Characterization of Maltose and Trehalose Transport in *Corynebacterium glutamicum*

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“Observation is a passive science,

experimentation an active one.”

Claude Bernard

Kurzzusammenfassung

Zur Produktion von Aminosäuren mit dem kommerziell bedeutsamen Bakterium *Corynebacterium glutamicum* kann aus Stärkehydrolysat gewonnene Maltose als gute und günstige Kohlenstoffquelle dienen. In der vorliegenden Arbeit wurde das Maltose/Maltodextrin Aufnahme-System MusIFGK_2-E von *C. glutamicum* identifiziert und charakterisiert; ein ABC Transporter mit einem K_m von 1,2 ± 0,2 µM und V_{max} von 26,2 ± 1,0 nmol/(min*mg TG). Es konnte gezeigt werden, dass das System kein Operon bildet und dass es von dem transkriptionellen Regulator RamA reguliert wird. Zusätzlich konnte eine regulatorische Verbindung zur Phosphotransferase System vermittelten Glukose Verstoffwechslung aufgezeigt werden. Weitere Analysen identifizierten das Protein MusI, welches zwar für die Aufnahme von Maltose essentiell, dessen Funktion aber noch unbekannt ist.

Neben der Bedeutung als Aminosäureproduzent ist *C. glutamicum* ein wichtiger Modellorganismus für die Zellwand und deren Synthese innerhalb der *Corynebacterineae*. In diesem Zusammenhang spielt Trehalose als Komponente von Mycolaten, wichtigen Bausteinen der Zellwand von Corynebakterien and Mycobakterien, eine bedeutende Rolle. In dieser Arbeit konnte zum ersten Mal gezeigt werden, dass *C. glutamicum*, im Gegensatz zu bereits publizierten Daten, mit einem Trehalose Aufnahme-System ausgestattet ist. Die Aufnahme wird über den ABC Transporter TusFGK_2-E mit einem K_m von 0,16 ± 0,02 µM und V_{max} von 2,5 ± 0,1 nmol/(min*mg TG) vermittelt. Trehalose kann anschließend durch TreS in Maltose umgewandelt werden und dann über den Maltose Abbauweg verstoffwechselt werden. Beachtenswerterweise konnte in dieser Arbeit ein zweiter Abbauweg für Trehalose / Maltose identifiziert werden. Dieser Weg beinhaltet die TreS vermittelte Umwandlung von Trehalose in Maltose, TreX vermittelte Maltose-1-Phosphate und anschließende α-Glucan Synthese durch GlgE, vergleichbar zu einem Abbauweg in *M. tuberculosis* (Kalscheuer *et al*., 2010a).

Diese Ergebnisse stellten die Hypothese von Tropis *et al*., 2005 in Frage, dass man zur Synthese von Trehalose-Mycolaten Biosynthese und Export von Trehalose benötigt. Mit Hilfe einer vierfach-Deletionsmutante, die weder fähig ist Trehalose zu synthetisieren noch aufzunehmen, konnte gezeigt werden, dass die Synthese von Trehalose-Mycolaten durch externe Zugabe des Zuckers, auch ohne die vorherige Aufnahme von Trehalose möglich ist. Dies zeigte dass die Trehalose Export Hypothese weiterhin Gültigkeit besitzt und dass das Trehalose Export-System von *C. glutamicum* noch zu identifizieren ist.

Abstract

For the production of amino acids with the commercially important Gram-positive bacterium *Corynebacterium glutamicum* maltose derived from starch hydrolysate can serve as a good and cheap carbon source. The present study identified and characterized the maltose/maltodextrin uptake system MusIFGK_2-E of *C. glutamicum* being an ABC transport system with a K_m of 1.2 ± 0.2 µM and a V_{max} of 26.2 ± 1.0 nmol/(min*mg cdm). Analyses showed that the system is not transcribed in an operon and that it is regulated by the transcriptional regulator RamA. In addition a regulatory connection to phosphotransferase system mediated glucose utilization was found. Further it was shown that the maltose uptake system possesses an additional protein MusI with unknown function that is important for the uptake of maltose.

Besides being an important amino acid producer, *C. glutamicum* is a major model organism for the cell envelope and its synthesis of the *Corynebacterineae*. In this context trehalose plays a crucial role as component of mycolates, important building blocks of the cell wall of corynebacteria and mycobacteria. This study showed for the first time that *C. glutamicum*, in contradiction to previously published data, is equipped with an uptake system for trehalose and is further capable to use trehalose for growth. The uptake of trehalose is mediated by the ABC transport system TusFGK_2-E with a K_m of 0.16 ± 0.02 µM and a V_{max} of 2.5 ± 0.1 nmol/(min*mg cdm). Trehalose can be utilized for growth via TreS mediated conversion into maltose and further degradation by the known maltose pathway. Notably, this study revealed a second pathway for the utilization of trehalose / maltose involving TreS mediated conversion of trehalose to maltose, TreX mediated maltose-1-phosphat formation and α-glucan formation by GlgE a pathway similar to the one found in *M. tuberculosis* (Kalscheuer *et al*., 2010a).

These results challenged the hypothesis of Tropis *et al*., 2005 that for the synthesis of trehalose mycolates biosynthesis and export of trehalose is needed. But with the help of a quadruple-mutant, that is neither able to synthesize nor to import trehalose, it was shown that the formation of trehalose mycolates is possible without the uptake of externally present trehalose leaving the trehalose export hypothesis valid and the trehalose export system of *C. glutamicum* to be identified.

Abbreviations

ABC	ATP-binding cassette
ADP	Adenosine 5'-diphosphate
AGM	Arabinogalactan mycolate
ATP	Adenosine 5'-triphosphate
ATCC	American Type Culture Collection
b	Bases
cAMP	Cyclic Adenosin Monophosphate
cdm	Cell dry matter
GMM	Glucose monomycolate
h	Hours
HPLC	High performance liquid chromotography
IPTG	Isopropyl β-D-1-thiogalactopyranoside
Kb	Kilo basepares
K_m	Michaelis constant
KPi	Potassium phosphate buffer
LB	Luria-Bertani
M	Molar
mM	Millimolar
OD	Optical density
OPA	Ortho-phthaldialdehyde
ORF	Open reading frame
PEP	Phosphoenolpyruvate
PCR	Polymerase chain reaction
PP-pathway	Pentose phosphate pathway
PTS	Phosphotransferase System
TDM	Trehalose dimycolate
TLC	Thin-layer chromatography
TM	*C. glutamicum* spontaneous-mutant capable to grow on trehalose
TMM	Trehalose monomycolate
TY	Tryptone Yeast (complex medium)
U	Units
V_{max}	Maximum velocity
(v/v)	Volume per volume
(w/v)	Weight per volume
WT	Wild type (*C. glutamicum* ATCC 13032)

1 Introduction

1.1 *Corynebacterium glutamicum*

The Gram-positive GC-rich bacterium *Corynebacterium glutamicum* was isolated in 1957 by Udaka und Kinoshita (Kinoshita *et al.*, 1957). *C. glutamicum* is a non-pathogenic member of the *Corynebacteriaceae*, who form together with the families *Mycobacteriaceae*, *Nocardiaceae*, *Gordoniaceae*, *Dietziaceae* und *Tsukamurellaceae* a suborder of the *Actinomycetales*, named *Corynebacterianeae* (Stackebrandt *et al*., 1997). Today *C. glutamicum* is commercially used for the industrial production of amino acids reaching an annual production of more than two million tons in 2005, mainly L-glutamic acid and L-lysine (Leuchtenberger *et al*., 2005).

Besides being an important amino acid producer, *C. glutamicum* is a major model organism for the cell envelope and its synthesis of the suborder *Corynebacterianeae*, including pathogenic members such as *Mycobacterium tuberculosis*, *Mycobacterium leprae* and *Corynebacterium diphtheriae*. Moreover, the 3.3 Mb genome of *C. glutamicum* was completely sequenced by independent research groups in Europe and Japan (Kalinowski *et al*., 2003; Ikeda and Nakagawa, 2003), making the organism a good target for genome wide genetic research.

1.2 Cell wall composition of corynebacteria

Members of the suborder of the *Corynebacterianeae* such as corynebacteria and mycobacteria show a unique cell envelope design which shows major differences compared to other Gram-positive bacteria. Their cell envelope comprises not only a peptidoglycan layer, with some additional polysaccharides or polyol phosphate polymers like in other gram-positive bacteria, but also an outer membrane which is typical for Gram-negative bacteria (Fig. 1). This additional membrane contains extractable lipids and long-chain mycolic acids that are covalently linked to peptidoglycan via a unique polysaccharide arabinogalactan network (Brennan & Nikaido, 1995). The innermost layer is the plasma membrane. In *C. glutamicum* it consists almost exclusively of phospholipids with negatively charged head-groups, namely phosphatidylglycerol, di-phosphatidylglycerol (cardiolipin), phosphatidylinositol and phosphatidylinositol mannosides. These lipids posses to over 90% palmitic ($C_{16:0}$) and oleic ($C_{18:1}$) acids as fatty acid chains (Hoischen & Krämer, 1990; Özcan *et al*., 2007). Adjacent to the plasma membrane a peptidoglycan layer, a common feature of Gram-positive bacteria, can be found forming the first layer of the cell wall skeleton. In *Corynebacterianeae* this layer is composed of β-1,4-linked N-acetylglucosamine and N-acetyl muramic acid residues linked

to tri- or tetrapeptides such as L-Ala-D-Glu-*meso*-diaminopimelic acid or L-Ala-D-Glu-*meso*-diaminopimelic acid-D-Ala (Schleifer & Kandler, 1972).

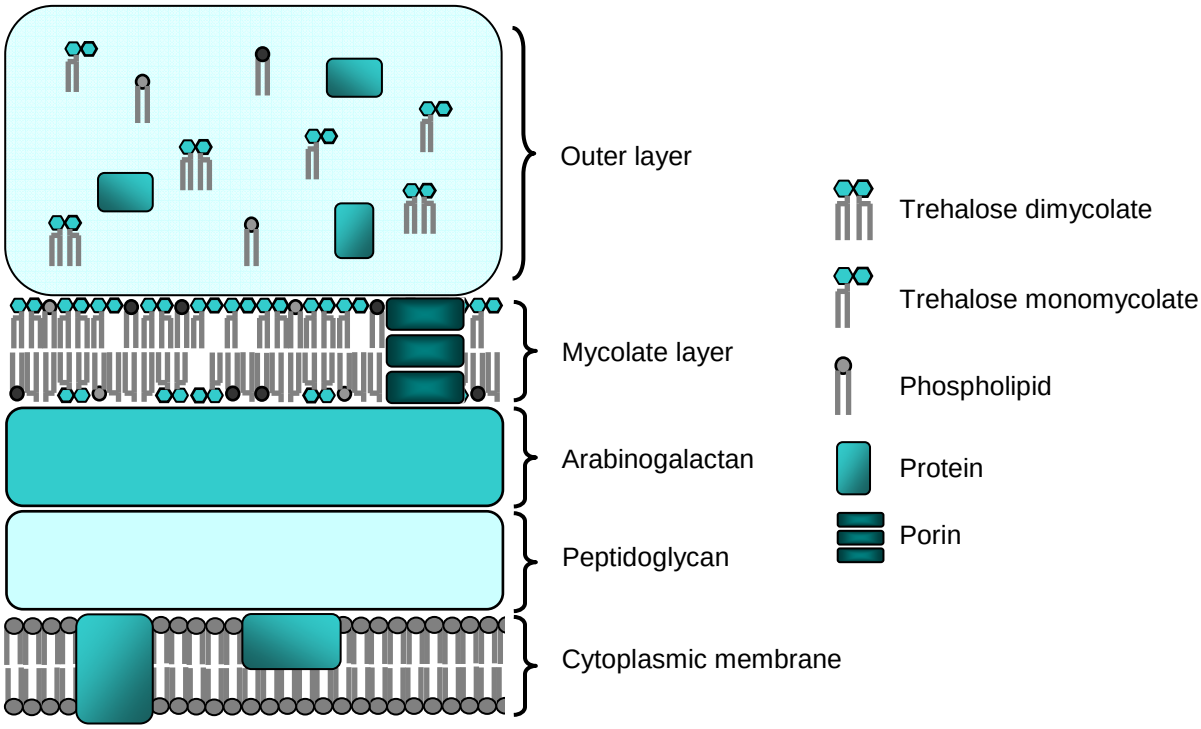

Figure 1: Cell wall structure of *Corynebacterianeae* according to Puech *et al*., 2001.

The heteropolysaccharide arabinogalactan forms the second layer, which is composed of a linear alternating β-D-galactofuranosyl backbone connected to a 3,5-branched α-D-arabinofuranosyl structure. The galactan domain of this layer is attached to peptidoglycan via a special “linker unit” and its arabinan domain is linked to mycolic acids, forming the mycolyl-arabinogalactan-peptidoglycan (mAGP) complex (Puech *et al*., 2001; Seidel *et al*., 2007).

Mycolic acids are 2-alkyl-branched, 3-hydroxylated long chain fatty acids which represent major and specific constituents of the third layer of the cell wall skeleton, the mycolate layer. The number of carbon atoms and the degree of desaturation of main and lateral chains vary according to the genus. In mycobacteria the fatty acid chains are very long ($C_{60\text{-}90}$), whereas the alkyl-chain in norcadia and corynebacteria is shorter (norcadomycolic acids $C_{44\text{-}60}$; corynomycolic acids $C_{22\text{-}36}$) (De Briel *et al*., 1992; Barry *et al*., 1998; Rafidinarivo *et al.*, 2009).

While this additional barrier in Gram-negative bacteria is a typical bilayer of phospholipid and lipopolysaccharide, in mycobacteria and corynebacteria the cell-wall-linked mycolates and corynomycolates certainly participate in this barrier since the disruption of genes that code for mycoloyltransferases causes a decrease in the amount of cell-wall-bound

mycolates and affects the permeability of the envelope of the mutants (Puech *et al*., 2000). Electron microscopy of freeze-fractured preparations and chemical analyses indicated that the inner part of the mycolate layer consists mainly of arabinogalactan mycolate and lesser amounts of extractable mycolate (Puech *et al*., 2001). Parts of the extractable mycolates are trehalose monomycolate (TMM) and trehalose dimycolate (TDM), which derive from the esterification of one or two mycolic acids with a molecule trehalose (Shimakata & Minatogawa, 2000; Tropis *et al*., 2005). The outer layer consists almost exclusively of these mycolates (TMM; TDM) (Daffé & Draper, 1998; Puech *et al*., 2001). Recently, Hoffmann and co-workers found with the help of cryo-electron tomography and the investigation of frozen-hydrated cryosections of *Mycobacterium smegmatis*, *Myobacterium bovis* and *C. glutamicum* that the outermost layer, the mycolate layer, is a morphologically symmetrical lipid bilayer (Hoffmann *et al*., 2008). This stands in contrast to the, so far preferred, model that the mycolate layer is asymmetrical.

Analogue to the outer membrane in Gram-negative bacteria, the mycolate layer of mycobacteria is thought to be responsible for the low permeability of the cell envelope (Brennan & Nikaido, 1995). To facilitate exchange of substances across this barrier, channel-forming proteins, so-called porins, such as PorA are necessary to allow the passage of hydrophilic substances (Costa-Riu *et al*., 2003; Hünten *et al*., 2005 a/b; Ziegler *et al*., 2008).

On the surface of this membrane-like structure corynebacteria posses an outer layer which is formed of up to 90% of polysaccharides, composed of glucose, arabinose and various lipids, mostly TDM and TMM, but also phospholipids such as phosphatidylinositol mannosides and phosphatidylglycerol (Puech *et al*., 2001).

