10 % (v/v)] and combining all cells in one Falcon tube, the cells were resuspended in 1 mL of ice-cold glycerol solution [10 % (v/v)] and aliquots of 50 µL were stored at - 80 °C until further use.

For transformation of electro-competent *E. coli* cells, the frozen aliquots were gently thawed on ice and properly mixed with 0.1 - 10 µg DNA. After incubation of around 10 minutes, this mixture was transferred to sterile electroporation cuvettes with an electrode distance of 0.2 cm. The electroporation was performed using a Gene Pulser electroporation system with the following settings: 2.5 kV, 25 µF and 200 Ω (Liebl *et al.*, 1989; Dower *et al.*, 1988). After electroporation, cells were transferred to a preheated (37 °C) 1.5 mL Eppendorf cup with 400 mL of 2x TY medium, incubated at 37 °C on a

rotary shaker at 120 rpm for 50 minutes and plated on 2x TY agar plates with appropriate concentrations of the respective antibiotic for selection of transformants.

2.7.2 Preparation and transformation of electro-competent *Corynebacterium glutamicum* cells

For preparation of electro-competent *C. glutamicum* cells, a method modified according to van der Rest *et al.* (1999) was used. 5 mL of an over night culture (50 mL BHIS) were used to inoculate 250 mL BHIS in a 1000 mL baffled Erlenmeyer flask. This culture was incubated at 28 °C on rotary shaker at 120 rpm until it reached an OD_{600} of around 1.75. After harvesting the cells by centrifugation (4,500 x g, 12 min, 4 °C), the supernatant was discarded and the cell pellets were washed four times with 20 mL of ice-cold glycerol solution [10 % (v/v)]. After the washing steps, cells were resuspended in 0.5 mL of ice-cold glycerol solution [10 % (v/v)] and aliquots of 150 µL were stored at - 80 °C until further use.

For transformation of electro-competent *C. glutamicum* cells, the frozen aliquots were gently thawed on ice and properly mixed with 0.1 - 10 µg DNA. After incubation for about 10 minutes, this mixture was transferred to sterile electroporation cuvettes with an electrode distance of 0.2 cm. After electroporation using a Gene Pulser electroporation system with following settings: 2.5 kV, 25 µF and 200 Ω (Liebl *et al.*, 1989), cells were mixed instantly with 1 mL of preheated (46 °C) BHIS medium. This mixture was transferred to 4 mL preheated (46 °C) BHIS medium in 15 mL tubes and incubated for 6 minutes at 46 °C. After this heat shock, cells were incubated at 28 °C on a rotary shaker at 120 rpm for 1 hour. Regenerated cells were plated on 2x TY agar plates with the respective antibiotic in appropriate concentration.

2.8 Chromosomal deletions in *Corynebacterium glutamicum*

For the deletion of genes in the chromosome of *C. glutamicum*, a method using allelic exchange by homologous recombination was used (Schäfer *et al.*, 1994). The first integration took place after transformation of the suicide vector pK19mobsacB with homologous sequences of the target gene (at the 3'- and 5'-ends of respective gene) into *C. glutamicum*. The electroporation was performed with the settings mentioned in 2.7.2. Selection of transformants, which had integrated the plasmid into the genome (first homologous recombination), was carried out on BHIS agar plates with 15 µg kanamycin/ml. The obtained integrants were transferred to 2x TY agar plates with 50 µg kanamycin/mL and incubated at 28 °C for 24 hours. Cells from this master plate were used to inoculate 5 mL 2x TY cultures. After cultivation of these cultures at 28 °C on a

rotary shaker at 120 rom for around 6 hours, cultures were diluted 1:100 and 1:1000 with cold 0.9 % (w/v) NaCl solution. 100 µL of these dilutions were plated on 2x TY agar plates with 10 % (w/v) sucrose and incubated at 28 °C for 24 to 48 hours. Only cells without a functional *sacB* gene were able to grow on this medium. Cells should lose their functional *sacB* gene by excising it out of their genome (second homologous recombination). Grown colonies were transferred on master plate. For identification of clones without integrated vector, clones from the second master plate were streaked out on 2x TY agar plates with 50 µg kanamycin/mL and on 2x TY agar plates with 10 % (w/v) sucrose and incubated at 28 °C for 24 hours. Clones, which excised the plasmid, were not able to grow in presence of kanamycin, but on 2x TY agar plates with 10 % (w/v) sucrose. The obtained colonies were tested by colony PCR for the deletion of fragments in the respective genes.

2.9 Construction of *Corynebacterium glutamicum* integration mutants

For promoter studies using a real-time luciferase system established by Tanja Laslo (Laslo, 2013), several *C. glutamicum* RESluxIN derivatives with the respective promoter fragments had to be constructed. *C. glutamicum* RESluxIN was constructed by Tanja Laslo (Laslo, 2013), is based on *C. glutamicum* RES167 and already possesses the genes *luxA*, *luxB* and *fre* integrated in copy B of the 16S rDNA. The *luxA* and *luxB* genes are coding for sub-units of a luciferase, while *fre* is coding for a FMN reductase. Promoter studies were performed using integration mutants with respective promoter fragments directly integrated upstream of *luxA*.

For the construction of *C. glutamicum* integration mutants, the same protocol as for chromosomal deletion in *C. glutamicum* was used without the step for second homologous recombination (see chapter 2.8). Possible positive clones carrying the desired promoter fragment upstream of *luxA* were tested by colony PCR with appropriate primers and the respective promoter sequence was verified by sequencing.

2.10 Determination of luciferase activities

For determination of luciferase activities, a modified method after Gupta *et al.* (2003) and established by Laslo (2013) was used. To determine the luminescence in the respective culture, samples from the cultures were taken and diluted with CgC minimal medium. 200 µL of these dilutions were added into a well of a LumiNunc™ 96-well-plate. After adding 40 µL of 50 mM n-decanal, measurements were performed using a Tecan infinite M200 plate reader and the Tecan i-control software. The incubation temperature was 30 °C and plates were shaken for 12 seconds with an amplitude of 6 followed by detection of

luminescence at a wavelength of 490 mm for 1 second. This cycle of shaking and luminescence detection was repeated at least 10 times. The relative light units (RLU) determined between minute 1 and 3 after starting the measurement were averaged. By dividing the average luminescence level by the OD_{600} of the culture at this time point, luciferase activities (in RLU/OD_{600}) were calculated.

2.11 DNA treatment

2.11.1 Preparation of chromosomal DNA from *C. glutamicum*

For preparation of chromosomal DNA from *C. glutamicum*, a modified method of Eikmanns *et al.* (1994) was used.

TE buffer

EDTA	1	mM
Tris	10	mM

The pH of the solution was adjusted with concentrated HCl to 7.6.

Lysis buffer

EDTA	2	mM
NaCl	400	mM
Tris	10	mM

The pH of the solution was adjusted with concentrated HCl to 8.2.

For isolation of chromosomal DNA from *C. glutamicum*, a 5 mL overnight culture (2x TY medium) was harvested by centrifugation (4,500 x g, 10 min, 4 °C). After discarding the supernatant, cells were washed twice with cold 10 mL TE buffer and finally resuspended in 1 mL of TE buffer with 15 mg lysozyme/ml. Incubation took place at 37 °C for at least 3 hours. After adding 3 mL lysis buffer, 220 µL 10 % (w/v) SDS and 150 µL proteinase K solution (20 mg/ml), the mixture was incubated at 37 °C overnight. 2 mL of saturated NaCl solution was added to denature proteins, which were precipitated by centrifugation (4,500 x g, 15 min, RT). The resulting supernatant was transferred into a new 15 mL Falcon tube. Adding of 10 mL precooled (- 20 °C) ethanol served for precipitation of DNA, which was fished with glass hooks. After washing in 70 % ethanol and following removal of residual ethanol by drying, DNA was resuspended in 200 µL of sterile H_20.
For purification of DNA, a phenol-chloroform-isoamyl alcohol [25:24:1 (v/v/v)] extraction

(modified after Green and Sambrook, 2012) was used. The DNA solution was mixed with the same volume of a phenol-chloroform-isoamyl alcohol solution [25:24:1 (v/v/v)]. Phase separation was obtained by centrifugation (16,060 x g, 8 min, RT) and upper aqueous phase was transferred to a new 1.5 mL Eppendorf cup. Lower organic phase and interphase were discarded. This step was repeated at least 3 times. As soon as no protein was visible at the interphase, residual phenol was removed by adding the same volume of a chloroform-isoamyl alcohol solution to the aqueous phase, mixing and centrifugation (16,060 x g, 10 min, RT). The upper aqueous phase was again transferred to a new 1.5 mL Eppendorf cup and mixed with 2.5 volumes of isopropanol. After centrifugation (16,060 x g, 10 min, RT), the supernatant was discarded and the DNA pellet was washed with 500 µL of 70 % (v/v) ethanol. Residual alcohol was removed by drying at 37 °C. Finally, DNA was resuspended in 100 µL of sterile H_2O.

2.11.2 Preparation of plasmid DNA

2.11.2.1 Preparation of plasmid DNA from *E. coli*

For preparation of plasmid DNA from *E. coli*, the E.Z.N.A. Plasmid Miniprep Kit I was used according to the instructions of manufacturer. Elution of DNA took place with 50 - 100 µL of sterile H_2O.

2.11.2.2 Preparation of plasmid DNA from *C. glutamicum*

For preparation of plasmid DNA from *C. glutamicum*, a method after Green and Sambrook (2012) was modified and used.

Solution A

Glucose	50	mM
Tris	25	mM
EDTA	10	mM

The pH of the solution was adjusted with concentrated HCl to 7.6.

Solution B

NaOH	0.2	mM
SDS	1	% (w/v)

The solution was prepared freshly for each preparation.

Solution C

Sodium acetate	3	M
Acetic acid	11.5	% (v/v)

For isolation of plasmid DNA from *C. glutamicum*, 5 mL overnight culture (2x TY medium with respective concentrations of antibiotic) was harvested by centrifugation (4,500 x g, 10 min, 4 °C). After discarding the supernatant and washing the pellets once with 1 mL of cold TE buffer (see chapter 2.11.1), cells were resuspended in 200 µL of ice-cold solution A. After adding 15 mg lysozyme/mL and 5 µL of RNase A (50 µg/ml), the mixture was incubated at 37 °C for at least 3 hours. Incubation on ice for 5 minutes took place after 400 µL of solution B has been added. Next step was the addition of 350 µL of ice-cold solution C. After another incubation on ice for 10 minutes, the mixture was centrifuged (17,949 x g, 15 minutes, 4 °C) and the supernatant was transferred in a new 1.5 mL Eppendorf cup. Precipitation of DNA was performed by adding 0.8 volumes of isopropanol. Again, centrifugation (16,060 x g, 15 min, RT) followed. The supernatant was discarded and plasmid DNA was washed with 70 % ethanol. Residual alcohol was removed by drying at 37 °C and DNA was resuspended in 100 µL of sterile H_2O. If required, plasmid DNA was purified by phenol-chloroform-isoamyl alcohol extraction (see chapter 2.11.1).

2.11.3 PCR

For amplification of DNA fragments, a standard PCR method (Green and Sambrook, 2012) was used. Construction of primers was performed using Clone Manager software or CLC Main Workbench software. Synthesis of primers was done by biomers.net GmbH (Ulm, Germany). All PCR techniques are based on one common program, which can be adjusted to respective use of the PCR.

PCR program:

1) Initial denaturation: 94 °C, 5 minutes
2) Denaturation: 94 °C, 30 seconds
3) Annealing: 47 - 60 °C (according to properties of the respective primers), 30 seconds
4) Elongation: 72 °C, 30 seconds - 5 minutes (according to length of expected product size and used polymerase)
5) Final elongation: 72 °C, 8 minutes
6) Hold: 4 °C

Steps 2 - 4 were repeated 32 - 38 times.

Standard PCR:

For amplification of DNA fragments, a DNA polymerase with proofreading capabilities was used. The typical 50 µL assay for this use contained:

template DNA (chromosomal or plasmid DNA)	10 – 100	ng
forward primer	0.3	µM (final concentration)
reverse primer	0.3	µM (f. c.)
each dNTP	200	µM (f. c.)
DMSO	2	µl
5x Phusion reaction buffer	10	µl
Phusion polymerase	1	U

Colony PCR:

For screening for positive mutants after transformation, a colony PCR was performed. In a colony PCR, the supernatants of lysed cells were used as template DNA. The supernatant was obtained by resuspending a single colony grown on an agar plate in 50 µL of sterile H_2O, heating this mixture at 99 °C for 15 minutes and centrifugation (16,060 x g, 8 minutes, RT). In contrast to standard PCR, the less sensitive *Taq* DNA polymerase S was used. The typical 25 µL assay for this use contained:

template DNA (supernatant of lysed cells)	10	µl
forward primer	0.3	µM (f. c.)
reverse primer	0.3	µM (f. c.)
each dNTP	200	µM (f. c.)
DMSO	1	µl
10x *Taq* DNA polymerase S buffer	2.5	µl
Taq DNA polymerase S	0.5	U

Crossover PCR:

For the generation of fused DNA fragments as used for the deletion of genes, crossover PCR was performed. The two DNA fragments were amplified in standard PCR with special primers. The reverse primer of the fragment containing the 5'-end of the target gene and the forward primer of the fragment containing the 3'end of the target gene had additional homologous sequences, which overlapped by at least 15 bp. After amplification and subsequent purification of both fragments, these PCR products were used in equal amounts (around 80 to 100 ng) as template in one crossover PCR reaction. The typical 50 µL assay for this use contained:

template DNA 1 (fragment containing the 5'-end of the target gene)	80 – 100	ng
template DNA 2 (fragment containing the 3'-end of the target gene)	80 – 100	ng
forward primer (forward primer of template DNA 1)	0.3	µM (f. c.)
reverse primer (forward primer of template DNA 2)	0.3	µM (f. c.)
each dNTP	200	µM (f. c.)
DMSO	2	µl
5x Phusion reaction buffer	10	µl
Phusion polymerase	1	U

2.11.4 Agarose gel electrophoresis

For separation of plasmids and PCR products by agarose gel electrophoresis, a method described by Green and Sambrook (2012) was used. Required buffers are listed.

50x TAE buffer

EDTA sodium salt	50	mM
Sodium acetate	500	mM
Tris	2	mM

The pH of the buffer was adjusted with concentrated HCl to 8.0.

6x Loading dye

Bromide phenol blue	0.25	% (w/v)
Glycerol	40	% (v/v)

0.8 % (w/v) agarose gels were prepared by solving 3.2 g agarose in 400 mL of 1x TAE buffer. The liquid agarose was poured into gel chamber and covered with 1x TAE buffer after agarose had hardened. Samples were mixed with 0.2 volumes of 6x loading dye and loaded into the gel pockets. As reference, 3 µL of a DNA marker was also loaded into one gel pocket. Separation of DNA fragments took place at 80 to 100 V. DNA was stained afterwards in an ethidium bromide solution (1µg/ml) and detected at 312 nm.

2.11.5 Purification of DNA fragments

For purification of DNA fragments from PCR assays or agarose gels, the NucleoSpin Gel and PCR clean up Kit was used according to instructions by manufacturer. Elution of DNA was performed with 20 - 100 µL of sterile H_2O.

2.11.6 Enzymatic manipulation of DNA

For construction of plasmids for overexpression or deletion of genes, PCR products and plasmid DNA had to be ligated. This was achieved by restriction digest of the PCR product and the vector with corresponding restriction enzymes and subsequent ligation. Digested plasmid DNA was also treated with FastAP thermosensitive alkaline phosphatase preventing religation of cut vector DNA. After restriction digest, DNA was separated with agarose gel electrophoresis and specific DNA fragments were detected to confirm successful and complete digestion. DNA of cut PCR product and plasmid was purified with NucleoSpin Gel and PCR clean up Kit and ligated using the T4 DNA ligase. After ligation, the mixture was used for transformation of electro-competent *E. coli* DH5α cells.

All restriction enzymes, FastAP thermosensitive alkaline phosphatase and T4 DNA ligase were obtained from Fermentas GmbH (St. Leon-Rot, Germany) together with the corresponding buffers. Assay volumes and concentrations of ingredients were used according to instruction by manufacturer.

2.11.7 Determination of DNA concentrations

For determination of DNA concentrations, 1 µL of respective DNA solutions were analysed using a NanoDrop 2000 Spectrophotometer and NanoDrop 2000 software.

2.12 RNA treatment

2.12.1 Preparation of total RNA

For the preparation total RNA, *C. glutamicum* cells were grown aerobically in baffled Erlenmeyer flasks with 50 mL of CgC medium. At the desired time points, cells were harvested by centrifugation (4,500 x g, 10 minutes, 4 °C) and the supernatant was discarded. Cells were immediately frozen in liquid nitrogen and stored at - 80 °C until further use.

For isolation of total RNA, cells were thawed on ice and resuspended in 2 mL of RLT buffer (Qiagen GmbH, Hilden, Germany) containing 10 µL 2-mercaptoethanol/ml. After transferring the cell suspension into 2 mL screw cap tubes with 250 mg of glass beads (diameter 0.1 mm), cells were disrupted using a RiboLyser (Hybaid) with 4 cycles of 20 seconds at level 6.5. Cell debris and glass beads were sedimented by centrifugation (20,800 x g, 20 minutes, 4 °C) and supernatants were transferred into new 1.5 mL Eppendorf cups. Purification of RNA was performed using the Qiagen RNeasy Midi Kit and following instructions by manufacturer. Elution took place with 150 µL RNase-free H_2O. To remove remaining DNA contaminations from the RNA preparations, DNase I was used according to the instructions of the manufacturer. Qiagen RNeasy Mini Kit was used according to instructions of the manufacturer to purify RNA. After elution with 50 to 60 µL RNase-free H_2O, a control PCR (following protocol of standard PCR) was performed to test the absence of DNA in the RNA preparation. If needed, the DNA digestion was repeated. RNA samples free of DNA were stored as 20 µL aliquots at - 80 °C until further use. For determination of RNA concentrations, a NanoDrop 2000 Spectrophotometer and NanoDrop 2000 software were used. If RNA was used for semi-quantitative real-time PCR, the RNA concentration was adjusted to 300 ng/µl.

2.12.2 Semi-quantitative real-time PCR

For semi-quantitative transcription analysis of the genes *cg1269* (*glgC*), *cg1268* (*glgA*), *cg1381* (*glgB*), *cg2310* (*glgX*), *cg1479* (*malP*) and *cg2289* (*glgP*), samples of *C. glutamicum* WT cultures grown in CgC minimal medium with 2 % glucose and two distinct phosphate concentrations (13 mM and 1.95 mM, corresponding to 100 % and 15 %, respectively, of the original concentration) were taken 6 and 12 hours after inoculation and prepared for RNA preparation as described above.

Primers for each gene were designed with CLC main workbench software and are listed in table 3. It was required that the lengths of the primers were about 20 bps, the difference in melting temperatures of corresponding primers must not exceed 2 °C, and the product formed by the respective primer pairs had to be about 180 bps and should be located 200

to 400 bps downstream of transcription start of the respective genes.

According to a method by Koch *et al.* (2005), semi-quantitative real-time PCR analysis was performed using a LightCycler and the SensiMix SYBR One-Step RT-PCR kit. The protocol started with addition of 1 µg of prepared RNA to the reaction mixture. After a reverse transcription step at 50 °C for 20 minutes and a subsequent denaturation step at 95 °C for 15 minutes, 55 cycles of PCR followed. Each cycle consisted of denaturation at 95 °C for 10 seconds, annealing at 55 °C for 20 seconds and elongation at 72 °C for 12 seconds.

Recording of melting curves was done by continuous measurement of fluorescence in a range from 65 - 95 °C with a heating rate of 0.1 °C per second. Comparison of fluorescence levels was performed by determination of the crossing point (CP) for each gene and each condition using the second-derivative maximum data analysis method (LightCycler software, version 3.5). All measurements were conducted with 2 technical replicates per measurement and 4 biological replicates per condition. Calculated mRNA levels of respective genes were normalized on total RNA amount. Relative changes in the transcription rate were determined as $2^{-\Delta CP}$ with ΔCP matching the difference of measured CPs for each condition.

2.13 Analytics

2.13.1 Determination of glycogen levels

The determination of intracellular glycogen content in *C. glutamicum* was performed enzymatically according the method described by Parrou and Francois (1997). 5 mL of culture were harvested and cells were washed twice with ice-cold TN buffer. Then, cells were resuspended in a total volume of 1 mL resuspension buffer. Resuspended cells were transferred to 2 mL screw top Eppendorf tubes with 250 mg silica beads. Glycosidic activity of cellular proteins was inactivated by incubation at 95 °C for 30 minutes. Cells were disrupted by the use of a RiboLyser (Hybaid) three times at maximum speed of 6.5 for 45 seconds without intermediate cooling. After separation of cell debris and silica beads from the supernatant by centrifugation with 13 000 g for 20 minutes, the supernatant was transferred to a 1.5 mL Eppendorf cup. Since the efficiency of cell disruption varied between performances of the experiments, cells of *C. glutamicum* WT were always treated in the same way as respective mutants strains.

Two 100 µL aliquots of each sample were transferred into new 1.5 mL Eppendorf cups (assays A and B) and 2 µL of Amyloglucosidase were added to assay A, while assay B was used as reference. Amyloglucosidase cleaves α-1,4-glycosidic and α-1,6-glycosidic linkages in glucose polymers. Hence, it is not possible to differ between linear

maltodextrins with α-1,4-glycosidic linkages and branched glycogen with α-1,4-glycosidic and α-1,6-glycosidic linkages using this assay. Both assays were incubated at 56 °C overnight. Then, the glucose concentrations in both assays were determined enzymatically using hexokinase / glucose-6-phosphate dehydrogenase mixture and following spectrophotometric quantification of formed NADPH at a wavelength of 340 nm. The glucose concentration in reference assay B was subtracted from the glucose concentration in assay A. Cell dry weight of *C. glutamicum* was calculated according to the OD_{600}. For *C. glutamicum*, an OD_{600} of 1 corresponds to a cell dry weight of 0.25 g/L (Börmann *et al*., 1992).

TN buffer

Tris	50	mM
NaCl	50	mM

The pH of the buffer was adjusted with concentrated HCl to 6.3.

Resuspension buffer

Potassium acetate	40	mM

The pH of the buffer was adjusted with concentrated acetic acid to 4.2.

2.13.2 Determination of sugars and organic acids in culture supernatants

For determination of amino acids in the supernatant of cultures, a method described by Wieschalka *et al.* (2012) was used. 1 mL of culture was harvested by centrifugation (16,060 x g, 10 minutes, RT). Supernatants were used for determination of glucose, maltose, acetate, lactate and succinate concentration by reversed-phase high performance liquid chromatography. The used system was an Agilent LC 1100 with an Agilent 1100 Variable Wavelength Detector at 215 nm (for detection of organic acids) and an Agilent 1100 Refractive Index Detector. Separation was carried out with an organic acid-resin (polystyrol-divinylbenzol copolymer) column from CS-Chromatographie under isocratic conditions at 40 °C for 38 minutes. As eluent 100 mM sulphuric acid was used with a flow of 0.4 ml/min. Quantification was carried out by 6-point calibration curve for each measured sugar and organic. For graphical analysis and calculation, the software Agilent Chemstation was used.

2.13.3 Determination of intracellular metabolite concentrations

Determination of intracellular metabolite concentrations was performed in cooperation with Nina Pfelzer and Marco Oldiges at the Research Center Jülich (Forschungszentrum Jülich). For determination of intracellular metabolite concentrations, a method based on Paczia *et al.* (2012) was used. Volumes of samples were calculated in a way that the biovolume formed by cells in the sample was about 20 µl. Biovolume was determined by Beckman Coulter Counter 3 and Coulter Multi software. For each culture three 50 mL Falcon tubes containing 60 % (v/v) methanol (three times the volume of sample volume) were prepared and stored at - 80 °C until further use. Shortly before use, the methanol was gently thawed on ice until the temperature was between - 50 and - 60 °C. Immediately after the samples were taken, they calculated volume was given to each of the prepared Falcon tubes, mixed and centrifuged (10304 x g, 5 minutes, - 20 °C). 1 mL of supernatant was sterile filtered (cellulose acetate filter, 0.22 µm) and stored at - 20 °C until further use. Remaining supernatant was discarded and cell pellets were resuspended in 1 mL of cold chloroform. (The volume of chloroform was fifty times the calculated biovolume formed by cells in the sample.) After resuspension, 1 mL of a cold methanol-TE buffer-mixture was added.

During the quenching, the actual biovolume was determined by a Coulter Counter and samples of supernatants for analysis were prepared. For this purpose, culture samples were centrifuged (16,060 x g, 10 minutes, RT) and supernatants were sterile-filtered. 250 µL of cell-free supernatant was added to a 2 mL Eppendorf cup with 750 µL of precooled methanol (-20 °C) and stored at - 20 °C.

For extraction of intracellular metabolites, Falcon tubes with cells, chloroform and methanol-TE buffer-mixture was shaken for two hours at - 20 °C on an overhead shaker (Reax 2, Heidolph). Afterwards, the mixture was transferred into a 2 mL Eppendorf cup and centrifuged (13,000 g, 5 minutes, - 20 °C). 1 mL of the upper methanol phase was sterile filtered and stored at - 20 °C until further use.

Analysis of extracts, supernatants of quenching and culture supernatants was carried out by LC-ESI-MS/MS. If required, samples were diluted with cold (- 20 °C) 50 % (v/v) methanol. Mixture with internal standard was performed during the preparation of the samples. All steps of the preparation were performed at a temperature below - 10 °C. Separation was carried out with a C18 column (synergy hydro) from Phenomenex at 60 °C and with a flow of 0.45 ml/min. As mobile phase a mixture of polar eluent (10 mM Tributylamin, 15 mM acetic acid, pH 4.95) and methanol as non-polar eluent was used according to the following elution profile:

Time	Solv. B
4.00	0.0
6.00	20.0
12.00	20.0
13.00	35.0
14.00	35.0
15.00	60.0
16.00	100.0
18.00	100.0
18.53	0.0

^{13}C-labeled analogues of AMP and NAD^+ were used as internal standard for AMP and NAD^+. These standards were also used for ADP, ATP, $NADH/H^+$, $NADP^+$ and $NADPH/H^+$. A Multiple Reaction Monitoring (MRM) module detected these molecules. Quantification of analytes was done by using a 6-point calibration curve.

The concentration in the supernatant c_{cs}, the concentration in the quenching supernatant c_{qs}, the concentration in the quenching extract c_{ext}, the biovolume V_{bv}, the sample volume V_{sample}, the volume of quenching solution V_{qs}, the volume of extraction solution V_{es} and the specific intracellular volume $V_{intraspec}$ were needed for the calculation of the intracellular concentration c_{intra} of the metabolite. Calculations were done using the following formula:

$$c_{intra} = \frac{(c_{qs} - c_{cs}) \times (V_{sample} + V_{qs}) + c_{ext} \times (V_{bv} + V_{es})}{V_{bv}}$$

With the determined intracellular concentrations of AMP, ADP, ATP, NAD^+, $NADH/H^+$, $NADP^+$ and $NADPH/H^+$, the energy charge, the catabolic reduction charge and the anabolic reduction charge were calculated.
The energy charge was calculated using the following formula:

$$EC = \frac{[ATP] + \frac{1}{2}[ADP]}{[ATP] + [ADP] + [AMP]}$$

The catabolic reduction charge was calculated using the following formula:

$$cRC = \frac{[NADH/H^+]}{[NAD^+] + [NADH/H^+]}$$

The anabolic reduction charge was calculated using the following formula:

$$aRC = \frac{[NADPH/H^+]}{[NADP^+] + [NADPH/H^+]}$$

2.14 Determination of cell size

Determination of cell sizes was performed in cooperation with Nina Pfelzer and Marco Oldiges at the Research Center Jülich (Forschungszentrum Jülich). For the determination of cell sizes during anaerobic cultivation and for determination of the intracellular volume of cells for the determination of intracellular metabolite concentrations, a method using a Coulter Counter was performed. Samples of culture were diluted with CASY ton to an OD_{600} of about 0.2. 200 µL of these dilutions were mixed with 9.8 mL of CASY ton and transferred into a beaker for analysis of cell sizes using the Beckman Coulter Counter 3. Signals were analysed with Coulter Multi software regarding sizes and number of cells.

3 Results

3.1 Characterisation of deletion mutants of genes involved in glycogen metabolism

3.1.1 Characterisation of single deletion mutants of genes involved in glycogen synthesis

C. glutamicum integration mutants of the genes *glgC*, *glgA* and *glgB*, respectively, have been constructed and characterized in previous studies (Seibold *et al.*, 2007; Tzvetkov *et al.*, 2003; Seibold *et al.*, 2011). Since integration mutants are less stable than deletion mutants and since it is not possible to transform integration mutants with plasmids carrying kanamycin resistance cassettes (e. g., for deletion/inactivation of additional genes), deletion strains of the genes *glgC*, *glgA* and *glgB*, respectively, were constructed, verified and investigated regarding growth and glycogen levels. For this purpose, the single deletion mutants were grown in CgC minimal medium with 2 % glucose and 2 % maltose, respectively, as carbon source.

3.1.1.1 Characterisation of *C. glutamicum* Δ*glgC*

The ADP-glucose pyrophosphorylase GlgC catalyses the reaction from glucose 1-phosphate and ATP to ADP-glucose and pyrophosphate. A *glgC* deletion mutant was constructed and compared to *C. glutamicum* WT regarding growth and glycogen levels in CgC minimal medium with 2 % glucose or 2 % maltose, respectively (Figure 2). When grown with glucose as carbon source, both strains showed a growth rate of about 0.39 h^{-1} (Figure 2A). *C. glutamicum* WT reached its maximum OD_{600} of about 37 after 12 hours, while *C. glutamicum* Δ*glgC* grew to a bit lower OD_{600} of around 33 after 10 hours. *C. glutamicum* WT accumulated up to approximately 60 mg glycogen per g cell dry weight (CDW) after 6 hours. After around 10 hours, glycogen levels decreased and after 48 hours the glycogen levels were as low as about 5 mg glycogen per g CDW. *C. glutamicum* Δ*glgC*, which should be unable to synthesize ADP-glucose as precursor for glycogen, accumulated almost no glycogen (< 4 mg glycogen per g CDW) during the whole experiment.

During growth in CgC minimal medium with 2 % maltose as carbon source, *C. glutamicum* WT and *C. glutamicum* Δ*glgC* reached both a maximal OD_{600} of around 33 after 10 hours (Figure 2B), the growth rates of both strains were similar (0.37 h^{-1} and 0.39 h^{-1},

respectively). *C. glutamicum* WT accumulated approximately 80 mg glycogen per g CDW after 4 hours, the levels decreased after 8 hours. In contrast to experiments with glucose as carbon source, *C. glutamicum* Δ*glgC* grown on maltose accumulated glycogen to concentrations of up to 60 mg glycogen per g CDW after 4 hours. The levels of glycogen dropped also after around 8 hours.

Taken together, when grown with glucose, *C. glutamicum* Δ*glgC* was not able to accumulate glycogen, but showed growth comparable to *C. glutamicum* WT. In contrast, *C. glutamicum* Δ*glgC* accumulated glycogen and grew like *C. glutamicum* WT during growth with maltose.

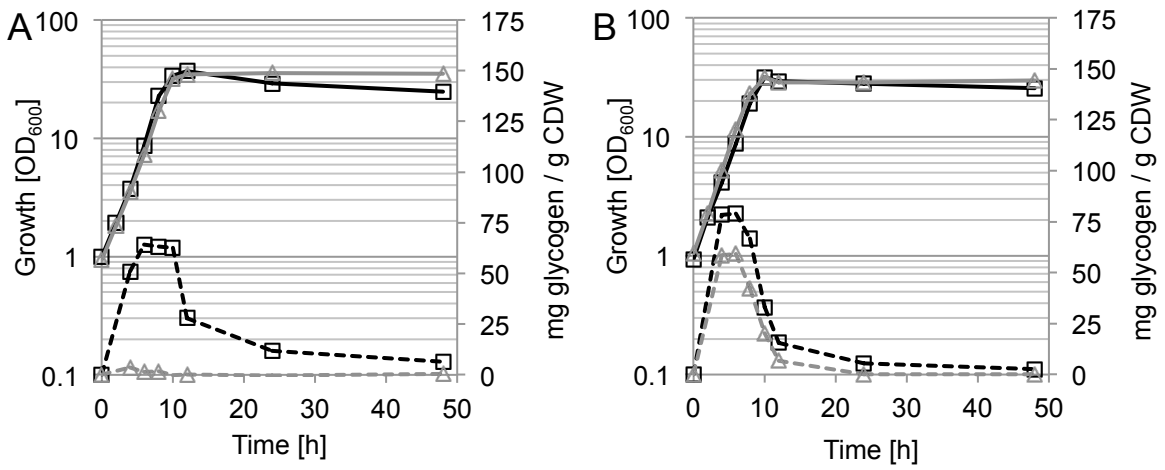


Figure 2: Growth (solid lines) and glycogen levels (dashed lines) of *C. glutamicum* WT (black open squares) and *C. glutamicum* Δ*glgC* (grey open triangles) in CgC minimal medium with 2 % glucose (A) or 2 % maltose (B) as carbon source. The Figure shows the results of one representative experiment. At least three independent experiments were performed, all three showing comparable results.

3.1.1.2 Characterisation of *C. glutamicum* Δ*glgA*

The glycogen synthase GlgA uses ADP-glucose to elongate pre-existing linear glucans. The *glgA* deletion mutant was compared to *C. glutamicum* WT regarding growth behaviour and glycogen levels in CgC minimal medium with 2 % glucose and with 2 % maltose as carbon source. When grown in minimal medium and glucose, *C. glutamicum* Δ*glgA* grew with a significant lower growth rate ($\mu = 0.23\ h^{-1}$) than *C. glutamicum* WT ($\mu = 0.37\ h^{-1}$) (Figure 3A). *C. glutamicum* WT reached the highest OD_{600} of around 39 at the beginning of stationary phase after 10 hours, while the maximal OD_{600} of *C. glutamicum* Δ*glgA* ($OD_{600} = 28$) was observed only after 24 hours. The highest glycogen levels of approximately 85 mg glycogen per g CDW were reached by *C.*

glutamicum WT after 10 hours of growth. *C. glutamicum* Δ*glgA* showed only in the first six hours very low levels of glycogen (< 8 mg glycogen per g CDW).

During growth in CgC minimal medium with maltose as carbon source, *C. glutamicum* WT and *C. glutamicum* Δ*glgA* showed similar growth rates (0.33 h^{-1} and 0.31 h^{-1}, respectively) reached the same maximal OD_{600} of 36 after 12 hours (Figure 3B). Both strains reached maximal glycogen levels between 80 and 85 mg per g CDW after 4 to 6 hours. *C. glutamicum* Δ*glgA* exhibited slightly slower degradation of glycogen than *C. glutamicum* WT.

In conclusion, the deletion of *glgA* in *C. glutamicum* led to the abolishment of glycogen accumulation and strongly impaired growth during cultivation with glucose. With maltose as carbon source, growth and glycogen accumulation of *C. glutamicum* Δ*glgA* were comparable to *C. glutamicum* WT.

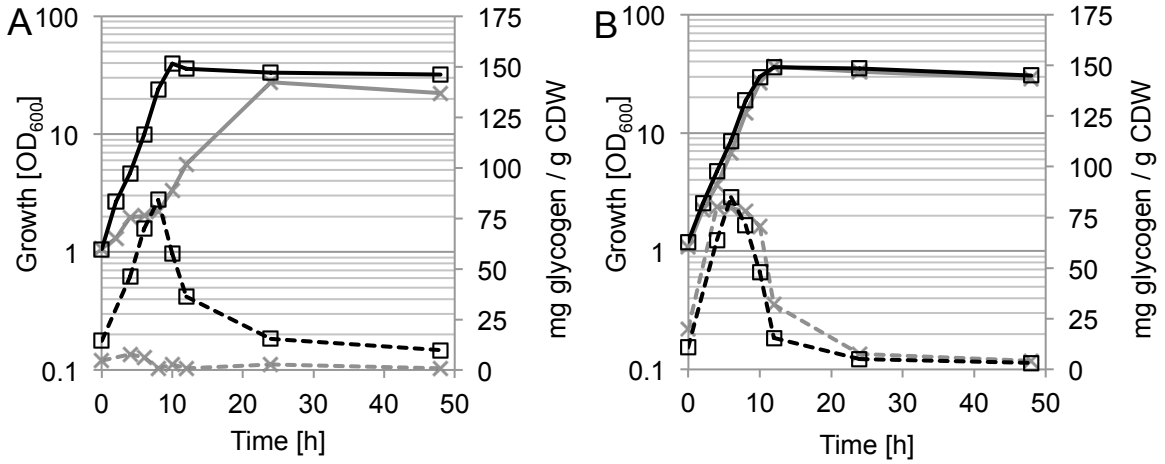


Figure 3: Growth (solid lines) and glycogen levels (dashed lines) of *C. glutamicum* WT (black open squares) and *C. glutamicum* Δ*glgA* (grey crosses) in CgC minimal medium with 2 % glucose (A) or 2 % maltose (B) as carbon source. The Figure shows the results of one representative experiment. At least three independent experiments were performed, all three showing comparable results.

3.1.1.3 Characterisation of *C. glutamicum* Δ*glgB*

The gene *glgB* encodes for a glycogen branching enzyme, which introduces alpha-1,6-glycosidic linkages into linear glucans, forming branched glycogen. The constructed *C. glutamicum* Δ*glgB* was analysed regarding growth behaviour and glycogen levels during growth in CgC minimal medium with 2 % glucose and with 2 % maltose as carbon sources (Figure 4A). With 2 % glucose, both strains showed identical growth rates (μ = 0.38 $h^{-}1$). *C. glutamicum* WT grew to an OD_{600} of around 36 after 10 hours, while the maximal OD_{600} = 31 of *C. glutamicum* Δ*glgB* was determined after 24 hours. Interestingly,

C. glutamicum Δ*glgB* had reached an OD_{600} of about 25 after 10 hours and showed this OD_{600} also after 12 hours, indicating the beginning of stationary phase. *C. glutamicum* WT showed the highest glycogen levels of around 80 mg per g CDW after 10 hours. Afterwards, the glycogen was degraded to levels of around 10 mg per g CDW. *C. glutamicum* Δ*glgB* reached only minor glycogen levels (< 10 mg glycogen per g CDW) after 6 hours, which was completely degraded after 12 hours of growth.

When grown with maltose as carbon source, the growth behaviour of the two strains *C. glutamicum* WT and *C. glutamicum* Δ*glgB* differed significantly (Figure 4B). While *C. glutamicum* WT exhibited a growth rate of 0.41 h^{-1} and reached an OD_{600} of about 30 after 12 hours, *C. glutamicum* Δ*glgB* showed a lower growth rate (0.22 h^{-1}) and the maximum OD_{600} of about 30 was determined only after 24 hours. The maxima of glycogen levels of both strains were about 75 mg per g CDW. While *C. glutamicum* WT reached the maximum between 4 and 8 hours, *C. glutamicum* Δ*glgB* reached it after 8 to 10 hours. In both strains the glycogen was degraded completely during further cultivation.

Taken together, *C. glutamicum* Δ*glgB* showed similar growth as *C. glutamicum* WT (slightly lower OD_{600}), but strongly reduced accumulation of glycogen, when grown with glucose. During cultivation with maltose, *C. glutamicum* Δ*glgB* displayed slower growth than *C. glutamicum* WT and delayed accumulation of glycogen.

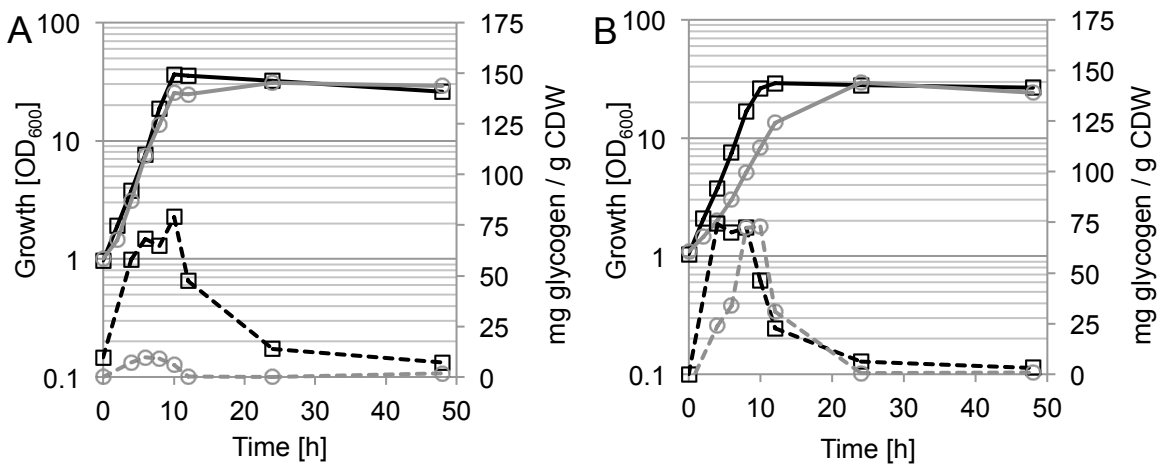


Figure 4: Growth (solid lines) and glycogen levels (dashed lines) of *C. glutamicum* WT (black open squares) and *C. glutamicum* Δ*glgB* (grey open circles) in CgC minimal medium with 2 % glucose (A) or 2 % maltose (B) as carbon source. The Figure shows the results of one representative experiment. At least three independent experiments were performed, all three showing comparable results.

3.1.2 Characterisation of single deletion mutants of genes involved in glycogen degradation

Previously a *C. glutamicum* integration mutant of *glgX* and a *C. glutamicum* deletion mutant of *malP* have been constructed and characterized (Seibold and Eikmanns, 2007; Seibold *et al.*, 2009). As mentioned before (see chapter 3.1.1), deletion mutants are more advantageous for further work than integration mutants. Therefore and to have a complete set of deletion mutants derived from the same parental strain, *C. glutamicum* single deletion mutants of *glgX*, *malP* and *glgP* were constructed, verified and investigated regarding growth and glycogen levels. For this purpose, the single deletion mutants were grown in CgC minimal medium with 2 % glucose and 2 % maltose, respectively, as carbon source.

3.1.2.1 Characterisation of *C. glutamicum* Δ*glgX*

The glycogen debranching enzyme GlgX catalyses the cleavage of alpha-1,6-glycosidic linkages in glycogen. *C. glutamicum* Δ*glgX* was compared regarding growth and glycogen metabolism to *C. glutamicum* WT during growth in CgC minimal medium with glucose and maltose as carbon source. When grown with glucose, *C. glutamicum* WT and *C. glutamicum* Δ*glgX* showed same growth rate (0.36 h^{-1}) and reached the stationary phase after 10 hours with ODs_{600} of 35 and 41, respectively (Figure 5A). *C. glutamicum* WT and *C. glutamicum* Δ*glgX* showed similar maximum glycogen levels of about 65 – 70 mg glycogen per g CDW.

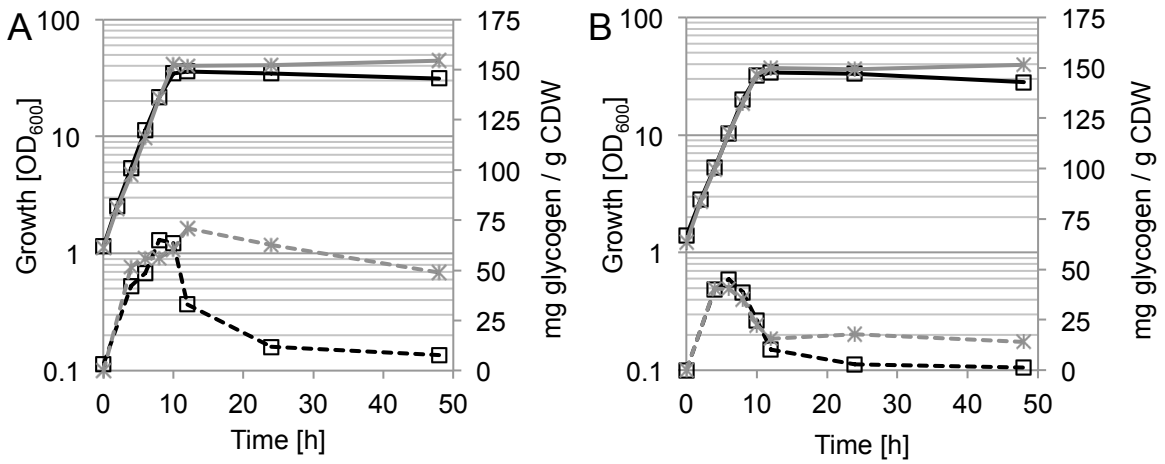


Figure 5: Growth (solid lines) and glycogen levels (dashed lines) of *C. glutamicum* WT (black open squares) and *C. glutamicum* Δ*glgX* (grey asterisks) in CgC minimal medium with 2 % glucose (A) or 2 % maltose (B) as carbon source. The Figure shows the results of one representative experiment. At least three independent experiments were performed, all three showing comparable results.

While *C. glutamicum* WT reached this maximum after 8 hours, *C. glutamicum* Δ*glgX* reached it after 12 hours. In contrast to *C. glutamicum* WT, in which glycogen levels dropped below 10 mg per g CDW after 48 hours, only a slight decrease in glycogen levels was observed in *C. glutamicum* Δ*glgX* (down to about 50 mg per g CDW).

During growth with maltose, *C. glutamicum* WT and *C. glutamicum* Δ*glgX* also showed similar growth rates (0.32 h^{-1} and 0.33 h^{-1}) and reached similar maximal ODs_{600} (34 and 37) (Figure 5B). *C. glutamicum* WT and *C. glutamicum* Δ*glgX* showed maximal glycogen levels of about 40 to 45 mg per g CDW after 4 to 6 hours and degraded glycogen to levels of 10 mg per g CDW and 15 mg per g CDW, respectively, after 12 hours. While *C. glutamicum* WT degraded glycogen almost completely in further cultivation (< 2.5 mg per g CDW after 48 hours), *C. glutamicum* Δ*glgX* kept glycogen levels at about 15 mg per g CDW for the last 36 hours of cultivation.

Taken together, the deletion of *glgX* in *C. glutamicum* did not lead to a change in growth rates, when grown on glucose or maltose. With glucose, the maximum OD_{600} of *C. glutamicum* Δ*glgX* was slightly higher than that of *C. glutamicum* WT. *C. glutamicum* Δ*glgX* showed only slight decrease in glycogen levels with glucose and incomplete degradation of glycogen with maltose.

3.1.2.2 Characterisation of *C. glutamicum* Δ*malP*

Previous studies by Seibold *et al.* (2007) suggested that the gene *glgP1* (in the following denoted as *malP*) codes for a maltodextrin phosphorylase MalP. The deletion mutant *C. glutamicum* Δ*malP* was constructed and analysed regarding growth and glycogen levels during growth in CgC minimal medium with 2 % glucose and with 2 % maltose. When glucose was used as carbon source, *C. glutamicum* Δ*malP* and *C. glutamicum* WT exhibited similar growth rates (0.39 h^{-1} and 0.36 h^{-1}) (Figure 6A). After 10 hours *C. glutamicum* WT and *C. glutamicum* Δ*malP* reached the stationary phase with ODs_{600} of 35 and 37, respectively. Similar glycogen levels of 65 – 70 mg per g CDW were reached by both strains after 8 hours. In *C. glutamicum* Δ*malP* glycogen was degraded slightly slower than in *C. glutamicum* WT. After 48 hours both strains showed glycogen levels below 5 mg per g CDW.

When grown in minimal medium with maltose, *C. glutamicum* Δ*malP* showed a growth behaviour clearly differing from the one of *C. glutamicum* WT (Figure 6B). While *C. glutamicum* WT exhibited exponential growth with a growth rate of 0.35 h^{-1} to an OD_{600} of about 32 after 10 hours, the growth of *C. glutamicum* Δ*malP* was only comparable to that of *C. glutamicum* WT in the first 4 hours. Afterwards, *C. glutamicum* Δ*malP* exhibited slower growth to an OD_{600} of about 20 after 12 hours (26 after 48 hours).

C. glutamicum WT reached the highest glycogen levels of approximately 65 mg per g CDW after 6 hours of growth and was able to degrade glycogen to concentrations below 2 mg per g CDW after 48 hours. In contrast, *C. glutamicum* Δ*malP* accumulated up to 130 mg glycogen per g CDW after 24 hours. After 48 hours, the glycogen concentrations were only slightly decreased to about 115 mg per g CDW.

In conclusion, *C. glutamicum* Δ*malP* showed during growth on glucose similar growth behaviour as *C. glutamicum* WT and only slightly slower glycogen degradation than *C. glutamicum* WT. When grown with maltose, *C. glutamicum* Δ*malP* exhibited impaired growth and was not able to degrade glycogen after reaching the maximal levels, which were about twice as high as these observed in *C. glutamicum* WT

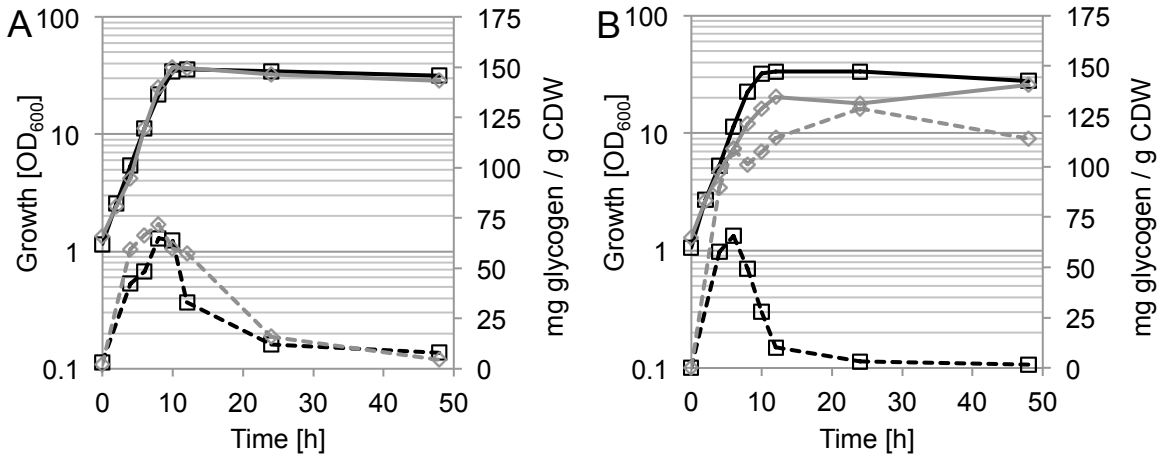


Figure 6: Growth (solid lines) and glycogen levels (dashed lines) of *C. glutamicum* WT (black open squares) and *C. glutamicum* Δ*malP* (grey open diamonds) in CgC minimal medium with 2 % glucose (A) or 2 % maltose (B) as carbon source. The Figure shows the results of one representative experiment. At least three independent experiments were performed, all three showing comparable results.

3.1.2.3 Characterisation of *C. glutamicum* Δ*glgP*

The gene *glgP2* (in the following denoted as *glgP*) encodes for a putative glycogen phosphorylase GlgP. Glycogen phosphorylases are able to cleave terminal glucose residues, which are linked to glycogen by alpha-1,4-glycosidic linkages in glycogen, thereby setting free glucose-1-phosphate. The deletion mutant *C. glutamicum* Δ*glgP* was compared to *C. glutamicum* WT with respect to growth behaviour and glycogen levels during cultivation in CgC minimal medium with 2 % glucose or with 2 % maltose. During growth with glucose, *C. glutamicum* WT and *C. glutamicum* Δ*glgP* showed similar growth rates (0.36 h^{-1} and 0.39 h^{-1}) and similar maximal ODs_{600} (35 and 37) after 10 hours (Figure 7A). *C. glutamicum* WT reached the highest glycogen levels of about 65 mg per g CDW after 8 hours. After 10 hours, degradation of glycogen started and after 48 hours the

glycogen levels were below 10 mg per g CDW. The maximal glycogen levels of *C. glutamicum* Δ*glgP* were slightly lower than in *C. glutamicum* WT (50 mg per g CDW after about 6 hours). The degradation of glycogen started after 12 hours and after 48 hours less than 5 mg glycogen per g CDW were determined in *C. glutamicum* Δ*glgP*.

With maltose as carbon source, *C. glutamicum* WT and *C. glutamicum* Δ*glgP* grew with similar growth rates (0.35 h^{-1} and 0.36 h^{-1}) (Figure 7B). While *C. glutamicum* Δ*glgP* reached a maximal OD_{600} of around 38 after 10 hours, *C. glutamicum* WT grew to a lower OD_{600} of about 33. The highest glycogen levels of about 65 mg per g CDW were determined for both strains after about 4 to 6 hours after inoculation. Afterwards, *C. glutamicum* WT and *C. glutamicum* Δ*glgP* degraded glycogen to levels of about 10 mg glycogen per g CDW after 12 hours and below 2 mg per g CDW after 48 hours.

In summary, the deletion of *glgP* in *C. glutamicum* led neither with glucose nor with maltose to significant changes in growth behaviour, glycogen accumulation or glycogen degradation in comparison to *C. glutamicum* WT.

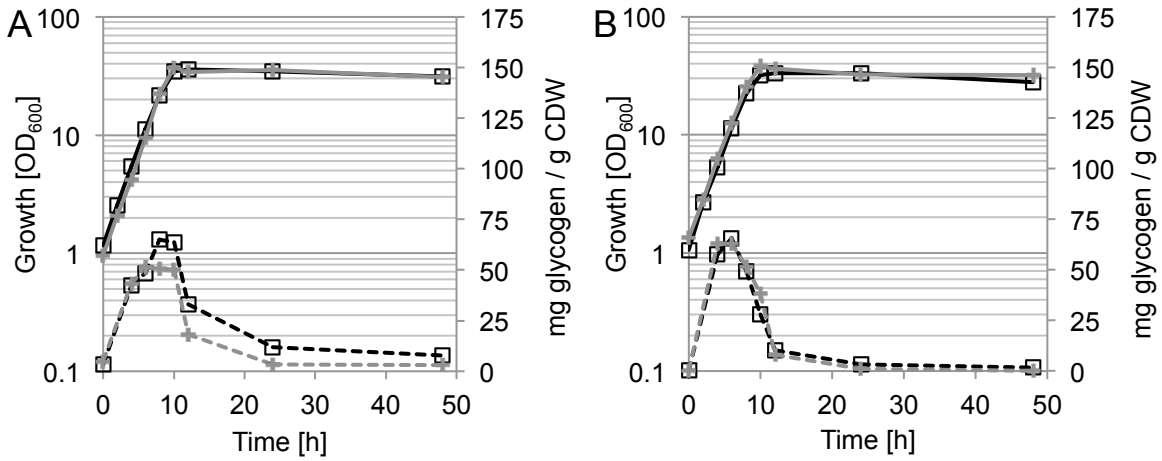


Figure 7: Growth (solid lines) and glycogen levels (dashed lines) of *C. glutamicum* WT (black open squares) and *C. glutamicum* Δ*glgP* (grey pluses) in CgC minimal medium with 2 % glucose (A) or 2 % maltose (B) as carbon source. The Figure shows the results of one representative experiment. At least three independent experiments were performed, all three showing comparable results.

3.1.3 Characterisation of *C. glutamicum* strains with multiple deletions of genes involved in glycogen degradation

For the construction of a strain, which is completely devoid of glycogen degradation, several double and a triple deletion mutant of genes involved in glycogen degradation were constructed and characterized with respect to growth and glycogen levels.

3.1.3.1 Characterisation of *C. glutamicum* Δ*glgX*Δ*malP*

C. glutamicum Δ*glgX*Δ*malP* was constructed and compared to *C. glutamicum* WT with regard to growth and glycogen levels during cultivation in CgC minimal medium with 2 % glucose or with 2 % maltose. When grown with glucose, *C. glutamicum* Δ*glgX*Δ*malP* showed similar growth as *C. glutamicum* Δ*glgX* (Figure 8A; Figure 5A). Maximal OD_{600} of *C. glutamicum* Δ*glgX*Δ*malP* was slightly higher than that of *C. glutamicum* WT and accumulated significantly more glycogen (about twice as much when compared to *C. glutamicum* WT). The glycogen levels of *C. glutamicum* Δ*glgX*Δ*malP* were even higher than the ones of *C. glutamicum* WT. When maltose was used as carbon source, *C. glutamicum* Δ*glgX*Δ*malP* showed growth comparable to the one of *C. glutamicum* Δ*malP* (Figure 8B; Figure 6B). After 4 hours, *C. glutamicum* Δ*glgX*Δ*malP* grew slower to an OD_{600} of only 13, which was unchanged after 24 hours. Between 24 and 48 hours, the OD_{600} of *C. glutamicum* Δ*glgX*Δ*malP* increased. The glycogen levels of *C. glutamicum* Δ*glgX*Δ*malP* were significantly higher than these of *C. glutamicum* WT and did not decrease.

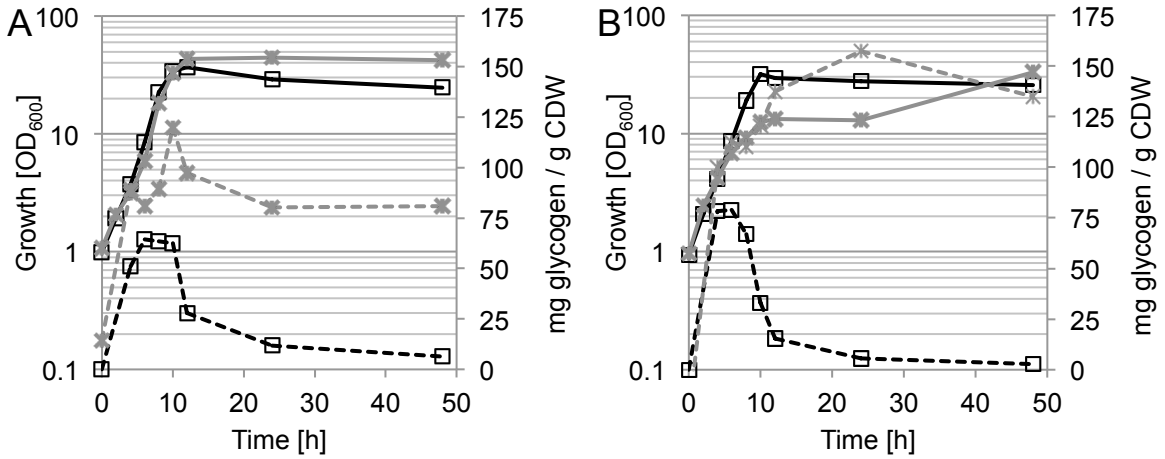


Figure 8: Growth (solid lines) and glycogen levels (dashed lines) of *C. glutamicum* WT (black open squares) and *C. glutamicum* Δ*glgX*Δ*malP* (grey asterisks) in CgC minimal medium with 2 % glucose (A) or 2 % maltose (B) as carbon source. The Figure shows the results of one representative experiment. At least three independent experiments were performed, all three showing comparable results.

To sum up, the simultaneous deletion of *malP* and *glgX* in *C. glutamicum* led with glucose to slightly higher maximum OD_{600} (as seen before for *C. glutamicum* Δ*glgX*), while the growth rate remained unchanged.

3.1.3.2 Characterisation of *C. glutamicum* Δ*glgX*Δ*glgP*

By deleting of the genes *glgP* (encoding a putative glycogen phosphorylase) and *glgX* (encoding glycogen debranching enzyme) the double deletion mutant *C. glutamicum* Δ*glgX*Δ*glgP* was constructed. This mutant was analysed with respect to growth and glycogen levels in minimal medium with 2 % glucose and 2 % maltose as carbon source. During growth with glucose, *C. glutamicum* Δ*glgX*Δ*glgP* showed the same growth behaviour as *C. glutamicum* Δ*glgX* reached higher glycogen levels than *C. glutamicum* WT, which decreased only marginally after reaching the maximal values. When maltose was used as carbon source, *C. glutamicum* Δ*glgX*Δ*glgP* showed the same growth as *C. glutamicum* Δ*glgX* and accumulated the same levels of glycogen, which were only partially degraded (Figure 9B; Figure 5B).
In conclusion, *C. glutamicum* Δ*glgX*Δ*glgP* showed on both substrates phenotypes similar to *C. glutamicum* Δ*glgX* and no additional effect by further deletion of *glgP*.

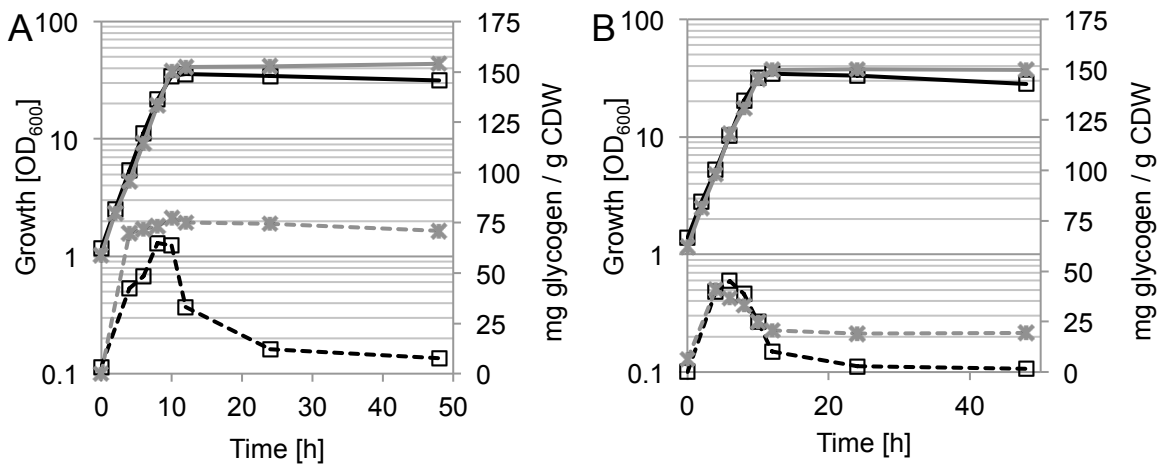


Figure 9: Growth (solid lines) and glycogen levels (dashed lines) of *C. glutamicum* WT (black open squares) and *C. glutamicum* Δ*glgX*Δ*glgP* (grey asterisks) in CgC minimal medium with 2 % glucose (A) or 2 % maltose (B) as carbon source. The Figure shows the results of one representative experiment. At least three independent experiments were performed, all three showing comparable results.

3.1.3.3 Characterisation of *C. glutamicum* Δ*glgP*Δ*malP*

The strain *C. glutamicum* Δ*glgP*Δ*malP* was constructed by deleting of the two genes *glgP* and *malP* coding for a putative glycogen phosphorylase and a maltodextrin phosphorylase, respectively. Newly strain was analysed with respect to growth behaviour and glycogen levels during growth in CgC minimal medium with 2 % glucose or 2 % maltose as carbon source. On glucose, *C. glutamicum* Δ*glgP*Δ*malP* showed similar growth and reached similar glycogen levels as *C. glutamicum* Δ*malP* (Figure 10A; Figure 6A). Glycogen was degraded slightly slower than in *C. glutamicum* WT. During growth with maltose, *C. glutamicum* Δ*glgP*Δ*malP* showed the same growth behaviour and similar glycogen levels as *C. glutamicum* Δ*malP* (Figure 10B; Figure 6B).
Altogether, *C. glutamicum* Δ*glgP*Δ*malP* showed with both carbon sources phenotypes similar to *C. glutamicum* Δ*malP* and no additional effect of further deletion of *glgP*.

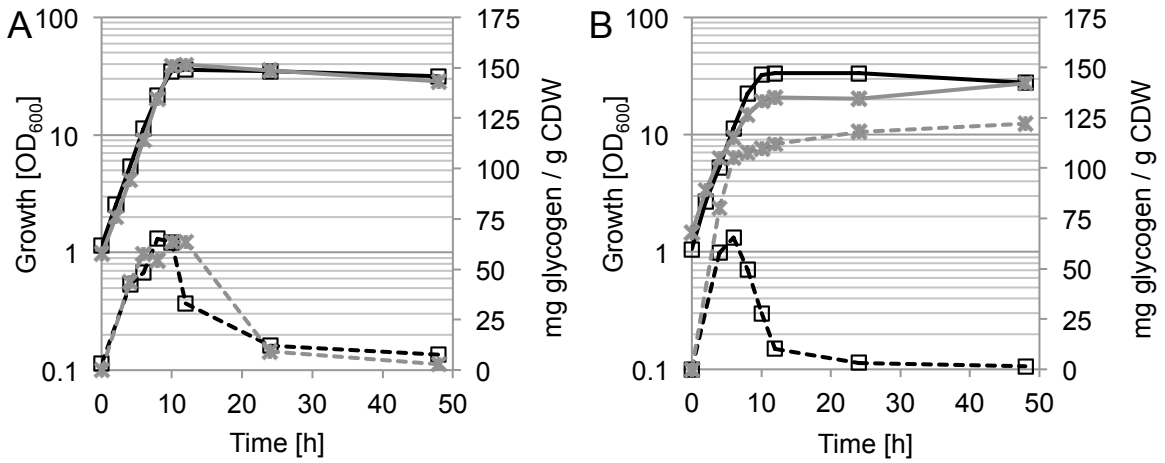


Figure 10: Growth (solid lines) and glycogen levels (dashed lines) of *C. glutamicum* WT (black open squares) and *C. glutamicum* Δ*glgP*Δ*malP* (grey asterisks) in CgC minimal medium with 2 % glucose (A) or 2 % maltose (B) as carbon source. The Figure shows the results of one representative experiment. At least three independent experiments were performed, all three showing comparable results.

3.1.3.4 Characterisation of *C. glutamicum* Δ*glgX*Δ*malP*Δ*glgP*

A triple deletion mutant of the three genes *glgX*, *malP* and *glgP* (*C. glutamicum* Δ*glgX*Δ*malP*Δ*glgP*) was constructed and compared in respect to growth behaviour and glycogen levels with *C. glutamicum* WT during cultivation in CgC minimal medium with 2 % glucose or with 2 % maltose. When grown on glucose, *C. glutamicum* Δ*glgX*Δ*malP*Δ*glgP* showed the same growth and comparable glycogen levels as *C. glutamicum* Δ*glgX*Δ*malP* (Figure 11A; Figure 8A). During growth on maltose, *C. glutamicum* Δ*glgX*Δ*malP*Δ*glgP* showed also the same growth and similar glycogen levels as *C. glutamicum* Δ*glgX*Δ*malP* (Figure 11B; Figure 8B).

Taken together, the triple deletion mutant *C. glutamicum* Δ*glgX*Δ*malP*Δ*glgP* showed on glucose as well as on maltose a phenotype comparable to the one of *C. glutamicum* Δ*glgX*Δ*malP*.

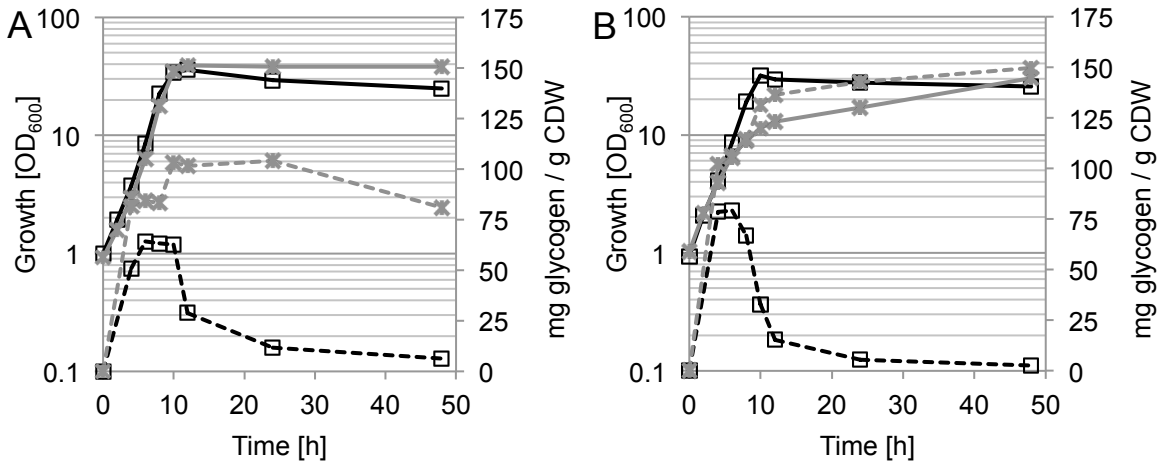


Figure 11: Growth (solid lines) and glycogen levels (dashed lines) of *C. glutamicum* WT (black open squares) and *C. glutamicum* Δ*glgX*Δ*glgP*Δ*malP* (grey asterisks) in CgC minimal medium with 2 % glucose (A) or 2 % maltose (B) as carbon source. The Figure shows the results of one representative experiment. At least three independent experiments were performed, all three showing comparable results.