1.3 Trehalose: An important component of the cell wall of *C. glutamicum*

It has been shown that mycolates are a crucial part of the cell envelope of Gram-positive bacteria belonging to the suborder of the *Corynebacterianeae*. These mycolates are formed from trehalose esterified by one or two mycolic acids and mycolic acids covalently linked to arabinogalactan (Brennan & Nikaido, 1995). As trehalose is involved not only in the synthesis of TMM and TDM, but also in the formation of arabinogalactan mycolate (AGM), trehalose was thought to be essential for these three components of the mycolate layer (Shimakata & Minatogawa, 2000).

In mycobacteria, the mycolate layer is the part of the cell envelope that causes the high resistance of these bacteria to most common antibiotics e.g. in *M. tuberculosis,* the causal agent of tuberculosis. Furthermore, trehalose dimycolate, also known as cord factor, plays a role in the persistence of this pathogenic bacterium in the host cell, presumably by

inhibiting the fusion between lysosomes and phagosomes containing the bacteria (Crowe *et al*., 1994). This makes it important to study the role of trehalose thoroughly in its close relative *C. glutamicum*, possibly opening new opportunities for drug development.

1.4 Trehalose synthesis in *C. glutamicum*

In *C. glutamicum* three different trehalose synthesis pathways have been identified TreYZ, OtsAB and TreS (Fig. 2). As only one of these pathways is present in most bacteria this fact underlines the importance of trehalose in the general physiology of mycobacteria and corynebacteria, e.g. as a crucial part of the cell envelope and during stress adaptation under certain conditions (Gebhardt, 2005; Tropis *et al*., 2005).

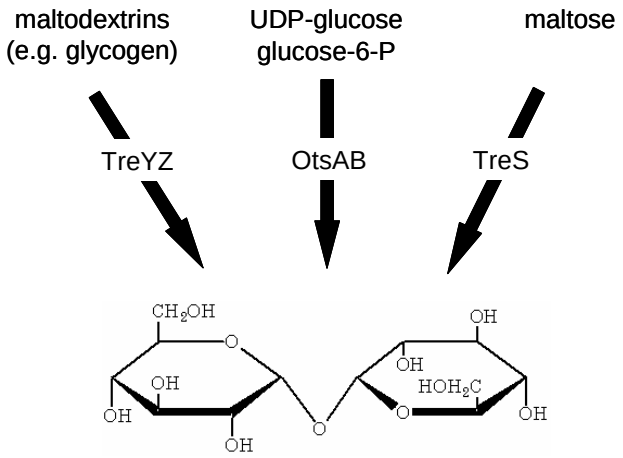

Figure 2: Trehalose synthesis pathways in *C. glutamicum*, according to Wolf *et al.*, 2003.

In *C. glutamicum* the TreYZ-pathway is the main trehalose synthesis pathway (Tzvetkov *et al*., 2003; Wolf *et al.*, 2003). It is responsible for the synthesis and the accumulation of trehalose as a response to hyperosmotic stress. In a two step reaction the terminal maltosyl-residue of maltodextrin is converted via the maltooligosyltrehalose synthase (TreY) into a trehalosyl unit which is then cut off by a maltooligosyltrehalose hydrolase (TreZ) leading to trehalose (Maruta *et al.*, 1996 a/b/c; Kim *et al*., 2000). The second pathway starts with the condensation of UDP-glucose and glucose-6-phosphate by the osmotically regulated trehalose synthesis (Ots) A enzyme yielding trehalose-6-phosphate which is then dephosphorylated to trehalose by OtsB (Argüelles, 2000). For the OtsAB-pathway in *Corynebacterium matruchotii* it was discussed by Shimakata and Minatogawa (2000) that the production of trehalose-6-phosphate by OtsA is important for the synthesis of trehalose monomycolate, an important component of the cell envelope. This conclusion seems only to be true for *C. matruchotii*, since it has been shown for *C. glutamicum* that a

strain deleted in the *otsA* gene was able to synthesize trehalose mycolate (Wolf *et al*., 2003). The OtsAB-pathway is more important for the synthesis of trehalose as compatible solute after a hyperosmotic shock, when the cells face carbon- and nitrogen-limitation at the same time (Gebhardt, 2005). This is underlined by the fact that the *otsA* gene is upregulated five-fold after an osmotic up-shift (Wolf *et al*., 2003).

The third way to synthesize trehalose is the TreS-pathway that is a one step reaction in which the trehalose synthase (TreS) catalyzes the conversion of maltose into trehalose. In *C. glutamicum* TreS is thought to be a possible substitute for a missing trehalase, since it has been shown in *in vitro* assays that it catalyzes maltose synthesis from trehalose and therefore, it can be important for the recycling of trehalose after an osmotic up-shift (Wolf *et al*., 2003).

1.5 Trehalose transport

For *C. glutamicum* it has been published that there is no uptake system present for trehalose (Gebhardt, 2005). This has been concluded by two observations / experiments. At first growth of *C. glutamicum* monitored for 10 h with trehalose as sole carbon source revealed that it is not able to utilize the disaccharide for growth (Wolf, 2002). A second approach measuring the uptake of [^{14}C]-trehalose strengthened the hypothesis that trehalose can not be taken up by *C. glutamicum*. A mutant deficient in trehalose synthesis (*C. glutamicum* Δ*otsA* Δ*treS* Δ*treY*) was able to incorporate external [^{14}C]-trehalose in TMMs, so that radioactivity was only detected in the cell wall , whereas there was no uptake into the cytoplasm detected, showing that the synthesis of TMM takes place in the cell envelope (Gebhardt, 2005). When the respective triple-mutant was supplemented with external trehalose, the cells were able to synthesize trehalose monomycolate, showing that external trehalose is important for mycolate synthesis in *C. glutamicum*. Further experiments revealed that the triple-mutant is also capable of using other sugars (glucose, maltose or maltotriose) for the synthesis of mycolated glycolipids. Thus it was stated that in principle mycolate synthesis is generally possible if an α-glucosyl-containing sugar is present in the medium (Tropis *et al*., 2005). From these experiments it was concluded that *C. glutamicum* must be equipped with a trehalose export system that facilitates the transport of trehalose across the plasma membrane, as trehalose is produced in the cytoplasm and the esterification with mycolic acids seems to take place in the cell envelope (Tropis *et al*., 2005).

Escherichia coli synthesizes internal trehalose as an osmoprotectant under high osmolarity, independent of the carbon source, and is also able to utilize trehalose as carbon source both under low and high osmolality (Boos *et al*., 1990). In *E. coli* the uptake of trehalose is facilitated by a phosphotransferase system (PTS) leading to trehalose-6-

phosphate (Boos *et al*., 1990). It continues with hydrolysis to trehalose and proceeds by splitting trehalose by an amylotrehalase, releasing one glucose residue with the simultaneous transfer of the other to a polysaccharide acceptor (Boos *et al*., 1990). In this organism the trehalose-specific permease (EII^{Tre}) is a permease of the EIIBC domain type that lacks a covalently bound EIIA domain. Instead, enzyme II^{Tre}-mediated phosphorylation of trehalose requires the activity of enzyme IIA^{Glc}, a component of the major glucose transport system (Klein *et al*., 1995). In addition to the uptake and the subsequent degradation of trehalose in the cytoplasm, *E. coli* also harbors a periplasmic trehalase TreA that is induced under high osmolarity to ensure the utilization of trehalose by hydrolysis to glucose and subsequent uptake via PTS. Under these conditions both uptake and internal hydrolysis of trehalose are turned off (Boos *et al*., 1987; Boos *et al*., 1990).

Phosphotransferase systems that facilitate the transport of trehalose have been found in several organisms including *Bacillus subtilis*, *Pseudomonas fluorescens*, *Salmonella typhimurium, Vibrio parahaemolyticus* and *Spiroplasma citri* (Kubota *et al*., 1979; Postma *et al*., 1986; Schöck & Dahl, 1996; Matthijs *et al*., 2000; André *et al*., 2003). In all those organisms the uptake of trehalose is tightly connected to other sugar import systems through common domains. The Gram-negative soil bacterium *Sinorhizobium meliloti* takes up trehalose via an ABC transport system with a trehalose/maltose-binding protein (Jensen *et al*., 2002). Such systems can also be found in *Streptomyces reticuli* and hyperthermophilic archaea such as *Thermococcus litoralis*, *Thermus thermophilus*, and *Sulfolobus solfataricus* (Xavier *et al*., 1996; Elferink *et al*., 2001; Schlösser 2000; Silva *et al*., 2005; Herman *et al*., 2006).

During the present work it was published that *M. tuberculosis* and *M. smegmatis* also possess an ABC transport system for the uptake of trehalose (Kalscheuer *et al*., 2010). Both systems are specific for trehalose and are thought to be important for the recycling of external trehalose derived as a byproduct during the biosynthesis of the mycolic acid cell envelope (Kalscheuer *et al*., 2010).

Saccharomyces cerevisiae possesses at least two different uptake mechanisms for trehalose; a high-affinity trehalose-H^+ symporter (*AGT1*) as well as a constitutive low-affinity transport system which is thought to be a facilitated diffusion process (Stambuk *et al.*, 1998; Plourde-Owobi *et al*., 1999). Both transporters could also mediate trehalose efflux under stress conditions (Stambuk *et al.*, 1998).

With regard to trehalose export systems, hardly anything is known. For *M. tuberculosis* it was suggested that TMMs are synthesized in the cytoplasm and afterwards transported via an ABC transporter outside the cell (Takayama *et al*., 2005). Such a mechanism would

avoid the transport of free trehalose but does not seem to be realized by *C. glutamicum* as mentioned previously.

1.6 Maltose metabolism in *C. glutamicum*

Maltose is a disaccharide formed by two α-1,4-glycosidic linked glucose molecules. High amounts of maltose arise from the enzymatic degradation of starch which can be an alternative as a cheap carbon source for the cultivation of various bacteria. Growth experiments with *C. glutamicum* in minimal medium containing maltose revealed that *C. glutamicum* can efficiently exploit maltose as carbon source (Seibold, 2007). Further it has been shown that maltose positively influences glucose utilization increasing the productivity of an amino acid producing *C. glutamicum* strain (Krause *et al*., 2010), making it even more interesting to study maltose metabolism.

In contrast to other Gram-positive bacteria the maltose/maltodextrin metabolism in *C. glutamicum* has not been fully investigated. The maltose metabolic pathway starts with the uptake of maltose by a so far unknown and not characterized transport system. The incoming maltose is then channeled into metabolism by the 4-α-glucanotransferase (MalQ), which catalyzes the transfer from maltosyl and longer dextrinyl residues onto maltose, producing glucose and maltodextrins (Kempkes, 2009).

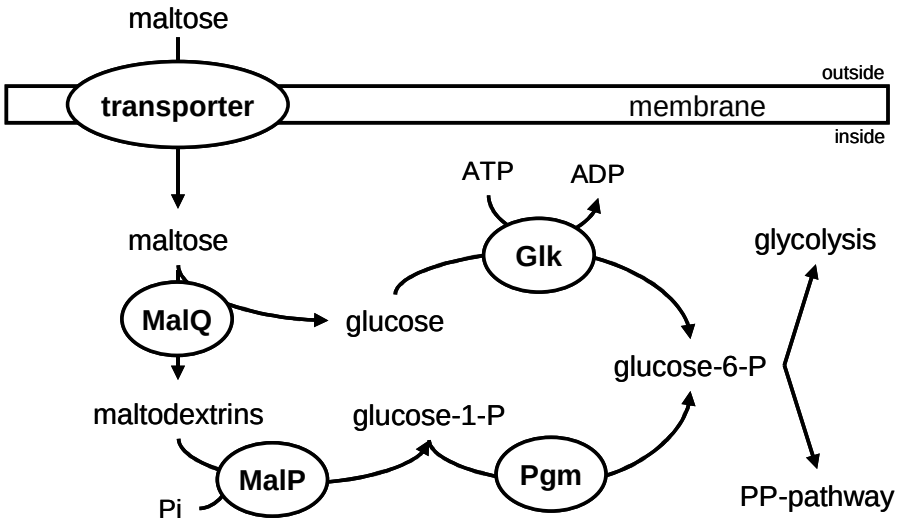

Figure 3: Maltose metabolism in *C. glutamicum*. MalQ - 4-α-glucanotransferase; MalP - maltodextrin phosphorylase; Glk - glucokinase; Pgm - phosphoglucomutase, after Seibold *et al.*, 2009.

The released glucose is then phosphorylated by the glucokinase (Glk) yielding glucose-6-phoshate which is then further metabolized (Fig.3). The maltodextrins are degraded by the

maltodextrin phosphorylase (MalP), which forms glucose-1-phoshate by sequential phosphorolysis of the non-reducing end of larger dextrins (Seibold *et al*., 2009).

The so far revealed maltose metabolism in *C. glutamicum* is very similar to the well studied maltose and maltodextrin metabolism of the Gram-negative bacterium *E. coli* and the archeaon *T. litoralis* (Boos & Shuman, 1998; Xavier *et al*., 1999), but it is rather different to the pathways found in other Gram-positive bacteria such as *Lactobacillus sanfrancisco* (Neubauer *et al*., 1994) and *B. subtilis* (Schönert *et al*., 2006). In *B. subtilis* maltose is taken up by a maltose specific PTS and becomes phosphorylated. The cytoplasmic maltose-phosphate is then hydrolyzed by an α-glucosidase (MalA), resulting in glucose and glucose-6-phosphate which is then channeled into metabolism (Schönert *et al*., 2006). In contrast, *L. sanfrancisco* takes up maltose by an H^+-symporter and cleaves it afterwards via a maltose phosphorylase into glucose and β-glucose-1-phosphate.

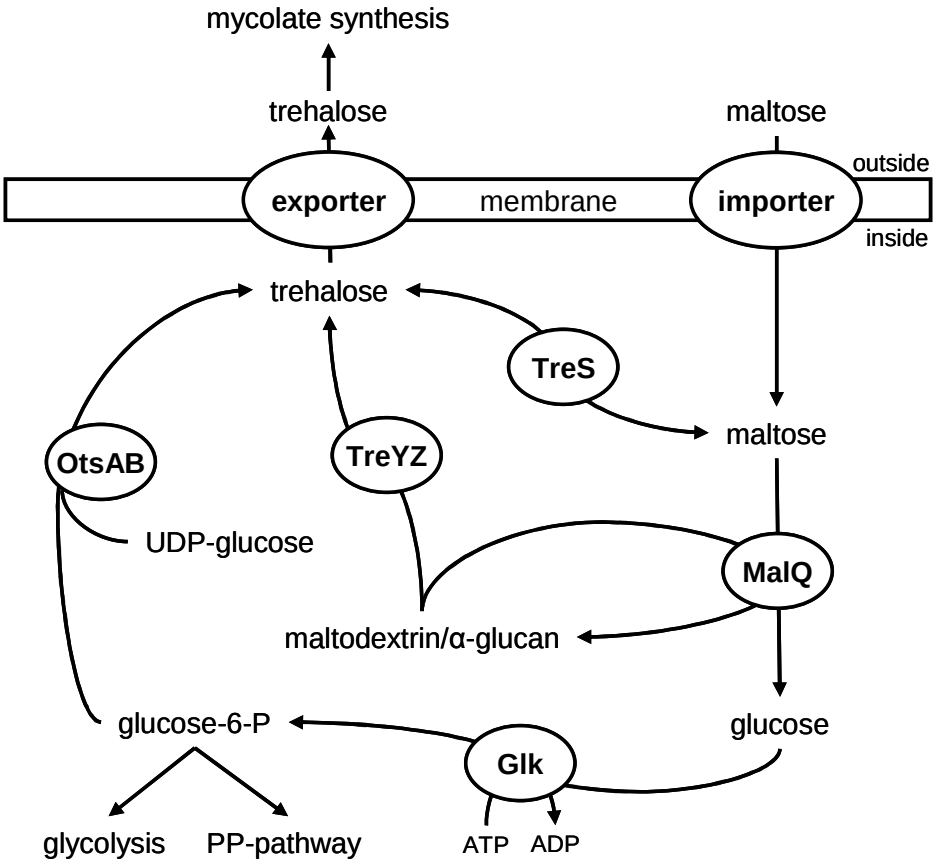

Figure 4: Interconnections between maltose and trehalose metabolic pathways. For the maltose metabolism an unknown import system, MalQ – 4-α-glucanotransferase, and Glk – glucokinase are needed. Trehalose synthesis starting from maltodextrins involves the maltooligosyltrehalose synthase (TreY) and the maltooligosyltrehalose trehalohydrolase (TreZ). The second pathway is catalysed by the trehalose-6-phosphat synthase (OtsA) and the trehalose-6-phosphate phosphatase (OtsB), respectively, starting with UDP-glucose and glucose-6-phosphate. The third pathway is catalyzed by TreS, with maltose-trehalose interconverting activity. The export of trehalose for mycolate synthesis is facilitated by an unknown transport system.