3.1.4 Comparison of constructed *Corynebacterium glutamicum* deletion strains

The maximal OD_{600}, the maximal growth rates and the effects on glycogen synthesis/degradation in the experiments with single and multiple deletion mutants of *C. glutamicum* are summarized in Tables 4 and 5.

On glucose, the deletion of genes involved in glycogen synthesis (*glgC*, *glgA*, *glgB*) led to abolishment or strong reduction of glycogen synthesis during growth on glucose (Table 4). The deletion of *glgX* led to strongly reduced degradation of glycogen, while *C. glutamicum* Δ*malP* showed only slightly reduced glycogen degradation. The combination of *glgX* and *malP* deletions in *C. glutamicum* Δ*glgX*Δ*malP* and *C. glutamicum* Δ*glgX*Δ*malP*Δ*glgP* led to an increase of the respective effects on glycogen degradation. Growth was only affected, when *glgA*, *glgB* or *glgX* were deleted.

Table 4: Overview of constructed deletion mutants and the respective maximal ODs_{600}, maximal growth rates, effects on glycogen accumulation / degradation during growth in minimal medium with 2 % glucose.

Strain	Max. OD	Max. μ	Glycogen accumulation	Glycogen degradation	Shown in Figure
C. glutamicum WT	35 - 39	0.35 - 0.40	normal	normal	
C. glutamicum Δ*glgC*	31 - 35	0.35 - 0.39	abolished	n. d.	2A
C. glutamicum Δ*glgA*	26 - 28	0.16 - 0.23	strongly reduced	n. d.	3A
C. glutamicum Δ*glgB*	28 - 31	0.36 - 0.40	strongly reduced	n. d.	4A
C. glutamicum Δ*glgX*	41 - 47	0.36 - 0.39	slightly elevated	strongly reduced	5A
C. glutamicum Δ*malP*	37 - 40	0.38 - 0.41	normal	slightly reduced	6A
C. glutamicum Δ*glgP*	37 - 41	0.39 - 0.41	normal	normal	7A
C. glutamicum Δ*glgX*Δ*malP*	36 - 40	0.35 - 0.39	elevated	strongly reduced	8A
C. glutamicum Δ*glgX*Δ*glgP*	40 - 44	0.35 - 0.39	slightly elevated	strongly reduced	9A
C. glutamicum Δ*malP*Δ*glgP*	39 - 41	0.39 - 0.40	normal	slightly reduced	10A
C. glutamicum Δ*glgX*Δ*malP*Δ*glgP*	39 - 44	0.35 - 0.40	strongly elevated	strongly reduced	11A

(n. d. = not determinable)

When grown with maltose, the respective deletion mutants showed different phenotypes compared to growth with glucose (Table 5). The deletion of *glgB* caused delayed accumulation of glycogen, while *C. glutamicum* Δ*glgX* degraded glycogen only partially. *C. glutamicum* Δ*malP* showed strongly reduced glycogen degradation. *C. glutamicum* Δ*glgX*Δ*malP* showed strongly reduced glycogen degradation. Deletion of *glgB* or *malP* led to a lower maximal OD_{600}, while deletion of *glgX* caused a slightly elevated OD_{600}.

Table 5: Overview of constructed deletions and the respective maximal ODs_{600}, maximal growth rates, effects on glycogen accumulation / degradation during growth in minimal medium with 2 % maltose.

Strain	Max. OD	Max. μ	Glycogen accumulation	Glycogen degradation	Shown in Figure
C. glutamicum WT	30 - 36	0.32 - 0.41	normal	normal	
C. glutamicum Δ*glgC*	31 - 36	0.32 - 0.35	normal	normal	2B
C. glutamicum Δ*glgA*	34 - 37	0.31 - 0.37	normal	normal	3B
C. glutamicum Δ*glgB*	28 - 31	0.23 - 0.25	delayed	normal	4B
C. glutamicum Δ*glgX*	37 - 43	0.32 - 0.35	normal	partially	5B
C. glutamicum Δ*malP*	26 - 32	0.21 - 0.24	elevated	strongly reduced	6B
C. glutamicum Δ*glgP*	34 - 40	0.33 - 0.37	normal	normal	7B
C. glutamicum Δ*glgX*Δ*malP*	33 - 38	0.14 - 0.22	strongly elevated	strongly reduced	8B
C. glutamicum Δ*glgX*Δ*glgP*	36 - 37	0.32 - 0.33	slightly elevated	partially	9B
C. glutamicum Δ*malP*Δ*glgP*	27 - 31	0.18 - 0.20	elevated	strongly reduced	10B
C. glutamicum Δ*glgX*Δ*malP*Δ*glgP*	30 - 33	0.18 - 0.21	strongly elevated	strongly reduced	11B

3.2 Influence of specific growth factors on glycogen levels in C. glutamicum

3.2.1 Influence of phosphate availability on glycogen levels

In the course of the studies described in this thesis, it was shown that *C. glutamicum* accumulates more glycogen under phosphate-limited conditions (0.13 mM) than under conditions of phosphate surplus (13mM) (Woo *et al.*, 2011). Since phosphate-limited cultures did not only display higher glycogen levels, but also impaired growth, it was unclear, if the phosphate limitation or the growth impairment caused the increase of glycogen levels. Hence, it was interesting to investigate, whether the reduced phosphate availability or the reduced growth were responsible for higher glycogen levels in these cultures. For this purpose, growth of *C. glutamicum* WT in CgC minimal medium with 2 % glucose as carbon source and variable concentrations of phosphate (1.3 mM, 1.95 mM, 2.6 mM, 3.25 mM, 3.9 mM and 13 mM) was monitored and glycogen levels of these cultures were determined. In Figure 12, the growth and the glycogen levels of *C. glutamicum* WT in CgC minimal medium with different phosphate concentrations are shown.

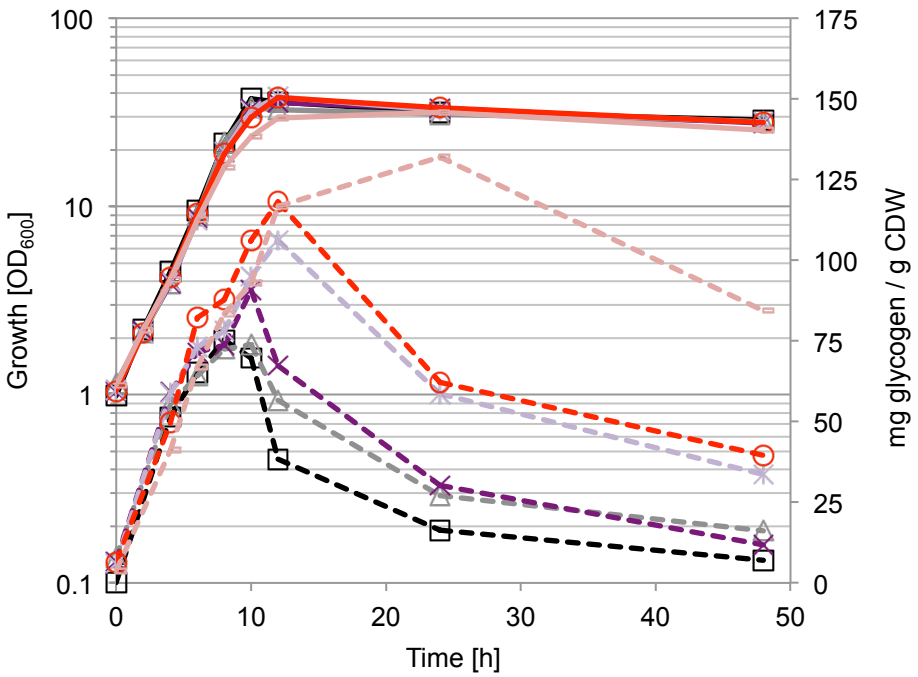


Figure 12: Growth (solid lines) and glycogen levels (dashed lines) of *C. glutamicum* WT grown in CgC minimal medium with different phosphate concentrations: 13 mM phosphate, black squares; 3.9 mM phosphate, grey triangles; 3.25 mM phosphate, purple crosses; 2.6 mM phosphate, light purple crosses; 1.95 mM, red circles; 1.3 mM, light red dashes; Shown are the averages of at least three independent cultivations.

In the cultures with phosphate concentrations of 13 mM (corresponding to 100 % of the original phosphate concentration) down to 1.95 mM (corresponding to 15 % of the original phosphate concentration) no differences in growth rate and maximal OD_{600} could be observed. The growth rates of the cultures with phosphate concentrations from 1.95 mM to 13 mM were between 0.36 h^{-1} and 0.39 h^{-1}, while the culture with 1.3 mM reached only a growth rate of 0.33 h^{-1}. The culture with the lowest phosphate concentration reached its maximal OD_{600} of around 30 after 12 hours, whereas all other cultures reached the maximal OD_{600} of 36 to 39 after 10 to 12 hours. It was observed that the glycogen levels in the various cultures rose with decreasing phosphate concentrations. In the cultures with 3.9 mM phosphate the highest glycogen levels of around 75 mg glycogen / g CDW were reached after 10 hours, which is comparable to cultures with normal phosphate concentrations of 13 mM (around 75 mg glycogen / g CDW after 8 hours). In minimal medium with a phosphate concentration of 3.25 mM, *C. glutamicum* WT reached 90 mg glycogen / g CDW after 10 hours, but final glycogen levels did not differ from cultures with 3.9 mM and 13 mM phosphate. In contrast, the final glycogen levels of the other cultures (phosphate concentrations of 2.6 mM, 1.95 mM and 1.3 mM) were much higher with around 35 mg glycogen / g CDW, around 40 mg glycogen / g CDW and around 85 mg glycogen / g CDW, respectively. While the cultures with 2.6 mM and 1.95 mM phosphate reached their maximal glycogen level of around 105 mg glycogen / g CDW and around 120 mg glycogen / g CDW, respectively, after 12 hours, the culture with 1.3 mM phosphate had a maximal glycogen level of around 130 mg glycogen / g CDW after 24 hours.

Taken together, the results show that decreasing phosphate concentrations in the range between 1.95 mM and 3.9 mM cause higher glycogen levels in *C. glutamicum* without affecting the growth behaviour.

3.2.2 Influence of nitrogen availability on glycogen levels

Previously, it was shown for a variety of organisms that lower concentrations of nitrogen sources in the medium can lead to elevated glycogen levels (Holme, 1957; Mulder *et al.*, 1962; Madsen, 1963). Since glycogen levels in *C. glutamicum* were shown to be affected positively by decreasing concentrations of phosphate (see. 3.2.1), the question arose, if similar effects could also be observed for lower concentrations of nitrogen sources. To investigate this issue, growth of *C. glutamicum* WT in CgC minimal medium with 2 % glucose as carbon source and variable concentrations of nitrogen sources (37.8 mM ammonium sulphate plus 82.5 mM urea, 22.7 mM ammonium sulphate plus 49.5 mM urea, 15.1 mM ammonium sulphate plus 33.0 mM urea, 7.6 mM ammonium sulphate plus 16.5 mM urea, 3.8 mM ammonium sulphate plus 8.3 mM urea, 1.5 mM ammonium

sulphate plus 3.3 mM urea – corresponding to 4 %, 10 %, 20 %, 40 %, 60 %, and 100 %, respectively, of the original concentrations of each nitrogen source) was monitored and glycogen levels of these cultures were determined. In Figure 13, growth and glycogen levels of these cultures with different concentrations of nitrogen are shown. In contrast to the experiments with different phosphate concentrations, the concentrations of nitrogen sources could not be altered to this large extent without affecting the growth behaviour of *C. glutamicum*. The cultures with 37.8 mM ammonium sulphate plus 82.5 mM urea (100 % of the original nitrogen conc.) and 22.7mM ammonium sulphate plus 49.5 mM urea (60 % of original conc.) showed comparable growth with a maximal OD_{600} of about 34 to 38 and growth rates of 0.38 h^{-1}. The cultures with lower concentrations of nitrogen sources showed lower maximal ODs_{600} (about 8 to 32) and lower growth rates (μ = 0.22 h^{-1} – 0.34 h^{-1}). Taken together, the reduction of nitrogen sources led to no significant effect in glycogen levels in *C. glutamicum*. Growth of *C. glutamicum* was affected as soon as the concentrations of ammonium sulphate and urea were lower than 22.7 mM and 49.5 mM, respectively.

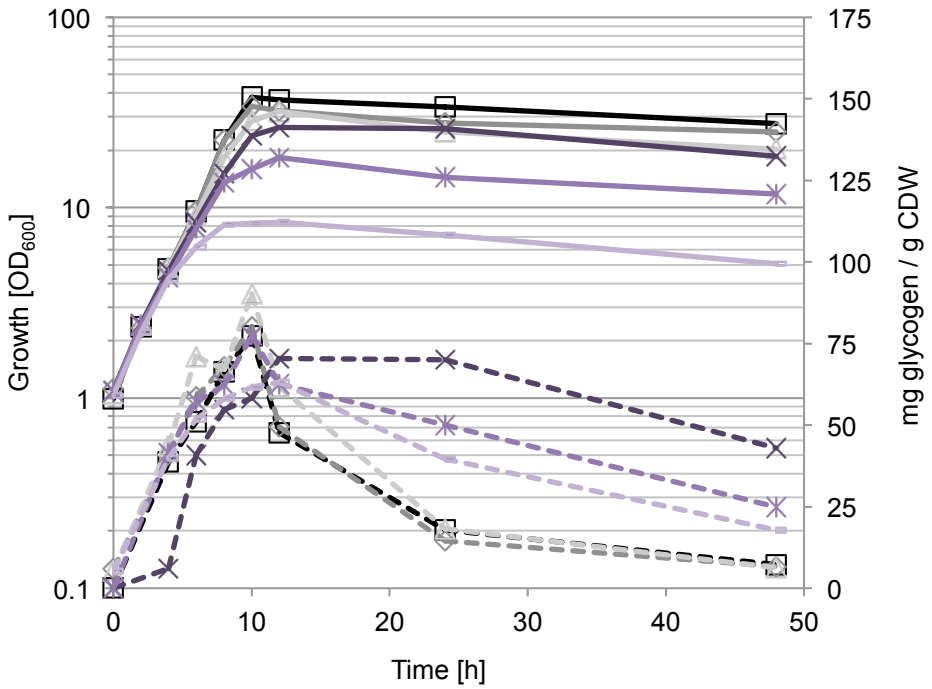


Figure 13: Growth (solid lines) and glycogen levels (dashed lines) of *C.* WT grown in CgC minimal medium with different concentrations of nitrogen sources: 37.8 mM ammonium sulphate plus 82.5 mM urea, black open squares; 22.7mM ammonium sulphate plus 49.5 mM urea, grey open diamonds; 15.1 mM ammonium sulphate plus 33.0 mM urea, light grey open triangles; 7.6 mM ammonium sulphate plus 16.5 mM urea, dark purple crosses; 3.8 mM ammonium sulphate plus 8.3 mM urea, purple asterisks; 1.5 mM ammonium sulphate plus 3.3 mM urea, light purple minus. Shown are the averages of at least three independent cultivations.

3.2.3 Influence of pH on glycogen levels

The extracellular pH is a critical factor for growth of bacteria. Previous studies showed that *C. glutamicum* is able to maintain an internal pH of about 7.5 at an extracellular pH of 6, but is affected by oxidative stress, which causes also changes in metabolic fluxes (Follmann *et al.*, 2009). The pH difference across the bacterial membrane is crucial for the proton motive force, which is responsible for the formation of ATP. The formation of glycogen consumes more ATP than what can be regained by degradation of glycogen (see introduction). Therefore, it was interesting to investigate, in which disturbances of the glycogen metabolism affect the growth of *C. glutamicum* under slightly acidic conditions. For examination of this question, *C. glutamicum* WT, *C. glutamicum* Δ*glgC* and *C. glutamicum* Δ*glgX* were grown using the DASGIP parallel fermenter system in CgC minimal medium with 2 % glucose and pH values of 7 and 6, respectively, and growth rates, maximal ODs_{600} and glycogen levels of respective strains were compared under both conditions.

C. glutamicum Δ*glgX* showed the same growth as *C. glutamicum* WT at a constant pH of 7 (OD_{600} of about 30; μ of about 0.33 h^{-1}) (Figure 14A). *C. glutamicum* Δ*glgX* accumulated about 120 mg glycogen per g CDW after 6 hours and glycogen was not degraded afterwards. In contrast, *C. glutamicum* accumulated only about 85 mg of glycogen per g CDW, which was degraded to about 5 mg per g CDW. At a constant extracellular pH of 7, *C. glutamicum* Δ*glgC* showed the identical growth behaviour as C. glutamicum WT (OD_{600} of about 36; μ of about 0.35 h^{-1}) (Figure 14B). As seen in shake flask experiments, *C. glutamicum* Δ*glgC* was not able to form any glycogen with glucose, while *C. glutamicum* WT accumulated about 100 mg glycogen per g CDW.

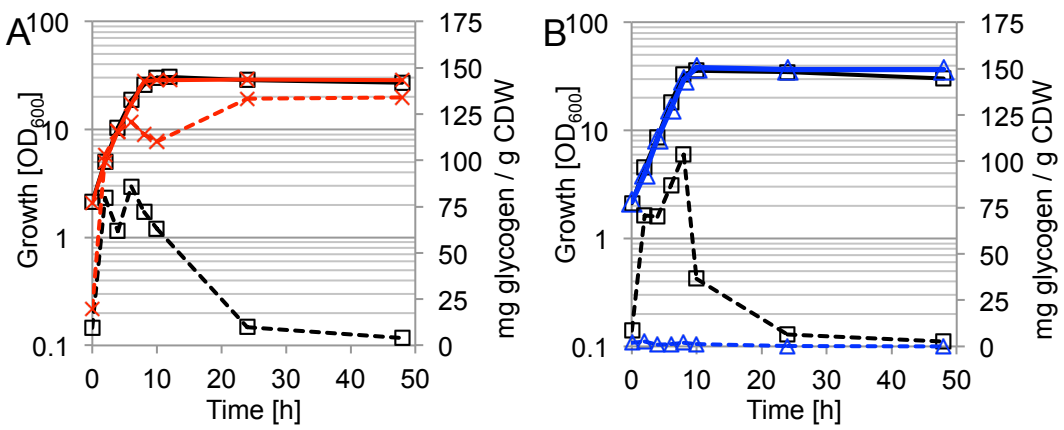


Figure 14: Growth (solid lines) and glycogen levels (dashed lines) of *C. glutamicum* WT (black open squares) and *C. glutamicum* Δ*glgX* (A; red open crosses) or *C. glutamicum* Δ*glgC* (A; blue open triangles) in DasGip parallel fermenter with CgC minimal medium with 2 % glucose and a constant pH of 7. The Figure shows the results of one representative experiment. At least three independent experiments were performed, all three showing comparable results.

At a constant pH value of 6, *C. glutamicum* Δ*glgX* grew with lower growth rate and reached a dramatically lower maximal OD_{600} than *C. glutamicum* WT (μ of 0.16 h^{-1} and 0.13 h^{-1}, respectively; OD_{600} of about 30 and 13, respectively) (Figure 15A). *C. glutamicum* Δ*glgX* reached higher glycogen levels than *C. glutamicum* WT (110 mg per g CDW and 70 mg per g CDW, respectively). *C. glutamicum* Δ*glgX* showed very slow degradation of glycogen. *C. glutamicum* Δ*glgC* reached the same maximal OD_{600} as *C. glutamicum* WT (OD_{600} of about 33), but showed significant higher growth rates than *C. glutamicum* WT (μ of 0.22 h^{-1} and 0.17 h^{-1}, respectively). *C. glutamicum* Δ*glgC* accumulated no glycogen, while *C. glutamicum* WT accumulated less glycogen than at a pH of 7 (about 60 mg per g CDW), which was degraded afterwards.

Taken together, disturbances in the glycogen metabolism (deletion of *glgC* and *glgX*, respectively) had no significant effect on growth at a constant pH of 7 in comparison to *C. glutamicum* WT. At a constant pH of 6, *C. glutamicum* Δ*glgC* showed a higher growth rate than *C. glutamicum* WT. Both strains reached comparable maximal OD_{600}. In contrast, *C. glutamicum* Δ*glgX* grew slower than *C. glutamicum* WT and reached a significant lower maximal OD_{600}.

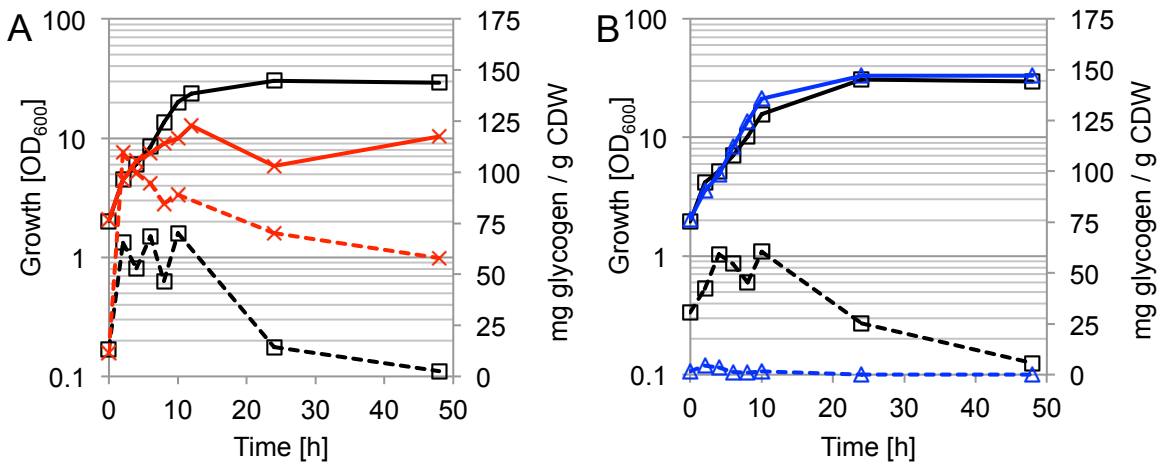


Figure 15: Growth (solid lines) and glycogen levels (dashed lines) of *C. glutamicum* WT (black open squares) and *C. glutamicum* Δ*glgX* (A; red open crosses) or *C. glutamicum* Δ*glgC* (B; blue open triangles) in DasGip parallel fermenter with CgC minimal medium with 2 % glucose and a constant pH of 6. The Figure shows the results of one representative experiment. At least three independent experiments were performed, all three showing comparable results. Experiment with *C. glutamicum* Δ*glgX* was performed only once.

3.2.4 Influence of anaerobic conditions on glycogen levels

To investigate, whether *C. glutamicum* is able to form glycogen under anaerobic conditions, *C. glutamicum* WT cells from a 2x TY overnight preculture were used to inoculate 50 mL CgC main cultures in 100 mL Schottglas bottles with nitrogen atmosphere to an OD of about 10. Samples were taken periodically for determination of OD_{600} and of glycogen levels at the respective time points. In Figure 16, the OD_{600} and glycogen levels of *C. glutamicum* WT under anaerobic conditions are shown. The OD_{600} rose from around 10 at the start of incubation to around 25 after 10 hours of incubation. Afterwards, the OD_{600} dropped to about 16 within 48 hours. The course of glycogen levels showed a similar behaviour in the first 10 hours. Glycogen accumulated to levels of about 90 mg glycogen per g CDW within the first 8 hours, roughly matching with the maximal OD_{600} after 10 hours. In contrast to OD_{600}, the glycogen levels did not change significantly from 10 hours to 48 hours of incubation.

Since glycogen levels correlated more or less with the OD_{600}, the same experiments were performed with *C. glutamicum* Δ*glgC* (which does not accumulate glycogen on glucose; see chapter 3.1.1.1) and with *C. glutamicum* Δ*glgX*Δ*malP*Δ*glgP* (which shows strongly impaired degradation of glycogen and high glycogen levels on glucose; see chapter 3.1.3.4), to test, whether the increase of OD_{600} of *C. glutamicum* WT is connected to the increase of the glycogen levels.

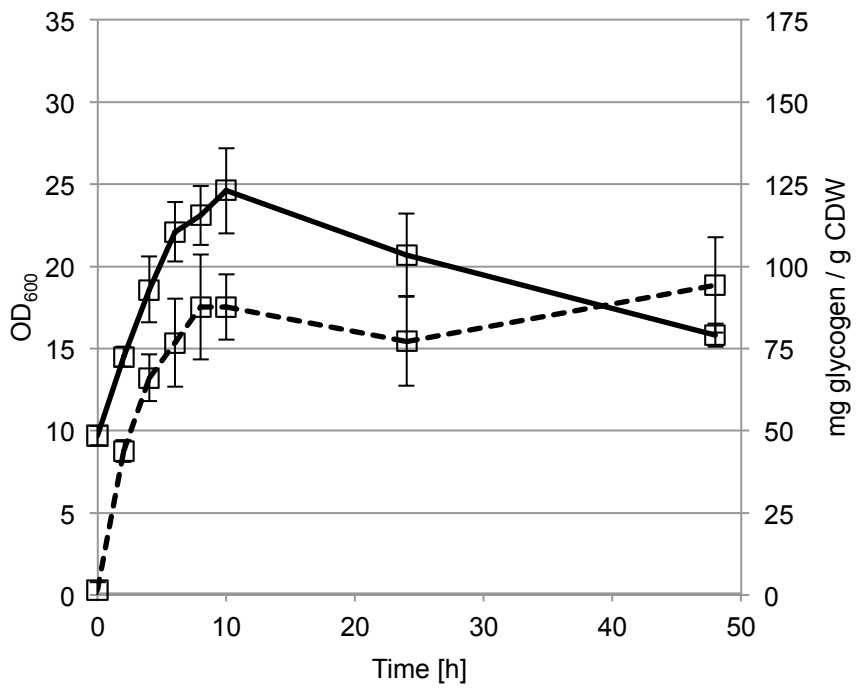


Figure 16: OD_{600} (solid line) and glycogen levels (dashed line) of *C. glutamicum* WT in CgC minimal medium under anaerobic conditions. The average values of three independent experiments are shown.

Figure 17 shows ODs_{600} and glycogen levels of *C. glutamicum* WT, *C. glutamicum* Δ*glgC* and *C. glutamicum* Δ*glgX*Δ*malP*Δ*glgP* incubated under anaerobic conditions. In contrast to *C. glutamicum* WT, *C. glutamicum* Δ*glgC* showed almost no increase of OD_{600} (from about 12 at beginning of the incubation to an OD_{600} of around 14 after 8 hours). As already seen in shake flask experiments under anaerobic conditions /see chapter 3.1.1.1), *C. glutamicum* Δ*glgC* did not accumulate glycogen. In contrast, the OD_{600} of *C. glutamicum* Δ*glgX*Δ*malP*Δ*glgP* increased under anaerobic conditions almost threefold from around 11 at the beginning of the incubation to about 32 after 24 hours. *C. glutamicum* Δ*glgX*Δ*malP*Δ*glgP* accumulated even slightly higher glycogen levels than *C. glutamicum* WT (about 110 mg glycogen / g CDW until 10 hours of incubation) and showed no glycogen degradation until 48 hours of incubation. Taken together, *C. glutamicum* Δ*glgC*, which did not accumulate glycogen, showed no significant increase of the OD_{600}, while *C. glutamicum* WT and *C. glutamicum* Δ*glgX*Δ*malP*Δ*glgP*, which accumulated glycogen, showed dramatic increase in OD_{600}.

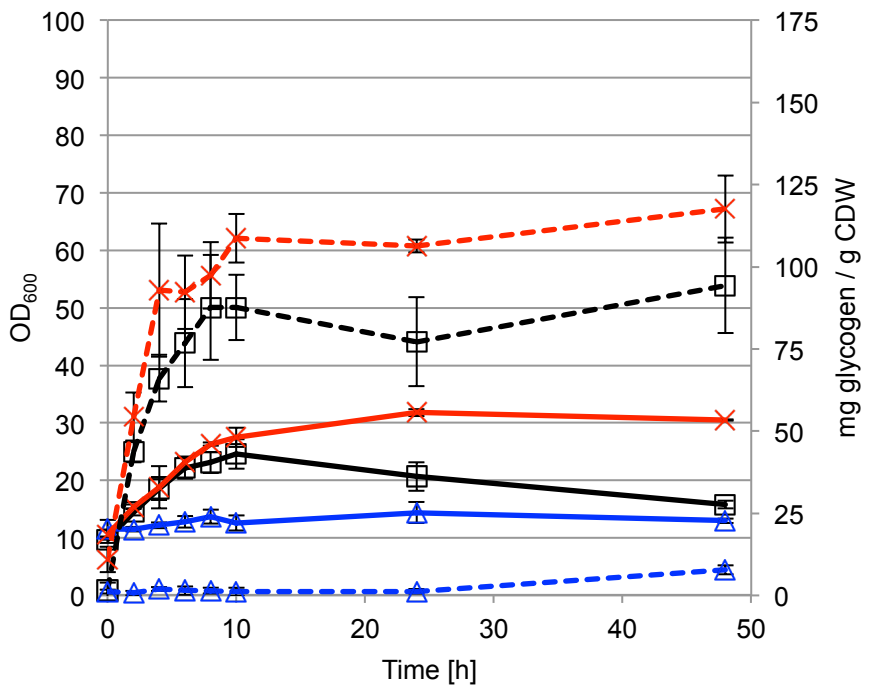


Figure 17: OD_{600} (solid line) and glycogen levels (dashed line) of *C. glutamicum* WT (black squares), *C. glutamicum* Δ*glgC* (blue triangles) and *C. glutamicum* Δ*glgX*Δ*malP*Δ*glgP* (red crosses) in CgC minimal medium under anaerobic conditions. The average values of three independent experiments are shown.

To investigate whether the increase of OD_{600} of *C. glutamicum* WT and *C. glutamicum* Δ*glgX*Δ*malP*Δ*glgP* is due to an increase of cell number of the respective strains, samples were taken directly after the start of the experiment and 10 hours of incubation, diluted 1:10^6 and plated out on 2x TY agar plates. Colony forming units (CFU) were counted and the ratio of CFU after 10 and 0 hours were compared with the ratio of OD_{600} at the respective time points. While the OD_{600}-ratio of *C. glutamicum* WT was about 2.4, the CFU-ratio was 1.4 and thus, significantly lower. The OD_{600}-ratio of *C. glutamicum* Δ*glgC* of about 1.1 was comparable to the CFU-ratio of roughly 0.9, while *C. glutamicum* Δ*glgX*Δ*malP*Δ*glgP* showed an even higher one than *C. glutamicum* WT with an OD_{600}-ratio of about 2.6. Interestingly, a CFU-ratio of about 0.6 indicated that less viable cells of *C. glutamicum* Δ*glgX*Δ*malP*Δ*glgP* were present after 10 hours than right at the beginning of the incubation. Taken together, *C. glutamicum* WT showed during anaerobic incubation a significant increase of OD_{600} and CFU, while *C. glutamicum* Δ*glgC* showed only slight changes in OD_{600} and CFU. In contrast, *C. glutamicum* Δ*glgX*Δ*malP*Δ*glgP* displayed significant higher OD_{600} after 10 hours, but a decrease in the number of CFU.

Since the number of viable cells of *C. glutamicum* WT, *C. glutamicum* Δ*glgC* and *C. glutamicum* Δ*glgX*Δ*malP*Δ*glgP* incubated under anaerobic conditions did not change in the same way as the OD_{600} did, it was interesting to follow the absolute cell number of the strains with normal glycogen metabolism, disturbed glycogen synthesis and disturbed glycogen degradation. For this purpose, *C. glutamicum* WT, *C. glutamicum* Δ*glgC* and *C. glutamicum* Δ*glgX* were incubated under anaerobic conditions and the total cell numbers were counted with a Helber counting chamber. In Figure 18 the percentage of the ratio of cell number to OD_{600} with reference to this ratio at the starting point is shown for *C. glutamicum* WT, *C. glutamicum* Δ*glgC* and *C. glutamicum* Δ*glgX* incubated under anaerobic conditions. The ratio of cell number to OD_{600} at the beginning of the anaerobic incubation was set as 100 % for all the three strains. The ratios at later time points are shown in percentage of this original ratio (Figure 18). The initial cell number to OD_{600} ratios were $3.6 \times 10^8 \pm 0.6 \times 10^8$ per mL for *C. glutamicum* WT (cell number: $2.8 \times 10^9 \pm 0.5 \times 10^9$ per ml; OD_{600}: 7.8 ± 0.8), $3.3 \times 10^8 \pm 0.4 \times 10^8$ per mL for *C. glutamicum* Δ*glgC* (cell number: $3.5 \times 10^9 \pm 0.6 \times 10^9$ per ml; OD_{600}: 10.6 ± 1.2) and $3.3 \times 10^8 \pm 0.5 \times 10^8$ per mL for *C. glutamicum* Δ*glgX* (cell number: $3.6 \times 10^9 \pm 0.6 \times 10^9$ per ml; OD_{600}: 11.1 ± 1.3). *C. glutamicum* WT and *C. glutamicum* Δ*glgX* showed a significant decrease of the cell number/OD_{600}-ratio to about 45 % within 6 to 8 hours. For *C. glutamicum* Δ*glgC*, this ratio only slightly dropped to about 90 % of the value within 10 hours and to about 80 % after 24 hours. These results indicated that the OD_{600} of *C. glutamicum* WT and *C. glutamicum* Δ*glgX* increased independently of the cell number.

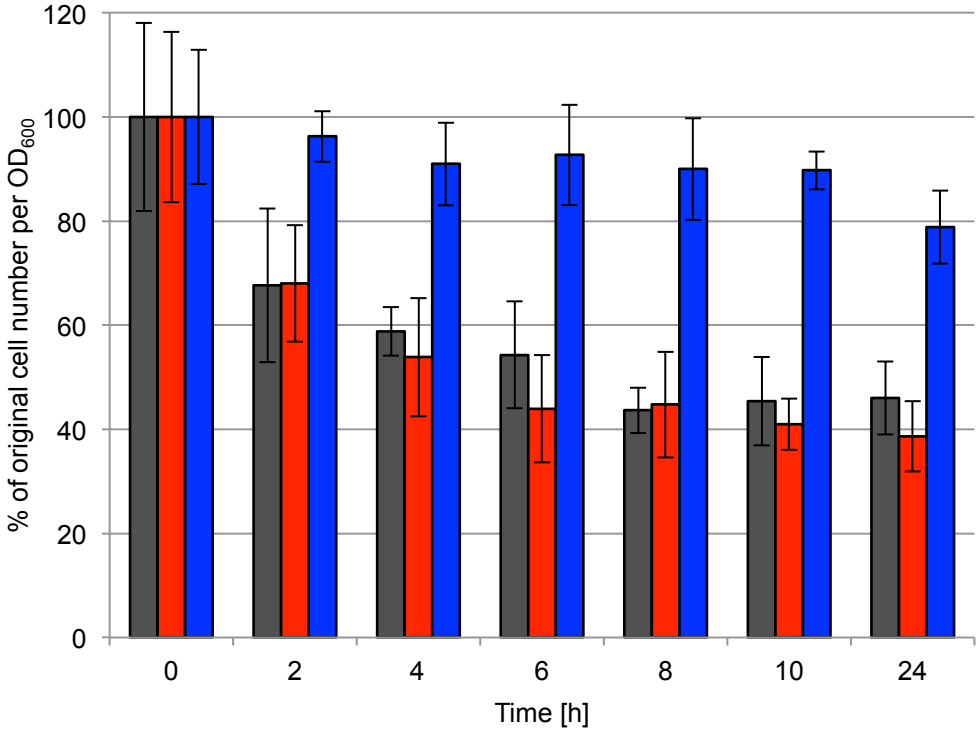


Figure 18: Ratio of cell number measured using Helber counting chamber and OD_{600}. Values in time points 2, 4, 6, 8, 10, 24 h are expressed as the percentage of the initial ratios measured at the beginning of cultivation in CgC minimal medium with 2 % glucose. Legend: *C. glutamicum* WT (dark grey), *C. glutamicum* Δ*glgC* (blue) and *C. glutamicum* Δ*glgX* (red). The average values of at least three independent cultivations are shown..

To test, whether an increase of the cell volume is responsible for the increase of the OD_{600}, *C. glutamicum* WT, *C. glutamicum* Δ*glgC* and *C. glutamicum* Δ*glgX* were incubated under anaerobic conditions and OD_{600}, cell number and cell volume were analysed using a Coulter Counter. With these data, cell number per OD_{600}, average volume per cell and total cell number were calculated. In Figure 19 these values are shown for *C. glutamicum* WT incubated anaerobic conditions. The initial values were $1.2 \times 10^9 \pm 0.1 \times 10^9$ per mL for the cell number, 6.5 ± 0.3 for the OD_{600}, $1.8 \times 10^8 \pm 0.2 \times 10^8$ per mL for the cell number to OD_{600} ratio, $3.1 \times 10^9 \pm 0.3 \times 10^9$ fL per mL for the total biovolume and 2.6 ± 0.2 fL for average cell volume.

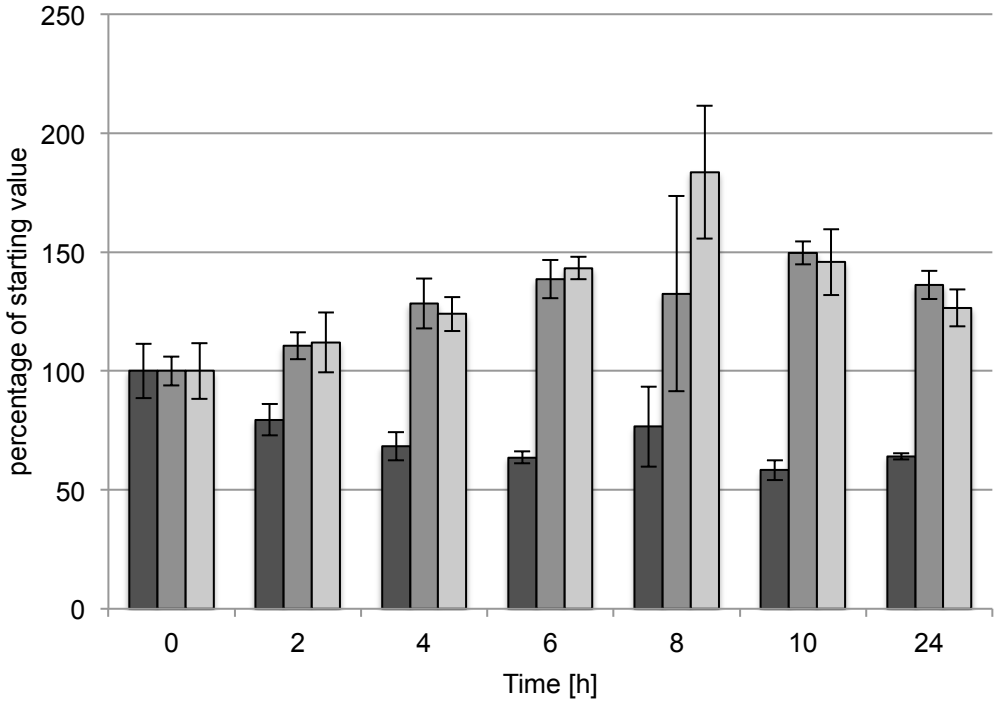


Figure 19: Ratio of counted (Coulter Counter) cell number to OD_{600} (dark grey), average cell volume (grey), total cell number (light grey) of *C. glutamicum* WT during growth in CgC minimal medium with 2 % glucose. All values are given as percentage of the original values at beginning of anaerobic incubation. The average values of at least three independent cultivations are shown.

As shown in experiments with Helber counting chamber, it was observed that the number of cells per OD_{600} decreased significantly after start of the incubation. In contrast to previous experiments, the ratio of cell number to OD_{600} dropped to about 60 % of the initial ratio at the starting point of incubation. Using the Coulter Counter it is possible to calculate also the average cell volume by dividing the total biovolume by the number of cells. The average cell volume of *C. glutamicum* WT incubated under anaerobic conditions increased within 10 hours to around 150 % of the average cell volume at the

starting point. It was also observed that the total cell number increased to around 180 % of the starting value within 8 hours. The total cell number dropped again to about 130 % after 24 hours.

In the same way, the ratio of cell number to OD_{600}, the average cell volume and the total cell number of *C. glutamicum* Δ*glgX* and of *C. glutamicum* Δ*glgC* incubated under anaerobic conditions were analysed (Figure 20 and Figure 21). The initial values were 1.7 x 10^9 ± 0.1 x 10^9 per mL for the cell number, 8.0 ± 0.6 for the OD_{600}, 2.1 x 10^8 ± 0.2 x 10^8 per mL for the cell number to OD_{600} ratio, 5.1 x 10^9 ± 0.4 x 10^9 fL per mL for the total biovolume and 3.0 ± 0.3 fL for average cell volume. Similarly to *C. glutamicum* WT, *C. glutamicum* Δ*glgX* showed under anaerobic conditions a significant decreased number-to-OD_{600}-ratio to around 45 % of the starting value after 8 hours. At this time point, the average cell volume of *C. glutamicum* Δ*glgX* had increased to around 130 % of the average cell volume at the beginning of the incubation. In contrast to experiments with *C. glutamicum* WT, increase during anaerobic incubation of total cell number of *C. glutamicum* Δ*glgX* was not observed. After 24 hours the total cell number reached around 110 % of the cell number at the start.

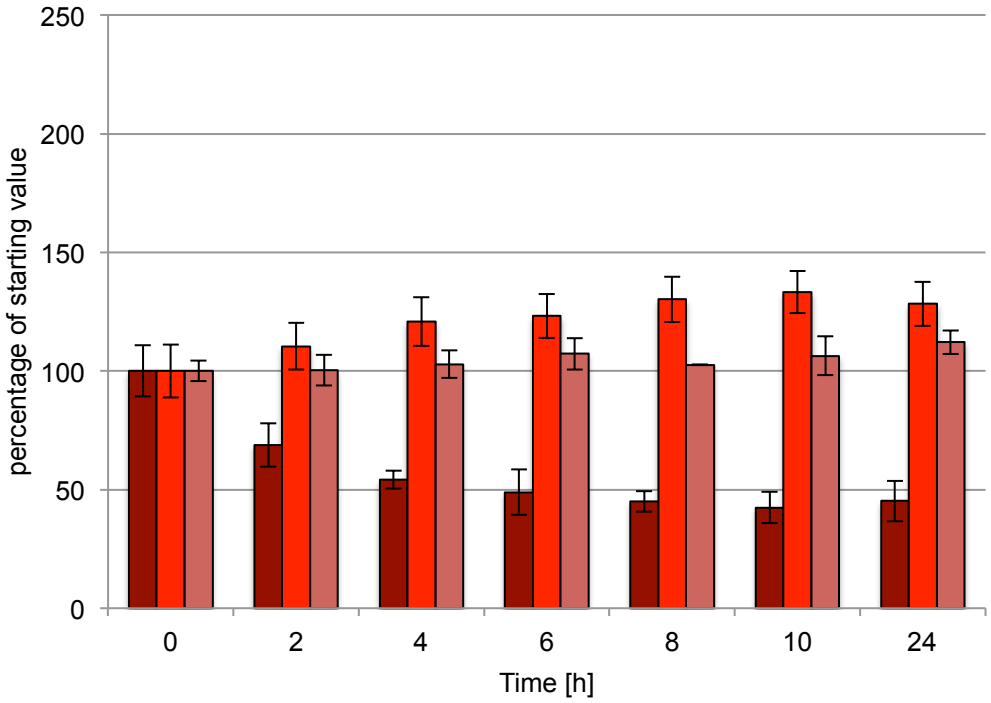


Figure 20: Ratio of counted (Coulter Counter) cell number to OD_{600} (dark red), average cell volume (red), total cell number (light red) of *C. glutamicum* Δ*glgX* during growth in CgC minimal medium with 2 % glucose. All values are given as percentage of the original values at beginning of anaerobic incubation. The average values of at least three independent cultivations are shown.

For *C. glutamicum* Δ*glgC*, the initial values were 1.7 x 10^9 ± 0.1 x 10^9 per mL for the cell number, 7.3 ± 0.2 for the OD_{600}, 2.3 x 10^8 ± 0.3 x 10^8 per mL for the cell number to OD_{600} ratio, 5.1 x 10^9 ± 0.2 x 10^9 fL per mL for the total biovolume and 3.0 ± 0.4 fL for average cell volume. The ratio of cell number to OD_{600}, the average cell volume and the total cell number of *C. glutamicum* Δ*glgC* showed no significant changes during anaerobic incubation (Figure 21). The ratio of cell number to OD_{600} of *C. glutamicum* Δ*glgC* under anaerobic conditions dropped only slightly to about 90 % of the starting value within 8 hours. The average cell volume also showed just a minor change with an increase within 8 hours to about 110 % of the starting value. In a similar way, the total cell number also showed only a slight increase after 4 hours to about 105 % of the initial value at the start of the anaerobic incubation. After 24 hours the total cell number had risen to 120 % of the starting value.

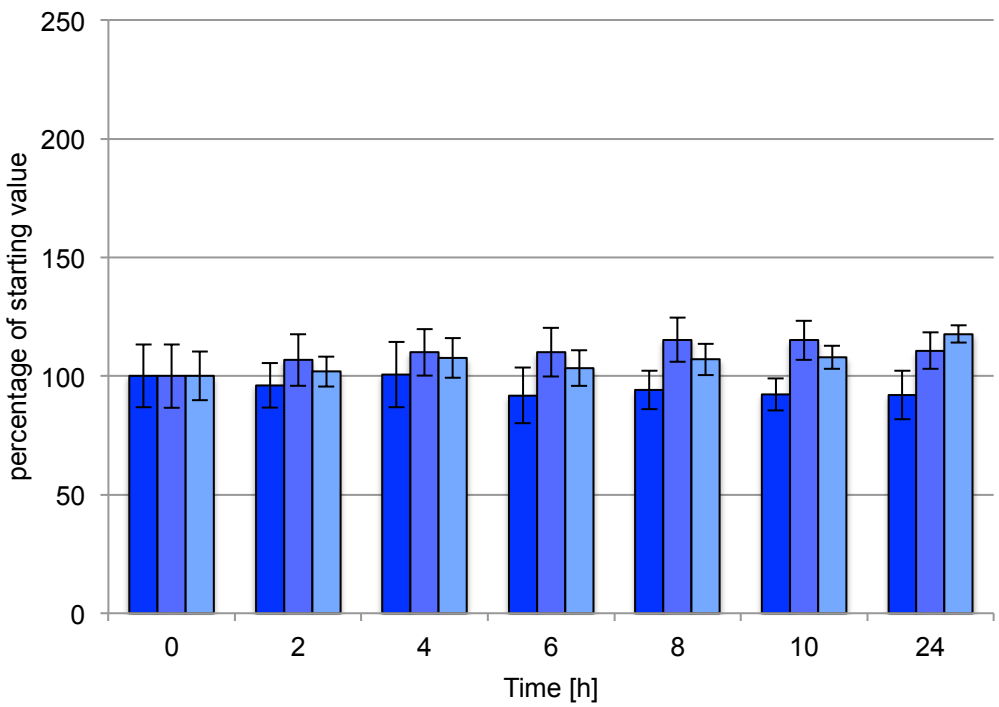


Figure 21: Ratio of counted (Coulter Counter) cell number to OD_{600} (dark blue), average cell volume (blue), total cell number (light blue) of *C. glutamicum* Δ*glgC* during growth in CgC minimal medium with 2 % glucose. All values are given as percentage of the original values at beginning of anaerobic incubation. The average values of at least three independent cultivations are shown.

Taken together, strains able to accumulate glycogen (*C. glutamicum* WT, *C. glutamicum* Δ*glgX*, *C. glutamicum* Δ*glgX*Δ*malP*Δ*glgP*) showed an increase of OD_{600} during incubation in CgC minimal medium with 2 % glucose under anaerobic conditions, whereas *C. glutamicum* Δ*glgC* (unable to form glycogen) showed no increase of OD_{600} under the same conditions. While glycogen accumulating strains displayed a significant decrease of the ratio of counted cell number to OD_{600} and significant increase of the cell volume, only *C. glutamicum* WT showed an increase of total cell number and number of viable cells. Strains not able to degrade glycogen (either because glycogen degrading enzymes are deleted or no glycogen was accumulated) showed a slight decrease in viable cell number and no changes in total cell numbers.

3.3 Regulation of glycogen metabolism

3.3.1 Quantitative real-time transcription analysis of genes involved in glycogen metabolism during growth with reduced phosphate concentrations

As shown in chapter 3.2.1, the glycogen levels in *C. glutamicum* WT grown with 1.95 mM phosphate (i. e. 15 % of the original phosphate concentration) were significantly higher than in *C. glutamicum* WT grown with “normal” phosphate concentrations of 13 mM (see chapter 3.2.1). These differences in glycogen levels could be caused by regulation on enzyme or expression level. For investigation of the expression levels, mRNA of *C. glutamicum* WT cells grown in CgC minimal medium with normal and reduced phosphate concentration were taken after 6 and 12 hours of growth and used to perform qRT-PCR and to compare the expression levels of the *glgX*, *glgC*, *glgB*, *glgA*, *malP* and *glgP* genes. In Figure 22 the expression levels of *C. glutamicum* WT grown with lower phosphate concentrations after 6 hours are shown in comparison to *C. glutamicum* WT grown with 13 mM phosphate after 6 hours. The genes responsible for glycogen synthesis – namely *glgC*, *glgB* and *glgA* – exhibited a higher expression in cultures grown with lower phosphate concentrations (about 60 %, 110 %, and 50 % higher, respectively, than in cultures with 13 mM phosphate). The *glgX* and *malP* genes showed about the same expression levels under both conditions. The expression of *glgP* was reduced, when the phosphate concentration was 1.95 mM instead of 13 mM.

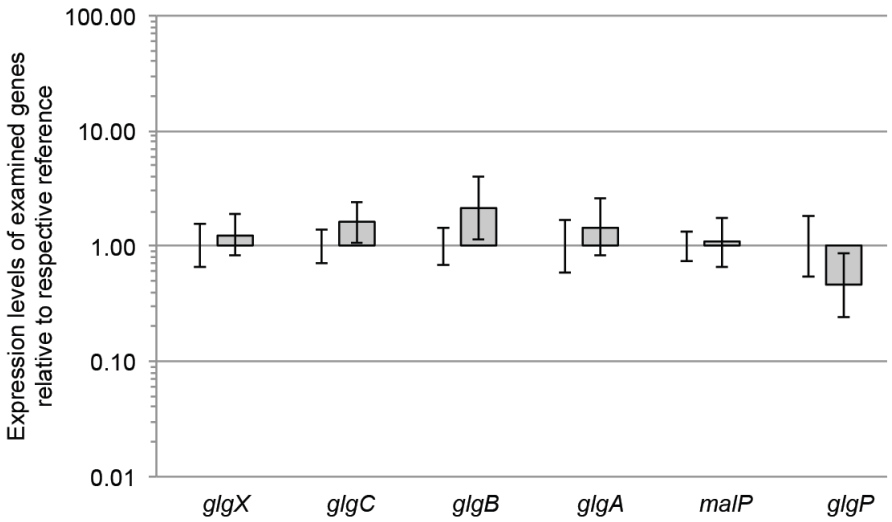


Figure 22: Change of mRNA levels of the genes *glgX*, *glgC*, *glgB*, *glgA*, *malP*, and glgP in *C. glutamicum* WT grown in CgC minimal medium with 1.95 mM phosphate after 6 hours of growth in comparison to *C. glutamicum* WT grown in CgC minimal medium with 13 mM phosphate after 6 hours of growth. For the comparison of the relative mRNA levels, semi-quantitative real-time RT-PCR was used. The mRNA levels in *C. glutamicum* WT grown in CgC minimal medium with normal phosphate concentration after 6 hours of growth were set to 1 ± standard deviation of all performed experiments. For the determinations of mRNA levels at least three biological replicates per condition and three technical replicates per biological replicate were performed.

When comparing the expression levels of the genes *glgX*, *glgC*, *glgB*, *glgA*, *malP*, and *glgP* in *C. glutamicum* WT grown with a phosphate concentration of 1.95 mM and in *C. glutamicum* WT grown with 13 mM phosphate after 12 hours of growth (shown in Figure 23), it was observed that the glycogen synthesis genes *glgC*, *glgB* and *glgA* showed higher expression levels with 1.95 mM phosphate (between about 90 % and 290 % higher) than with 13 mM phosphate. G*lgX* also displayed higher expression (about 115 %) with 1.95 mM phosphate than with 13 mM phosphate. In contrast to samples after 6 hours of growth, *malP* showed lower expression (decrease of approximately 50 %) after 12 hours with 1.95 mM phosphate than with 13 mM phosphate, while *glgP* showed to higher expression (increase of about 40 %).

Taken together, the analysis of expression levels of glycogen metabolism genes in minimal medium with 13 mM and 1.95 mM phosphate after 6 and 12 hours of growth showed that the reduction of phosphate concentration led to increased expression of most genes coding for enzymes involved in glycogen metabolism. The expression levels of the *glgX*, *glgC*, *glgB* and *glgA* genes were higher at both time points, whereas the expression level of the *malP* gene was higher only after 12 hours. The *glgP* gene showed a lower expression level after 6 hours and a higher expression level after 12 hours.

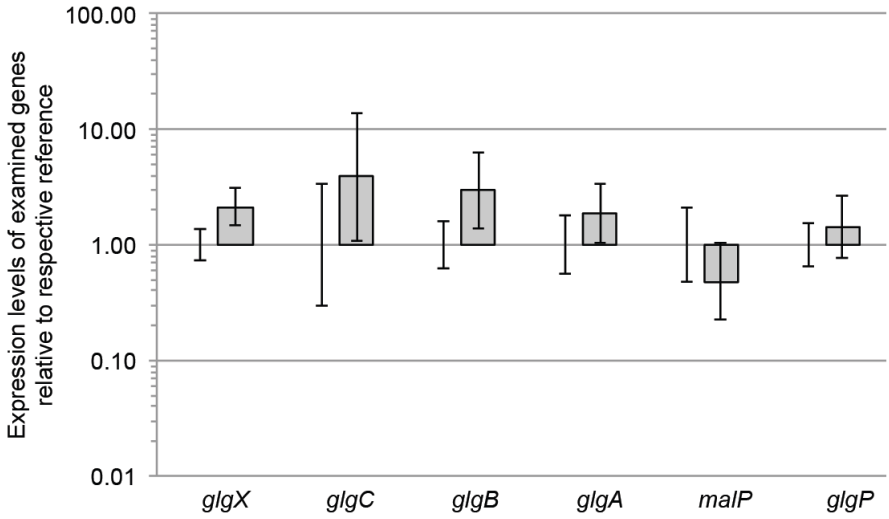


Figure 23: Change of mRNA levels of the genes *glgX*, *glgC*, *glgB*, *glgA*, *malP*, and glgP in *C. glutamicum* WT grown in CgC minimal medium with 1.95 mM phosphate after 12 hours of growth in comparison to *C. glutamicum* WT grown in CgC minimal medium with 13 mM phosphate after 12 hours of growth. For the comparison of the relative mRNA levels, semi-quantitative real-time RT-PCR was used. The mRNA levels in *C. glutamicum* WT grown in CgC minimal medium with normal phosphate concentration after 6 hours of growth were set to 1 ± standard deviation of all performed experiments. For the determinations of mRNA levels at least three biological replicates per condition and three technical replicates per biological replicate were performed.

3.3.2 *In vivo* and *real-time* transcription analysis of genes involved in glycogen metabolism using an integrated luciferase system

For the investigation of the transcriptional regulation of the glycogen synthesis genes *glgC* and *glgA*, and the glycogen degradation gene *glgX* in the course of growth on glucose or maltose, the reporter strains *C. glutamicum* RESluxIN_$p_{glgC(GTG)}$, *C. glutamicum* RESluxIN_p_{glgA} and *C. glutamicum* RESluxIN_p_{glgX2} were constructed and used for analysis of promoter activities of the respective genes (Schnell, 2013). The reporter system is based on the genomic integration of the *luxA* and *luxB* genes (coding for a luciferase from *Photorhabdus luminescens*) and the *fre* gene (coding for a FMN reductase from *Escherichia coli*) into copy B of the 16S rDNA of *C. glutamicum* RES167 resulting in the strain *C. glutamicum* RESluxIN. The expression of these genes is dependent on the promoter, which is integrated directly upstream of the *luxA* gene (Laslo, 2013). By determination of luminescence (relative light units per OD_{600}) of the tested strains, the activity of the respective promoters can be compared to each other.

3.3.2.1 Promoter activity of *glgC*

In Figure 24A, the luminescence levels and the growth of *C. glutamicum* RESluxIN_$p_{glgC(GTG)}$ in CgC minimal medium with 2 % glucose are shown. Growth rate and maximal OD_{600} of *C. glutamicum* RESluxIN_$p_{glgC(GTG)}$ were 0.35 h^{-1} and about 30, respectively, and were comparable to those of *C. glutamicum* RESluxIN (0.37 h^{-1} and about 34, respectively; data not shown). The luminescence levels of *C. glutamicum* RESluxIN during the whole experiment were below 0.1 x 10^3 RLU/OD_{600} (data not shown). *C. glutamicum* RESluxIN_$p_{glgC(GTG)}$ reached the highest luminescence levels of about 2.9 x 10^3 RLU/OD_{600} right after inoculation. The luminescence levels dropped below 1.5 x 10^3 RLU/OD_{600} after 2 hours of growth. After reaching luminescence levels of about 3.0 x 10^2 RLU/OD_{600}, no change in luminescence was observed until the end of the experiment.

When grown in CgC minimal medium with 2 % maltose, *C. glutamicum* RESluxIN_$p_{glgC(GTG)}$ reached a growth rate of 0.33 h^{-1} and a maximal OD_{600} of about 22 after 10 hours (Figure 24B). *C. glutamicum* RESluxIN grew to a maximal OD_{600} of about 29 after 12 hours with a growth rate of 0.34 h^{-1} (data not shown). As seen for experiments with 2 % glucose as carbon source, *C. glutamicum* RESluxIN_$p_{glgC(GTG)}$ showed the highest luminescence right after inoculation. The luminescence levels dropped from about 3.4 x 10^3 RLU/OD_{600} at the start to around 0.8 x 10^3 RLU/OD_{600} after 4 hours. The luminescence levels fell below 0.5 x 10^3 RLU/OD_{600} after 6 hours of growth. The reference *C. glutamicum* RESluxIN showed only luminescence levels of 0.1 x 10^3 RLU/OD_{600} and below (data not shown). Taken together, it was observed that the highest luminescence levels of *C. glutamicum* RESluxIN_$p_{glgC(GTG)}$ occurred right after the inoculation with both carbon sources.

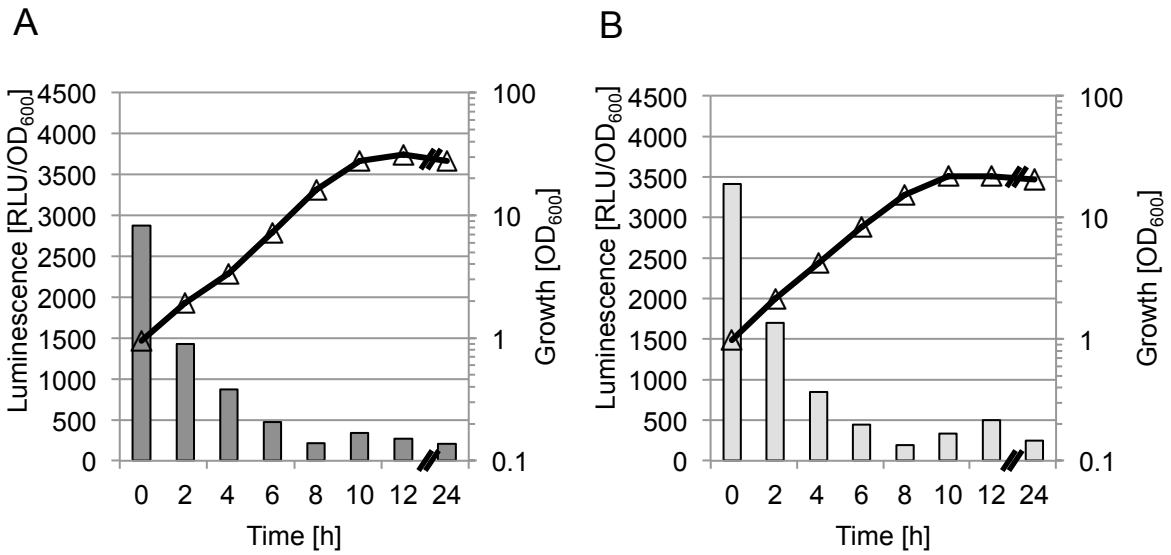


Figure 24: Luciferase activities (columns) and growth (triangles) of *C. glutamicum* RESluxIN_p$_{glgC(GTG)}$ in CgC minimal medium with 2 % glucose (A) or 2 % maltose (B) as carbon source. The Figure shows the results of one representative experiment. At least three independent experiments were performed, all three showing comparable results.

3.3.2.2 Promoter activity of *glgA*

For the analysis of the promoter activity of *glgA*, *C. glutamicum* RESluxIN_p$_{glgA}$ was grown in CgC minimal medium with 2 % glucose or 2 % maltose, while growth and luminescence levels were monitored. During growth with 2 % glucose, *C. glutamicum* RESluxIN_p$_{glgA}$ showed growth to a maximal OD_{600} of about 30 after 10 hours (Figure 25A) with a growth rate of 0.38 h^{-1}. While the growth rate was the same as that of *C. glutamicum* RESluxIN, the maximal OD_{600} of *C. glutamicum* RESluxIN_p$_{glgA}$ was lower than that of *C. glutamicum* RESluxIN (about 35; data not shown). The highest luminescence levels of almost 1.7 x 10^3 RLU/OD_{600} were reached after 2 hours of growth. After 4 hours the luminescence dropped below 0.5 x 10^3 RLU/OD_{600}. The luminescence of *C. glutamicum* RESluxIN was below 0.1 x 10^3 RLU/OD_{600} at all time points (data not shown).

When grown in minimal medium with 2 % maltose (Figure 25B), *C. glutamicum* RESluxIN_p$_{glgA}$ grew with growth rate of 0.36 h^{-1} to a maximal OD_{600} of about 33 after 12 hours, while *C. glutamicum* RESluxIN grew with a growth rate of 0.34 h^{-1} to a maximal OD_{600} of about 31 after 12 hours (data not shown). In contrast to growth with 2 % glucose, the luminescence level of *C. glutamicum* RESluxIN_p$_{glgA}$ at the beginning of growth was almost as high as after 2 hours. At this time point the luminescence maximum of around 1.5 x 10^3 RLU/OD_{600} was reached. After 4 hours the luminescence dropped below 0.5 x 10^3 RLU/OD_{600}.

To conclude, *C. glutamicum* RESluxIN_p$_{glgA}$ showed highest luminescence levels after about 2 hours of growth. While the luminescence levels were significantly lower right after inoculation than after 2 hours with glucose as carbon source, the luminescence levels with maltose as carbon source were after 2 hours just slightly higher than directly after inoculation. As seen for *C. glutamicum* RESluxIN_p$_{glgC(GTG)}$, the luminescence levels of *C. glutamicum* RESluxIN_p$_{glgA}$ dropped quickly after their maximum values.

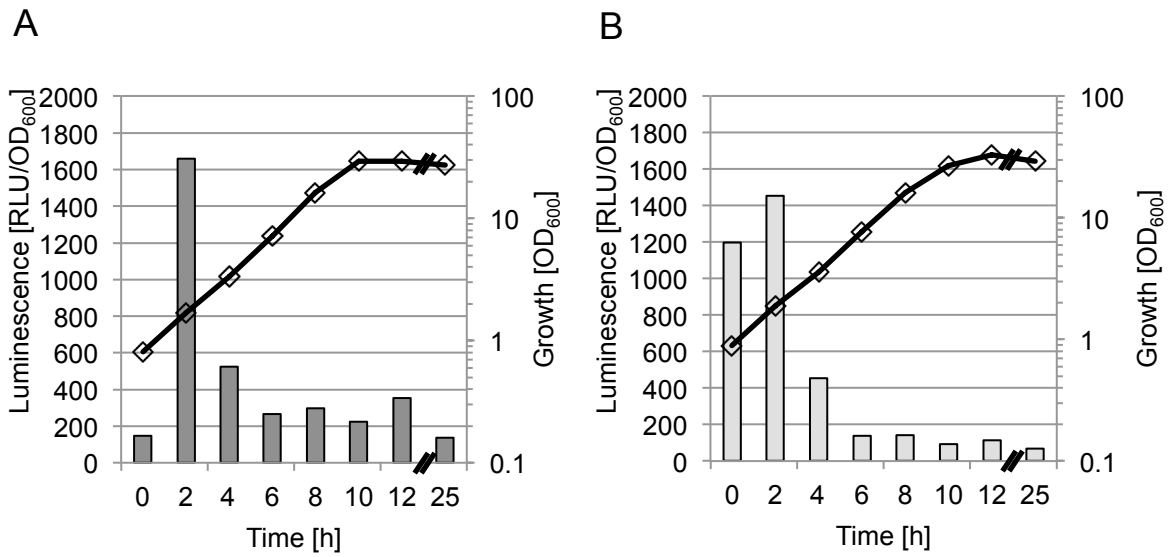


Figure 25: Luciferase activities (columns) and growth (diamonds) of *C. glutamicum* RESluxIN_p$_{glgA}$ in CgC minimal medium with 2 % glucose (A) or 2 % maltose (B) as carbon source. The Figure shows the results of one representative experiment. At least three independent experiments were performed, all three showing comparable results.

3.3.2.3 Promoter activity of *glgX*

To investigate the *glgX* promoter activity, *C. glutamicum* RESluxIN_p$_{glgX2}$ was also grown in CgC minimal medium with 2 % glucose or 2 % maltose as carbon source. Growth was monitored by determination by OD_{600} and the promoter activity was analysed by determining luminescence levels.

With 2 % glucose as carbon source, *C. glutamicum* RESluxIN_p$_{glgX2}$ exhibited a growth rate of 0.38 h^{-1} and grew to a maximum DO_{600} of about 33 after 12 hours (Figure 26A; for comparison *C. glutamicum* RESluxIN: μ = 0.38 h^{-1}, max. OD_{600} = about 35; data not shown). *C. glutamicum* RESluxIN_p$_{glgX2}$ showed the highest luminescence of about 1.0 x 10^3 RLU/OD_{600} at the beginning of growth. The luminescence levels ranged from 0.6 x 10^3 to 0.75 x 10^3 RLU/OD_{600} until after 6 hours of growth. The luminescence of *C. glutamicum* RESluxIN was below 0.1 x 10^3 RLU/OD_{600} at all time points (data not shown).

In minimal medium with 2 % maltose, *C. glutamicum* RESluxIN_p$_{glgX2}$ grew to a maximal OD_{600} of about 30 after 12 hours of growth with a growth rate of 0.35 h^{-1} (Figure 26B). *C. glutamicum* RESluxIN grew with a growth rate of 0.34 h^{-1} to a maximal OD_{600} of about 30 after 12 hours (data not shown). Similar to results with minimal medium with 2 % glucose, *C. glutamicum* RESluxIN_p$_{glgX2}$ reached the highest luminescence at the beginning of growth (around 1.2 x 10^3 RLU/OD_{600}). Between 2 and 6 hours of growth, the luminescence levels were around 0.6 x 10^3 RLU/OD_{600}. After 8 hours of growth, the luminescence dropped below 0.4 x 10^3 RLU/OD_{600}. The luminescence levels of *C. glutamicum* RESluxIN were below 0.1 x 10^3 RLU/OD_{600} throughout the whole experiment.