Interestingly, the metabolic pathways for maltose and trehalose are closely interconnected in *C. glutamicum* (Fig. 4). Incoming maltose can be converted to trehalose by TreS and it has also been proposed that trehalose derived after an osmotic up-shift might be recycled by TreS via maltose (Wolf *et al*., 2003). Furthermore, MalQ derived maltodextrins are connected to trehalose synthesis via the TreYZ-pathway and the OtsAB-pathway uses glucose-6-phosphate derived during maltose metabolism (Fig. 4). The synthesized trehalose again is important for the cell envelope structure, as building block of mycolates (Tropis *et al*., 2005).

1.7 General transport mechanisms

Protein facilitated transport processes can proceed via two principal mechanisms, a passive and an active one. The pore- or channel-facilitated diffusion enables the thermodynamically favored transport of substances along an existing concentration gradient (from high to low concentrations). These proteins can be addressed as water filled pores that enable the passage of unspecific substances or they function as specific channels over biological membranes, presenting passive transport processes. A good example is LamB, the specific diffusion pore for maltose and maltodextrins, in the outer membrane of *E. coli* (Boos & Shuman, 1998).

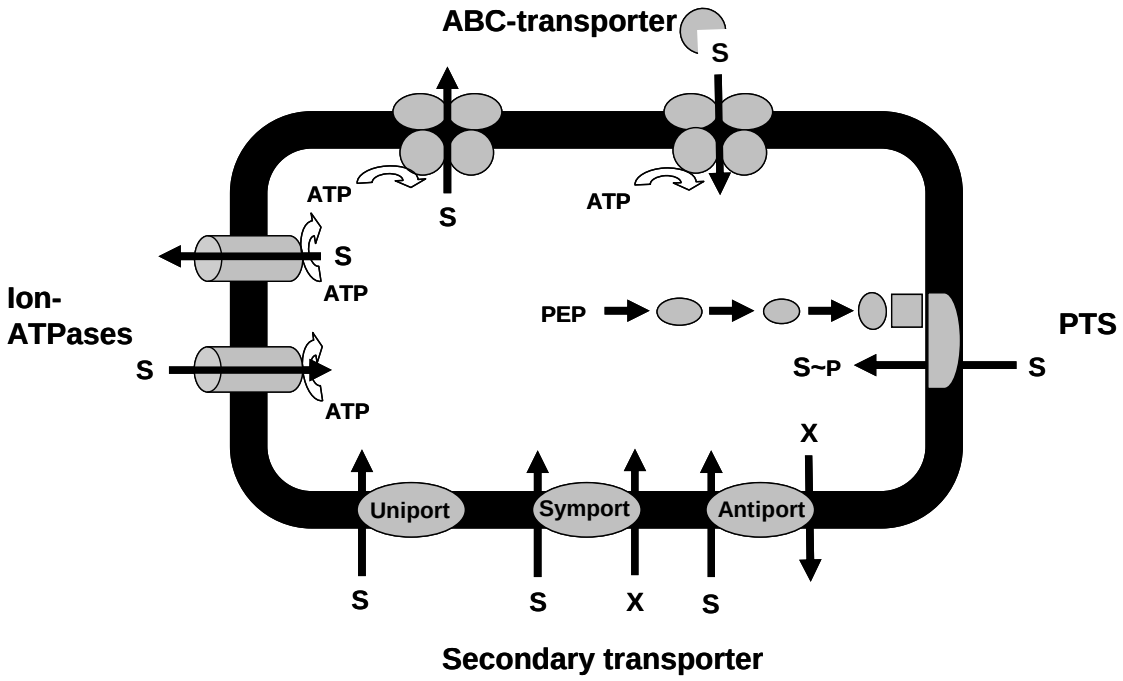

Figure 5: Important classes of bacterial transport systems. S - substrate; X - ion (e.g. H^+, Na^+), which gradient drives substrate transport; ATP - adenosine 5'-triphosphate; PEP – phospoenolpyruvate, according to Schneider, 2000.

When substances are accumulated in the cytoplasm against an existing concentration gradient (from low to high concentrations) the uptake has to be coupled to an energy dependent step. These active transport processes can be grouped in different classes (Fig. 5). Primary active transport systems utilize the free energy of ATP hydrolysis for the transport of substances. Ion-ATPases, such as detoxication-systems in bacteria for heavy metals (e.g. Cd^{2+} or AsO_4^{3-}; Schneider, 2000) and ABC transporter (see 1.8) belong to this class of transport systems. Secondary active transport systems couple the transport of substances to an existing electrochemical gradient (mostly ions such as H^+ and Na^+). In principle three different secondary active transport mechanisms exist.

During a uniport, also known as facilitated diffusion, which is in fact a passive transport process, transport is driven by the concentration gradient of the transported substance in only one direction, e.g. found in *S. cerevisiae* for the uptake of xylose (Saloheimo *et al*., 2007). During a symport two substances are transported in the same direction over biological membranes, using the energy derived from the transport of the co-substance down its concentration gradient. A well studied example is the lactose permease LacY of *E. coli* that transports lactose in symport with H^+ ions (Kaback *et al*., 2010). On the contrary one substrate is transported in one direction at the same time as co-transporting another substance in the other direction during an antiport (Fig. 5).

Exclusive to bacteria are the phosphotransferase systems (PTS) for sugar uptake, like the main uptake system for glucose in *C. glutamicum* (Moon *et al*., 2005), where the source of energy is phospoenolpyruvate (PEP). The phosphoryl group of PEP is transferred to the imported sugar via several proteins; this process is called group translocation. As the transported sugar is chemically modified during the transport process a concentration gradient is provided preventing return transport of the substrate (Fig. 5).

1.8 The maltose uptake system MalFGK$_2$-E of *E. coli*

The so far best studied of all maltose/maltodextrin transport systems is the ABC transporter MalFGK$_2$-E of *E. coli* which serves as a model for ATP-binding cassette importers in general (Bordignon *et al*., 2010). ATP-binding cassette (ABC) transporters are found in all three domains of life and form one of the largest protein superfamilies of paralogous sequences. They are common membrane proteins that facilitate the uptake and export of a large variety of substrates ranging from sugars, amino acids, peptides, vitamins, ions, and even polypeptides across biological membranes.

ABC transport systems share a common structural organization comprising two transmembrane domains (TMDs) and two nucleotide-binding domains (NBDs). The TMDs form the translocation pore and determine the substrate specificity. The NBDs bind and hydrolyze ATP, thereby generating conformational changes that are coupled to the TMDs

and ultimately lead to substrate translocation. Transmembrane domains and nucleotide-binding domains can be arranged as separate proteins, like in prokaryotes, as half-transporters or as a single polypeptide chain. Prokaryotic ABC transport systems that facilitate the uptake of substances have been shown to require a third component that is important for substrate capture and initiation of the transport cycle addressed to as solute-binding protein (SBP) (Eitinger *et al*., 2011).

The maltose/maltodextrin uptake system MalFGK$_2$-E of *E. coli* comprises at least five genes which encode the proteins building the system. LamB is a specific outer membrane porin, which is important for the uptake of maltose into the periplasm under low external maltose concentrations. The maltose binding protein MalE, which is located in the periplasm, forms the substrate recognition site and binds maltose and maltodextrins with high affinity (Boos & Shuman, 1998). The membrane spanning part consists of the two permeases MalF and MalG. On the cytoplasmic side they are joined by two MalK proteins, the ATP-hydrolyzing subunits of the transporter, which provide the necessary energy for the active transport (Fig. 6) (Dippel & Boos, 2005).

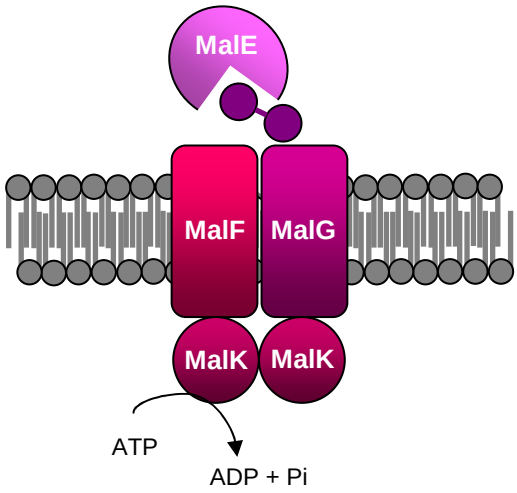

Figure 6: The maltose uptake system MalFGK$_2$-E of *E. coli*.

1.9 Thesis objectives

The first part of this work aimed to characterize and identify the maltose uptake system of *C. glutamicum* with the help of biochemical methods and to identify possible regulatory interconnections. So far it has been published that the inactivation of the four genes encoding enzyme II permeases of the phosphotransferase systems in *C. glutamicum* had no effect on growth of the respective mutants in minimal medium containing maltose (Moon *et al*., 2005). Further it was proposed that intracellular maltose and not maltose-phosphates functions as substrate for the key enzyme 4-α-glucanotransferase of the proposed maltose metabolisation pathway (Seibold *et. al*., 2009), excluding that maltose is taken up via PTS. This stands in contrast to the observations that PTS-enzymes EI and Hpr, two proteins of the PTS phosphorylation cascade, are necessary for growth on maltose as sole carbon source (Parche *et al*., 2001). Although maltose uptake seemed likely to be facilitated by an H^+-symporter or ABC-transporter the involvement of a phosphotransferase system had to be considered as well.

The second part of this project aimed the identification and characterization of the trehalose export system of *C. glutamicum*. The disaccharide trehalose has been shown to be important for *C. glutamicum* as a component of mycolates which make up a crucial part of the outer membrane of the *Corynebacterianeae*. Already published data indicated that mycolate biosynthesis is dependent on external trehalose, which is most probably exported by *C. glutamicum* via an unknown and not characterized transport system (Tropis *et al*., 2005). But the export mechanism of the internal produced trehalose has not been elucidated yet. As trehalose is of such major importance in terms of cell wall synthesis for *C. glutamicum*, there is increasing interest identifying the responsible export system. After the identification of this system its impact on cell envelope synthesis should be revealed.

2 Materials and Methods

2.1 Bacterial strains, plasmids and oligonucleotides

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

Table 1: Bacterial strains used in this study.

Strain	Genotype	Reference
E. coli		
DH5α*mcr*	*endA1 supE44 thi-1 λ-recA1 gyrA96 relA1 deoR Δ(lacZYA-argF) U196 ∈80DlacZ ΔM15mcrA Δ(mmr hsdRMS mcrBC*)	Grant *et al*., 1990
C. glutamicum		
ATCC 13032	Wild type	Abe *et al.*, 1967
Δ*malQ*	Wild type with a chromosomal deletion in the *malQ* gene	this study
Δ*malQ* Δ*treS*	Wild type with chromosomal deletions in the *malQ* and *treS* genes	this study
Δ*ptsG*	Wild type with a chromosomal deletion in the *ptsG* gene	this study
Δmus	Wild type with a chromosomal deletion from the *cg2703* to *cg2708* gene (including *cg2704*, *cg2705* and *cg2707*)	this study
Δtus	Wild type with a chromosomal deletion from the *cg0831* to *cg0835* gene (including *cg0832*, *cg0833* and *cg0834*)	this study
IM*musK*	Wild type with a chromosomal insertion in the *cg2708* gene	this study
IM*musI*	Wild type with a chromosomal insertion in the *cg2701* gene	this study
IM*musG*	Wild type with a chromosomal insertion in the *cg2703* gene	this study
IM*musE*	Wild type with a chromosomal insertion in the *cg2705* gene	this study
IM*cg2707*	Wild type with a chromosomal insertion in the *cg2707* gene	this study
Δ*otsA* Δ*treS* Δ*treY*	Wild type with chromosomal deletions in the *otsA*, *treS* and *treY* genes	Wolf *et al*., 2003

Table 1 (continued)

Strain	Genotype	Reference
C. glutamicum		
Δ*otsA* Δ*treS* Δ*treY* Δtus	Wild type with chromosomal deletions in the *otsA*, *treS* and *treY* and from the *cg0831* to *cg0835* gene (including *cg0832*, *cg0833* and *cg0834*)	this study
Δ*hpr*	Wild type with a chromosomal deletion in the *hpr* gene	Lindner *et al*., 2011
Δ*treS*	Wild type with chromosomal deletion in the *treS* gene	Wolf *et al*., 2003
IM*treX*	Wild type with a chromosomal insertion in the *treX* gene	Ute Meyer, this study
Δ*malQ* IM*treX*	Wild type with a chromosomal deletion in the *malQ* gene and a chromosomal insertion in the *treX* gene	Ute Meyer, this study
trehalose mutant	Wild type spontaneous mutant growing on trehalose, unknown genotype	this study
Res 167	Wild type with chromosomal deletions in the *cglIM*, *cglIR*, *cglIIR*, and Nx_R genes	Tauch *et al*., 2002
Δ*sugR*	Wild type with chromosomal deletion in the *sugR* gene	Engels & Wendisch, 2007
RG1	Wild type with chromosomal deletion in the *ramB* gene	Gerstmeier *et al.*, 2004
RG2	Wild type with chromosomal deletion in the *ramA* gene	Cramer *et al*., 2006

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

Table 2: Plasmids used in this study

Plasmid	Description	Reference
pK19mobsacB	deletion vector, Kan^R, $oriV_{E.c.}$, *oriT*, *mob*, *sacB*	Schäfer *et al*.,1994
pK19mobsacB_ MalQDel	pK19mobsacB derivative, containing up- and downstream regions of the *malQ* gene	Seibold, 2008
pK19mobsacB_ ptsGDel	pK19mobsacB derivative, containing up- and downstream regions of the *ptsG* gene	this study

Table 2 (continued)

Plasmid	Description	Reference
pK19mobsacB_ musDel	pK19mobsacB derivative, containing up- and downstream regions of the *cg2703* and the *cg2708* gene, respectively	this study
pK19mobsacB_ tusDel	pK19mobsacB derivative, containing up- and downstream regions of the *cg0831* and the *cg0835* gene, respectively	this study
pDrive	cloning vector; Kan^R, *lacZ*	Qiagen, Hilden
pDrive_IM*musI*	pDrive derivative, containing an internal region of the *cg2701* gene for inactivation	this study
pDrive_IM*musG*	pDrive derivative, containing an internal region of the *cg2703* gene for inactivation	this study
pDrive_IM*musE*	pDrive derivative, containing an internal region of the *cg2705* gene for inactivation	this study
pDrive_IM*musK*	pDrive derivative, containing an internal region of the *cg2708* gene for inactivation	this study
pDrive_IM*cg2707*	pDrive derivative, containing an internal region of the *cg2707* gene for inactivation	this study
pDrive_IM*treX*	pDrive derivative, containing an internal region of the *treX* gene for inactivation	Ute Meyer, this study
pXMJ19	shuttle vector, P_{tac}, $lacI^q$, Cm^R	Jakoby *et al*., 1999
pXMJ19_*musFGK-E*	pXMJ19 derivative, carrying the genes from *cg2703* to *cg2708* (including *cg2704*, *cg2705* and *cg2707*) for expression	this study
pXMJ19_*tusFGK-E*	pXMJ19 derivative, carrying the genes from *cg0831* to *cg0835* (including *cg0832*, *cg0833* and *cg0834*) for expression	this study
pXMJ19_*musI*	pXMJ19 derivative, carrying the gene *cg2701* for expression	this study
pXMJ19_*treS*	pXMJ19 derivative, carrying the gene *treS* for expression	this study
pEKEx2	shuttle vector, Kan^R, $oriV_{E.\ c.}$, $oriV_{C.\ g.}$, P_{tac} , $laqI^q$	Eikmanns *et al*., 1991

All oligonucleotides used in this study are listed in table 3.