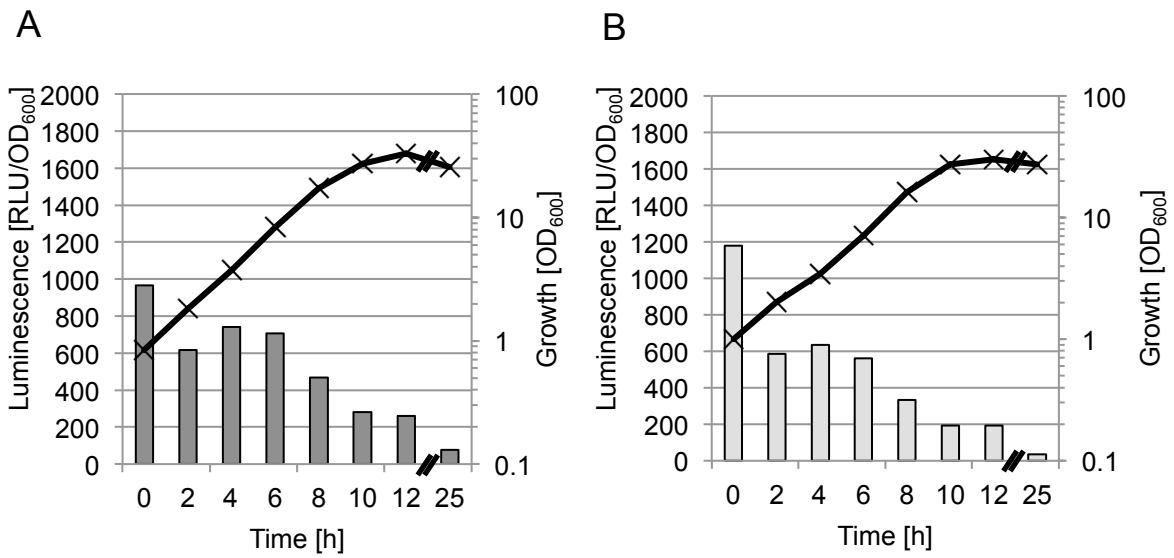


Figure 26: Luciferase activities (columns) and growth (crosses) of *C. glutamicum* RESluxIN_p$_{glgX2}$ in CgC minimal medium with 2 % glucose (A) or 2 % maltose (B) as carbon source. The Figure shows the results of one representative experiment. At least three independent experiments were performed, all three showing comparable results.

In conclusion, while the luminescence levels of *C. glutamicum* RESluxIN_p$_{glgX2}$ were not as high as those of *C. glutamicum* RESluxIN_p$_{glgA}$ and *C. glutamicum* RESluxIN_p$_{glgC(GTG)}$, the luminescence was longer on similar levels than in *C. glutamicum* RESluxIN_p$_{glgA}$ and *C. glutamicum* RESluxIN_p$_{glgC(GTG)}$. These findings indicate that the *glgA* and especially the *glgC* gene are expressed significantly higher, than the *glgX* gene at the beginning of growth, whereas *glgX* seems to be longer expressed than *glgA* and *glgC*.

3.4 Effects of disturbed glycogen metabolism on production behaviour

3.4.1 Effects of disturbed glycogen metabolism on the production of organic acids

To investigate, whether the production of organic acids by *C. glutamicum* under anaerobic conditions is influenced by abolition of glycogen synthesis or glycogen degradation, the production behaviour of *C. glutamicum* WT, *C. glutamicum* Δ*glgC* and *C. glutamicum* Δ*glgX*Δ*malP*Δ*glgP* in CgC minimal medium with 2 % glucose was examined. Cell suspensions of these strains were incubated in Schottglas bottles with CgC minimal medium and 2 % glucose under nitrogen atmosphere and the OD_{600}, the glycogen levels and the concentrations of glucose, succinate, lactate and acetate were determined.

As seen in previous experiments, *C. glutamicum* WT and *C. glutamicum* Δ*glgX*Δ*malP*Δ*glgP* showed an increase in OD_{600} from about 10 to 27 and 32, respectively, and glycogen concentrations to 100 and 110 mg glycogen per g CDW, respectively, while *C. glutamicum* Δ*glgC* exhibited neither an increase of the OD_{600} nor of the glycogen levels (Figure 27A and Figure 27B, respectively). In cell suspensions of *C. glutamicum* WT, the OD_{600} decreased significantly after 10 hours to about 16 after 48 hours and glycogen levels dropped also to about 85 mg per g CDW after 48 hours.

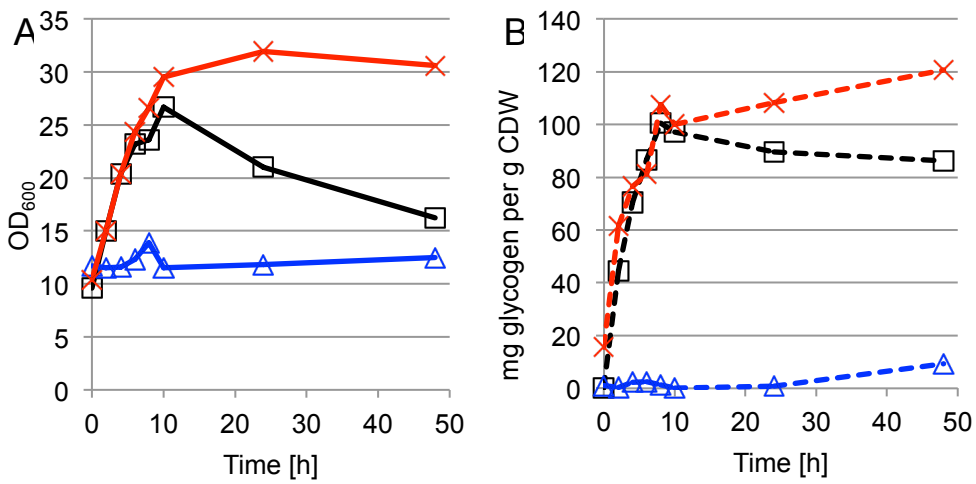


Figure 27: OD_{600} (A) and glycogen levels (B) of *C. glutamicum* WT (black squares), *C. glutamicum* Δ*glgC* (blue triangles) and *C. glutamicum* Δ*glgX*Δ*malP*Δ*glgP* (red crosses) in CgC minimal medium with 2 % glucose during incubation under anaerobic conditions. The Figure shows the results of one representative experiment. At least three independent experiments were performed, all three showing comparable results.

In contrast, the OD_{600} of *C. glutamicum* Δ*glgX*Δ*malP*Δ*glgP* remained about 30 and glycogen levels even slightly increased to glycogen levels of about 120 mg per g CDW. While the glucose concentrations in cell suspension of *C. glutamicum* WT and *C. glutamicum* Δ*glgX*Δ*malP*Δ*glgP* dropped in a similar way to about 5 mM within 48 hours, cell suspensions of *C. glutamicum* Δ*glgC* showed still higher glucose concentrations after 24 and 48 hours (Figure 28A). *C. glutamicum* WT and *C. glutamicum* Δ*glgX*Δ*malP*Δ*glgP* produced around 50 mM succinate and 20 mM acetate after 48 hours of incubation (Figure 28B and Figure 28D, respectively). *C. glutamicum* Δ*glgC* produced only slightly less succinate and acetate after 48 hours. *C. glutamicum* WT and *C. glutamicum* Δ*glgC* both produced around 140 mM lactate after 48 hours, while *C. glutamicum* Δ*glgX*Δ*malP*Δ*glgP* reached slightly lower concentrations in the same time (Figure 28C).

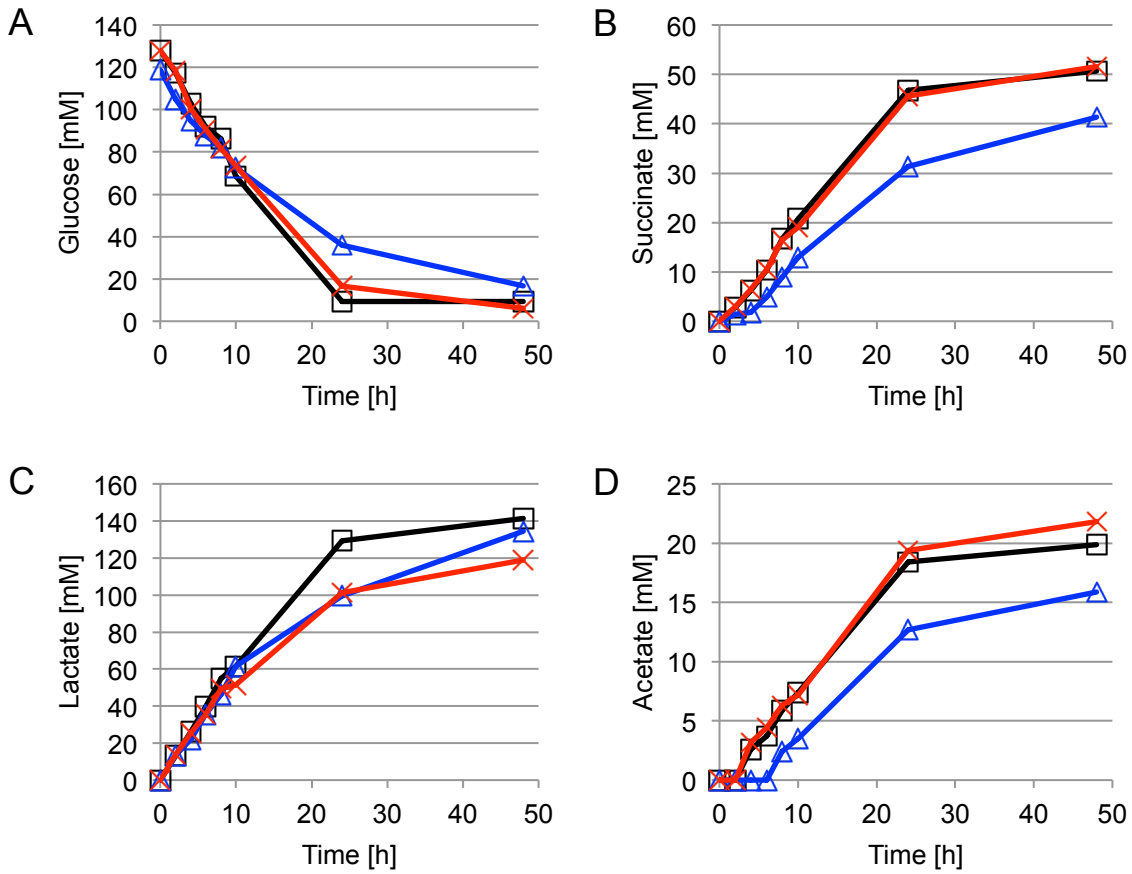


Figure 28: Concentrations of glucose (A), succinate (B), lactate (C) and acetate (D) in cell suspensions of *C. glutamicum* WT (black open squares), of *C. glutamicum* Δ*glgC* (blue open triangles) and of *C. glutamicum* Δ*glgX*Δ*malP*Δ*glgP* (red open crosses) in CgC minimal medium with 2 % glucose during incubation under anaerobic conditions. The Figure shows the results of one representative experiment. At least three independent experiments were performed, all three showing comparable results.

Taken together, it was observed that glucose consumption of *C. glutamicum* Δ*glgC* was slightly slower than of *C. glutamicum* WT and *C. glutamicum* Δ*glgX*Δ*malP*Δ*glgP*. The final concentrations of succinate and acetate were similar for all strains, with *C. glutamicum* Δ*glgC* showing slightly lower concentrations. The final concentration of lactate was slightly lower for *C. glutamicum* Δ*glgX*Δ*malP*Δ*glgP* than for the other strains. These results suggest that abolition of glycogen synthesis slightly negatively affects the succinate and acetate production, while abolition of glycogen degradation only marginally influences the lactate production.

3.4.2 Glycogen levels and production pattern of the succinate producer *C. glutamicum ΔaceE Δpqo ΔldhA ΔC–T ilvN ΔalaT ΔavtA* (*C. glutamicum* ELB-P) with different carbon sources

For the investigation of a link between production of organic acids and glycogen levels, the strain *C. glutamicum ΔaceE Δpqo ΔldhA ΔC–T ilvN ΔalaT ΔavtA* (*C. glutamicum* ELB-P) was chosen. Under anaerobic conditions *C. glutamicum* ELB-P is able to produce pyruvate in significant concentrations (Wieschalka *et al.*, 2012). In contrast, the same strain can be used for the production of succinate under anaerobic conditions (Wieschalka *et al.*, 2013). The *aceE* gene coding for the E1p subunit of the pyruvate dehydrogenase complex has been deleted in this strain. Because of this deletion, the strain requires acetate for growth and is not able to grow with glucose as sole carbon source. When incubated in CgC minimal medium with 2 % glucose under anaerobic conditions, *C. glutamicum* ELB-P showed increasing OD_{600} from about 16 to maximal values of about 24 between 6 and 10 hours after start of incubation (Figure 29A). Interestingly, the maximum of OD_{600} coincided with the maximal glycogen concentrations between 40 and 50 mg per g CDW (Figure 29B). It should also be noted, that the glycogen levels of *C. glutamicum* ELB-P dropped significantly from the maximum after about 10 hours to about 10 mg per g CDW after 30 hours. *C. glutamicum* WT showed no decrease of glycogen levels under anaerobic conditions (see chapter 3.2.4). Since *C. glutamicum* ELB-P is not able to grow with glucose as sole carbon source, the OD_{600} and glycogen levels of *C. glutamicum* ELB-P were also investigated during incubation in CgC minimal medium with 2 % glucose and 0.5 % acetate under anaerobic conditions. During this incubation with two carbon sources, *C. glutamicum* ELB-P showed an increase of OD_{600} from 14 to 18 in the first 4 hours (Figure 29A). During the remaining incubation, the OD_{600} ranged from 16 to 18. However, *C. glutamicum* ELB-P accumulated only minor glycogen concentrations of around 5 mg per g CDW, when incubated with glucose and acetate as carbon source (Figure 29B).

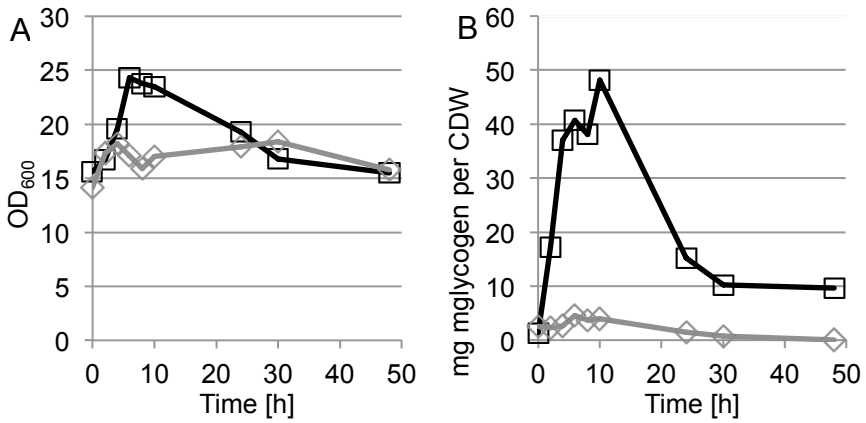


Figure 29: OD_{600} (A) and glycogen levels (B) of *C. glutamicum ΔaceE Δpqo ΔldhA ΔC–T ilvN ΔalaT* ∆*avtA* during incubation in CgC minimal medium under anaerobic conditions with 2 % glucose (black open squares) or 2 % glucose plus 0.5 % acetate (grey open diamonds) as carbon source. The Figure shows the results of one representative experiment. At least three independent experiments were performed, all three showing comparable results.

The glucose concentrations dropped in cell suspensions with glucose as sole carbon source and with glucose and acetate as carbon sources in a similar way (Figure 30A). In cell suspensions of *C. glutamicum* ELB-P with acetate as additional carbon source, the acetate concentration dropped from about 80 mM at the start to around 65 mM after 48 hours, while in cell suspensions of *C. glutamicum* ELB-P with 2 % glucose as sole carbon source minor concentrations (below 4 mM) could be detected just after 8 and 10 hours of incubation (data not shown). *C. glutamicum* ELB-P without acetate as additional carbon source produced slightly more succinate than with acetate as additional carbon source (95 and 80 mM, respectively; Figure 30B). Both cell suspensions showed no differences in the production of lactate with about 4 mM (Figure 30C). Cell suspension of *C. glutamicum* ELB-P without acetate produced almost 20 mM of pyruvate after 24 hours, whereas cell suspensions of *C. glutamicum* ELB-P with acetate as additional carbon source reached only about 12 mM after 10 hours (Figure 30D). Taken together, *C. glutamicum* ELB-P showed with glucose as sole carbon source glycogen accumulation and degradation. The addition of acetate as carbon source led to abolition of glycogen accumulation and minor changes in product formation.

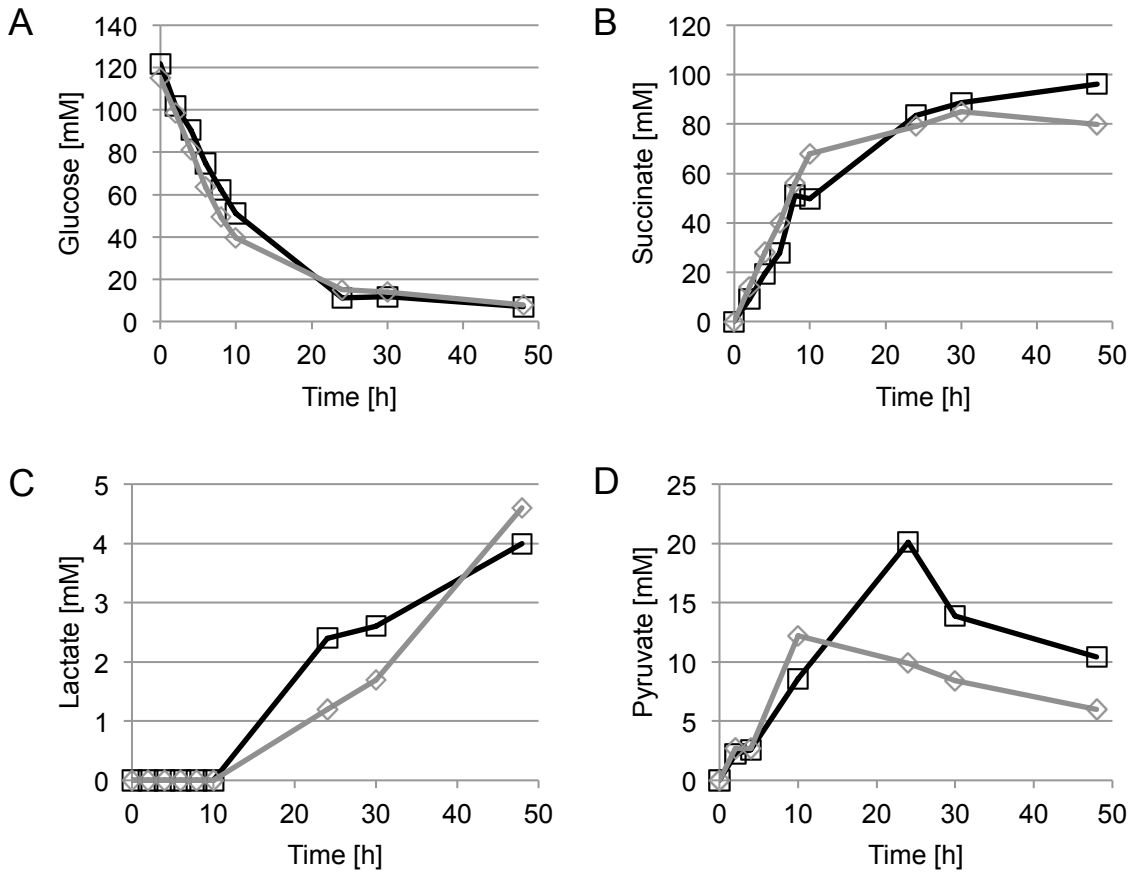


Figure 30: Concentrations of glucose (A), succinate (B), lactate (C) and pyruvate (D) of *C. glutamicum ΔaceE Δpqo ΔldhA ΔC–T ilvN ΔalaT* △*avtA* during incubation in CgC minimal medium under anaerobic conditions with 2 % glucose (black open squares) or 2 % glucose plus 0.5 % acetate (grey open diamonds) as carbon source. The Figure shows the results of one representative experiment. At least three independent experiments were performed, all three showing comparable results.

3.5 Physiological role of glycogen metabolism in *C. glutamicum*

3.5.1 Effect of disturbed glycogen metabolism on energetic parameters in *C. glutamicum*

Since synthesis of glycogen consumes ATP, which cannot be completely regenerated by degradation of glycogen (see Figure 1), it was interesting to investigate, how the absence of a functional glycogen synthesis affects the availability of ATP in *C. glutamicum* Δ*glgC* in comparison to *C. glutamicum* WT. It was also examined, whether the disruption of *glgX* had an effect on the availability of ATP in comparison to *C. glutamicum* WT. In case of a disturbed degradation of glycogen by deletion of *glgX*, glucose polymers with α-1,6-glycosidic linkages cannot be degraded (Seibold and Eikmanns, 2007). As a consequence, some glucose molecules cannot be oxidized by glycolysis or the pentose phosphate pathway, but persist as these glucose polymers with α-1,6-glycosidic linkages. For the investigation of the ATP availability, the so-called "energy charge" was used as criterion (see chapter 2.13.3). Intracellular concentrations of AMP, ADP and ATP were determined in *C. glutamicum* WT, *C. glutamicum* Δ*glgC* and *C. glutamicum* Δ*glgX* and used for the calculation of energy charges. As shown in Figure 31, *C. glutamicum* WT, *C. glutamicum* Δ*glgC* and *C. glutamicum* Δ*glgX* exhibited after 6 hours of growth an energy charge of about 0.8 ± 0.07.

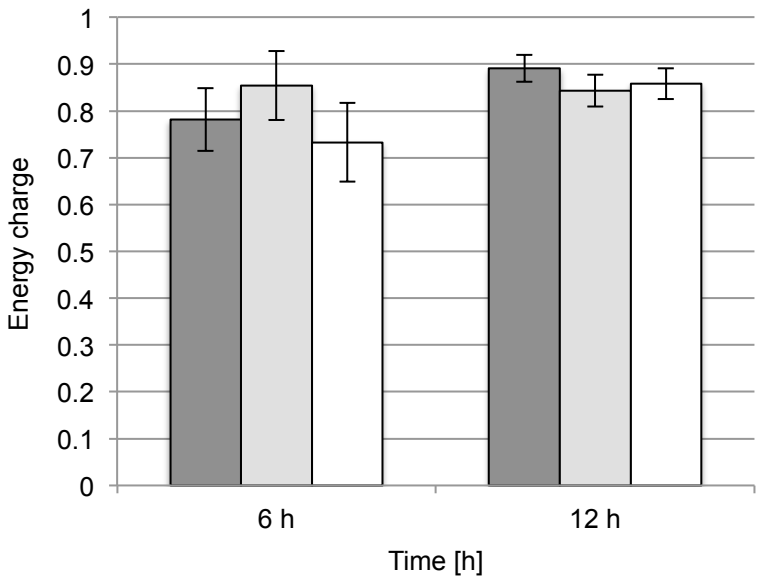


Figure 31: Energy charges of *C. glutamicum* WT (dark grey), *C. glutamicum* Δ*glgC* (light grey) and *C. glutamicum* Δ*glgX* (white) after 6 hours and 12 hours, respectively, of growth in CgC minimal medium with 2 % glucose as carbon source. The Figure shows the average values of at least three biological replicates with two technical replicates per biological replicate.

The energy charges after 12 hours were between 0.85 and 0.9 for all three strains tested. From these results, it can be assumed that disturbances of the glycogen synthesis (deletion of *glgC*) or disturbances of the glycogen degradation (deletion of *glgX*) have only marginal or no effects on the energy charge of the strains.

Disturbances in glycogen metabolism might affect glycolysis or the pentose phosphate pathway, which might also have an effect on the formation of reduction equivalents. Therefore, it was also interesting to investigate, whether disturbances in glycogen metabolism (glycogen synthesis/degradation) have influence on the concentrations of $NADH/H^+$ and $NADPH/H^+$. For these examinations, the samples taken for analysis of intracellular levels of AMP, ADP and ATP were used. The anabolic reduction charge (aRC) is a criterion for the availability of $NADPH/H^+$ (see chapter 2.13.3). The aRC of *C. glutamicum* WT was about 0.08 (Figure 32) during the middle exponential growth phase (after 6 hours). *C. glutamicum* Δ*glgC* displayed a slightly higher aRC value (about 0.14), *C. glutamicum* Δ*glgX* showed by trend a lower aRC value of around 0.03 after 6 hours of growth. In the early stationary phase after 12 hours, *C. glutamicum* Δ*glgC* again tendentially exhibited the highest aRC with about 0.015. To this time point of growth, the aRC values of *C. glutamicum* WT and *C. glutamicum* Δ*glgX* were approximately 0.006 and 0.007, respectively.

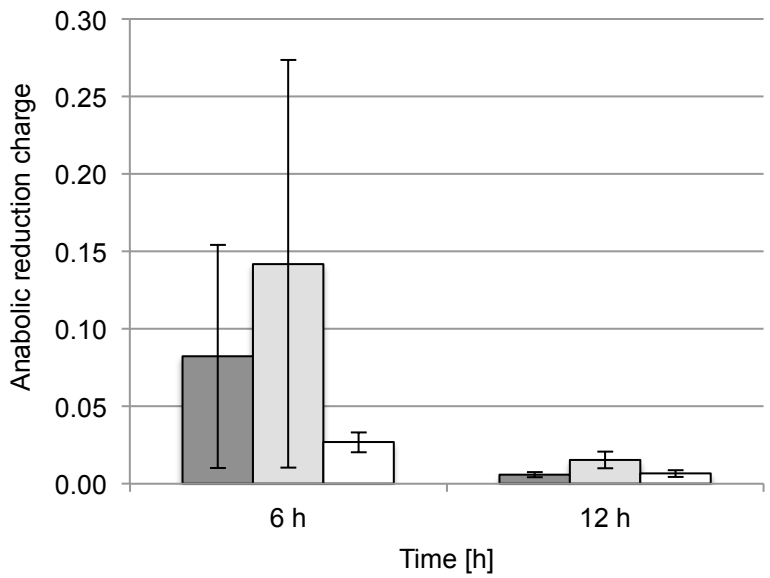


Figure 32: Anabolic reduction charges of *C. glutamicum* WT (dark grey), *C. glutamicum* Δ*glgC* (light grey) and *C. glutamicum* Δ*glgX* (white) after 6 hours and 12 hours, respectively, of growth in CgC minimal medium with 2 % glucose as carbon source. The Figure shows the average values of at least three biological replicates with two technical replicates per biological replicate.

The catabolic reduction charge (cRC), which is a criterion for the availability of NADH/H$^+$ (see chapter 2.13.3), exhibited for all three strains similarities to the aRC (Figure 33). *C. glutamicum* Δ*glgC* showed the highest cRC values of about 0.12 after 6 hours of growth, while *C. glutamicum* WT *and C. glutamicum* Δ*glgX* showed by trend lower values of about 0.10 and 0.06, respectively. After 12 hours of growth the highest cRC of approximately 0.025 was reached by *C. glutamicum* Δ*glgC* followed by *C. glutamicum* Δ*glgX* with about 0.017 and *C. glutamicum* WT with about 0.012.

To conclude, although *C. glutamicum* Δ*glgC* showed a trend to reach higher aRC and cRC values and *C. glutamicum* Δ*glgX* tended to have lower aRC and cRC values than *C. glutamicum* WT, it is not possible to make a clear statement about the influence of disturbances in the glycogen metabolism on the availability of the reduction equivalents NADH/H$^+$ and NADPH/H$^+$. Since there was no obvious trend in the single determinations of the intracellular metabolite concentrations of *C. glutamicum* WT, *C. glutamicum* Δ*glgC* and *C. glutamicum* Δ*glgX*, it has to assumed that disturbances in the glycogen metabolism have no or only marginal effects on the availability of the reduction equivalents NADH/H$^+$ and NADPH/H$^+$.

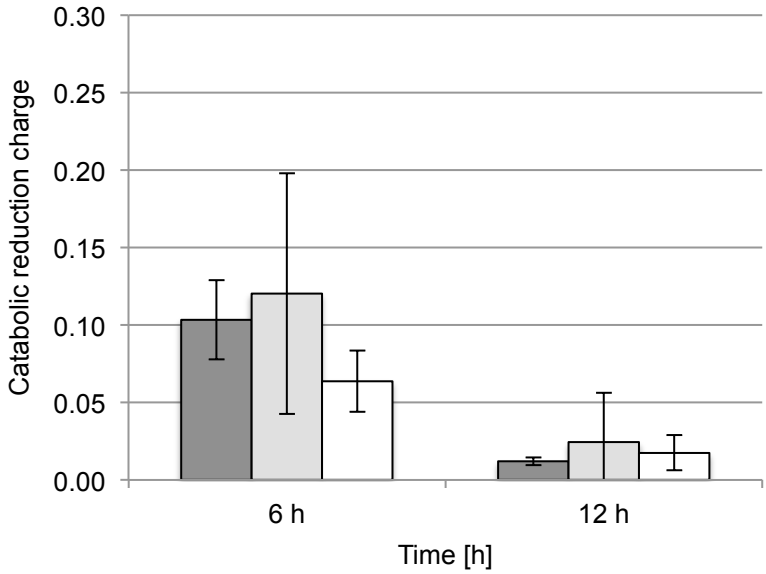


Figure 33: Catabolic reduction charges of *C. glutamicum* WT (dark grey), *C. glutamicum* Δ*glgC* (light grey) and *C. glutamicum* Δ*glgX* (white) after 6 hours and 12 hours, respectively, of growth in CgC minimal medium with 2 % glucose as carbon source. The Figure shows the average values of at least three biological replicates with two technical replicates per biological replicate.

3.5.2 Effect of disturbed glycogen metabolism on survival of *C. glutamicum*

It has been reported that glycogen can increase the survival rate of several bacterial species under different environmental conditions (Bourassa and Camilli, 2009; Busuioc *et al.*, 2009; Sambou *et al.*, 2008; McMeechan *et al.*, 2005). Although it has been mentioned that inactivation of *glgC* in *C. glutamicum* had no effect on the viability for growth on plates (Seibold *et al.*, 2007), the effects of a deletion of *glgC* and of *glgX* in *C. glutamicum* on the viability of *C. glutamicum* in liquid media were tested. To investigate this, *C. glutamicum* WT, *C. glutamicum* Δ*glgC* and *C. glutamicum* Δ*glgX* were grown in CgC minimal medium with 2 % glucose and with 1 % acetate.

3.5.2.1 Effect of disturbed glycogen metabolism on survival of *C. glutamicum* after growth with glucose as carbon source

For the first set of experiments, cells grown for 48, 72 or 120 hours on glucose were used for re-inoculation of new cultures in CgC minimal medium with 2 % glucose and the viable cell number was compared by following the OD_{600} in the freshly inoculated cultures. During the first cultivation (precultures for re-inoculation), *C. glutamicum* WT, *C. glutamicum* Δ*glgC* and *C. glutamicum* Δ*glgX* showed similar growth behaviour, although the final ODs_{600} were slightly higher (OD_{600} = about 48) with *C. glutamicum* Δ*glgX* and somewhat lower (OD_{600} = about 35) with C. glutamicum ΔglgC, when compared to C. glutamicum WT (OD_{600} = about 40) (Figure 34A). All three strains grew with comparable growth rates of 0.36 – 0.37 h^{-1}. After 48 hours of growth in the first culture with CgC minimal medium and 2 % glucose, cells of each strain were harvested for re-inoculation of new cultures with CgC minimal medium and 2 % glucose (Figure 34B). While *C. glutamicum* WT and *C. glutamicum* Δ*glgC* showed again similar growth behaviour, *C. glutamicum* Δ*glgX* differed in its growth behaviour in the first culture. While the two previous strains showed only one growth phase, *C. glutamicum* Δ*glgX* showed two growth phases, i. e. from 0 to 6 hours and from 8 to 12 hours with growth rates of around 0.25 h^{-1} and 0.35 h^{-1}, respectively. The maximal OD_{600} of around 45 was reached by *C. glutamicum* Δ*glgX* after 12 hours.

The results of the growth experiments with the cells pregrown for 72 hours in medium with 2 % glucose are shown in Figure 34C. *C. glutamicum* WT entered stationary phase after 11 hours with an OD_{600} of about 41. This strain showed no lag phase and grew with a growth rate of about 0.32 h^{-1}. In contrast, *C. glutamicum* Δ*glgC* showed delayed exponential growth starting about 6 hours after inoculation. This strain grew with a growth rate of 0.32 h^{-1} and reached stationary phase after 14 hours with a maximal OD_{600} of about 35. *C. glutamicum* Δ*glgX* again exhibited biphasic-like growth. The first growth

phase was linear and lasted from 0 to around 6 hours, the second (μ = 0.33 h^{-1}) lasted from about 10 to 18 hours. After 18 hours of growth the stationary phase was reached with an OD_{600} of about 45.