Table 3: Oligonucletides used in this study. Restriction sites are underlined; linker regions are highlighted in bold, T7 promoter sequences are shown in italics.

Oligonucleotide	Sequence (5'-3')	Function
MalQRegioFor	GATAGGCACGCTCACTG	verification of *malQ* deletion
MalQRegioRev	GGTCTCTGCGACGAATG	verification of *malQ* deletion
PtsG_DEL1	GTCGACGGGCATAATCTGACAGTGTG	*ptsG* deletion
PtsG_DEL2	**CTTATAAATTTGGAGTGTGAAGGTTATTGCGTG** GGACGCCAAGAACTGATGGG	*ptsG* deletion
PtsG_DEL3	**CACGCAATAACCTTCACACTCCAAATTTATAAG** CCGCTGACTTCATTCGATCC	*ptsG* deletion
PtsG_DEL4	GGATCCTAAGGACGCCATGTCAAACC	*ptsG* deletion
cg2703_Del1BamHI	GGGGATCCAACCGCGAACTGCT	*mus* deletion, *BamH*I
cg2703_Del2	**CTTATAAATTTGGAGTGTGAAGGTTATTGCGTG** CGAACGATCCGTAGTGTGAG	*mus* deletion
cg2703_Del3	**CACGCAATAACCTTCACACTCCAAATTTATAAG** ACGACTACCTGCTGCCATAC	*mus* deletion
cg2703_Del4BamHI	GGGGATCCTGCCTTGGCAATGA	*mus* deletion, *BamH*I
cg0831_Del1BamHI	GGGGATCCGGCGATGCCATTGT	*tus* deletion, *BamH*I
cg0831_Del2	**CTTATAAATTTGGAGTGTGAAGGTTATTGCGTG** GGCAGCTTAGCCAACTTC	*tus* deletion
cg0831_Del3	**CACGCAATAACCTTCACACTCCAAATTTATAAG** CGCCAACTATCCAACACCTC	*tus* deletion
cg0831_Del4BamHI	GGGGATCCTGCAACCGCTCCAT	*tus* deletion, *BamH*I
dptsG_test for	TCGTACGGTGTGGTTAAG	verification of *ptsG* deletion
dptsG_test rev	AGTATGCACCGCGTAATC	verification of *ptsG* deletion
d2703_test for	TAGATGGCGCACAGTGACTC	verification of *mus* deletion
d2703_test rev	TAACTACCGCAACACCGATG	verification of *mus* deletion
cg2703for	TCTAGAATGGCGCACAGTGACTCACTT	expression of *mus*, *Xba*I

Table 3 (continued)

Oligonucleotide	Sequence (5'-3')	Function
cg2703rev	ACCGGTCGAGTATGCGATTCATGGTT	expression of *mus*, *Age*I
cg0831for	GCTCTAGATGCGTTCTGCTCCTGACCTT	expression of *tus*, *Xba*I
cg0831rev	CGGGATCCTTTGCGTTGCGATTCGGATT	expression of *tus*, *BamH*I
Cg2701_sonde1	TTCGCTGACCTAGTCATCGT	inactivation + RNA probe + RT-PCR of *cg2701*
Cg2701_sonde2	*GGGCCCTAATACGACTCACTATAGGG* ACTGCGAGGAAGAACAGGTA	inactivation + RNA probe + RT-PCR of *cg2701*
Cg2703_sonde1	GCTTCTTGGAGGCCACATTG	inactivation + RNA probe + RT-PCR of *cg2703*
Cg2703_sonde2	*GGGCCCTAATACGACTCACTATAGGG* TATCGCGGTTACCGTTGGAG	inactivation + RNA probe + RT-PCR of *cg2703*
Cg2705_sonde1	TCATCACTTGGCAGGACTAC	inactivation + RNA probe + RT-PCR of *cg2705*
Cg2705_sonde2	*GGGCCCTAATACGACTCACTATAGGG* ACTCAGCCATGGAATCAGAC	inactivation + RNA probe + RT-PCR of *cg2705*
Cg2707_sonde1	CCTATTCCGCCTATCTCGTC	inactivation + RT-PCR of *cg2707*
Cg2707_sonde2	*GGGCCCTAATACGACTCACTATAGGG* GGCGATAGTCGGTTCGTATT	inactivation + RT-PCR of *cg2707*
Cg2708_sonde1	ACTGAAGATCGCCGGCAAGT	inactivation + RNA probe + RT-PCR of *cg2708*
Cg2708_sonde2	*GGGCCCTAATACGACTCACTATAGGG* ATTATCCTCCGGCGTCATGG	inactivation + RNA probe + RT-PCR of *cg2708*
cg2701_for	GGGGATCCTTCTCCACGCAGAGGCACAT	expression of *cg2701*, *BamH*I
cg2701_rev	GGGGATCCATGACGTGGATACCACTACC	expression of *cg2701*, *BamH*I

Table 3 (continued)

Oligonucleotide	Sequence (5'-3')	Function
cg0832_IN 1	GGATCCTCAGACGGCACCAT	RNA probe of *cg0832*
cg0832_sonde2	*GGGCCCTAATACGACTCACTATAGGG* GTGCGGAAGAGTA	inactivation + RNA probe of *cg0832*
cgtreS XbaI	GCTCTAGAAGGCGAAAGTGGTGAAAGAC	expression of *treS*, *Xba*I
cgtreSrev KpnI	GCGGTACCTTGTTGATGCGCACTACAGC	expression of *treS*, *Kpn*I
ptsG-NB-T7_for	CAAACTGACGACGACATC	RNA probe of *ptsG*
ptsG-NB-T7_rev	*GGGCCCTAATACGACTCACTATAGGG* TGGCAGGAAGTAGAAGAC	RNA probe of *ptsG*
treX-IM-for	CACCGCCGACAATAAAGC	inactivation of *treX*
treX-IM-rev	GTGGATGCGTTGGATGTG	inactivation of *treX*
2704_SP1	AAGTTGGTACCGCGGAGT	5'-RACE
2704_SP2	AGGCGAAGATGTTGACTG	5'-RACE
2704_SP3	CACGATGAACGGCACCAA	5'-RACE
2705_SP1	CCTGCCAAGTGATGAAGC	5'-RACE
2705_SP2	TTCGTCCTTGCCAATCTC	5'-RACE
2705_SP3	AACATCGCCTTCCTCGTC	5'-RACE
2708_SP1	ATGGCGATGTCACGGTCA	5'-RACE
2708_SP2	ACGTCCTTGTCTCCGATGAA	5'-RACE
2708_SP3	AACTCGCCATCGGCGATT	5'-RACE

2.2 Media and culture conditions

E. coli as well as pre-cultures of *C. glutamicum* WT and its derivatives were cultivated aerobically in LB or TY complex medium (Sambrook & Russel, 2001) at 37℃ and 30℃, respectively. For the main-culture the cells of an overnight pre-culture were washed twice with 0.9% (w/v) NaCl before inoculating. *C. glutamicum* main-cultures were grown under aerobic conditions at 30℃ as 50 ml cultures in 500 ml baffled shaking flasks at 125 rpm in CGC minimal medium (Eikmanns *et al.*, 1991) with the addition of 1% - 2% (w/v) carbon source as indicated in the Results and Discussion section.

For the selection of integration mutants and expression plasmids 25 µg ml^{-1} chloramphenicol and/or 50 µg ml^{-1} kanamycin was added. For induction of the expression plasmid pXMJ19 and its derivatives 250 mM IPTG was added to the medium. Growth of bacterial cultures was measured photometrically at 600 nm (OD_{600}).

2.3 Molecular biology methods

2.3.1 DNA digestion, ligation and purification

Standard techniques such as DNA digestion (enzymes purchased from New England Biolabs; Schwalbach), ligation using T4 ligase (New England Biolabs, Schwalbach) and plasmid DNA purification (NucleoSpin® Plasmid DNA Purification kit; Macherey-Nagel, Düren) were performed according to the manufacturer's manuals.

2.3.2 Competent cells and transformation

Competent *E. coli* cells were prepared and transformed according to Inoue *et al.*, 1990. Competent *C. glutamicum* cells were prepared as described by Liebl *et al*., 1989, and transformed by electroporation (2.5 kV, 600 Ω, 2.5 µF) with a MICRO PULSER™ (Bio-Rad, München).

2.3.3 Polymerase chain reaction

The amplification of specific DNA fragments was performed by PCR (polymerase chain reaction, Mullis *et al*., 1986) using the PRECISOR High-Fidelity DNA Polymerase, the EconoTaq® PLUS GREEN 2X Master Mix (BioCat GmbH, Heidelberg) and the Taq polymerase from NEB (New England Biolabs, Schwalbach) according to the manufacturer's manuals. Oligonucleotides were obtained from Eurofins MWG Operon (Ebersberg, Germany).

2.3.4 Agarose gel electrophoresis

Gel electrophoresis of DNA was performed using 0.9% agarose gels in 1 x TAE buffer (1 x TAE: 0.04 M Tris, 0.5 mM EDTA, pH 7.5 adjusted with acetic acid) as described by Sambrook *et al*. ,1989. After electrophoresis, DNA was stained with ethidium bromide. For detection of stained DNA, the Image Master VDS system (Amersham Biosciences, Freiburg) was used. DNA was isolated from agarose gels using the NucleoSpin® Extract kit (Macherey-Nagel, Düren) according to the manufacturer's manual.

2.3.5 Construction of *C. glutamicum* mutant strains

For the construction of *C. glutamicum* deletions the method of allelic replacement described by Schäfer *et al.*, 1994, was used. Derivatives of the vector pK19mobsacB were constructed using the method of *cross over* PCR after Link *et al.*, 1997. For the allelic replacement, competent *C. glutamicum* cells were transformed with pK19mobsacB derivatives by means of electroporation (van der Rest *et al*., 1999). Plasmid integration into the genome was verified by selecting kanamycin-resistant and sucrose-sensitive colonies (the expression of the sacB gene encoding the levan sucrase is toxic in sucrose-

containing media). To promote re-excision of the plasmid DNA, positive clones were grown overnight in LB broth containing 25 µg ml^{-1} kanamycin, washed and cultivated in CgXII without phosphate and ammonium source containing 0.5% glucose for 6 h. The starved cells were plated on LB agar supplemented with 10% (w/v) sucrose in different dilutions, usually between 10^{-3} and 10^{-5}. Positive colonies obtained from the subsequent selection (Km^S, Suc^R) were tested for allelic replacement by PCR. Single or double deletion strains were used instead of *C. glutamicum* WT to generate strains carrying multiple gene deletions. For the construction of strains deleted in trehalose uptake 1.5% trehalose was added to the selection agar.

For the construction of inactivation mutants pDrive derivatives were constructed with internal parts of the target genes. The gene was inactivated by inserting the vector via homologous recombination into the gene in the genomes of *C. glutamicum* WT (Schäfer *et al*. 1994). In short, competent WT cells were transformed with pDrive derivatives by means of electroporation (van der Rest *et al*., 1999). Plasmid integration into the genome was verified by selecting kanamycin-resistant colonies and PCR.

The trehalose spontaneous-mutant was derived by cultivating *C. glutamicum* WT in minimal medium with 4% (w/v) trehalose as sole carbon source at 30℃ for several days until the culture started to grow. Cells from this culture were repeatedly cultivated with trehalose to gain a *C. glutamicum* strain that grows fast with trehalose.

2.3.6 RNA hybridization experiments

RNA hybridization experiments were performed as described in Möker *et al*., 2004. In short, for purification of RNA from *C. glutamicum* cells were harvested in the exponential growth phase, broken with glass beads in a Fast-Prep® 120 instrument (Q-Biogene, Heidelberg), and the RNA purified from the disrupted cells with the NucleoSpin® RNA II kit (Macherey-Nagel, Düren) according to the manufacturer's manual. For the construction of RNA anti-sense probes of the desired gene intragenic fragments of a size of ~ 500 bp were amplified with primers carrying the T7 RNA polymerase promoter sequence (Tab. 3). The resulting DNA fragments were used for DIG-11-dUTP-labeled anti-sense RNA generation by *in vitro* transcription using T7 RNA polymerase.

For Northern Blot analyses total RNA from *C. glutamicum* WT was separated on an agarose gel containing 17% (v/v) formaldehyde and transferred onto a nylon membrane. RNA was cross-linked to the membrane by means of ultraviolet irradiation. Hybridization with the constructed probes and detection steps were carried out according to the DIG application manual (Roche Applied Science, Mannheim). Chemiluminescence was detected with the CCD camera of the LAS 1000 CH system (Fuji, Sraubenhardt).

Changes in gene transcription were monitored by RNA hybridization experiments using total RNA transferred to a nylon membrane using a Minifold Slot-Blot system (Schleicher

and Schuell, Dassel). Hybridization and detection steps were carried out according to the Northern Blot analyses.

2.3.7 5'-RACE

The transcriptional start points were determined by means of the 5'-RACE technique (rapid amplification of 5'-cDNA ends). The 5',3'-RACE Kit (Roche Applied Science, Mannheim) was used according to the manufacturer's manual. First strand cDNA was synthesized from total RNA (up to 2 mg) using a gene-specific primer (annealing ~ 250 bp downstream of the translational start point), reverse transcriptase and dNTPs. The mixture was incubated for 1 h at 55°C and then the single-stranded cDNA was purified from unincorporated primers and nucleotides. Subsequently, terminale transferase was used to add a homopolymeric A-tail to the 3'-end of the single-stranded cDNA, thereby tagging the 5'-end or transcriptional start point of the respective mRNA. Tailed DNA was then amplified by PCR using an oligo-dT-anchor primer (supplied by the manufacturer) and a gene-specific primer. The obtained cDNA was further re-amplified by a second PCR with a nested gene-specific primer and the oligo-dT-anchor primer. The resulting PCR product was used as template in a sequencing reaction to determine the last nucleotide upstream of the poly A-tail, which represents the 5'-end of the respective mRNA (Möker *et al*., 2004).

2.3.8 RT-PCR

For the determination of whether genes are transcribed monocistronically or in an operon RT-PCRs (reverse transcription polymerase chain reaction) was used. Total cDNA was generated as described earlier and used for PCR with specific primer combinations (Tab. 3), in additional reactions DNA and RNA were used as control.

2.4 Biochemical methods

2.4.1 HPLC analyses

For the quantitative analysis of carbohydrates, amino acids and organic acids HPLC (high performance liquid chromatography) analyses on VWR/Hitachi EliteLaChrom systems were used. Cells were separated from the growth medium by centrifugation and analysis was performed from the supernatant.