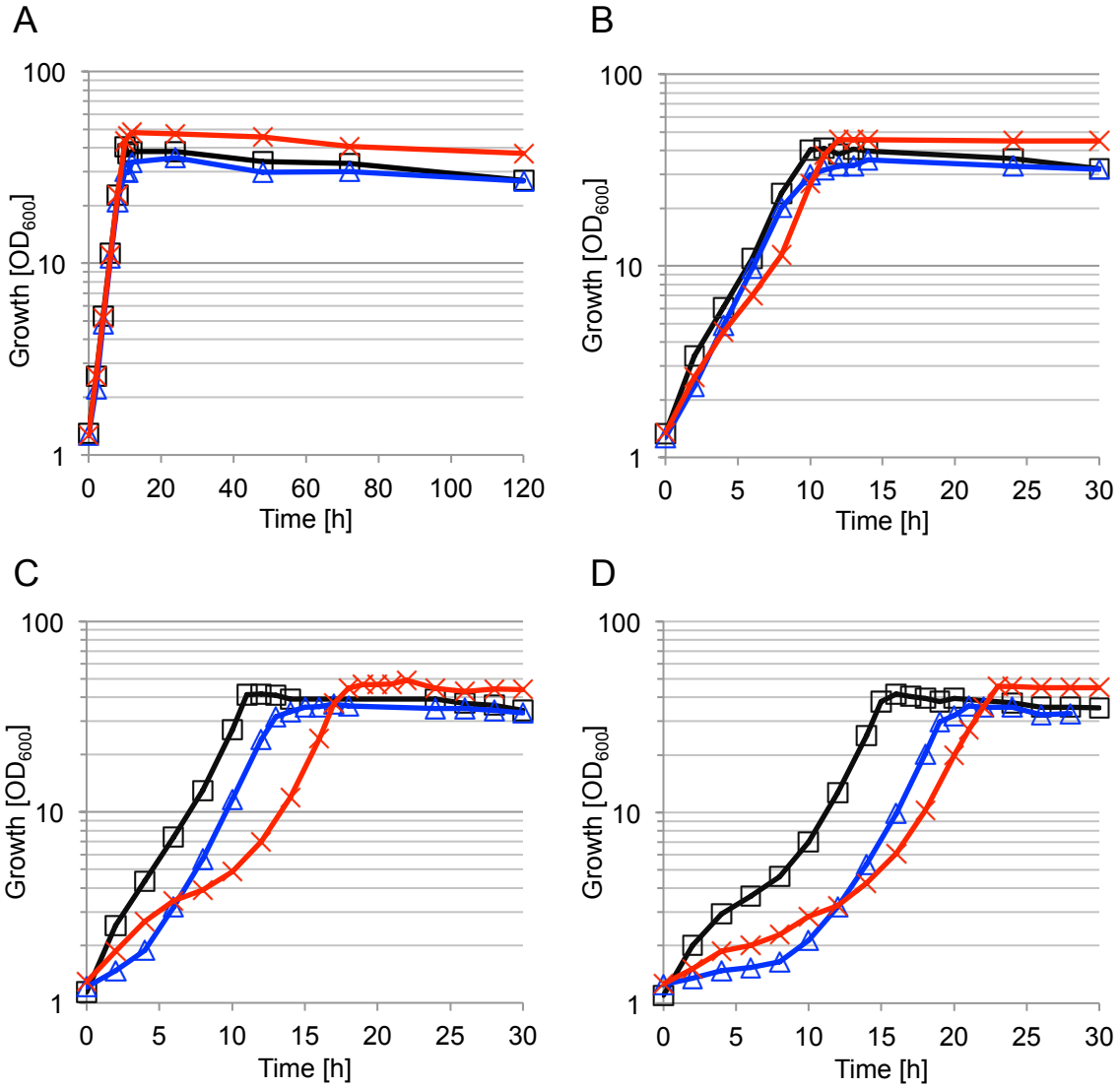


Figure 34: **Growth of *C. glutamicum* WT (black), *C. glutamicum* Δ*glgC* (blue) and *C. glutamicum* Δ*glgX* (red) in CgC minimal medium with 2 % glucose after precultivation in 2x TY complex medium is shown in (A).** These cultures served as precultures for re-inoculation of new cultures in CgC minimal medium with 2 % glucose. Re-inoculation was performed after 48, 72 and 120 hours of growth, respectively. Growth of *C. glutamicum* WT (black), *C. glutamicum* Δ*glgC* (blue) and *C. glutamicum* Δ*glgX* (red) in CgC minimal medium with 2 % glucose after precultivation for 48, 72 and 120 hours in CgC minimal with 2 % glucose is shown in **(B)**, **(C)** and **(D)**, respectively. Each Figure shows the results of one representative experiment. At least four independent experiments were performed, all four showing comparable results.

When cells were taken 120 hours after inoculation to re-inoculate fresh CgC minimal medium with 2 % glucose, all three strains showed growth behaviour very different to the one in the first cultures (Figure 34D). *C. glutamicum* WT showed two growth phases from 0 to 6 hours ($\mu = 0.15\ h^{-1}$) and from 8 to 16 hours ($\mu = 0.34\ h^{-1}$). The maximal OD_{600} of about 42 was reached after 16 hours of growth. *C. glutamicum* Δ*glgC* showed a lag-phase of about 10 hours. Afterwards, *C. glutamicum* Δ*glgC* grew with a growth rate of 0.32 h^{-1} and reached the highest OD_{600} of around 36 after 21 hours. *C. glutamicum* Δ*glgX* showed linear growth until about 12 hours, then it grew exponentially ($\mu = 0.30\ h^{-1}$). The stationary phase was reached after 23 hours with maximal OD_{600} of around 46.

The results show that *C. glutamicum* WT has advantages in growth after elongated cultivation time in comparison to *C. glutamicum* strains, which were not able to accumulate (*C. glutamicum* Δ*glgC*) or degrade (*C. glutamicum* Δ*glgX*) glycogen.

3.5.2.2 Effect of disturbed glycogen metabolism on survival of *C. glutamicum* after growth with acetate as carbon source

C. glutamicum forms significantly less glycogen, when grown with acetate (Seibold et al., 2010). To investigate, whether the lower glycogen levels due to growth with acetate have an influence on the survival of *C. glutamicum*, survival experiments with 1 % acetate as carbon source were performed. Cells of *C. glutamicum* WT, *C. glutamicum* Δ*glgC* and *C. glutamicum* Δ*glgX* were grown in CgC minimal medium with 1 % acetate and used after 24, 48 and 72 hours for re-inoculation of fresh cultures with 2 % glucose as carbon source and the viable cell number was compared by following the OD_{600} in the freshly inoculated cultures. In the first cultures with 1 % acetate as carbon source (Figure 35A), the three strains *C. glutamicum* WT, *C. glutamicum* Δ*glgC* and *C. glutamicum* Δ*glgX* showed the same growth rate between 0.33 and 0.34 h^{-1} and reached the maximum OD_{600} of around 15 after 10 hours of growth.

After 24 hours of growth with 1 % acetate, cells were taken for re-inoculation of fresh cultures with 2 % glucose (Figure 35B). As seen in the experiments with 2 % glucose in the first culture, *C. glutamicum* WT and *C. glutamicum* Δ*glgC* showed similar growth rates ($\mu = 0.32\ h^{-1}$). *C. glutamicum* Δ*glgX* showed two growth phases from 0 to about 10 hours ($\mu = 0.20\ h^{-1}$) and from about 10 to 14 hours ($\mu = 0.36\ h^{-1}$). All strains reached maximal ODs_{600} as seen as in growth experiments with 2 % glucose (Figure 35A).

When cells were harvested for re-inoculation after growth for 48 hours with acetate as carbon source, the three strains *C. glutamicum* WT, *C. glutamicum* Δ*glgC* and *C. glutamicum* Δ*glgX* showed growth behaviour significantly differing from the previous one (Figure 35C). While each strain reached again maximal ODs_{600} comparable to the

ones from growth experiments with 2 % glucose, it was observed that all strains displayed two growth phases. *C. glutamicum* WT showed the first growth phase from 0 to about 12 hours ($\mu = 0.13\ h^{-1}$) and the second from about 14 to 18 hours ($\mu = 0.36\ h^{-1}$). *C. glutamicum* Δ*glgC* exhibited linear growth from 0 to about 12 hours and exponential growth from 14 to about 22 hours ($\mu = 0.34\ h^{-1}$). *C. glutamicum* Δ*glgX* showed linear growth phase from 0 to 18 hours and grew exponentially from about 18 to 24 hours ($\mu = 0.28\ h^{-1}$).

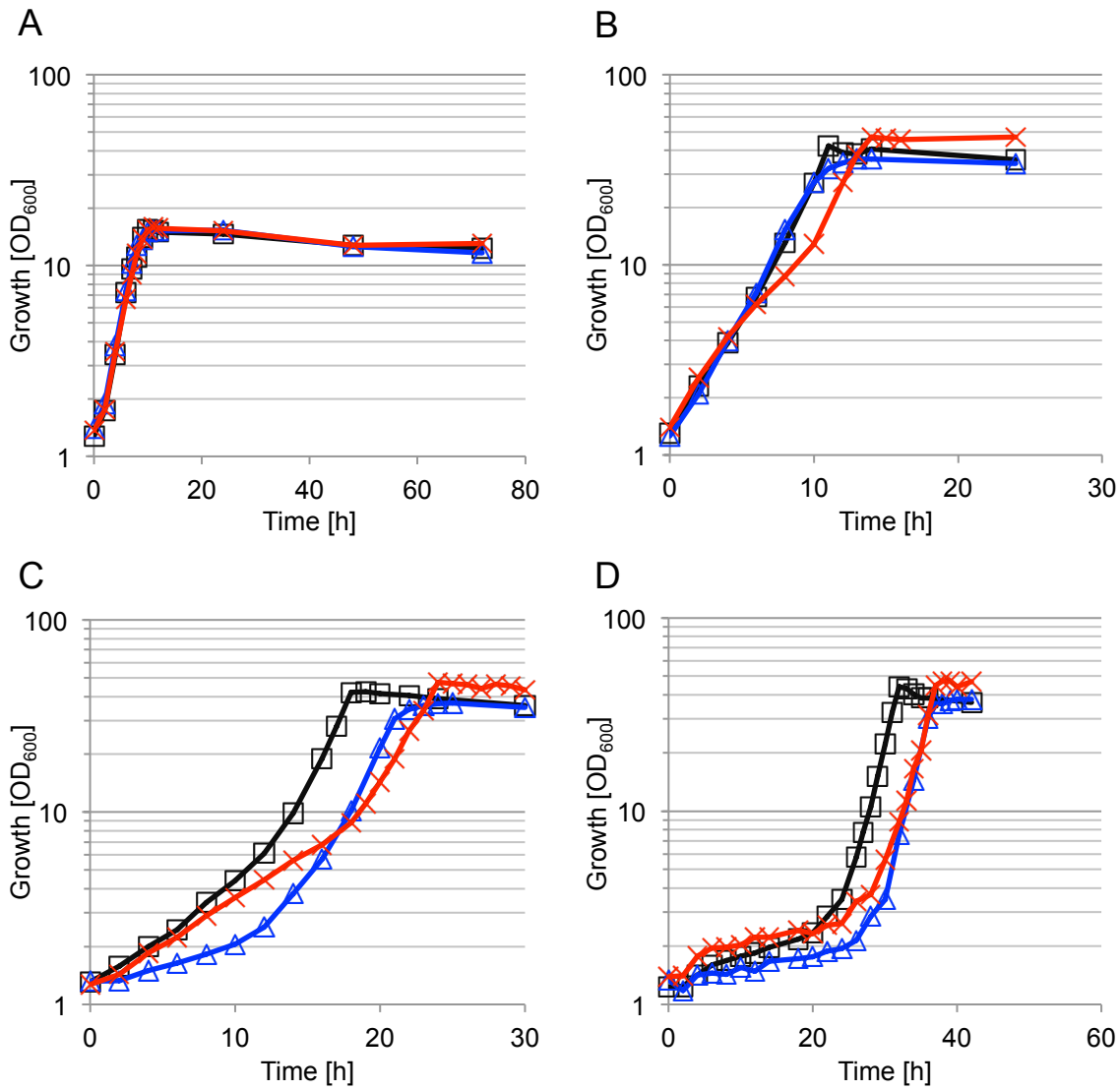


Figure 35: Growth of *C. glutamicum* WT (black), *C. glutamicum* Δ*glgC* (blue) and *C. glutamicum* Δ*glgX* (red) in CgC minimal medium with 1 % acetate after precultivation in 2x TY complex medium is shown in (A). These cultures served as precultures for re-inoculation of new cultures in CgC minimal medium with 2 % glucose. Re-inoculation was performed after 24, 48 and 72 hours of growth, respectively. Growth of *C. glutamicum* WT (black), *C. glutamicum* Δ*glgC* (blue) and *C. glutamicum* Δ*glgX* (red) in CgC minimal medium with 2 % glucose after precultivation for 24, 48 and 72 hours in CgC minimal with 1 % glucose is shown in **(B)**, **(C)** and **(D)**, respectively. Each Figure shows the results of one representative experiment. At least four independent experiments were performed, all four showing comparable results.

C. glutamicum WT, *C. glutamicum* Δ*glgC* and *C. glutamicum* Δ*glgX* exhibited considerably delayed growth, when re-inoculated after growth in CgC minimal medium with 1 % acetate for 72 hours (Figure 35D). All strains showed again the distinct maximal ODs_{600} from previous re-inoculations. *C. glutamicum* WT grew linearly from inoculation to about 24 hours and exponentially from 26 to 32 hours ($\mu = 0.35\ h^{-1}$). *C. glutamicum* Δ*glgC* showed a lag-phase until 28 hours and exponential growth from about 30 hours until 37 hours ($\mu = 0.33\ h^{-1}$). *C. glutamicum* Δ*glgX* showed also a lag-phase from 0 to 28 hours and grew from 30 to about 38 hours exponentially ($\mu = 0.32\ h^{-1}$).

In conclusion, all three *C. glutamicum* WT, *C. glutamicum* Δ*glgC* and *C. glutamicum* Δ*glgX* showed similar behaviour after re-inoculation from cultures with acetate as carbon source as after re-inoculation from cultures with glucose as carbon source (Figure 38A-D). The effects of the respective deletions were observed to earlier time points of re-inoculation than after re-inoculation from cultures with glucose as carbon source. In general, *C. glutamicum* WT showed the best and fastest growth, while strains with disturbed glycogen metabolism showed slower growth rates and longer lag-phase than *C. glutamicum* WT.

4 Discussion

The glycogen metabolism in *C. glutamicum* and factors involved were subject of various studies (Tzvetkov *et al.*, 2003; Padilla *et al.*, 2004a; Seibold *et al.*, 2007; Seibold and Eikmanns, 2007; Seibold *et al.*, 2009; Seibold *et al.*, 2010; Woo *et al.*, 2010; Seibold *et al.*, 2011; Seibold and Eikmanns, 2013). In these studies it was shown that *C. glutamicum* is able to accumulate and degrade glycogen, but does not use it as carbon storage in the stationary phase. By inactivation of enzymes presumably involved in glycogen synthesis – namely Pgm, GlgC, GlgA and GlgB – it was confirmed that *C. glutamicum* uses the glycogen synthesis pathway known from other organisms during growth with glucose as carbon source (Seibold and Eikmanns, 2013; Seibold *et al.*, 2007; Tzvetkov *et al.*, 2003; Seibold *et al.*, 2011; see Figure 1). During growth with maltose as carbon source, *C. glutamicum* uses a different pathway via MalQ to accumulate glycogen (Seibold, 2007; Seibold *et al.*, 2009). By studies using a *C. glutamicum* mutant with inactivated *glgB* gene, it was also shown that GlgB is involved in maltose metabolism (Seibold, 2007; Seibold, personal information). The present study aimed at the confirmation of the role of enzymes (namely GlgC, GlgA, GlgB, GlgX, MalP and GlgP) in the glycogen metabolism during growth on the substrates glucose and maltose. Additionally, identification of factors affecting glycogen metabolism was done by investigating the influence of nitrogen and phosphate availability, of anaerobic conditions and of lower extracellular pH on the glycogen metabolism in *C. glutamicum*. To gain further insight into the regulation of the glycogen metabolism, promoter studies of the *glgC*, *glgA* and *glgX* genes with a newly developed *in vivo* real time luciferase reporter assay and semi-quantitative RT-PCR under glycogen level-elevating conditions were performed. Last, but not least, approaches were done to investigate the involvement of glycogen metabolism with energetic parameters and survival rate in *C. glutamicum* with disturbed glycogen metabolism.

The model of glycogen metabolism in *C. glutamicum* is based on data from *E. coli* and *M. tuberculosis*. Glycogen metabolism in *E. coli* was subject of various investigations and has been thoroughly examined (Preiss and Romeo, 1994; reviewed in Preiss, 1984, 1996). A phosphoglucomutase forms glucose 1-phosphate from glucose 6-phosphate (Joshi and Handler, 1964; Regni *et al.*, 2006; Schramm *et al.*, 2010). An ADP-glucose pyrophosphorylase converts glucose 1-phosphate to ADP-glucose consuming one ATP molecule. As first step of a unique pathway, this reaction is controlled allosterically by various intermediates of the central metabolism (reviewed by Preiss, 1984 and 1996; Ballicora *et al.*, 2003; Ball and Morell, 2003). ADP-glucose is substrate for a glycogen synthase, which transfers a glucose residue onto already existing linear maltodextrins using energy of cleaving ADP and glucose (Sheng *et al.*, 2009ab). These linear maltodextrins contain only α-

1,4-glycosidic linkages. The α-1,6-glycosidic links are introduced by actions of a glycogen branching enzyme (Boyer and Preiss, 1977). Three enzymes are involved in the glycogen degradation in *E. coli*. Glycogen phosphorylases cleave glucose residues from the non-reducing ends of the glucose polymers, forming glucose 1-phosphate (Alonso-Casajús *et al.*, 2006). The resulting glucose 1-phosphate can be converted to glucose 6-phosphate by a phosphoglucomutase and fed into central metabolism. Since the glycogen phosphorylase is not able to cleave α-1,6-glycosidic linkages and is sterically hindered from cleaving α-1,4-glycosidic links near α-1,6-glycosidic linkages, so-called phosphorylase-limited dextrins (PL-dextrins) are formed. Cleaving of the α-1,6-glycosidic linkages by a glycogen debranching enzyme forms new targets for the glycogen phosphorylase (Dauvillée *et al.*, 2005). A maltodextrin phosphorylase is able to degrade linear maltodextrins (formed by the glycogen debranching enzyme) and connects maltose metabolism with glycogen metabolism (reviewed in Boos and Shuman, 1998; Dippel *et al.*, 2005). Maltose is taken up by an ABC transporter and by action of MalQ a glucose residue is transferred to already existing linear maltodextrins, thereby elongating them. The remaining glucose moiety is phosphorylated by an intracellular glucokinase and the formed glucose 6-phosphate is channelled into glycolysis. MalP cleaves the linear maltodextrins at the non-reducing ends forming glucose 1-phosphate. MalZ can also recognize and cleave linear maltodextrins, but forms glucose. Glucose 1-phosphate is converted to glucose 6-phosphate, while glucose is phosphorylated by an intracellular glucokinase forming glucose 6-phosphate, which is then oxidized in the glycolysis. Also, glycogen phosphorylase cannot replace the activity of maltodextrin phosphorylase for degradation of formed maltodextrins. It was shown that enzymes of maltose metabolism (MalQ and MalP) are involved in the glycogen formation in *E. coli* on glucose and that maltotriose formed from glycogen can induce genes of the maltose metabolism in *E. coli* (Dippel *et al.*, 2005; Park *et al.*, 2000).

In *E. coli*, genes of glycogen metabolism are organized in a single transcriptional unit *glgBXCAP* with additional promoters (Montero *et al.*, 2011), whereas the genes are scattered in *C. glutamicum*. In *E. coli*, the glycogen synthesis is regulated on transcriptional, translational and post-translational level. On the transcriptional level, the cAMP-dependent catabolite repression system and the stringent response are involved in regulation of *glgA* and *glgC* transcription (Romeo and Preiss, 1989; Romeo *et al.*, 1990). The CsrA protein (part of the carbon storage regulation system) is able to prevent the translation of *glgC* (Baker et al., 2002). Also, an allosterical control of GlgC was reported (Ballicora *et al.*, 2003). Last, but not least, it was reported that reduction of nutrients required for growth led to elevated glycogen levels, indicating regulation of glycogen metabolism (Holme and Palmstierna, 1956b; Preiss and Romeo, 1989).

In the beginning of this work, *glgC*, *glgA* and *glgB* deletion mutants as well as *glgX*, *malP* and *glgP* deletion mutants were constructed and analysed regarding growth and glycogen levels. Comparing the glycogen metabolism of *E. coli* on glucose, with the results of the experiments with *glgC*, *glgA* and *glgB* deletion mutants, confirmed the proposed pathway of glycogen synthesis via ADP-glucose pyrophosphorylase, glycogen synthase and glycogen branching enzyme during growth with glucose (see Figure 1; Tzvetkov *et al.*, 2003; Seibold *et al.*, 2007; Seibold *et al*., 2011). All three deletion mutant strains showed no or dramatically decreased glycogen accumulation during growth in minimal medium with glucose (Figure 2A, Figure 3A, Figure 4A) corroborating their role in glycogen synthesis. Also, *glgX* and *malP* deletion mutants showed impairment in glycogen degradation as previously observed in integration/deletion mutants confirming their role in glycogen degradation during growth of *C. glutamicum* with glucose (Seibold and Eikmanns, 2007; Seibold *et al.*, 2009). The role of *glgP* in glycogen metabolism remains uncertain, since no significant phenotype was observed.

The maltose metabolism in *C. glutamicum* (see Figure 1) is also mainly in agreement with the maltose metabolism of *E. coli*. Additionally, it was previously shown that *C. glutamicum* is able to form glycogen during growth on maltose, but it is unclear, whether these polymers were linear or branched (Seibold, 2007; Seibold *et al.*, 2009). Maltose is taken up by an ABC transporter system MusEFGK$_2$I and the 4-alpha-glucanotransferase MalQ transfers one glucose residue to already existing, linear maltodextrins setting free the other glucose moiety (Henrich *et al.*, 2013; Seibold *et al.*, 2009; see Figure 1). The free glucose is phosphorylated to glucose 6-phosphate by a glucokinase Glk (Lindner *et al.*, 2010). MalP cleaves glucose residues from linear maltodextrins forming glucose 1-phosphate, which can be converted to glucose 6-phosphate (Seibold *et al.*, 2009; Seibold and Eikmanns, 2013). Glucose 6-phosphate then is fed into the central metabolism. Theoretically, glycogen could be formed from glucose 1-phosphate by actions of GlgC, GlgA and GlgB or from maltose by actions of the 4-alpha-glucanotransferase MalQ and GlgB. In addition to data from *E. coli*, a third pathway for glycogen synthesis was described in *M. tuberculosis* and *M. smegmatis* using a maltose kinase, a maltosyl transferase and a glycogen branching enzyme (Elbein *et al.*, 2010; Kalscheuer *et al.*, 2010). In *C. glutamicum,* the *glgE* gene codes for a maltosyl transferase and forms an operon with *glgB* (Seibold *et al.*, 2011). Other studies suggest that the *treX* encodes a maltokinase, which is required for the first step of the third pathway for glycogen synthesis during growth on maltose (Henrich, 2011; Seibold, personal communication).

In the present study, the role of the *glgC*, *glgA*, *glgB*, *glgX*, *malP* and *glgP* genes in glycogen metabolism of *C. glutamicum* during growth on maltose were tested. The results indicate that

GlgC is not involved in the formation of maltodextrins/glycogen in *C. glutamicum* with maltose as carbon source (Figure 2B), since the respective deletion mutant shows the same growth and the same glycogen levels as *C. glutamicum* WT. An integration mutant *C. glutamicum* WT-IMC showed also the same growth as *C. glutamicum* WT on maltose, but slightly decreased glycogen levels (Seibold, 2007). Also, the results of this thesis suggest that GlgA is not involved in glycogen formation on maltose. In contrast, studies in *E. coli* showed that a GlgA-negative mutant did not accumulate glycogen with maltose as substrate, but this effect was attributed to MalP activity, which degraded glucose polymers faster than they were formed (Park *et al.*, 2010). The present study indicates that GlgB uses linear maltodextrins derived from the MalQ- or the putative TreX-GlgE-pathway and is not dispensable for glycogen formation on maltose. As on glucose, GlgX is still an important factor for the degradation of glycogen with maltose as carbon source, but is less important than with glucose. While absence of MalP had only slight effects on the degradation of glycogen on glucose, it is the major degradation enzyme of the glucose polymer formed on maltose. GlgP plays – as seen in *E. coli* – no role in glycogen metabolism of *C. glutamicum* on maltose.

In the following, the phenotypes of each deletion mutant and the respective role of each gene in glycogen metabolism during growth of *C. glutamicum* with glucose or with maltose are discussed more thoroughly. As reported previously for the integration mutant *C. glutamicum* WT-IMC, *C. glutamicum* Δ*glgC* demonstrated the same growth rate as the *C. glutamicum* WT and reached slightly lower maximal OD_{600} than *C. glutamicum* WT (Seibold *et al.*, 2007; Figure 2A). These findings confirm that the accumulation of glycogen is not essential for the normal growth of *C. glutamicum*. Looking at the model of glycogen metabolism in *C. glutamicum*, the abolition of glycogen synthesis by deletion of GlgC could lead to higher availability of glucose 6-phosphate for glycolysis or pentose phosphate pathway in the cell (Figure 1). Assuming this might be the case, faster growth rates of strains unable to synthesize glycogen in comparison to strains accumulating glycogen would be expected. A possible explanation for the unchanged growth rate and even lower maximum OD_{600} of *C. glutamicum* Δ*glgC* is that the capability of glycolysis, PPP or other pathways required for growth are already maxed out in *C. glutamicum* WT under present growth conditions and therefore the higher concentrations of glucose 6-phosphate can not be used for generation of ATP or other compounds needed for growth. Consistent with that assumption, *C. glutamicum* Δ*glgC* showed the same glucose uptake rate as *C. glutamicum* WT (data not shown). This means that the same glucose concentrations are taken up by *C. glutamicum* WT and *C. glutamicum* Δ*glgC*, but both strains show the same growth behaviour. Since *C. glutamicum* WT spends in contrast to *C. glutamicum* Δ*glgC* some part of the glucose for

glycogen synthesis, one has to assume that this surplus of glucose in *C. glutamicum* Δ*glgC* is somehow lost and not used for growth. Under these circumstances, the phenomenon of so-called "extended overflow metabolism" could occur (Paczia *et al.*, 2013). This phenomenon describes the formation of glycolytic intermediates due the excess of carbon and limited enzyme activity, which then accumulate intracellularly and can be transported outside of the cell. As soon as carbon source starts to be limited again, the intermediates are transported inside of the cell and metabolized. It could be the case that part of these intermediates end up in *C. glutamicum* Δ*glgC* for other purposes than as in *C. glutamicum* WT leading to different carbon distributions in these strains. Two possible by-products, which are formed when the capacity of the glyceraldehyde 3-phosphate dehydrogenase is exceeded, are glycerol and dihydroxyacetone (Kiefer *et al.*, 2003). These by-products can be produced by *C. glutamicum* under certain conditions, but are not consumed without overexpression of respective genes (Dominguez *et al.*, 1998; Meiswinkel *et al.*, 2013). Neither supernatants nor cell extracts of *C. glutamicum* Δ*glgC* grown in minimal medium with glucose were tested for the formation of these by-products. The accumulation of glycerol and dihydroxyacetone (and the subsequent loss of carbon) could explain the unchanged growth rate and the slightly lower maximum OD_{600} of *C. glutamicum* Δ*glgC* on glucose in comparison with *C. glutamicum* WT. Also, it was shown in other experiments that accumulation of glycogen has influence on the OD_{600} of *C. glutamicum* (see chapter 3.2.4). Since *C. glutamicum* WT accumulates glycogen in contrast to *C. glutamicum* Δ*glgC*, the higher OD_{600} of *C. glutamicum* WT could be caused by the higher glycogen levels and not by higher cell numbers.

C. glutamicum Δ*glgA* exhibited impaired growth rate and lower maximal OD_{600} than *C. glutamicum* WT in minimal medium with glucose (Figure 3A). The *glgA* gene encodes the glycogen synthase GlgA, which catalyses the transfer of a glucose residue to a linear α-1,4-glycosidic-linked maltodextrin. For this reaction, GlgA uses ADP-glucose formed by the ADP-glucose pyrophosphorylase GlgC as substrate, thereby setting free ADP. A mutant strain with deletion of *glgA* is unable to use ADP-glucose for elongation of already existing linear maltodextrins. A negative effect on growth in mutants with disrupted glycogen/starch synthase has also been reported in cyanobacteria and plants (Suzuki *et al.*, 2010; Ragel *et al.*, 2013). In *Arabidopsis thaliana* plants it was observed that a mutant, which was lacking two out of four starch synthases (corresponding to glycogen synthases), showed strongly impaired growth and dramatically increased levels of ADP-glucose (Ragel *et al.*, 2013). The ADP-glucose pyrophosphorylase- or phosphoglucomutase-lacking mutants of *Arabidopsis* showed slower growth than the wild-type, but better than the starch synthase-deficient mutants (Ragel *et al.*, 2013). By additional deletion of genes for the ADP-glucose

pyrophosphorylase or the phosphoglucomutase, respectively, in the glycogen/starch synthase-lacking *Arabidopsis* mutants, the growth was comparable to that of *Arabidopsis thaliana* mutants lacking only ADP-glucose pyrophosphorylase or only the phosphoglucomutase, respectively, presumably by limiting the formation of ADP-glucose itself or the precursor glucose-1-phosphate (Ragel *et al.*, 2013). A similar approach was done in *C. glutamicum* by simultaneous deletion of *glgC* and *glgA*. The double deletion mutant *C. glutamicum* Δ*glgA*Δ*glgC* showed the same growth behaviour as *C. glutamicum* Δ*glgC* indicating that this hypothesis is also true in *C. glutamicum* (Seibold, personal communication). A *pgm* deletion mutant of *C. glutamicum* showed no impaired growth on glucose, since the residual phosphoglucomutase activity by at least one additional isoenzyme is sufficient for growth (Seibold and Eikmanns, 2013). Thus, a deletion of *pgm* in *C. glutamicum* Δ*glgA* might not be able to abolish the effects of *glgA* deletion completely, since ADP-glucose might be still formed. In *Arabidopsis* it is assumed that the irreversible reaction of the ADP-glucose pyrophosphorylase leads to an imbalance of adenine nucleotide pools thereby locking away nucleotides and carbon for other metabolic processes (Ragel *et al.*, 2013). This might also be the reason for the delayed growth of *C. glutamicum* Δ*glgA* in minimal medium with glucose as carbon source.
Another explanation for the impaired growth of *C. glutamicum* Δ*glgA* could be a regulatory effect due to the accumulation of ADP-glucose. It was shown that ADP-glucose has an inhibitory effect on the MalP activity (Seibold, personal communication) and it is unlikely that MalP is the only enzyme affected, since deletion of *malP* led to no growth impairment, but slower glycogen degradation.

In contrast to previously published results of *C. glutamicum* IMglgB with inactivated glycogen branching enzyme (Seibold *et al.*, 2011), the deletion mutant *C. glutamicum* Δ*glgB* grew indeed with the same growth rate as *C. glutamicum* WT in minimal medium with glucose, but did not reach the same maximal OD_{600} (Fig 4A). It remains unclear, why the deletion of *glgB* has a negative effect on the maximal OD_{600} of *C. glutamicum*. Accumulation of linear maltodextrins and subsequent degradation of glucose 1-phosphate should be still possible without GlgB (see Figure 1). A temporary accumulation and degradation is shown by the increase and decrease of low levels of glycogen during cultivation of *C. glutamicum* Δ*glgB* in minimal medium with glucose as carbon source (Figure 4A). Thus, *C. glutamicum* Δ*glgB* should not lose any carbon and should show normal growth. It is unknown why the OD_{600} of *C. glutamicum* Δ*glgB* increases again after reaching stationary phase. Glycogen as possible carbon source can be excluded, since no glycogen is present after reaching stationary phase (Figure 4A). However, the results confirmed the role of GlgB as important enzyme in the synthesis of glycogen with glucose as precursor.

The growth of *C. glutamicum* Δ*glgX* showed no differences in growth rate in comparison with *C. glutamicum* WT, but slightly higher OD_{600} during growth with glucose (Figure 5A). The integration mutant *C. glutamicum* WT-ImX showed slower growth and lower OD_{600} than *C. glutamicum* WT. It is unclear what causes the difference in growth between the deletion and integration mutant, but is likely to assume that it is due to the nature of the gene inactivation in both strains. The fact that *C. glutamicum* WT and *C. glutamicum* Δ*glgX* show the same growth rate confirms that the growth is not impaired by the disturbed glycogen degradation. Therefore, it is likely that *C. glutamicum* has possibilities to compensate this disturbance. A compensation mechanism would also be in agreement with the role of glycogen as "carbon capacitor" (Seibold and Eikmanns, 2007 and 2013). A "carbon capacitor" should not be essential for growth under optimal growth conditions, since it is only important for sudden changes of the conditions. The higher OD_{600} of *C. glutamicum* Δ*glgX* could be due higher glycogen levels, since experiments with *C. glutamicum* Δ*glgC* and *C. glutamicum* Δ*glgX* under anaerobic conditions showed that glycogen also affects OD_{600} (see chapter 3.2.4). In this case, *C. glutamicum* Δ*glgX* would have the same or a lower number of cells than *C. glutamicum* WT during cultivation, but the higher levels of glycogen would increase the OD_{600}. The strongly impaired degradation of glycogen in *C. glutamicum* Δ*glgX* confirms the role of the glycogen debranching enzyme GlgX as first enzyme of glycogen degradation (Seibold and Eikmanns, 2007). Since *C. glutamicum* Δ*glgX* is unable to degrade the α-1,6-glycosidic linkages of glycogen, there are only few starting points for the glycogen phosphorylase and no linear glucans/maltodextrins are generated, which could be substrate for the maltodextrin phosphorylase. Consequently, the degradation of glycogen is slowed down in *C. glutamicum* Δ*glgX*.

As reported before, *C. glutamicum* Δ*malP* showed the same growth as *C. glutamicum* WT in minimal medium with glucose (Seibold *et al.*, 2009). MalP was described as maltodextrin phosphorylase and therefore, it was assumed that the apparent slower degradation of glycogen in *C. glutamicum* Δ*malP* on glucose was caused by the slower degradation of linear maltodextrins (which cannot be discerned from glycogen by the method used for determination of glycogen levels). New insights suggest that activity of MalP does not vary significantly for longer linear maltodextrins or branched glycogen and should be classified as glycogen phosphorylase due to allosteric regulation by ADP-glucose, since in general, glycogen phosphorylases are allosterically regulated, whereas maltodextrin phosphorylases are regulated on transcriptional level (Seibold, personal communication; Schinzel and Nidetzky, 1999). Since glycogen phosphorylase activity was determined in *C. glutamicum* Δ*malP* (Seibold *et al.*, 2009), at least one enzyme catalysing these reactions is present in cell

extracts of *C. glutamicum*. A possible candidate was the gene product of *glgP* (Seibold *et al.*, 2009). However, the deletion of *glgP* led to no observable phenotype in *C. glutamicum* during cultivation on glucose. Also additional deletions of *glgP* in *C. glutamicum* Δ*glgX*, *C. glutamicum* Δ*malP* or *C. glutamicum* Δ*glgX*Δ*malP* showed no effect on glycogen degradation or glycogen synthesis indicating that GlgP plays no important role in glycogen metabolism under the tested conditions (Figure 9A, Figure 10A, Figure 11A). Therefore, it remains uncertain whether the gene product of *glgP* has any role in glycogen metabolism of *C. glutamicum* at all. Glycogen phosphorylase activity was observed in *C. glutamicum* Δ*malP* (Seibold *et al.*, 2009). Also, no significant decreases in glycogen phosphorylase activity were observed in *C. glutamicum* Δ*glgP* (Horn, 2011). It is unlikely that one enzyme could compensate the loss of the other, since *C. glutamicum* Δ*malP*Δ*glgP* showed the same phenotype as *C. glutamicum* Δ*malP*. So far, it is unclear which enzyme could be responsible for glycogen phosphorylase activity besides MalP.