Carbohydrates and organic acids were separated via a pre-column (ChromCart 30x4 mm CC30/4 Nucleogel Sugar 810-H, Macherey-Nagel, Düren) and an ion-exchange main-column (300x7.8 mm Nucleogel Sugar 810H, Macherey-Nagel, Düren), at 40 °C with a

flow rate of 0.5 ml/min. The mobile phase was 0.01 M H_2SO_4 and detection was obtained via an UV- and RI-detector.

Amino acid, mainly glutamate, samples were separated after automated fluorescent derivatization by ortho-phthaldialdehyd/mercaptopropionic acid (OPA) via a reversed phase pre-column (Multospher 40x4 mm, CS-Chromatographie, Macherey-Nagel, Düren) and a reversed phase main-column (Nucleodur RP-18, 125x4 mm, Macherey-Nagel, Düren), at 35 °C with a flow rate of 0.8 ml/min. The mobile phase was a mixture of solvent A [40 mM sodium acetate, 0.06% sodium azide, 5% (v/v) MeOH:acetonitrile (1:1)] and solvent B [MeOH:acetonitrile (1:1)]. A solvent gradient was used ranging from 5% solvent B in the beginning to 100% solvent B at the end of a run. Detection was obtained via a fluorescence detector.

2.4.2 Extraction of glycolipids

For the qualitative analysis of trehalose mycolate and glucose monomycolate, lipids were extracted from wet cells of 50 ml cultures for 16 h with $CHCl_3/CH_3OH$ [1:2, (v/v)] at room temperature. This procedure was repeated with $CHCl_3/CH_3OH$ [1:1, (v/v)] and $CHCl_3/CH_3OH$ [2:1, (v/v)]. The organic phases were pooled and dried. The crude lipid extracts were re-extracted in $CHCl_3/H_2O$ [8:2, (v/v)]. The lower organic phases were collected, evaporated to dryness to yield the crude lipid extracts from each strain and comparatively examined using TLC (thin layer chromatography) on silica gel-coated plates (Durasil-25, 0.25 mm thickness, Macherey-Nagel, Düren) developed with $CHCl_3/CH_3OH/H_2O$ [65:25:4, (v/v/v)]. Glycolipids were visualized by spraying plates with 0.2% anthrone in concentrated H_2SO_4, followed by heating to 110°C. Blue spots indicate the presence of glycolipids.

2.4.3 TLC for maltose and trehalose separation

To separate maltose and trehalose 5 µl of culture supernatant were examined via TLC (thin layer chromatography) on silica gel-coated plates (Durasil-25, 0.25 mm thickness, Macherey-Nagel, Düren) with butanol/pyridine/H_2O [70:30:10, (v/v/v)] as mobile phase. Sugars were visualized by spraying plates with 0.2% anthrone in concentrated H_2SO_4, followed by heating to 110°C.

2.4.4 Screening assay

For the susceptibility screening assay cells from an overnight culture were used. After the optical density was adjusted to 0.1, 3 µl of the cultures were spotted on CGC agar plates containing 1.5% (w/v) fructose, 25 µg ml^{-1} kanamycin and 1.1 µg ml^{-1} ethambutol with and without the addition of 1.5% (w/v) trehalose. Afterwards the plates were incubated at 30°C for 48 hours.

2.4.5 Enzyme assay

To reveal the identity of a produced carbohydrate an enzymatic assay using trehalase and maltase was performed. 50 µl of the supernatant was mixed with 50 µl reaction buffer (400 mM Na^+-acetate pH 5.2, for trehalase and 200 mM citrate pH 6.6, for maltase) before 3 µl trehalase (1.7 U/ml) and 5 µl maltase (5.8 U/ml) was added, respectively, followed by an overnight incubation at 37°C. As control for enz yme activity trehalose and maltose were treated accordingly. The so gained samples were examined by TLC.

2.4.6 Glutamate production

For glutamate production cells were cultivated in CGXII minimal medium (Keilhauer *et al*., 1993) with 2% (w/v) glucose or trehalose, respectively. Glutamate excretion was induced during the exponential growth phase (OD_{600} 5 - 6) by addition of 6 U/ml Penicillin G. Concentration of glutamate in the supernatant was determined by HPLC analysis.

2.4.7 Radioactive transport assays

For measuring maltose, glucose and trehalose uptake *C. glutamicum* cultures were grown in liquid medium, as indicated in the Results and Discussion section, at 30°C up to the exponential growth phase. Subsequently, cells were washed two times with ice cold CGC (pH 6.8) medium, and stored on ice until the measurements took place. Before the transport assay, cells were incubated for 3 min at 30°C; the reaction was started by addition of different concentrations of the substrate for determination of kinetic parameters and 50 µM of the substrate for subsequent experiments. Inhibitors were given to the setup at indicated concentrations 30 s before the measurements were started. For trehalose uptake measurements 2.5 mM glucose for energetization was given to each experiment. At given time intervals (15, 30, 45, 60, and 90 s), 200 µl samples were filtered through glass fibre filters (Typ F; Millipore, Eschborn) and washed twice with 2.5 ml of 100 mM LiCl. The radioactivity of the sample was determined using scintillation fluid (Rotiszinth; Roth) and a scintillation counter (LS 6500; Beckmann, Krefeld). Kinetic parameters as well as standard errors were derived from nonlinear regressions according the Michaelis-Menten equation.

[^{14}C]-maltose (specific activity 679 mCi $mmol^{-1}$; Amersham, Braunschweig)
[^{14}C]-glucose (specific activity 360 mCi $mmol^{-1}$; Moravek Biochemicals, Brea, USA)
[^{14}C]-trehalose (specific activity 600 mCi $mmol^{-1}$; Trenzyme GmbH, Konstanz)

3 Results

3.1 Maltose uptake in *Corynebacterium glutamicum*

The Gram-positive bacterium *Corynebacterium glutamicum* is known to be able to grow on a variety of different carbon sources such as sugars, organic acids and alcohols (Arndt & Eikmanns, 2008; Blombach & Seibold, 2010). For cultivation the disaccharide maltose serves as an excellent substrate for *C. glutamicum* (Seibold *et al*., 2009).

Although the metabolic pathway for maltose utilization has been described, involving maltodextrins and glucose formation catalyzed by the 4-α-glucanotransferase MalQ, maltodextrin degradation via the maltodextrin phosphorylase MalP and the α-phosphoglucomutase Pgm, and glucose phosphorylation by the glucose kinase Glk (Seibold *et al.*, 2009), little is know about the uptake of maltose. Genome sequence analyses of *C. glutamicum* ATCC 13032 revealed the presence of about 260 putative transport proteins (Kalinowski *et al*., 2003; Ikeda & Nakagawa, 2003; Winnen *et al*., 2005), the majority of these are classed as secondary carriers and primary transporters (117 and 106, respectively). Moreover, 183 further proteins with unknown function are predicted to have at least 3 transmembrane helices and thus might function as a transporter as well (Winnen *et al*., 2005). Furthermore, four phosphotransferase system enzyme II genes are present in *C. glutamicum*. As the inactivation of the respective genes had no effect on growth of maltose, the uptake of maltose is thought to be facilitated by a transporter of another category (Moon *et al*., 2005). This contradicts the observations that the inactivation of EI and HPr has a negative effect on maltose utilization in *C. glutamicum* (Parche *et al*., 2001) and that maltose has a positive effect on the expression of *ptsG* (Engels & Wendisch, 2007).

3.1.1 Maltose positively influences glucose utilization

Most bacteria cultivated in media containing two or more carbon sources adapt their metabolism to the utilization of their preferred substrate. This successive utilization of substrates is mediated by regulatory mechanisms summarized as carbon catabolite repression and is often represented by a biphasic growth behavior (Deutscher, 2008; Görke & Stülke, 2008). Such diauxic growth can be observed e.g. for *Escherichia coli* cultivated with glucose plus lactose or maltose. On substrate mixtures *C. glutamicum* co-metabolizes most common carbon sources and shows monophasic growth. Sequential utilization of substrates by *C. glutamicum* causing biphasic growth has only been observed for the mixtures of glucose plus glutamate, glucose plus ethanol, and acetate plus ethanol (Krämer *et al*., 1990; Arndt & Eikmanns, 2007; Arndt *et al*., 2008). When

C. glutamicum is cultivated with a mixture of glucose plus acetate, the glucose uptake rate is reduced about twofold when compared to growth on glucose as sole carbon source and this effect is probably due to transcriptional repression of *ptsG* by SugR (Wendisch *et al*., 2000; Engels & Wendisch, 2007). On the other hand, when *C. glutamicum* is grown with a mixture of glucose plus maltose *ptsG* expression has been shown to be about twice as high as observed in cells grown with glucose as sole carbon source and this effect was independent of SugR (Engels & Wendisch, 2007).

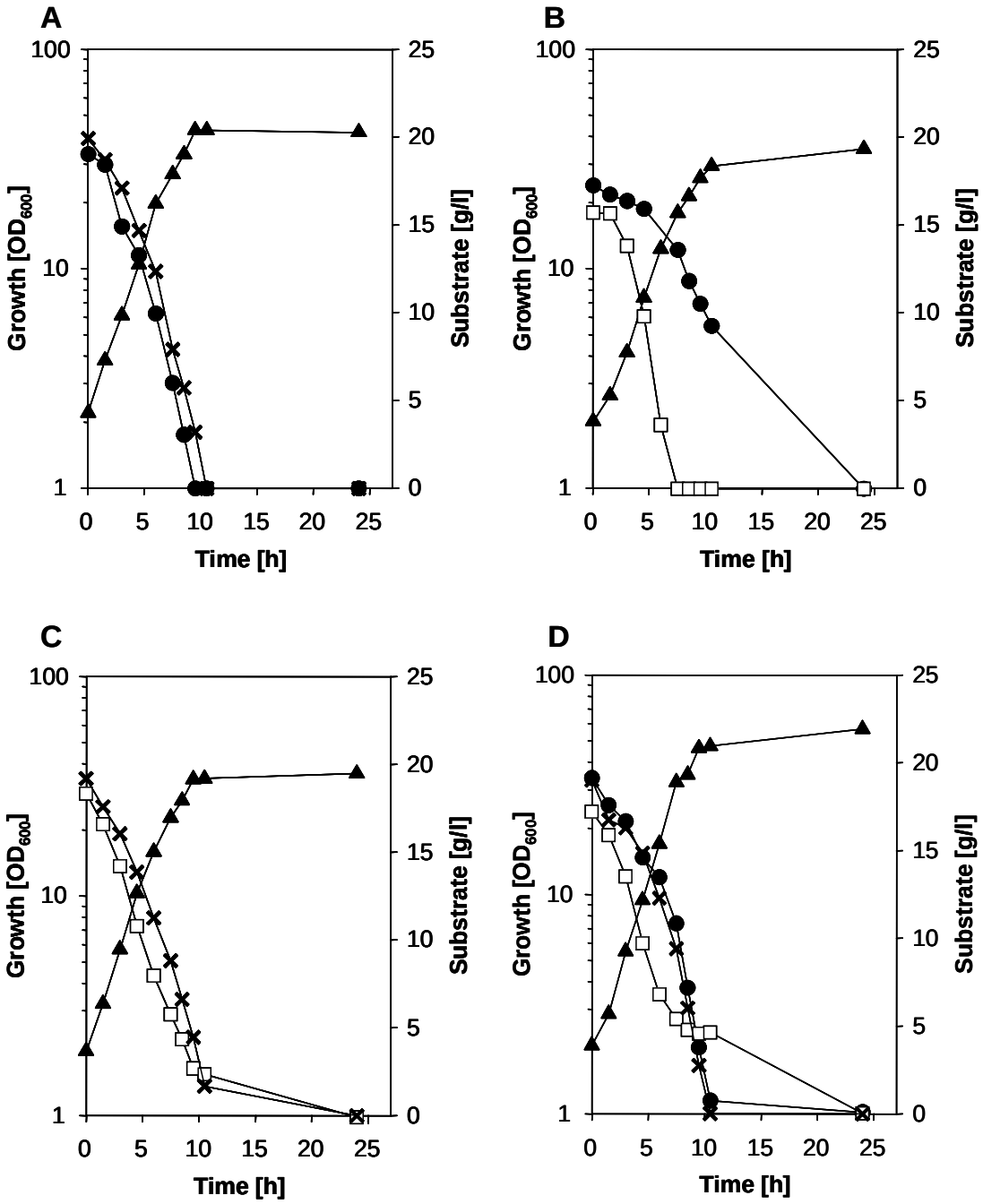

Figure 7: Growth of *C. glutamicum* WT in minimal medium with mixed carbon sources. (A) 2% glucose plus 2% maltose, (B) 2% glucose plus 2% acetate, (C) 2% maltose plus 2%acetate, (D) 2% glucose plus 2% maltose and 2% acetate. Growth of: (▲) *C. glutamicum* WT. Consumption of: (●) glucose, (×) maltose, (□) acetate.

To see whether this effect has a positive influence on glucose utilization under mixed carbon source cultivations growth experiments were performed. *C. glutamicum* grew on glucose and maltose as substrates with an accelerated growth rate of 0.43 ± 0.05 h^{-1}, compared to single carbon source cultivation (Krause *et al*., 2010), until it slowed down at an optical density of 15 probably due to limitations in aeration (Fig. 7, A). Furthermore, an increased final optical density of 42 ± 2 was reached, and both sugars were completely consumed at about the same time point (Fig. 7, A). Both sugars can be consumed in parallel excluding the presence of catabolite repression by glucose of the maltose metabolism, as it is observed for *E. coli* (Deutscher, 2008; Görke & Stülke, 2008). So far, co-utilization of substrates such as glucose and acetate has already been reported for *C. glutamicum*. However, a similar increase in the growth rate for the mixture of glucose and maltose has so far not been observed with other substrate mixtures. For the mixture of glucose and acetate decreased glucose consumption was detected and the growth rate of 0.38 ± 0.05 h^{-1} was not elevated compared to single carbon source cultivations (Fig. 7, B). For the mixture of maltose and acetate an elevated growth rate of 0.46 ± 0.06 h^{-1} was detected, accompanied with the co-metabolization of both substrates (Fig. 7, C). Addition of maltose to the medium containing glucose plus acetate caused accelerated glucose utilization during the early and mid exponential growth phase (Fig. 7, D). Moreover, the growth rate of the culture was higher compared to cultivation with only glucose plus acetate (0.44 ± 0.05 h^{-1} and 0.38 ± 0.05 h^{-1}, respectively).

To examine effects of maltose addition on *ptsG* expression in *C. glutamicum*, RNA hybridization experiments with labeled *ptsG* RNA as probe were performed.

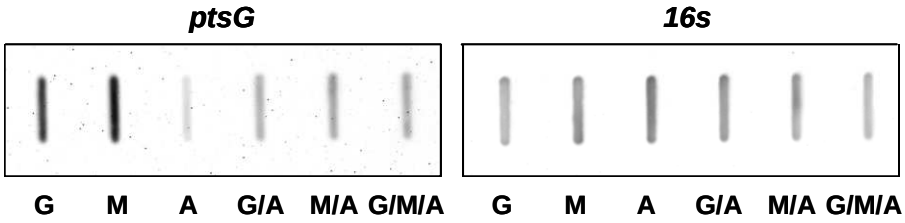

Figure 8: RNA hybridization experiments of *C. glutamicum* WT cultivated with different carbon sources. RNA levels of *ptsG* and *16s* were monitored with DIG-labeled antisense-RNA probes. G = glucose, M = maltose, A = acetate.

In samples from cells cultivated with maltose as sole carbon source *ptsG* expression was increased to about 130% compared to the expression level during cultivation with glucose as sole carbon source (Fig. 8). When cultivated with acetate as sole carbon source, *ptsG* expression was reduced to about 25%. However, the reduced expression of *ptsG* in cells cultivated in minimal medium with acetate or with glucose plus acetate (about 45% *ptsG*

expression) could partially be relieved by the addition of maltose to the culture broth, resulting in about 75% expression. These data match the recent observations of Engels and Wendisch (2007) that expression of a *ptsG-cat* fusion in *C. glutamicum* was highest when cells were cultivated in minimal medium with maltose as sole carbon source.

To examine the effect of *ptsG* inactivation on maltose utilization the strain *C. glutamicum* Δ*ptsG* was constructed via homologous recombination and the deletion was verified by PCR. *C. glutamicum* Δ*ptsG* was used for growth experiments in minimal medium with maltose. The data show that the *ptsG* deficient strain was not impaired for growth with maltose; the strain grew with a rate of 0.37 ± 0.01 h^{-1} to an optical density of 20 utilizing all the given maltose (Fig. 9), comparable to the WT under the same conditions (compare Fig. 14, A), excluding an influence of PtsG on maltose utilization.

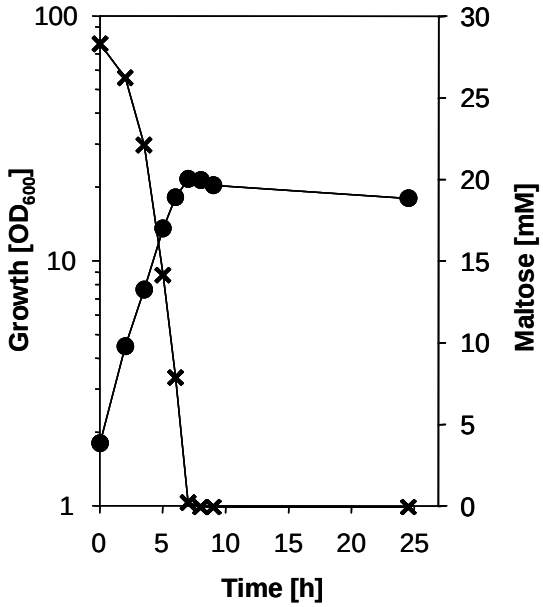

Figure 9: Growth of *C. glutamicum* Δ*ptsG* in minimal medium with 1% maltose. Growth of: (●) *C. glutamicum* Δ*ptsG*. Consumption of: (✕) maltose.

3.1.2 Biochemical characterization of glucose uptake in *C. glutamicum*

To have a closer look at glucose uptake in *C. glutamicum* radioactive assays with [^{14}C]-glucose were established for the biochemical characterization. *C. glutamicum* WT cells were cultivated in CGC minimal medium with 1.5% glucose to mid-exponential growth phase, harvested and washed twice in ice cold CGC minimal medium, before they were used for the measurements.

To reveal the biochemical parameters K_m and V_{max} for glucose uptake, transport assays with substrate concentrations ranging from 1 µM to 110 µM were performed. Plotting the

data of *C. glutamicum* WT according to the Michaelis-Menten equation revealed a saturation kinetic (Fig. 10, A).

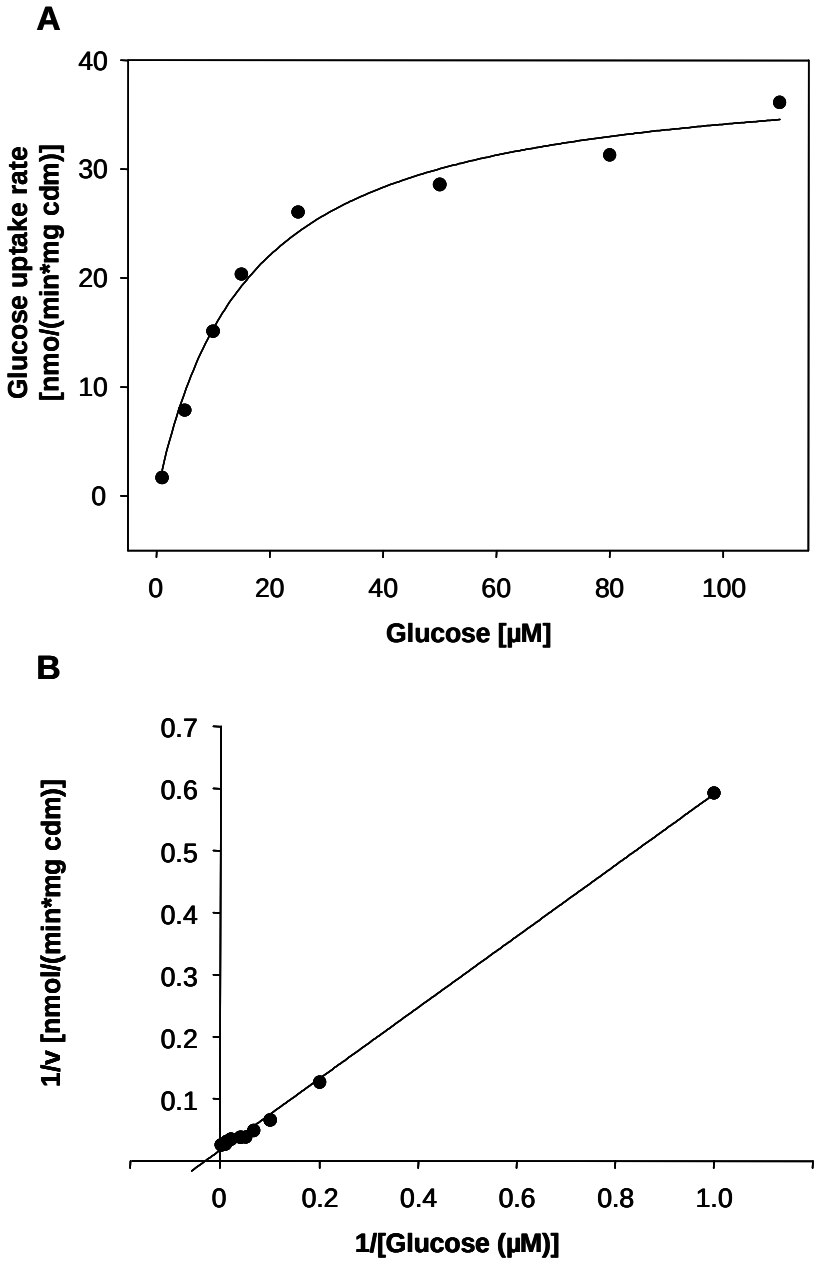

Figure 10: Biochemical characterization of glucose uptake in *C. glutamicum* WT. [^{14}C]-glucose uptake rates were determined with different concentrations (1 - 110 µM). (A) Data plotted according to Michaelis-Menten equation, (B) Data plotted according to Lineweaver-Burk.

As a graphical method for analysis of the Michaelis-Menten equation the data were plotted according to Lineweaver-Burk in a double reciprocal plot (Fig. 10, B). Kinetic parameters were derived by using the Sigma Plot 10.0 software, revealing a K_m of 14 ± 3 µM and a V_{max} of 35 ± 3 nmol/(min*mg cdm). There was no indication of a second system besides the phosphotransferase system (PTS), such as biphasic dependence of the uptake rate

on substrate concentration, although it has been shown that *C. glutamicum* strains with inactivated *ptsG* were still able to grow with glucose when high substrate concentrations were present (Moon *et al*., 2005; Moon *et al*., 2007). Interestingly, Lindner and co-workers (2011) were able to identify PTS independent glucose utilization by the two inositol permeases IolT1 and IolT2 which require high glucose concentrations to support half-maximal growth rates (Lindner *et al*., 2011). The biochemical parameters for glucose uptake were measured under conditions far below the concentration optimum for IolT1 and IolT2, so that the presented K_m and V_{max} values just represent glucose uptake via PTS.

3.1.3 Biochemical characterization and first regulatory insight of the maltose uptake system of *C. glutamicum*

For the biochemical characterization of maltose uptake in *C. glutamicum* radioactive transport assays with [^{14}C]-maltose were established. Therefore, *C. glutamicum* WT cells were cultivated in CGC minimal medium with 1.5% glucose to mid-exponential growth phase, harvested and washed twice in ice cold CGC minimal medium, before they were used for the measurements. To reveal the biochemical parameters K_m and V_{max} for the maltose uptake, transport assays with maltose concentrations ranging from 0.5 µM to 30 µM were performed. Plotting the data of *C. glutamicum* WT according to the Michaelis-Menten equation revealed a saturation kinetic (Fig. 11 A). As a graphical method for analysis of the Michaelis-Menten equation the data were plotted according to Lineweaver-Burk in a double reciprocal plot (Fig. 11, B). Kinetic parameters were derived from nonlinear regression by using the Sigma Plot 10.0 software, revealing a K_m of 1.2 ± 0.2 µM and a V_{max} of 26.2 ± 1.0 nmol/(min*mg cdm). There was no indication of a second system, such as biphasic dependence of the uptake rate on substrate concentration, showing that maltose uptake is facilitated by a single high affinity transport system.

To identify the transport mechanism, three different inhibitors were added to the experimental setup. The addition of the two ionophores valinomycin and nigericin was used to destroy the membrane potential driving secondary active transport processes. To eliminate ATP driven transport sodium vanadate, a phosphate analogue blocking ATP binding sites, was used. The data show that after the addition of valinomycin and nigericin the maltose uptake rate dropped to 75% [17.0 ± 5.0 nmol/(min*mg cdm)] of the initial rate of 22.7 ± 3.4 nmol/(min*mg cdm) (Fig. 12; blue, red). Taking the absolute errors into account the uptake rates for untreated and treaded cells overlap so that a direct dependence of the maltose uptake system on the membrane potential was unlikely. The data from the vanadate experiments show that the maltose uptake rate dropped to 49% [11 ± 5 nmol/(min*mg cdm)] of the initial rate (Fig. 12, green). These results indicate that

the uptake of maltose in *C. glutamicum* is probably facilitated by a primary active transport system. Together with the data showing that a phosphotransferase system is not involved in the uptake of maltose, it is most likely taken up by a high affinity ABC transport system.

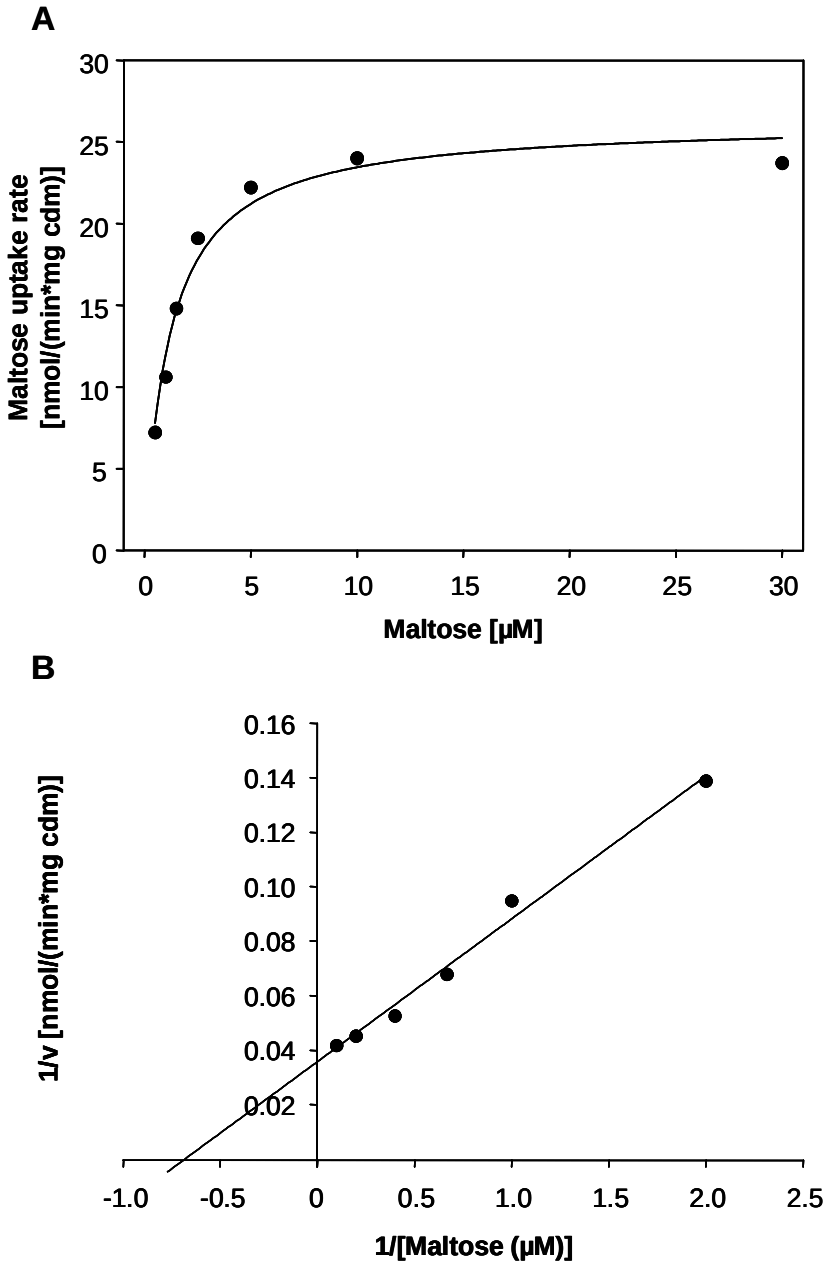

Figure 11: Biochemical characterization of maltose uptake in *C. glutamicum* WT. [^{14}C]-maltose uptake rates were determined with different concentrations (0.5 - 30 µM). (A) Data plotted according to Michaelis-Menten equation, (B) Data plotted according to Lineweaver-Burk.

To test if the maltose uptake system is expressed when *C. glutamicum* is cultivated with different carbon sources, uptake rates from cells cultivated in minimal medium with glucose, maltose and acetate were compared. The data show that the maltose uptake rate was highest in cells cultivated with glucose [22.7 ± 3.4 nmol/(min*mg cdm)] (Fig. 13, blue). In cells cultivated with maltose the rate was only 75% [17.0 ± 6.4 nmol/(min*mg

cdm)] of the glucose cultivated cells and in cells cultivated with acetate the rate was even lower [47%, 10.7 ± 1.0 nmol/(min*mg cdm)] (Fig. 13; red, green). These results were rather surprising, showing that the maltose uptake is not reduced in cells cultivated with glucose. Moreover, the maltose uptake system seemed not to be induced by maltose addition.

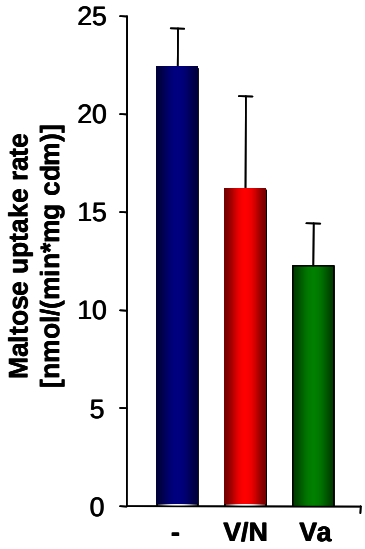

Figure 12: Effect of inhibitors on maltose uptake rates of *C. glutamicum* WT. Inhibitors were added 30 s before the reaction was started by the addition of 50 µM [^{14}C]-maltose. (-, blue) no inhibitor, (V/N, red) 20 µM valinomycin and 5µM nigericin; (Va, green) 20 mM sodium vanadate

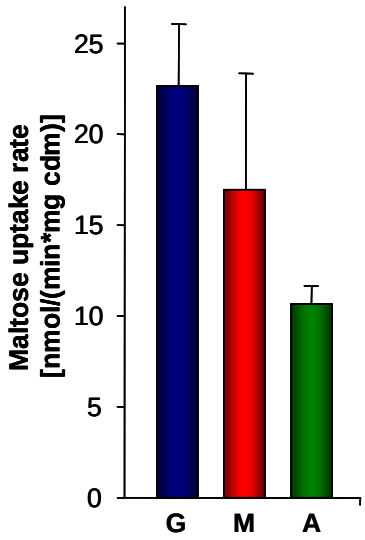

Figure 13: Maltose uptake rates of *C. glutamicum* WT cultivated with different carbon sources. Cells were cultivated in minimal medium with different carbon sources. The reaction was started by the addition of 50 µM [^{14}C]-maltose. (G, blue) glucose, (M, red) maltose, (A, green) acetate.