The results of the analysis of growth and glycogen levels of deletion mutants suggest that GlgC and GlgA play no role in the glycogen metabolism in *C. glutamicum* during growth on maltose and that maltodextrins, which are substrate for GlgB, are formed on a different pathway. In the following the phenotypes of the other deletion mutants are discussed. As observed for the integration mutant *C. glutamicum* WT-IMB (Seibold, 2007), the deletion mutant *C. glutamicum* Δ*glgB* showed lower growth rates than *C. glutamicum* WT during growth on maltose. Interestingly, the glycogen levels of *C. glutamicum* Δ*glgB* were not lower than *C. glutamicum* WT, but the maximum was reached at a later time point. The reasons for that difference in glycogen accumulation between integration and deletion mutant are unknown. In a previous work it was assumed that the growth impairment of the *C. glutamicum* with inactivated GlgB might be due to an involvement of GlgB in the maltose metabolism as possible 4-α-glucanotransferase (Seibold, 2007). But analysis of *C. glutamicum* Δ*malQ* and *C. glutamicum* Δ*malQ*Δ*glgB* showed that GlgB is not involved in maltose metabolism in that way (Seibold, personal communication). During the postulated glycogen synthesis pathway on maltose via the maltokinase TreX, the maltosyl transferase GlgE and the glycogen branching enzyme GlgB, the intermediate maltose 1-phosphate is formed (and normally consumed right away), which showed toxic effects in *M. tuberculosis* strains with deletions of GlgE and GlgB (Kalscheuer *et al.*, 2010). In *C. glutamicum* the *glgB* and *glgE* genes form an operon (Seibold *et al.*, 2011). If the TreX-GlgE-GlgB pathway is used for glycogen synthesis during growth of *C. glutamicum* on maltose, the observed growth impairment of *C. glutamicum* Δ*glgB* might be caused by lower GlgE activity and consequently higher maltose 1-phosphate concentrations. Possible reasons for lower GlgE activities might a polar effect by the deletion of *glgB* or enzymatic disbalances. In fact,

preliminary results indicate that this pathway is used during growth on maltose and that the formation of maltose 1-phosphate might be responsible for growth impairment, since deletion of *treX* abolished these effects (Seibold, personal communication).

C. glutamicum Δ*glgX* showed the same growth rate and OD_{600} as *C. glutamicum* WT during growth on maltose, whereas the integration mutant *C. glutamicum* WT-IMX showed slower growth and lower OD_{600} (Seibold, 2007). The growth effect in *C. glutamicum* WT-IMX might be due to the integrated kanamycin cassette and not due to the disturbed glycogen metabolism. As already observed in minimal medium with glucose, *glgX* is not essential, which is in agreement with the proposed role of glycogen as “carbon capacitor”. The fact that a part of glycogen/maltodextrin is not degraded in *C. glutamicum* Δ*glgX* confirms that at least a part of the glucose polymers formed on maltose is indeed branched glycogen. The loss of the glycogen debranching enzyme GlgX causes that α-1,6-glycosidic linkages are not cleaved. Consequently, the substrate for maltodextrin and glycogen phosphorylases is limiting.

Previous studies revealed that MalP activity is required in the late exponential phase during growth on maltose and that *C. glutamicum* Δ*malP* shows a strong reduction in growth rate after about 7 hours of growth on maltose (Seibold *et al.*, 2009). The deletion of *treX* coding for a maltokinase in *C. glutamicum* Δ*malP* abolished the growth impairment indicating that the accumulation of maltose 1-phosphate might be the reason for slower growth (Seibold, personal communication). Due to the growth phenotype and the remaining glycogen phosphorylase activity in the deletion mutant, MalP was designated as maltodextrin phosphorylase leaving GlgP as candidate for a glycogen phosphorylase (Seibold *et al.*, 2009). The absence of a phenotype in growth or glycogen levels of *C. glutamicum* Δ*glgP* during growth on glucose or on maltose suggest that GlgP has no involvement in glycogen metabolism under the tested conditions. The possibility that MalP could compensate its activity in the GlgP-negative strain was ruled out, since *C. glutamicum* Δ*malP*Δ*glgP* did not differ from *C. glutamicum* Δ*malP* in any tested aspect. Additional, it was found that the MalP enzyme is inhibited by ADP-glucose and shows similar activities with linear maltodextrins as with branched glycogen (Seibold, personal communication).

Reduction of nitrogen concentration (as essential nutrient for growth) in the medium was shown to cause an increase in glycogen levels of *Arthrobacter* and *E. coli* (Zevenhuizen, 1966; Holme and Palmstierna, 1956b; Preiss and Romeo, 1989). Since little is known about the regulation of the glycogen metabolism and possible stimuli for a regulatory effect in *C. glutamicum*, it was investigated, whether a reduction of the nitrogen source availability

affects the glycogen metabolism in *C. glutamicum*. For this purpose, growth experiments with gradually decreased concentrations of the two nitrogen sources ammonium sulphate and urea from 37.8 mM and 82.5 mM, respectively (corresponding to 100 % of nitrogen source concentration), to 1.5 mM and 3.3 mM, respectively (corresponding to 4 % of the originally used nitrogen source concentration), were performed and glycogen levels of the respective cultures were determined (see chapter 3.2.2). The present study shows that decreasing the concentrations of ammonium sulphate and urea has no effect on glycogen levels. In other organisms it was also reported that the increase of glycogen levels due to the reduction of nitrogen availability was concomitant with the decrease of nitrogen-containing compounds (Zevenhuizen, 1966). The minimal medium CGXII for *C. glutamicum* contains a four times higher ammonium sulphate concentration compared to CgC, which was used in the present study, and is used for the production of amino acids. Having these two facts in mind, it is interesting to ask whether an increase of nitrogen concentrations could lead to lower glycogen levels in *C. glutamicum*.

Another important element for cellular compounds is phosphorus. In *C. glutamicum* it was observed that the reduction of the phosphate concentration from 13 mM to 0.13 mM led to a significant increase in glycogen levels (Woo *et al.*, 2010). In this study, the authors described a 3- to 6-fold upregulation of the *pgm* and *glgC* genes under the strongly reduced phosphate concentration. Since the growth of *C. glutamicum* was strongly affected with 0.13 mM phosphate, it was tempting to test whether the effect on glycogen levels and expression levels of *pgm* and *glgC* is caused by the reduced phosphate concentration itself or by the impaired growth under these conditions. Therefore, growth experiments with gradually decreasing phosphate concentrations (ranging from 13 mM to 1.3 mM phosphate) with determination of glycogen levels in *C. glutamicum* were performed. It was observed that the reduction of phosphate to 3.25 mM and below led to increased glycogen levels in an inverse-proportional manner (see chapter 3.2.1). With 1.95 mM phosphate, *C. glutamicum* was only able to grow similarly to experiments with 13 mM phosphate. Lower phosphate concentrations affected growth even more negatively. Therefore, the phosphate concentrations of 13 mM and 1.95 mM were used for the semi-quantitative *real-time* transcription analysis of the genes *glgC*, *glgA*, *glgB*. *glgX*, *malP* and *glgP*, which are all coding for enzymes (putatively) involved in glycogen metabolism.
When expression levels of these genes were compared in mid-exponential growth phase under both phosphate concentrations, it was found that *glgC*, *glgA* and *glgB* showed higher expression levels with reduced phosphate concentration, while *glgX* and *malP* showed only small changes in expression levels (Figure 23). GlgP showed even lower expression levels with 1.95 mM phosphate than with 13 mM. This finding that genes coding for enzymes of

glycogen synthesis show higher mRNA levels, is consistent with higher glycogen levels, since more mRNA copies generally lead to higher enzyme levels of corresponding enzymes. The increase in *glgC* expression also confirms the data from CAT assays, which showed higher expression of glgC under phosphate-limited conditions (Woo *et al.*, 2010).

Furthermore, in cells grown with 13 mM phosphate the expression levels of all genes dropped in the early stationary phase as compared to mid-exponential phase of at least about 50 % (not shown). This indicates that most of the mRNA of these genes has been degraded and the levels of corresponding enzymes might be lower, too. In contrast, with 1.95 mM phosphate the decrease of mRNA levels were not as drastic as with 13 mM phosphate, except for *malP*, which showed mRNA levels even lower than with 13 mM phosphate (not shown). Therefore, the higher glycogen levels observed with 1.95 mM phosphate might be due to increased mRNA levels (relative to experiments with 13 mM phosphate) of genes coding for enzymes in glycogen synthesis and the decreased expression of *malP* coding for the maltodextrin phosphorylase, which in involved in glycogen degradation (not shown).

The transcriptional response of *C. glutamicum* to phosphate limitation was investigated previously and the changed expression of several genes was reported (Ishige *et al.*, 2003; Kočan *et al.*, 2006). The two-component system carrying out the phosphate-starvation induced (psi) response was identified as PhoS-PhoR system (Kočan *et al.*, 2006). The genes investigated in the present study were not reported as affected by phosphate limitation or deletion of the PhoS-PhoR system. So far, the only regulators known to be involved in glycogen metabolism in *C. glutamicum* are the two master regulators RamA and RamB (Seibold *et al.*, 2010). It was shown that RamA regulates the transcription of *glgC* and *glgA* positively, while RamB regulates the expression of *glgA* negatively (Seibold *et al.*, 2010). The regulatory effects on both genes were rather weak under the tested conditions. In contrast, in *C. glutamicum*, no regulator involved in glycogen metabolism besides RamA and RamB has been found.

Another set of experiments regarding the regulation of glycogen metabolism was performed by an *in vivo* and *real-time* transcription analysis using an integrated luciferase system. With this system, the activity of promoter sequences of *glgC*, *glgA* and *glgX* were analysed during growth in minimal medium with glucose and with maltose as carbon source. All promoter sequences showed similar luminescence profiles on glucose and on maltose. Since it was shown that deletions of *glgC* and *glgA* in *C. glutamicum* led with maltose to the same glycogen levels as observed for *C. glutamicum* WT indicating no involvement of the respective gene products in glycogen synthesis with maltose as carbon source (Figure 2B and 3B), it is surprising that *glgC* and *glgA* are expressed on maltose in the same way as on

glucose. So far, the advantages by transcription of *glgA* and *glgC* with maltose as sole carbon source are unclear.

The time-dependent promoter activity profile of *glgC* is particularly curious, since Seibold and co-workers reported no significant change in this promoter activity of *glgC* using a plasmid-based CAT reporter assay (Seibold *et al.*, 2007). In the same study, it was reported that specific enzyme activity of the ADP-glucose phosphorylase showed its maximum in the early exponential phase. The discrepancies in this and the present study might be based on the differences in the reporter assays. The reporter system used in this study was established as a *real-time* and *in vivo* reporter system, which is integrated into the genome (Laslo, 2013). The transcription of the reporter gene coding for a luciferase is under control of the respective promoter and activity is determined by luminescence in relation to OD_{600}. In contrast, the CAT reporter system is based on the plasmid pET2 with a high copy number (leading to more reporter enzyme) and the activity is determined by CAT activity relative to total protein (Vasicová *et al.*, 1998). Since the chloramphenicol acetyltransferase has a long half-life of about 50 hours, it is not suited for the observation of kinetic expression changes during growth experiments with *C. glutamicum* (Mülhardt, 2007). The half-life of the luciferase used in the present study is about 6 to 8 hours (Laslo, 2013). The mentioned differences might be the reason for the differing results regarding the activity of the *glgC* promoter. Using improved luciferase reporter system (with shortened half-life of the luciferase) might be an even better way to analyse promoter activity during growth of *C. glutamicum*.

Since the promoter activities of *glgC*, *glgA* and *glgX* were highest directly after inoculation in minimal medium with glucose or maltose, the present data suggests that the change from complex medium to minimal medium with glucose or maltose as carbon source is a signal for the transcription of the *glgC*, *glgA* and *glgX* genes. Possible advantages are that *C. glutamicum* is able to react to changes of environmental conditions easier, when the carbon capacitor glycogen is present. Functional enzymes of the glycogen metabolism are the requirement for glycogen accumulation. By allosteric regulation of glycogen synthesis and degradation, *C. glutamicum* would be able to react instantly on sudden changes of environmental conditions.

Being a soil bacterium, *C. glutamicum* has to face not only changes in the concentration of phosphorus and nitrogen sources, but also changes in the extracellular pH. Previously, it was reported that *C. glutamicum* shows significant lower growth rates at an extracellular pH of 6 than at an extracellular pH around 8 (Follmann *et al.*, 2009). The authors also showed that *C. glutamicum* is able to maintain an intracellular pH of about 7.5 in the range of extracellular pH 6 to 9. To test the influence of pH stress on glycogen metabolism and the possible role of

glycogen metabolism in a stress response, *C. glutamicum* WT, *C. glutamicum* Δ*glgC* and *C. glutamicum* Δ*glgX* were grown with glucose under the constant pH values of 6 and 7, while growth was followed and glycogen levels were determined. The results show that the deletions of *glgC* or *glgX* have no influence on growth of *C. glutamicum* in minimal medium with a constant pH of 7. *C. glutamicum* WT, *C. glutamicum* Δ*glgC* and *C. glutamicum* Δ*glgX* showed similar maximal OD_{600} and growth rates were almost identical and only slightly lower than in shake flask experiments (see chapters 3.1.1, 3.1.2 and 3.2.3). Therefore, it is very likely that under these conditions disturbances in the glycogen metabolism are of no or minor importance in *C. glutamicum*. In contrast, the deletion of *glgC* in *C. glutamicum* led to a significantly higher growth rate under a constant extracellular pH of 6 (Figure 14). Since the experiment with *C. glutamicum* Δ*glgX* was performed only once, the results should be handled with care, but is very likely that the deletion of *glgX* in *C. glutamicum* led to lower maximal OD_{600} and growth rate under an extracellular pH of 6. The reasons for the growth benefit of *C. glutamicum* Δ*glgC* in comparison with the other two strains are unknown so far.

Another environmental factor, which can play an important role for soil bacteria, is the availability of oxygen. In absence of oxygen and nitrate, *C. glutamicum* can utilize glucose and produce organic acids such as succinic lactic and acetic acid, while no growth was reported (Inui *et al.*, 2004b). If nitrate is available under anaerobic conditions, *C. glutamicum* is able to grow using nitrate as terminal electron acceptor (Nishimura *et al.*, 2007). The authors also showed that OD_{610} of *C. glutamicum* ATCC13032 and *C. glutamicum* R increased about two-fold during anaerobic conditions. Besides the production of lactate and succinate, anaerobic metabolism was studied with an aim of commercial production of amino acids such as alanine and valine, and of isobutanol (Yamamoto *et al.*, 2012; Hasegawa *et al.*, 2013; Blombach *et al.*, 2011).

The present study was part of the project “SysEnCor”. One goal of this project was the establishment and improvement of a metabolic flux model. In the course of the project, the assumption was made based on data by metabolic flux model that *C. glutamicum* forms glycogen under anaerobic conditions (Elisabeth Zelle, personal communication). To test this hypothesis, the glycogen levels and ODs_{600} of *C. glutamicum* WT, *C. glutamicum* Δ*glgC* and *C. glutamicum* Δ*glgX*Δ*malP*Δ*glgP* were determined during anaerobic conditions. Results of these experiments confirmed the predictions of the metabolic flux model and are in concordance with the observations made by Radoš *et al.*, 2014. *C. glutamicum* WT and *C. glutamicum* Δ*glgX*Δ*malP*Δ*glgP* showed increased glycogen levels during anaerobic conditions. Surprisingly, it was also found that strains accumulating glycogen increased OD_{600} values for about 2.5- to 3-fold, whereas the OD_{600} of *C. glutamicum* Δ*glgC* stayed approximately the same. To exclude the possibility that the strains increased number of living

cells, the colony forming units of each strain were determined right after the start of the incubation and after 10 hours. The results showed that the ratio of OD_{600} after 10 hours to OD_{600} after inoculation was not comparable with the ratio calculated by using CFU values at the same time points. While *C. glutamicum* showed a slightly increased number of CFU after 10 hours, *C. glutamicum* Δ*glgC* and *C. glutamicum* Δ*glgX*Δ*malP*Δ*glgP* showed decreased numbers of CFU. This might be a hint that strains with disturbances in the glycogen metabolism are impaired regarding survival or fitness under anaerobic conditions. It was shown for other bacteria that glycogen could increase the survival possibility under various conditions (Bourassa and Camilli, 2009; Busuioc *et al.*, 2009; Sambou *et al.*, 2008; McMeechan *et al.*, 2005). Also, in the present study results suggest that *C. glutamicum* strains with functional glycogen metabolism are less affected by elongated starvation (see chapter 3.5.2). In contrast, previous studies with a *C. glutamicum* integration mutant in *glgC* displayed no impairment in survival (Seibold *et al.*, 2007). Since *C. glutamicum* WT does not degrade glycogen during observed anaerobic condition, glycogen metabolism itself might be not involved in the higher survival rate. It can be assumed that the disturbances in glycogen metabolism affect other pathways or cell compounds directly or indirectly allowing a better adaptation to anaerobic conditions. A possible candidate for a cell compound affected by glycogen metabolism is trehalose, which is an important part of the cell wall and plays also a major role in response to osmotic stress in *C. glutamicum* and related bacteria (Chandra *et al.*, 2011; Tzvetkov *et al.*, 2003). It might be possible that utilization of glycogen by *C. glutamicum* WT facilitates growth on complex medium agar plates (which are used for the determination of the CFU) after anaerobic conditions – as it was shown for liquid minimal media after prolonged carbon starvation in the present study. In this case, the lower number of CFU of the strains with disturbed glycogen metabolism on complex medium agar plates might be due to worse growth on these agar plates and not due to a lower number of living cells in the anaerobic cultures.

For further investigation of this phenomenon of rising OD_{600} under anaerobic conditions, the ratios of counted number to OD_{600} in cell suspensions of *C. glutamicum* WT, *C. glutamicum* Δ*glgC* and *C. glutamicum* Δ*glgX* were determined with two methods. One set of experiments was done by determination the cell number with a Helber counting chamber. The other set was done using a Coulter Counter. In the second set of experiments the biovolume (volume of all cells) and the total cell number were also determined. The results of the first set suggested that under anaerobic conditions the OD_{600} of all strains raised more than the cell number. *C. glutamicum* Δ*glgC* showed only minor changes in OD_{600} and cell number resulting also in a low change of the ratio of OD_{600} to cell number. In contrast, *C. glutamicum* strains accumulating glycogen showed increasing ratios of OD_{600} to cell number. This means that the OD_{600} increased much more than the cell number in the investigated cell

suspensions. These results were confirmed by the second set of experiments performed with a Coulter Counter. *C. glutamicum* WT and *C. glutamicum* Δ*glgX* (both of which accumulated glycogen) showed increasing ratios of OD_{600} to cell number indicating that the OD_{600} raised under anaerobic conditions independently of the cell number. *C. glutamicum* Δ*glgC* showed almost no change in this ratio. The experiments also revealed that the average biovolume per cell of *C. glutamicum* WT and *C. glutamicum* Δ*glgX* increased under anaerobic conditions about 30 to 50 % (Figures 19 and 20), whereas cells of *C. glutamicum* Δ*glgC* displayed no significant change in average biovolume. Since the increased average cell volume under anaerobic conditions was only reported for strains accumulating glycogen, it can be suggested that glycogen affects the biovolume. As mentioned above, it was reported that *C. glutamicum* Δ*glgX* (accumulating, but not degrading glycogen) showed an increase in cell size under anaerobic conditions. Seibold *et al.* (2009) showed that *C. glutamicum* Δ*malP*, which showed increased glycogen/maltodextrin levels during growth on maltose, displayed abnormal cell shapes, increased cell sizes and increased number of dead cells in culture. The reason for the increased volume of *C. glutamicum* cells accumulating glycogen/maltodextrins is so far not known. The experiments with the Coulter Counter showed that all three strains also increased in total cell number, *C. glutamicum* WT showing the highest increase. Since it is not possible to discern between living and dead cells using the Coulter Counter, it is unclear, to which part these cells are alive. Former experiments regarding the CFU of *C. glutamicum* Δ*glgC* and of *C. glutamicum* Δ*glgX*Δ*malP*Δ*glgP* after during anaerobic incubation (see above) indicated that the respective deletions led to worse survival rate under anaerobic conditions than *C. glutamicum* WT. It cannot be ruled out that strains with disturbances in glycogen metabolism grow worse on complex medium plates after anaerobic conditions.

Since the deletions of genes coding for enzymes in the glycogen metabolism led to the observed changes in cell volume, total cell number and viability during anaerobic incubation, it was interesting to investigate possible influences on the production of organic acids under these conditions. It was shown that *C. glutamicum* WT produced the organic acids such as succinic, lactic and acetic from anaerobic glucose metabolism (Inui *et al.*, 2004b). *C. glutamicum* Δ*glgX*Δ*malP*Δ*glgP* differed insignificantly from *C. glutamicum* WT in the consumption of glucose and the production of succinate, lactate and acetate. Similarly, observed differences in glucose consumption and organic acid production by *C. glutamicum* Δ*glgC* were not significant. Succinic and lactic acid produced under anaerobic conditions derive from pyruvate and are formed by re-oxidation of $NADH/H^+$, which is formed during glycolysis. Acetate also derives from pyruvate, but $NADH/H^+$ is formed during production. The carbon flux model described by Radoš and co-workers showed similar glycogen levels

under both lactate and succinate producing conditions (Radoš *et al*., 2014), thus an impact of redox status of the cell on glycogen accumulation should not be very probable.

Since it was shown that glycogen accumulation influences the OD_{600} rather than the cell number, it is difficult to compare the glucose consumption rates of *C. glutamicum* WT, *C. glutamicum* Δ*glgC* and *C. glutamicum* Δ*glgX*Δ*malP*Δ*glgP*. In the first 10 hours *C. glutamicum* Δ*glgC* consumed glucose in a similar way compared to the other strains. Since *C. glutamicum* Δ*glgC* is not able to use glucose for the formation of glycogen, it was assumed that this strain might be able to form respective organic acids faster than *C. glutamicum* WT and *C. glutamicum* Δ*glgX*Δ*malP*Δ*glgP.* Analysis of organic acid production also invalidated this assumption.

The observation that the optical density of *C. glutamicum* changes dramatically if incubated anaerobically with glucose, led to suspicion that cells might still be in a microaerobic environment, and thus growing. *C. glutamicum ΔaceE Δpqo ΔldhA ΔC–T ilvN ΔalaT ΔavtA*, an acetate auxotroph that is not able to grow with glucose as sole carbon source, was incubated anaerobically with glucose or glucose plus acetate, to test this assumption (Wieschalka *et al.*, 2012). Interestingly, when incubated with glucose alone, *C. glutamicum ΔaceE Δpqo ΔldhA ΔC–T ilvN ΔalaT ΔavtA* showed an increase in OD_{600}, with glycogen being accumulated. As soon as glycogen levels dropped, OD_{600} also decreased. When incubated with glucose plus acetate, only marginal levels of glycogen were determined and OD_{600} showed no significant changes. Since *C. glutamicum ΔaceE Δpqo ΔldhA ΔC–T ilvN ΔalaT ΔavtA* is not able to increase in living cell number without acetate, these observations pointed out that changes in OD_{600} are indeed not to be traced back to changes in number of living cells, but to changes in glycogen levels. The differences in glycogen levels between *C. glutamicum ΔaceE Δpqo ΔldhA ΔC–T ilvN ΔalaT ΔavtA* and *C. glutamicum* WT might be a consequence of the altered carbon fluxes in the deletion strain. In many other bacteria, glycogen synthesis is controlled by small intermediates of the central metabolic pathways, which are signs for the energetic state of the cell (reviewed in Ballicora *et al.*, 2003). Effects of gene deletions on the formation of various products under anaerobic conditions were described earlier (Inui *et al.*, 2004a; Hasegawa et al., 2013). Since no analysis of intracellular metabolites was performed in *C. glutamicum ΔaceE Δpqo ΔldhA ΔC–T ilvN ΔalaT ΔavtA*, it is unclear which metabolites could be responsible for the change in glycogen levels.

The suspensions of *C. glutamicum ΔaceE Δpqo ΔldhA ΔC–T ilvN ΔalaT ΔavtA* showed no difference in organic acid production pattern, when fed with glucose or glucose plus acetate. Therefore, the most interesting finding was that the glycogen accumulation was completely abolished when acetate was also supplied in the medium. In this case, the acetate concentration in the supernatant changed only marginal during the incubation indicating very slow uptake/metabolism. The involvement of the two master regulators RamA and RamB has

been shown earlier (Seibold *et al.*, 2010). In that study it was also reported that promoter activity of *glgC* was lower with acetate or with glucose plus acetate than with glucose alone. Therefore, it is assumed that the high concentration of acetate in the supernatant has influence at least on the transcription of *glgC* in these experiments. Also, further reactions of acetate could influence the sensitive regulation of glycogen metabolism by changing concentrations of other intracellular metabolites.

The formation of glycogen consumes one ATP per one molecule glucose of incorporated or added glucose, which cannot be regained by degradation of glycogen (see Figure 1). Therefore, abolishment of glycogen synthesis or glycogen degradation could involve the intracellular levels of energetic parameters (such as ATP, $NADH/H^+$, $NADPH/H^+$) in *C. glutamicum*. Levels of ATP or reduction equivalents are critical for the formation of chemicals by *C. glutamicum*. For example, the formation of amino acids such as L-histidine or L-arginine consumes high amounts of ATP (Kulis-Horn *et al.*, 2013). Therefore, the increase of the ATP or reduction equivalent levels is one goal to generate more efficient production strains. Since the involvement of glycogen metabolism with energetic parameters is unknown, it was investigated in this study, how abolishment of glycogen synthesis or glycogen degradation influences the intracellular availability of ATP. In other studies, it was also reported that an increase of $NADPH/H^+$ availability is advantageous for the formation of L-valine (Bartek *et al.*, 2010). Changes in the glycogen metabolism could influence the intracellular abundance of glucose 6-phosphate. Hence, the flux through the PPP together with the formation of $NADPH/H^+$ might be also subject of change. The analysis of the energy charge (which is a criterion for the ATP availability), of the catabolic reduction charge (as a criterion for the $NADH/H^+$ availability) and of the anabolic reduction charge (as a criterion for the $NADPH/H^+$ availability) showed that disturbances in the glycogen synthesis and in the glycogen degradation had no significant influence on the criteria.

Previously, glycogen metabolism was also described as glycogen futile cycling in *Fibrobacter* (Matheron *et al.*, 1998). Futile cycling was also mentioned in context with energy spilling reactions (Russell, 2007). The role of glycogen as carbon capacitor was proposed under growth conditions (Seibold and Eikmanns, 2007), while Radoš and co-workers show that under anaerobic atmosphere ^{13}C-labeled glycogen synthesis occurred in parallel with unlabelled glycogen breakdown, *i.e*. futile cycling (Radoš *et al.,* 2014). An extended analysis of intracellular metabolites in *C. glutamicum* WT, *C. glutamicum* Δ*glgC* and *C. glutamicum* Δ*glgX* could help to investigate the influence on intracellular pathways more detailed and to confirm or disprove proposed roles of glycogen in *C. glutamicum*.

Due to the reported involvement of glycogen in survival and persistence in other bacteria, it was tested if disturbances in the glycogen metabolism of *C. glutamicum* affect the growth behaviour in minimal medium after prolonged starvation (Bourassa and Camilli, 2009; Busuioc *et al.*, 2009; Sambou *et al.*, 2008; McMeechan *et al.*, 2005). Experiments were performed with glucose and with acetate as carbon source due to different glycogen levels on these substrates. In case of both substrates it became obvious that *C. glutamicum* WT had growth advantages over *C. glutamicum* Δ*glgC* and *C. glutamicum* Δ*glgX* (Figures 34 and 35). The respective time points for re-inoculation after starvation were chosen due to preliminary experiments, which showed no influence on growth after one day of starvation and the same growth after 4 days of starvation as after 3 days of starvation (data not shown). With longer starvation time in minimal medium with glucose, the growth impairment became more dramatic for all three strains, but *C. glutamicum* WT showed the best growth throughout the whole experiment. A similar observation was made when acetate was the carbon source in the first culture – with other time points of re-inoculation. Based on the observations, it is assumed that the presence of usable glycogen in the cells (*C. glutamicum* Δ*glgC* was not able to accumulate, *C. glutamicum* Δ*glgX* was not able to degrade) is advantageous in the beginning of growth. In *E. coli* it was shown that glycogen acts as primary source during the lag phase (Yamamotoya *et al.*, 2012). Assuming a similar role in *C. glutamicum*, it becomes clear that *C. glutamicum* Δ*glgC* and *C. glutamicum* Δ*glgX* showed more defective growth than *C. glutamicum* WT. Analysis of glycogen levels in the first culture that served as inoculum showed that glycogen levels dropped only in *C. glutamicum* WT between 2 and 3 days of starvation (data not shown). Between 3 and 5 days of starvation no change in glycogen levels of *C. glutamicum* WT was observed. As expected, *C. glutamicum* Δ*glgC* showed no glycogen and *C. glutamicum* Δ*glgX* showed no glycogen degradation. Similar observations were made, when acetate was the carbon source in the first culture. Since *C. glutamicum* Δ*glgC* and *C. glutamicum* Δ*glgX* showed different growth as *C. glutamicum* WT and since the glycogen levels in *C. glutamicum* WT cells starved for 3 days did not differ from cells starved for 5 days (but the growth after re-inoculation differed greatly) it seems likely that glycogen metabolism is involved in survival, but is not the only factor in survival. A candidate for involvement is polyphosphate as it was detected in *C. glutamicum* and it was set in relation to survival in other bacteria before (Lambert *et al.*, 2002; Klauth *et al.*, 2006; Nikel *et al.*, 2013; Singh *et al.*, 2013). Polyphosphate is known for its role as energy storage compound, but also for its role as regulator in metabolism (reviewed in Achbergerová and Nahálka, 2011). Experiments in this thesis showed that reduction of phosphate led to elevated glycogen metabolism indicating connections between glycogen and polyphosphate metabolism (see chapter 3.2.1). Therefore, it was assumed that polyphosphate might be one of the factors playing a role in survival and growth after starvation. Preliminary experiments

showed no differences of phosphate reduced first cultures to normal first cultures. For further investigation of the involvement of phosphate in survival, experiments should be repeated with determinations of polyphosphate levels and lower phosphate concentrations.

With results of the present study, it was possible to clarify the roles of the *glgC*, *glgA*, *glgB*, *glgX*, *malP* and *glgP* gene in glycogen metabolism of *C. glutamicum* on glucose or maltose. Also phosphate and oxygen availability were identified as stimuli in the glycogen metabolism. Effects of disturbed glycogen metabolism on growth, on anaerobic production behaviour and on energetic parameters were ruled out under the tested conditions, but a connection of glycogen metabolism to growth was confirmed under slightly acidic extracellular pH and after prolonged starvation.

However, the present study gave rise to other questions. The analysis of deletion mutants and of glycogen levels under anaerobic conditions indicates a connection between glycogen levels and OD_{600}. A detailed analysis of cell number and OD_{600} of *C. glutamicum* WT, *C. glutamicum* Δ*glgC* and *C. glutamicum* Δ*glgX* during growth on glucose should be performed to investigate whether the higher OD_{600} of glycogen accumulating strains can be deduced to higher cell number or to higher glycogen levels. In this context, analysis of living cell number of *C. glutamicum* WT, *C. glutamicum* Δ*glgC* and *C. glutamicum* Δ*glgX* under anaerobic conditions should be performed by live/dead cell imaging to investigate, how disturbances in glycogen metabolism affect the survival under the tested conditions.

Also, it is still unclear, why *C. glutamicum* Δ*glgA* grows so badly with glucose as carbon source. Analysis of intracellular metabolite levels (especially of ADP-glucose) should be performed to identify possible accumulations of metabolites. Additionally, the overexpression of an ADP-glucose pyrophosphohydrolase, which hydrolyses ADP-glucose to glucose-1-phosphate and AMP, could be done to test, if an accumulation of ADP-glucose is responsible for this phenotype. In *E. coli* it was shown that this enzyme is involved in glycogen metabolism (Morena-Bruna *et al.*, 2001) and it was also reported that activities of this enzyme were detected in crude cell extracts of *C. glutamicum* (Padilla *et al.*, 2004b). Low activities of ADP-glucose pyrophosphohydrolase might allow an impaired growth as seen in cultures of *C. glutamicum* Δ*glgA* in minimal medium with glucose as sole carbon source.

Last, but not least, further efforts should be done for the identification of possible regulators of glycogen metabolism. One approach would make use of the finding that phosphate reduction leads to higher glycogen levels. It would be interesting to perform DNA microarrays or DNA affinity chromatography under phosphate reduced conditions with respective probes of genes coding for enzymes involved in glycogen metabolism.

References

Abe, S., K. I. Takayama, S. Kinoshita. 1967. Taxonomical studies on glutamic acid producing bacteria. J. Gen. Appl. Microbiol. 13: 279-301.

Achbergerová, L., and J. Nahálka. 2011. Polyphosphate – an ancient energy source and active metabolic regulator. Microb. Cell. Fact. 10: 63.