Based on the observation that the maltose uptake rate is lower in cells cultivated with acetate in contrast to cells cultivated with glucose and maltose, respectively, three known transcriptional regulators were considered as being involved in the regulation of maltose

uptake in *C. glutamicum*: i) RamA, a transcriptional activator of the acetate and ethanol metabolism, ii) RamB, a transcriptional repressor of the acetate and ethanol metabolism and iii) SugR, a transcriptional regulator of sugar uptake (Auchter *et al*., 2010; Engels & Wendisch, 2007). At first growth of the respective deletion mutants in minimal medium with maltose was compared to growth of the WT.

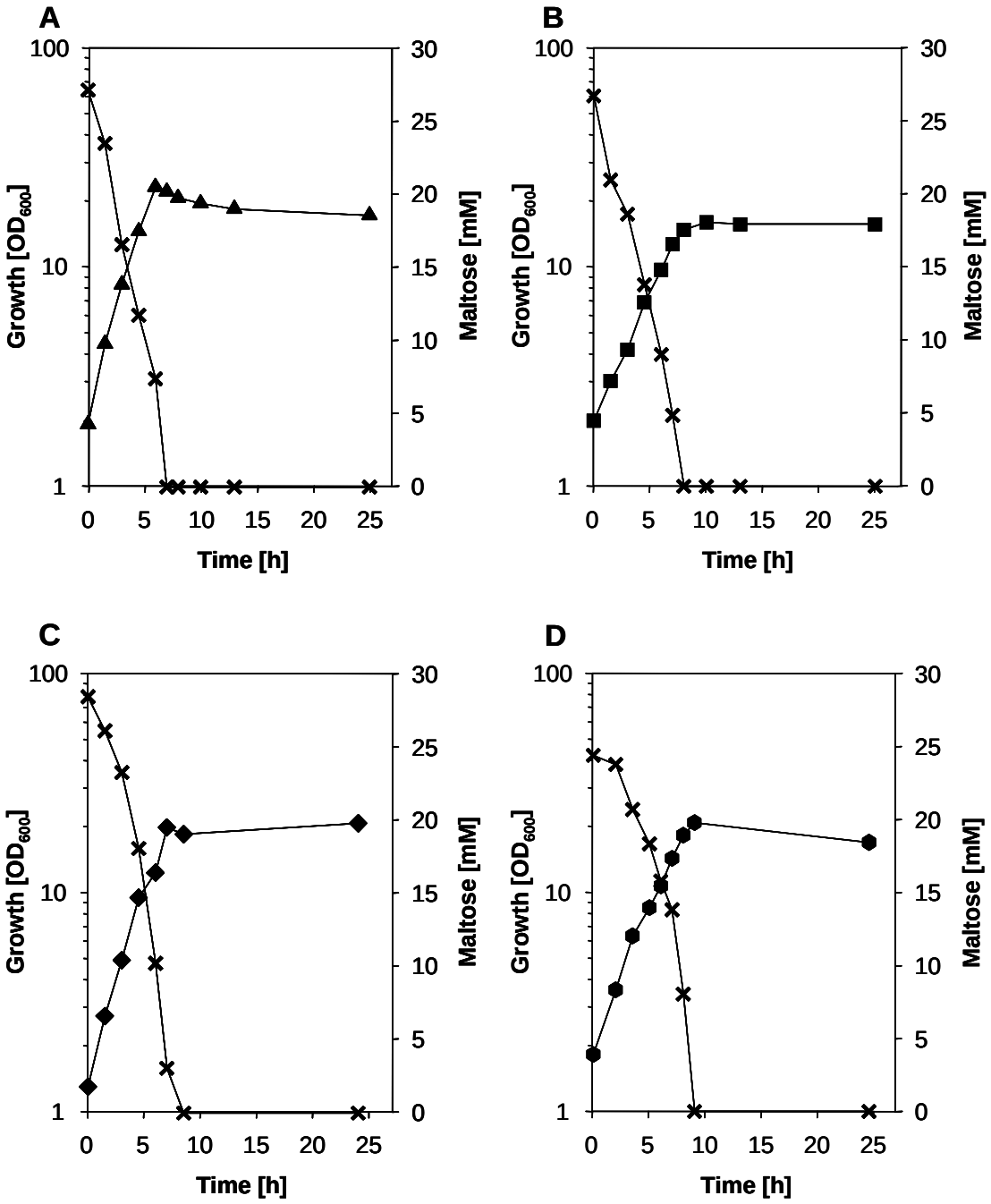

Figure 14: Growth of *C. glutamicum* strains in minimal medium with 1% maltose. Growth of: (A) (▲) *C. glutamicum* WT, (B) (■) *C. glutamicum* RG2 (Δ*ramA*), (C) (◆) *C. glutamicum* RG1 (Δ*ramB*), (D) (⬢) *C. glutamicum* Δ*sugR*. Consumption of: (✕) maltose.

The RamA deficient strain *C. glutamicum* RG2 showed a reduced growth rate with maltose of 0.27 ± 0.01 h^{-1} compared to the WT rate of 0.39 ± 0.1 h^{-1} (Fig. 14; A, B). Growth of the RamB deficient strain *C. glutamicum* RG1 was not significantly reduced and the growth rate of 0.35 ± 0.01 h^{-1} was comparable to the WT (Fig. 14, C). The SugR deletion strain *C. glutamicum* Δ*sugR* did also show a decreased growth rate of 0.27 ± 0.01 h^{-1} like the *ramA* deletion strain (Fig. 14, D). All strains consumed the given maltose and reached final optical densities after 24 h of 18 ± 1 without strain specific differences. The data from the growth experiments indicated that RamA and SugR might be involved in the regulation of the maltose uptake system of *C. glutamicum*. To have a closer look at maltose uptake in the deletion strains, radioactive transport measurements were performed after different cultivation conditions (Tab. 4).

Table 4: Maltose uptake rate in different *C. glutamicum* strains in dependence of the cultivation conditions. Cells were cultivated in TY rich medium (TY) overnight before they were cultivated in minimal medium with 1.5% glucose (Glu) and 1.5% maltose (Mal), respectively or they were exclusively cultivated in the respective medium. *C. glutamicum* RG1 (Δ*ramB*), *C. glutamicum* RG2 (Δ*ramA*), (n.d.) not determined.

	Maltose uptake rate [nmol/(min*mg cdm)]				
pre / main culture	TY / Glu	TY / Mal	Glu / Glu	Mal / Mal	TY / TY
C. glutamicum WT	30.3 ± 2.4	17.5 ± 0.2	22.7 ± 3.4	17.0 ± 6.4	29.4 ± 2.0
C. glutamicum RG1	18.9 ± 1.5	7.2 ± 0.1	19.0 ± 0.9	12.6 ± 8.0	n.d.
C. glutamicum RG2	9.1 ± 0.3	17.6 ± 1.3	1.8 ± 0.9	7.8 ± 0.9	37.7 ± 2.2
C. glutamicum Δ*sugR*	10.8 ± 0.4	9.9 ± 0.2	8.3 ± 0.5	6.3 ± 1.1	21.3 ± 1.4

Notably, the maltose uptake rate was higher in all cells that were cultivated in TY medium overnight before they were cultivated in minimal medium with the respective carbon source irrespective of the strain. Highest transport rates were observed in cells that were exclusively cultivated in the rich medium TY. Under this condition the uptake rate in the WT cell rose to 29.4 ± 2.0 nmol/(min*mg cdm) compared to 22.7 ± 3.4 nmol/(min*mg cdm) in glucose adapted cells (Tab. 4). Looking at the different strains it is striking that in the RG2 mutant the maltose uptake rate was as low as 1.8 ± 0.9 nmol/(min*mg cdm) in cells adapted to glucose and as high as 37.7 ± 2.2 nmol/(min*mg cdm) in cells exclusively cultivated on TY medium. Interestingly, this strain showed a comparable maltose uptake rate to the WT when cultivated with maltose after a TY pre-culture [17.6 ± 1.3 nmol/(min*mg cdm) and 17.5 ± 0.2 nmol/(min*mg cdm), respectively] but showed slowed growth with maltose (Fig. 14, B). The Δ*sugR* mutant did also show reduced maltose uptake rates compared to the WT under all conditions tested, and elevated transport rates

after cultivation on TY rich medium (Tab. 4). The RamB deficient strain RG1 showed unexpected transport rates which were lower than in the WT under comparable conditions (Tab. 4).

Taken together, the data show that *C. glutamicum* posses a high affinity transport system for the uptake of maltose with a K_m of 1.2 ± 0.2 µM and a V_{max} of 26.2 ± 1.0 nmol/(min*mg cdm), that is most probably an ABC transporter. Transport rates were high when cells were cultivated with maltose and glucose, respectively, but low on acetate. Furthermore, growth with maltose as well as maltose transport rates were influenced in strains deleted in *ramA* and *sugR*, whereas growth with maltose was not influenced in a *ramB* deletion strain, although maltose transport rates were decreased.

3.1.4 Identification of the maltose uptake system of *C. glutamicum*

The biochemical characterization of maltose uptake in *C. glutamicum* indicated that the maltose uptake system is a high affinity ABC transporter. From the 23 annotated ABC transport systems, in *C. glutamicum*, with a periplasmic component, four were annotated as possible sugar transporters (*cg0043-cg0046*, *cg0831-cg0835*, *cg1568-cg1571* and *cg2703-cg2708*). Radioactive transport measurements further revealed that the maltose uptake rate is high in with glucose and low in with acetate grown cells, and low in *C. glutamicum* RG2 (Δ*ramA*) cultivated with glucose. Therefore, DNA micro-array data from the PhD thesis of Annette Cramer (2006) were used, in which the differential gene expression in *C. glutamicum* WT after cultivation with glucose and acetate, respectively, and the RG2 mutant were compared. With the help of these data one candidate for the maltose uptake system was identified, as the genes encoding the transport systems were down-regulated in WT cells cultivated on acetate as well as in the RG2 mutant (Cramer, 2006). This system was annotated as ABC transporter possessing two permease components (*cg2703*, *cg2704*); one maltose- / solute-binding protein (*cg2705*) and an ATPase component (*cg2708*). In addition to these components, which are necessary to build an ABC transporter, the systems includes two hypothetical proteins (*cg2701*, *cg2707*) between the other genes (Fig. 15).

Figure 15: Genes encoding the maltose uptake system of *C. glutamicum*. *MusK* (*cg2708*) = ATPase component, *cg2707* = hypothetical protein, *musE* (*cg2705*) = maltose-binding protein, *musF* (*cg2704*) = permease, *musG* (*cg2703*) = permease, *cg2701* = hypothetical protein. All annotations are predicted, according to Kalinowski *et al*., 2003.

To test if the genes *cg2703-cg2708* indeed encode the maltose uptake system, a deletion mutant was constructed in which the genes from *cg2703* to *cg2708* were deleted by homologous recombination using the plasmid pK19mobsacB_musDel. The deletion was verified by PCR and the so constructed mutant named *C. glutamicum* Δmus. Subsequently, the deletion mutant was used for growth experiments in minimal medium with maltose and glucose, respectively.

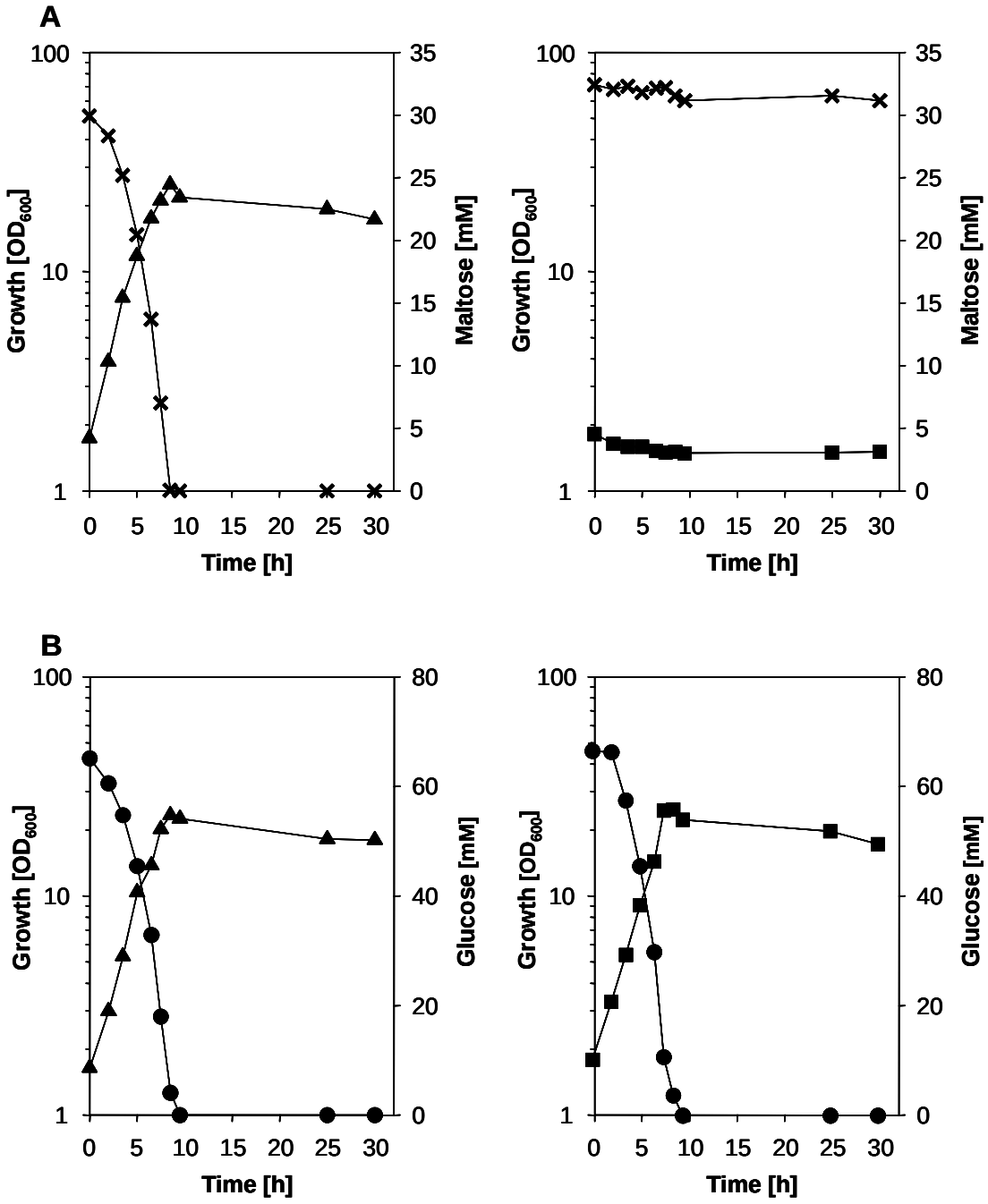

Figure 16: Growth of *C. glutamicum* WT and *C. glutamicum* Δmus in minimal medium with 1% maltose (A) and 1% glucose (B). Growth of: (▲) *C. glutamicum* WT, (■) *C. glutamicum* Δmus. Consumption of: (●) glucose, (✕) maltose.