Ajinomoto Co. 2012. Food products business. URL: http://www.ajinomoto.com/en/ir/pdf/Food-Oct2012.pdf

Ajinomoto Co. 2013. Feed-use amino acid business. URL: http://www.ajinomoto.com/en/ir/pdf/Feed-useAA-Oct2013.pdf

Alonso-Casajus, N., D. Dauvillée, A. M. Viale, F. J. Muñoz, E. Baroja-Fernandez, M. T. Moran-Zorzano, G. Eydallin, S. Ball, J. Pozueto-Romero. 2006. Glycogen phosphorylase, the product of the *glgP* gene, catalyses breakdown by removing glucose units from the nonreducing ends in *Escherichia coli*. J. Bacteriol. 188: 5266-5272.

Arndt, A., M. Auchter, T. Ishige, V. F. Wendisch, B. J. Eikmanns. 2008. Ethanol catabolism in *Corynebacterium glutamicum*. J. Mol. Microbiol. Biotechnol. 15: 222-233.

Arndt, A., and B. J. Eikmanns. 2007. The alcohol dehydrogenase gene *adhA* in *Corynebacterium glutamicum* is subject to carbon catabolite repression. J. Biotechnol. 189: 7408-7416.

Arndt, A., B. and J. Eikmanns. 2008. Regulation of carbon metabolism in *Corynebacterium glutamicum*. In Corynebacteria: Genomics and Molecular Biology. Burkovski, A., (ed.). pp. 155-182. Caister Academic Press, Norfolk, UK.

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

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

Bartek, T., B. Blombach, E. Zönnchen, P. Makus, S. Lang, B. J. Eikmanns, M. Oldiges. 2010. Importance of NADPH supply for improved L-valine formation in *Corynebacterium glutamicum*. Biotechnol. Prog. 26: 361-371.

Bayan, N., C. Houssin, M. Chami, G. Leblon. 2003. Mycomembrane and S-layer: two important structures of *Corynebacterium glutamicum* cell envelope with promising biotechnology applications. J. Biotechnol. 104: 55-67.

Becker, J., C. Klopprogge, C. Wittmann. 2008. Metabolic responses to pyruvate kinase deletion in lysine producing *Corynebacterium glutamicum*. Microb. Cell. Fact. 7: 8.

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

Blombach, B., T. Riester, S. Wieschalka, C. Ziert, J. W. Youn, V. F. Wendisch, B. J. Eikmanns. 2011. *Corynebacterium glutamicum* tailored for efficient isobutanol production. Appl. Environ. Microbiol. 77: 3300-3310.

Blombach, B., and G. M. Seibold. 2010. Carbohydrate metabolism in *Corynebacterium glutamicum* and applications for the metabolic engineering of L-lysine production strains. Appl. Microbiol. Biotechnol. 86: 1313-1322.

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

Bourassa, L., and A. Camilli. 2009. Glycogen contributes to the environmental persistence and transmission of *Vibrio cholerae*. Mol. Microbiol. 72: 124-138.

Busuioc, M., K. Mackiewicz, B. A. Buttaro, P. J. Piggot. 2009. Role of intracellular polysaccharide in persistence of *Streptococcus mutans*. J. Bacteriol. 191: 7315-7322.

Carpinelli, J., R. Krämer, E. Agosin. 2006. Metabolic engineering of *Corynebacterium glutamicum* for trehalose overproduction: role of the TreYZ trehalose biosynthetic pathway. Appl. Environ. Microbiol. 72: 1949-1955.

Daffé, M. 2005. The cell envelope of Corynebacteria. In: Handbook of *Corynebacterium glutamicum*. Eggeling, L., and M. Bott (eds.). pp. 121-148. CRC Press, Boca Raton, FL. USA.

Dauvillée, D., I. S. Kinderf, Z. Li, B. Kosar-Hashemi, M. S. Samuel, L. Rampling, S. Ball, M. K. Morell. 2005. Role of the *Escherichia coli glgX* gene in glycogen metabolism. J. Bacteriol. 187: 1465-1473.

Dippel, R., T. Bergmiller, A. Böhm, W. Boos. 2005. The maltodextrin system of Escherichia coli: glycogen-derived endogenous induction and osmoregulation. J. Bacteriol. 187: 8332-8339.

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

Dover, L. G., A. M. Cerdeño-Tárraga, M. J. Pallen, J. Parkhill, G. S. Besra. 2004. Comparative cell wall core biosynthesis in the mycolated pathogens, *Mycobacterium tuberculosis* and *Corynebacterium diphtheriae*. FEMS Microbiol. Rev. 28: 225–250

Dower, W. J., J. F. Miller, C. W. Ragsdale. 1988. High efficiency transformation of *E. coli* by high voltage electroporation. Nucleic Acids Res. 16: 6127-6145.

Eikmanns, B. J., M. Metzger, D. Reinscheid, M. Kircher, H. Sahm. 1991. Amplification of three threonine biosynthesis genes in *Corynebacterium glutamicum* and its influence on carbon flux in different strains. Appl. Microbiol. Biotechnol. 34: 617-622.

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

Funke, G., A. von Graevenitz, J. E. Clarridge III, K. A. Bernard. 1997. Clinical microbiology of coryneform bacteria. Clin. Microbiol. Rev. 10: 125-159.

Gibbons, R. J., and B. Kapsimalis. 1963. Synthesis of intracellular iodophilic polysaccharide by *Streptococcus mitis*. Arch. Oral. Biol. 8: 319-329.

Green, M. R., and J. Sambrook. 2012. Molecular cloning: A laboratory manual, 4th edition. Cold Spring Harbor Press, Cold Spring Harbor, NY. USA

Gupta, R. K., S. S. Patterson, S. Ripp, M. L. Simpson, G. S. Sayler. 2003. Expression of the *Photorhabdus luminescens lux* genes (*luxA, B, C, D, and E*) in *Saccharomyces cerevisiae*. FEMS Yeast Res. 4: 305-313.

Hanahan, D. 1983. Studies on transformation of *Escherichia coli* with plasmids. J. Mol. Biol. 166: 557-580.

Hasegawa, S., M. Suda, K. Uematsu, Y. Natsuma, K. Hiraga, T. Jojima, M. Inui, H. Yukawa. 2013. Engineering of *Corynebacterium glutamicum* for high-yield L-valine production under oxygen deprivation conditions. Appl. Environ. Microbiol. 79: 1250-1257.

Hasegawa, S., K. Uematsu, Y. Natsuma, M. Suda, K. Hiraga, T. Jojima, M. Inui, H. Yukawa. 2012. Improvement of the redox balance increases L-valine production by *Corynebacterium glutamicum* under oxygen deprivation conditions. Appl. Environ. Microbiol. 78: 865-875.

Henrich, A., N. Kuhlmann, A. W. Eck, R. Krämer, G. M. Seibold. 2013. Maltose uptake by the novel ABC transport system MusEFGK$_2$I causes increased expression of *ptsG* in *Corynebacterium glutamicum*. J. Bacteriol. 195: 2573-2584.

Holme, T. 1957. Continuous culture studies on glycogen synthesis in *Escherichia coli* strain B. Acta Chem. Scand. 11: 763-775.

Holme, T., and H. Palmstierna. 1956a. Changes in glycogen and nitrogen-containing compounds in *Escherichia coli* B during growth in deficient media. I. Nitrogen and carbon starvation. Acta Chem. Scand. 10: 578-586.

Holme, T., and H. Palmstierna. 1956b. On the glycogen in *Escherichia coli B*; its synthesis and breakdown and its specific labeling with ^{14}C. Acta Chem. Scand. 10: 1557-1562.

Horn, S. 2011. Untersuchungen zum Glycogenabbau in *Corynebacterium glutamicum*. Bachelor thesis. Institute for Microbiology and Biotechnology, University of Ulm.

Ikeda, M., and S. Nakagawa. 2003. The *Corynebacterium glutamicum* genome: Features and impacts on biotechnological processes. Appl. Microbiol. Biotechnol. 62: 99-109.

Inui, M., H. Kawaguchi, S. Murakami, A. A. Vertès, H. Yukawa. 2004. Metabolic engineering of *Corynebacterium glutamicum* for fuel ethanol production under oxygen-deprivation conditions. J. Mol. Microbiol. Biotechnol. 8: 243-254.

Inui, M., S. Murakami, S. Okino, H. Kawaguchi, A. A. Vertès, H. Yukawa. 2004. Metabolic analysis of *Corynebacterium glutamicum* during lactate and succinate productions under oxygen deprivation conditions. J. Mol. Microbiol. Biotechnol. 7: 182-196.

Inui, M., M. Suda, S. Okino, H. Nonaka, L. G. Puskás, A. A. Vertès, H. Yukawa. 2007. Transcriptional profiling of *Corynebacterium glutamicum* metabolism during organic acid production under oxygen deprivation conditions. Microbiology. 153: 2491-2504.

Jetten, M. S., and A. J. Sinskey. 1995. Purification and properties of oxaloacetate decarboxylase from *Corynebacterium glutamicum*. A. van. Leeuw. J. Microb. 67: 221-227.

Jojima, T., M. Fujii, E. Mori, M. Inui, H. Yukawa. 2010. Engineering of sugar metabolism of *Corynebacterium glutamicum* for production of amino acid L-alanine under oxygen deprivation. Appl. Microbiol. Biot. 87: 159-165.

Kalinowski, J., B. Bathe, D. Bartels, N. Bischoff, M. Bott, A. Burkovski, N. Dusch, L. Eggeling, B. J. Eikmanns, L. Gaigalat, A. Goesmann, M. Hartmann, K. Huthmacher, R. Krämer, B. Linke, A. C. McHardy, F. Meyer, B. Mockel, W. Pfefferle, A. Pühler, D. A. Rey, C. Rückert, O. Rupp, H. Sahm, V. F. Wendisch, I. Wiegrabe, A. Tauch. 2003. The complete *Corynebacterium glutamicum* ATCC 13032 genome sequence and its impact on the production of L-aspartate-derived amino acids and vitamins. J. Biotechnol. 104: 5-25.

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

Kaur, D., M. E. Guerin, H. Škovierová, P. J. Brennan, M. Jackson. 2009. Biogenesis of the cell wall and other glycoconjugates of *Mycobacterium tuberculosis*. Adv. Appl. Microbiol. 69: 23-78

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

Kinoshita, S., S. Udaka, M. Shimono. 1957. Studies on the amino acid fermentation. Production of L-glutamic acid by various microorganisms. J. Gen. Appl. Microbiol. 3: 193-205.

Klauth, P., S. R. Pallerla, D. Vidaurre, C. Ralfs, V. F. Wendisch, S. M. Schoberth. 2006. Determination of soluble and granular inorganic polyphosphate in *Corynebacterium glutamicum*. Appl. Microbiol. Biotechnol. 72: 1099-1106.

Koch, D. J., C. Rückert, D. A. Rey, A. Mix, A. Pühler, J. Kalinowski. 2005. The role of the *ssu* and *seu* genes of *Corynebacterium glutamicum* ATCC 13032 in the utilization of sulfonates and sulfonate esters as sulfur sources. Appl. Environ. Microbiol. 71: 6104-6114.

Krause, F. S., A. Henrich, B. Blombach, R. Krämer, B. J. Eikmanns, G. M. Seibold. 2010. Increased glucose utilization in *Corynebacterium glutamicum* by use of maltose, and its application for the improvement of L-valine productivity. Appl. Environ. Microbiol. 76: 370-374

Kulis-Horn, R. K., M. Persicke, J. Kalinowski. 2014. Histidine biosynthesis, its regulation and biotechnological application in *Corynebacterium glutamicum*. Microb. Biotechnol. 7: 5-25.

Lambert, C., D. Weuster-Botz, R. Weichenhain, E. W. Kreutz, A. A. de Graaf, S. M. Schoberth. 2002. Monitoring of inorganic polyphosphate dynamics in *Corynebacterium glutamicum* using a novel oxygen sparger for real time ^{31}P *in vivo* NMR. Acta Biotechnol. 22: 245-260.

Laslo, T. 2013. D-arabitol metabolism in *Corynebacterium glutamicum*. Doctoral thesis. Institute for Microbiology and Biotechnology, University of Ulm.

Laslo, T., P. von Zaluskowski, C. Gabris, E. Lodd, C. Rückert, P. Dangel, J. Kalinowski, M. Auchter, G. M. Seibold, B. J. Eikmanns. 2012. Arabitol metabolism of *Corynebacterium glutamicum* and its regulation by AtlR. J. Bacteriol. 194: 941-955.

Leuchtenberger, W., K. Huthmacher, K. Drauz. 2005. Biotechnological production of amino acids and derivatives: current status and prospects. Appl. Microbiol. Biotechnol. 69: 1-8.

Liebl, W. 2005. *Corynebacterium* Taxonomy. In: Handbook of *Corynebacterium glutamicum*. Eggeling, L., and M. Bott (eds.). pp. 9-34. CRC Press, Boca Raton, FL. USA.

Liebl, W., A. Bayerl, B. Schein, U. Stillner and K. H. Schleifer. 1989. High efficiency electroporation of intact *Corynebacterium glutamicum* cells. FEMS Microbiol. Lett. 53: 299-303.

Lindner, S. N., S. Knebel, S. R. Pallerla, S. M. Schoberth, V. F. Wendisch. 2010. *Cg2091* encodes a polyphosphate/ATP-dependent glucokinase of *Corynebacterium glutamicum*. Appl. Microbiol. Biotechnol. 87: 703-713.

Litsanov, B., A. Kabus, M. Brocker, M. Bott. 2012. Efficient aerobic succinate production from glucose in minimal medium with *Corynebacterium glutamicum*. Microb. Biotechnol. 5: 116-128.

Martin, M. C., D. Schneider, C. J. Bruton, K. F. Chater, C. Hardisson. 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.

Matheron, C., A. M. Delort, G. Gaudet, E. Forano, T. Liptaj. 1998. ^{13}C and ^{1}H nuclear magnetic resonance study of glycogen futile cycling in strains of the genus *Fibrobacter*. Appl. Environ. Microbiol. 64: 74-81.

McMeechan, A., M. A. Lovell, T. A. Cogan, K. L. Marston, T. J. Humphrey, P. A. Barrow. 2005. Glycogen production by different *Salmonella enterica* serotypes: contribution of functional *glgC* to virulence, intestinal colonization and environmental survival. Microbiology. 151: 3969-3977.

Meiswinkel, T. M., D. Rittmann, S. N. Lindner, V. F. Wendisch. 2013. Crude glycerol-based production of amino acids and putrescine by *Corynebacterium glutamicum*. Bioresource Technol. 145: 254-258.

Micklinghoff, J. C., K. J. Breitinger, M. Schmidt, R. Geffers, B. J. Eikmanns, F. C. Bange. 2009. Role of the transcriptional regulator RamB (Rv0465c) in the control of the glyoxylate cycle in *Mycobacterium tuberculosis*. J. Bacteriol. 191: 7260-7269.

Mills, J. A., K. Motichka, M. Jucker, H. P. Wu, B. C. Uhlik, R. J. Stern, M. S. Scherman, V. D. Vissa, F. Pan, M. Kundu, Y. F. Ma, M. McNeil. 2004. Inactivation of the mycobacterial rhamnosyltransferase, which is needed for the formation of the arabinogalactan-peptidoglycan linker, leads to irreversible loss of viability. J. Biol. Chem. 279: 43540-43546.

Mimitsuka, T., H. Sawai, M. Hatsu, K. Yamada. 2007. Metabolic engineering of *Corynebacterium glutamicum* for cadaverine fermentation. Biosci. Biotech. Bioch. 71: 2130-2135.

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

Moreno-Bruna, B., E. Baroja-Fernández, F. J. Muñoz, A. Bastarrica-Berasategui, A. Zandueta-Criado, M. Rodriguez-López, I. Lasa, T. Akazawa, J. Pozueta-Romero. 2001. Adenosine diphosphate sugar pyrophosphatase prevents glycogen biosynthesis in *Escherichia coli*. Proc. Natl. Acad. Sci. USA. 98: 8128-8132.

Mülhardt, C. 2007. Expression systems. In The experimenter series: Molecular biology and genomics. Translation of: Experimentator: Molekularbiologie, 4th edition. Edited by C. Mülhardt; translated by E. W. Beese. pp. 203-206. Elsevier Academic Press. Boston, USA.

Mulder, E. G., M. H. Deinema, W. L. van Veen, L. P. Zevenhuizen. 1962. Polysaccharides, lipids and poly-β-hydroxybutyrate in microorganisms. Recl. Trav. Chim. Pay-B. 81: 797-809.

Nara, T., H. Samejima, S. Kinoshita. 1964. Effect of penicillin on amino acid fermentation. Agr. Biol. Chem. 28: 120-124.

Neijssel, O. M., E. T. Buurman M. J. Teixeira de Mattos. 1990. The role of futile cycles in the energetics of bacterial growth. Biochim. Biophys. Acta. 1018: 252-255.

Nikaido, H., I. D. Pokrovskaya, L. Reyes, A. K. Ganesan, J. A. Hall. 1994. Maltose transport system of Escherichia coli as a member of ABC transporters. In: Phosphate in microorganisms: cellular and molecular biology. A. Torriani, E. Yagil, and S. Silver (eds.). pp. 91–96. American Society for Microbiology, Washington, D.C. USA.

Nikel, P. I., M. Chavarría, E. Martínez-García, A. C. Taylor, V. de Lorenzo. 2013. Accumulation of inorganic polyphosphate enables stress endurance and catalytic vigour in *Pseudomonas putida* KT2440. Microb. Cell. Fact. 12: 50.

Nishimura, T., A. A. Vertès, Y. Shinoda, M. Inui, H. Yukawa. 2007. Anaerobic growth of *Corynebacterium glutamicum* using nitrate as a terminal electron acceptor. Appl. Microbiol. Biotechnol. 75: 889-897.

Okino, S., R. Noburyu, M. Suda, T. Jojima, M. Inui, H. Yukawa. 2008. An efficient succinic acid production process in a metabolically engineered *Corynebacterium glutamicum* strain. Appl. Microbiol. Biotechnol. 81: 459-464.

Okino, S., M. Suda, K. Fujikura, M. Inui, H. Yukawa. 2008. Production of L-lactic acid by *Corynebacterium glutamicum* under oxygen deprivation. Appl. Microbiol. Biotechnol. 78: 449-454.

Padilla, L., S. Morbach, R. Krämer, E. Agosin. 2004. Impact of heterologous expression of *Escherichia coli* UDP-glucose pyrophosphorylase on trehalose and glycogen synthesis in *Corynebacterium glutamicum*. Appl. Environ. Microbiol. 70: 3845-3854.

Padilla, L., R. Krämer, G. Stephanopoulos, E. Agosin. 2004. Overproduction of trehalose: Heterologous expression of *Escherichia coli* trehalose-6-phosphate synthase and trehalose-6-phosphate phosphatase in *Corynebacterium glutamicum*. Appl. Environ. Microbiol. 70: 370-376.

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

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

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

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

Paczia, N., A. Nilgen, T. Lehmann, J. Gätgens, W. Wiechert, S. Noack. 2012. Extensive exometabolome analysis reveals extended overflow metabolism in various microorganisms. Microb. Cell. Fact. 11: 122.

Radmacher, E., A. Vaitsikova, U. Burger, K. Krumbach, H. Sahm, L. Eggeling. 2002. Linking central metabolism with increased pathway flux: L-valine accumulation by *Corynebacterium glutamicum*. Appl. Environ. Microbiol. 68: 2246-2250.

Radoš, D., D. L. Turner, L. L. Fonseca, A. L. Carvalho, B. Blombach, B. J. Eikmanns, A. R. Neves, H. Santos. 2014. Carbon flux analysis by ^{13}C nuclear magnetic resonance to determine the effect of CO_2 on anaerobic succinate production by *Corynebacterium glutamicum*. Appl. Environ. Microbiol. 80: 3015-3024.

Ragel, P., S. Streb, R. Feil, M. Sahrawy, M. G. Annunziata, J. E. Lunn, S. Zeeman, Á. Mérida. 2013. Loss of starch granule initiation has a deleterious effect on the growth of *arabidopsis* plants due to an accumulation of ADP-glucose. Plant Physiol. 163: 75-85.

Romeo, T., J. Black, J. Preiss. 1990. Genetic regulation of glycogen biosynthesis 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 J. Preiss. 1989. Genetic regulation of glycogen biosynthesis in *Escherichia coli*: In vitro effects of cyclic AMP and guanosine 5´-diphosphate 3´-diphosphate and analysis of *in vivo* transcripts. J Bacteriol. 171: 2773-2782.

Russell, J. B. 2007. The energy spilling reactions of bacteria and other organisms. J. Mol. Microbiol. Biotechnol. 13: 1-11.

Sahm, H., L. Eggeling, B. J. Eikmanns, R. Krämer. 1996. Construction of L-lysine-, L-threonine-, and L-isoleucine-overproducing strains of *Corynebacterium glutamicum*. Ann. NY. Acad. Sci. 782: 25-39.

Sambou, T., P. Dinadayala, G. Stadthagen, N. Barilone, Y. Bordat, P. Constant, F. Levillain, O. Neyrolles, B. Gicquel, A. Lemassu, M. Daffé, M. Jackson. 2008. Capsular glucan and intracellular glycogen of *Mycobacterium tuberculosis*: Biosynthesis and impact on the persistence in mice. Mol. Microbiol. 70: 762-774.

Sasaki, M., T. Jojima, M. Inui, H. Yukawa. 2010. Xylitol production by recombinant *Corynebacterium glutamicum* under oxygen deprivation. Appl. Microbiol. Biotechnol. 86: 1057-1066.

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

Schinzel, R., and B. Nidetzky. 1999. Bacterial alpha-glucan phosphorylases. FEMS Microbiol. Lett. 171: 73-79.

Schneider, J., and V. F. Wendisch. 2010. Putrescine production by engineered *Corynebacterium glutamicum*. Appl. Microbiol. Biotechnol. 88: 859-868.

Schneider, J., K. Niermann, V. F. Wendisch. 2011. Production of the amino acids L-glutamate, L-lysine, L-ornithine and L-arginine from arabinose by recombinant *Corynebacterium glutamicum*. J. Biotechnol. 154: 191-198.

Seibold, G.M., K. J. Breitinger, R. Kempkes, L. Both, M. Krämer, S. Dempf, B. J. Eikmanns. 2011. The *glgB*-encoded glycogen branching enzyme is essential for glycogen accumulation in *Corynebacterium glutamicum*. Microbiology. 157: 3243-3251.

Seibold, G. M., S. Dempf, J. Schreiner, B. J. Eikmanns. 2007. Glycogen formation in *Corynebacterium glutamicum* and role of ADP-glucose pyrophosphorylase. Microbiology. 153: 1275-1285.

Seibold, G. M., and B. J. Eikmanns. 2007. The *glgX* gene product of *Corynebacterium glutamicum* is required for glycogen degradation and for fast adaptation to hyperosmotic stress. Microbiology. 153: 2212-2220.

Seibold, G. M., C. T. Hagmann, M. Schietzel, D. Emer, M. Auchter, J. Schreiner, B. J. Eikmanns. 2010. The transcriptional regulators RamA and RamB are involved in the regulation of glycogen synthesis in *Corynebacterium glutamicum*. Microbiology. 156: 1256-1263.

Seibold, G. M., M. Wurst, B. J. Eikmanns. 2009. Roles of maltodextrin and glycogen phosphorylases in maltose utilization and glycogen metabolism in *Corynebacterium glutamicum*. Microbiology. 155: 347-358.

Seibold, G. M., and B. J. Eikmanns. 2013. Inactivation of the phosphoglucomutase gene *pgm* in *Corynebacterium glutamicum* affects cell shape and glycogen metabolism. Biosci. Rep. 33: 645-654.

Shiio, I., S. I. Otsuka, M. Takahashi. 1962. Effect of biotin on the bacterial formation of glutamic acid. I. Glutamate formation and cellular permeability of amino acids. J. Biochem. 51: 56-62.

Silberbach, M., A. Hüser, J. Kalinowski, A. Pühler, B. Walter, R. Krämer, A. Burkovski. 2005. DNA microarray analysis of the nitrogen starvation response of *Corynebacterium glutamicum*. J. Biotechnol. 119: 357-367.

Singh, R., M. Singh, G. Arora, S. Kumar, P. Tiwari, S. Kidwai. 2013. Polyphosphate deficiency in *Mycobacterium tuberculosis* is associated with enhanced drug susceptibility and impaired growth in guinea pigs. J. Bacteriol. 195: 2839-2851.

Smith, K., K. Cho, J. Liao. 2010. Engineering *Corynebacterium glutamicum* for isobutanol production. Appl. Microbiol. Biotechnol. 87: 1045-1055.

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

Suzuki, E., H. Ohkawa, K. Moriya, T. Matsubara, Y. Nagaike, I. Iwasaki, S. Fujiwara, M. Tsuzuki, Y. Nakamura. 2010. Carbohydrate metabolism in mutants of the cyanobacterium *Synechococcus elongatus* PCC 7942 defective in glycogen synthesis. Appl. Environ. Microbiol. 76: 3153-3159.

Takeno, S., J. Ohnishi, T. Komatsu, T. Masaki, K. Sen, M. Ikeda. 2007. Anaerobic growth and potential for amino acid production by nitrate respiration in *Corynebacterium glutamicum*. Appl. Microbiol. Biotechnol. 75: 1173-1182.

Takinami, K., H. Yoshii, H. Tsuri, H. Okada. 1965. Biochemical effects of fatty acid and its derivatives on L-glutamic acid fermentation. Part III. Biotin-tween 60 relationship in the accumulation of L-glutamic acid and the growth of *Brevibacterium lactofermentum*. Agric. Biol. Chem. 29: 351-359.

Tauch, A., O. Kirchner, B. Löffler, S. Götker, A. Pühler and J. Kalinowski. 2002. Efficient electrotransformation of *Corynebacterium diphtheriae* with a mini-replicon derived from *Corynebacterium glutamicum* plasmid pGA. Curr. Microbiol. 45: 362-367.

Tropis, M., X. Meniche, A. Wolf, H. Gebhardt, S. Strelkov, M. Chami, D. Schomburg, R. Krämer, S. Morbach, M. Daffé. 2005. The crucial role of trehalose and structurally related oligosaccharides in the biosynthesis and transfer of mycolic acids in Corynebacterineae. J. Biol. Chem. 280: 26573–26585.

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

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

Vasicová, P., Z. Abrhámová, J. Nesvera, M. Pátek, H. Sahm, B. Eikmanns. 1998. Integrative and autonomously replicating vectors for analysis of promoters in *Corynebacterium glutamicum*. Biotechnol. Tech. 12: 743-746.

Wang, L., and M. J. Wise. 2011. Glycogen with short average chain length enhances bacterial durability. Naturwissenschaften. 98: 719-729

Wendisch, V. F., M. Bott, B. J. Eikmanns. 2006a. Metabolic engineering of *Escherichia coli* and *Corynebacterium glutamicum* for biotechnological production of organic acids and amino acids. Curr. Opin. Microbiol. 9: 268-274.

Wendisch, V. F., M. Bott, J. Kalinowski, M. Oldiges, W. Wiechert. 2006. Emerging *Corynebacterium glutamicum* systems biology. J. Biotechnol. 124: 74-92.

Wieschalka, S., B. Blombach, B. J. Eikmanns. 2012. Engineering *Corynebacterium glutamicum* for the production of pyruvate. Appl. Microbiol. Biotechnol. 94: 449-459.

Wieschalka, S., B. Blombach, M. Bott, B. J. Eikmanns. 2013. Bio-based production of organic acids with *Corynebacterium glutamicum*. Microb Biotechnol. 6: 87-102.

Wilson, W. A., P. J. Roach, M. Montero, E. Baroja-Fernandez, F. J. Munoz, G. Eydallin, A. M. Viale, J. Pozueta-Romero. 2010. Regulation of glycogen metabolism in yeast and bacteria. FEMS Microbiol. Rev. 34. 952–985.

Wolf, A., R. Krämer, S. Morbach. 2003. Three pathways for trehalose metabolism in *Corynebacterium glutamicum* ATCC13032 and their significance in response to osmotic stress. Mol. Microbiol. 49: 1119-1134.

Woo, H. M., S. Noack, G. M. Seibold, S. Willbold, B. J. Eikmanns, M. Bott. 2010. Link between phosphate starvation and glycogen metabolism in *Corynebacterium glutamicum*, revealed by metabolomics. Appl. Environ. Microbiol. 76: 6910-6919.

Yamamoto, S., W. Gunji, H. Suzuki, H. Toda, M. Suda, T. Jojima, M. Inui, H. Yukawa. 2012. Overexpression of genes encoding glycolytic enzymes in *Corynebacterium glutamicum* enhances glucose metabolism and alanine production under oxygen deprivation conditions. Appl. Environ. Microbiol. 78: 4447-4457.

Yamamotoya, T., H. Dose, Z. Tian, A. Fauré, Y. Toya, M. Honma, K. Igarashi, K. Nakahigashi, T. Soga, H. Mori, H. Matsuno. 2012. Glycogen is the primary source of glucose during the lag phase of *E. coli* proliferation. Biochim. Biophys. Acta. 1824: 1442-1448.

Yasuda, K., T. Jojima, M. Suda, S. Okino, M. Inui, H. Yukawa. 2007. Analyses of the acetate-producing pathways in *Corynebacterium glutamicum* under oxygen-deprived conditions. Appl. Microbiol. Biotechnol. 77: 853-860.

Zevenhuizen, L. P. 1966. Formation and function of the glycogen-like polysaccharide of *Arthrobacter*. A. van. Leeuw. J. Microb. 32: 356-372.

Abbreviations

A	adenine
bp	base pairs
°C	degree Celsius
C	cytosine
Ca	Calcium
cAMP	cyclic adenosine monophosphate
CAT	chloramphenicol acetyltransferase
cDNA	complementary deoxyribonucleic acid
cm	centimetre
CO_2	carbon dioxide
CoA	coenzyme A
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dw	dry weight
e.g.	exempli gratia (= for example)
et al.	et alii (= and others)
F	farad
fL	femtolitre
g	gram
G	guanine
h	hour
HPLC	high performance liquid chromatography
i.e.	id est (= that is)
k	kilo
kDa	kilodalton
L	litre
µ	growth rate
µF	microfarad
µg	microgram
µL	microlitre
µm	micrometre
M	molar (mol/l)
mcs	multiple cloning site
mg	milligram
Mg	magnesium
min	minute
mL	millilitre
mm	millimetre

mM	millimolar
mol	mole
mRNA	messenger ribonucleic acid
N_2	nitrogen
NAD^+	nicotinamide adenine dinucleotide (oxidized)
$NADH/H^+$	nicotinamide adenine dinucleotide (reduced)
$NADP^+$	nicotinamide adenine dinucleotide phosphate (oxidized)
$NADPH/H^+$	nicotinamide adenine dinucleotide phosphate (reduced)
ng	nanogram
nm	nanometre
%	per cent
Ω	Ohm (electric resistance)
OD_{600}	optical density at 600 nm wavelength
pH	negative decade logarithm of the proton concentration
RLU	relative light units
RNA	ribonucleic acid
RNase	ribonuclease
RP	reversed phase
rpm	rounds per minute
rRNA	ribosomal ribonucleic acid
RT	reverse transcriptase
T	thymine
TL	translational start
TS	transcriptional start
U	unit
UV	ultraviolet
V	volt
v	volume
w	weight
x g	acceleration in multiples of acceleration of gravity

Publications

Laslo, T., P. von Zaluskowski, C. Gabris, E. Lodd, C. Rückert, P. Dangel, J. Kalinowski, M. Auchter, G. Seibold, BJ. Eikmanns. 2012. Arabitol metabolism of *Corynebacterium glutamicum* and its regulation by AtlR. J. Bacteriol. 194: 941-955.

Contributions of others to this work

The construction of the single deletion mutants *C. glutamicum* Δ*glgC*, *C. glutamicum* Δ*glgX* and *C. glutamicum* Δ*glgP* was done in collaboration with Dr. Verena Bückle-Vallant and Dr. Katrin Breitinger. The construction of the double deletion mutant *C. glutamicum* Δ*glgX*Δ*glgP* was done in collaboration with Sabrina Horn. The construction of *C. glutamicum* Δ*glgB* and *C. glutamicum* Δ*malP* was done in collaboration with Dr. Gerd Seibold. The construction and analysis of growth and glycogen levels of *C. glutamicum* Δ*glgA* was done in collaboration with Alissa Grässer and Nina Rettich. The construction of *C. glutamicum* Δ*glgX*Δ*malP* was done in collaboration with Melanie Leitte.

The investigation of OD_{600}, glycogen levels and production behaviour of *C. glutamicum* WT, *C. glutamicum* Δ*glgC* and *C. glutamicum* Δ*glgX*Δ*malP*Δ*glgP* under anaerobic conditions was done in collaboration with Elisa Heuel.

The experiments on the influence of nitrogen and phosphate availability were done in collaboration with Kristina Ruhland.

Semi-quantitative RT-PCR experiments were performed in collaboration with Kristina Ruhland and Dr. Christian Rückert at the Center for Biotechnology (CeBiTec) in Bielefeld.

Investigations on the connection of disturbances in the glycogen metabolism and growth under pH stress were done in collaboration with Alissa Grässer.

Promoter analyses of the *glgC*, *glgA* and *glgX* genes were performed in collaboration with Leonie Schnell.

The experiments on total cell number and biovolume of *C. glutamicum* WT, *C. glutamicum* Δ*glgC* and *C. glutamicum* Δ*glgX* under anaerobic conditions and on the intracellular concentrations of energetic parameters were done at the Research Center Jülich (Forschungszentrum Jülich) in collaboration with Nina Pfelzer.

Danksagung

Aus Gründen des Datenschutzes wurde die Danksagung auf den Seiten 123 und 124 entfernt.

Curriculum vitae

Aus Gründen des Datenschutzes wurde der Curriculum vitae auf den Seiten 125 und 126 entfernt.

Erklärung

Ich versichere, dass ich die Arbeit selbständig und ohne Benutzung anderer als die hier angegebenen Quellen angefertigt habe. Alle Ausführungen, die wörtlich oder sinngemäß übernommen wurden, sind mit bestem Wissen und Gewissen als solche gekennzeichnet.

Weiterhin erkläre ich, dass die vorliegende Arbeit weder vollständig noch in Auszügen einer anderen Fakultät mit dem Ziel vorgelegt wurde, einen akademischen Titel zu erwerben. Ich bewerbe mich hiermit erstmalig um den Doktorgrad der Naturwissenschaften der Universität Ulm.

Ulm, Mai 2014

Philipp Rola Alexander von Zaluskowski