Growth of the WT and the deletion mutant with maltose showed that the deletion mutant was not able to grow and did not utilize the added maltose, whereas the WT grew with a growth rate of 0.36 ± 0.01 h^{-1} and utilized all maltose (Fig. 16, B). Growth with glucose was similar for the WT and Δmus with final optical densities of 18.2 ± 1.0 after 24 h and growth rates during the exponential growth phase of 0.38 ± 0.05 h^{-1} and 0.35 ± 0.02 h^{-1}, respectively (Fig. 16, B). To support the findings of the growth experiments, radioactive uptake measurements were performed showing that the deletion mutant was not able to take up maltose (Tab. 5). These results indicated that the maltose uptake system of *C. glutamicum* is encoded by *cg2703-cg2708*.

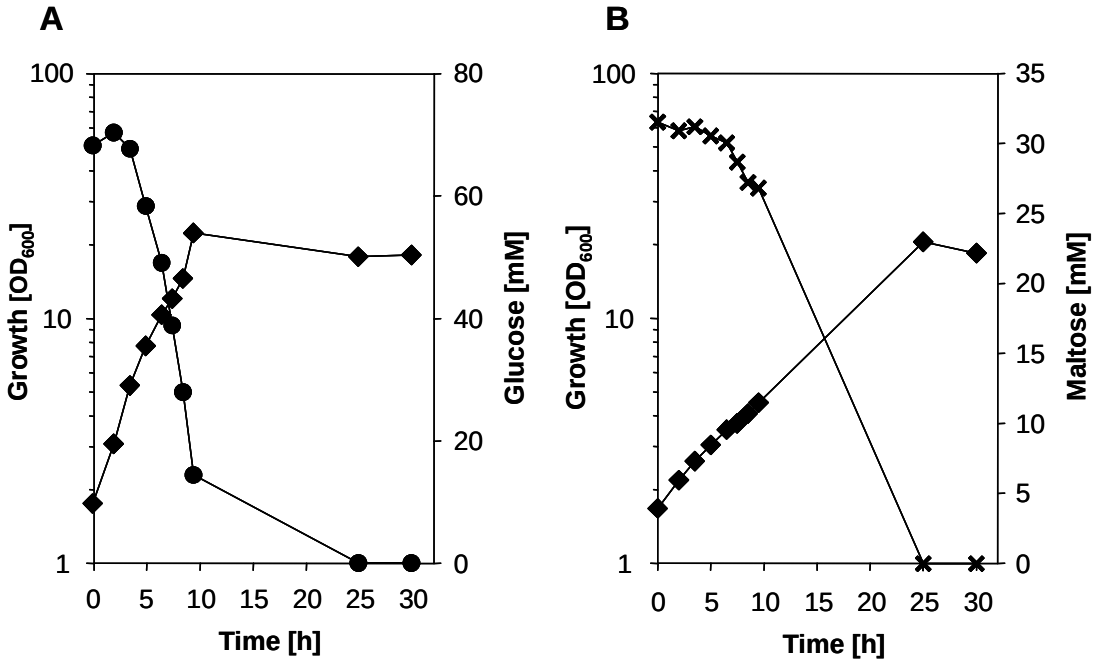

Figure 17: Growth of *C. glutamicum* Δmus (pXMJ19_*musFGK-E*) in minimal medium with 1% glucose (A) and 1% maltose (B). Growth of: (◆) *C. glutamicum Δmus* (pXMJ19_*musFGK-E*). Consumption of: (●) glucose, (✕) maltose.

To show that the genes *cg2703-cg2708* indeed encode the uptake system for maltose in *C. glutamicum*, the expression plasmid pXMJ19_*musFGK-E* was constructed, carrying the genes *cg2703*, *cg2704*, *cg2705*, *cg2707* and *cg2708*, and introduced into *C. glutamicum* Δmus. Growth experiments with *C. glutamicum Δmus* (pXMJ19_*musFGK-E*) showed that the mutant regained the ability to grow in minimal medium with maltose as sole carbon source, utilizing all maltose and reaching a final optical density of 19 ± 2, however with a reduced growth rate of 0.12 ± 0.04 h^{-1} compared to the growth of the WT (Fig. 17, B). Growth with glucose as sole carbon source was also slowed in *C. glutamicum* Δmus (pXMJ19_*musFGK-E*) as the growth rate was reduced to 0.27 ± 0.05 h^{-1} (Fig. 17, A). In addition to growth experiments with maltose, radioactive uptake measurements were performed to analyze the complementation of *C. glutamicum* Δmus. And indeed, the strain

C. glutamicum Δmus (pXMJ19_*musFGK-E*) regained the ability to take up maltose, although with a reduced rate of 5.6 ± 0.2 nmol/(min*mg cdm) when cultivated with maltose and 2.0 ± 0.1 nmol/(min*mg cdm) when cultivated with glucose (Tab. 5).

Table 5: Maltose uptake rates of *C. glutamicum* WT (pXMJ19) and *C. glutamicum* Δmus (pXMJ19_*musFGK-E*) cultivated in minimal medium with 1.5% glucose and 1.5% maltose, respectively. (n.d.) not detected.

	Maltose uptake rate [nmol/(min*mg cdm)]		
C. glutamicum	ATCC13032 (pXMJ19)	Δmus (pXMJ19)	Δmus (pXMJ19_*musFGK-E*)
Glucose	21.1 ± 0.1	n.d.	2.0 ± 0.1
Maltose	11.1 ± 0.1	n.d.	5.6 ± 0.2

Taken together the results reveal that the maltose uptake system of *C. glutamicum* is encoded by the genes *cg2703-cg2708*. Therefore, the system was named maltose uptake system MusFGK$_2$-E, according to the maltose uptake system of *E. coli* MalFGK$_2$-E, comprising two genes for permease subunits *musG* and *musF* (*cg2703*, *cg2704*); the gene for a maltose-binding protein *musE* (*cg2705*) and a gene for an ATPase subunit *musK* (*cg2708*).

3.1.5 Transcriptional organization of the maltose uptake system MusFGK$_2$-E

To characterize the transcriptional organization of the transport system MusFGK$_2$-E Northern Blot analyses were performed to test if all genes are transcribed in one operon. To do so DIG-labeled RNA probes for the genes *musK*, *musE*, *musG* and *musI* (*cg2701*; a hypothetical protein very close to *musG*) were constructed. The Northern Blot showed single transcripts of approximately 1800 bases for the genes *musK* and *musE* indicating that they are transcribed monocistronically, whereas transcripts of about 3000 bases were detected for *musG* and *musI*, indicating that they are on the same transcript forming an operon most probably with *musF*, which has not been used for probe synthesis, shown by the length of the detected fragment (Fig. 18, A). To support these data reverse transcription (RT)-PCRs were performed. Therefore, the prepared RNA was transcribed into cDNA with the help of a reverse transcriptase. Afterwards, PCRs with different combinations of gene specific primers were performed using DNA as the positive control, RNA as the negative control and cDNA as substrate for the identification of transcripts. With the cDNA products from *musK*, *cg2707*, *musE*, *musG* and *musI* (Fig. 18; C, primer

combinations AB; CD; EF; GH; IJ) were obtained and also a longer product using primers from the genes *musG* and *musI* (Fig. 18; C, primer combination GJ).

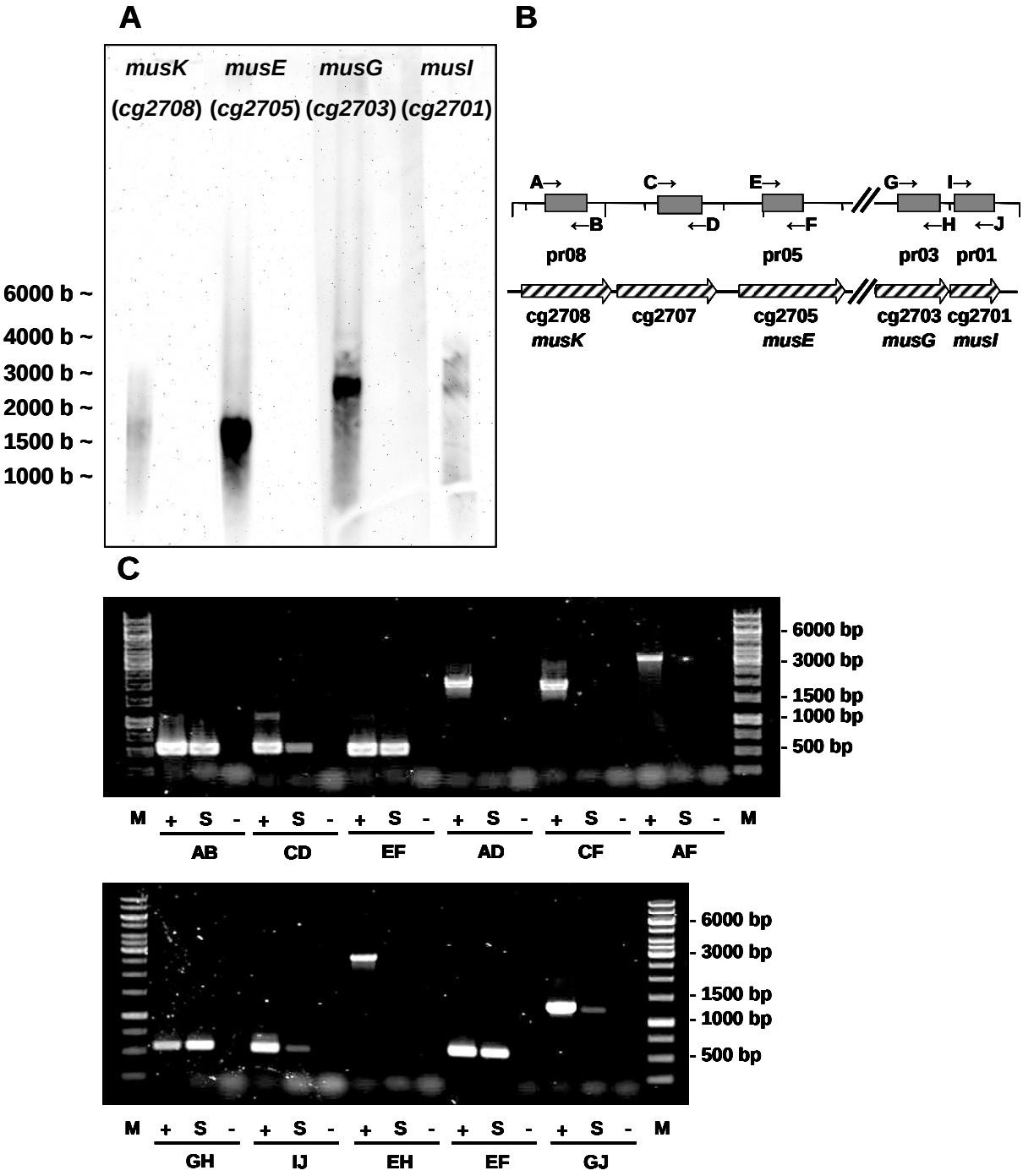

Figure 18: Transcriptional organisation of *musFGK-E* and *musI* of *C. glutamicum*. (A) Northern Blot, (B) localization of the used probes and primers, (C) RT-PCR. Pr08 = probe *musK*; pr05 = probe *musE*; pr03 = probe *musG*; pr01 = probe *musI*; A-J = primer; A, B in *musK*; C, D in *cg2707*; E, F in *musE*; G, H in *musG*; I, J in *musI*; M = marker; + = DNA (positive control); - = RNA (negative control); S = cDNA.

All other primer combinations e.g. from the genes *musK* and *musE* (Fig. 18; C, primer combination AF) or from *cg2707* and *musE* (Fig. 18; C, primer combination CF) did not result in any products. This supports the results from the Northern Blot analyses where *musE* and *musG* showed not to be on one transcript (Fig. 18, A).

Looking at the organization of the genes encoding the maltose uptake system, it is interesting to see that there are more genes present than needed for forming an ABC transport system. Most of these systems consist out of one or two genes encoding permease subunits and one gene encoding an ATPase subunit. Import systems further include another gene encoding a substrate specific binding protein. All these components are encoded by the ORFs of the maltose uptake system, but in addition there are two more genes (*cg2707*, *musI*) annotated as hypothetical proteins, with unknown functions. For *musI*, Northern Blot analyses already indicated that this gene forms an operon with *musK*. To reveal which genes are essential for catalyzing maltose uptake single genes were inactivated by integration of pDrive derivatives carrying internal fragments of the respective genes. The constructed mutants were tested by PCR and used for growth experiments in minimal medium with maltose as sole carbon source.

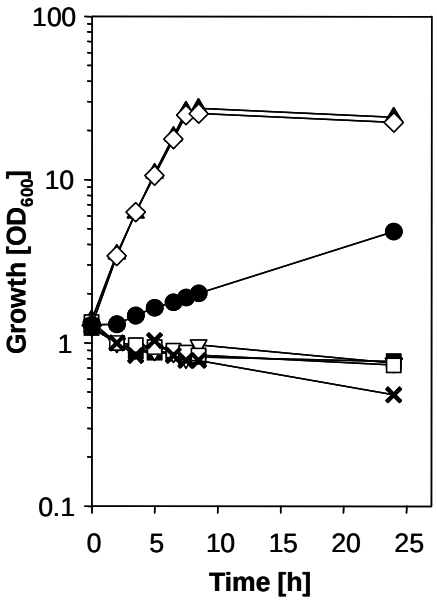

Figure 19: Growth of *C. glutamicum* integration mutants in minimal medium with 1% maltose. Growth of: (▲) *C. glutamicum* WT, (■) *C. glutamicum* Δmus, (▽) *C. glutamicum* IM*musI*, (□) *C. glutamicum* IM*musG*, (✕) *C. glutamicum* IM*musE*, (◇) *C. glutamicum* IM*cg2707*, (●) *C. glutamicum* IM*musK*.

The growth experiments with the integration mutants revealed that *C. glutamicum* IM*cg2707* was able to grow with maltose as sole carbon source like the WT (Fig. 19; ◇,

▲), with growth rates of 0.36 h^{-1} and 0.37 h^{-1}, respectively. In contrast to IM*cg2707* the mutants IM*musI*, IM*musG* and IM*musE* (Fig. 19; ▽, □, ✕) were not able to grow in minimal medium with maltose resembling the deletion mutant Δmus (Fig. 19, ■). Interestingly, the integration mutant IM*musK*, in which the ATPase subunit was knocked out, showed impaired growth with maltose but was still able to grow with a growth rate of 0.07 h^{-1} (Fig. 19, ●). This is most probably due to the fact that ATPase subunits from other ABC transport systems can compensate for the loss of the original one to certain extent, like it has been shown for the ATPase MsmX in *B. subtilis* (Ferreira *et al*., 2010).

The growth data were supported by radioactive uptake measurements, showing that IMcg*2707* was able to take up maltose with a rate of 27.5 ± 2.8 nmol/(min*mg cdm) and IM*musK* with a low rate of 2.5 ± 0.5 nmol/(min*mg cdm), whereas no uptake was detected in the other mutants (Tab. 6).

Table 6 Maltose uptake rates of integration mutants. Cells were cultivated in minimal medium with 1.5% glucose for the uptake measurements. (n.d.) not detected.

Strain	Maltose uptake rate [nmol/(min*mg cdm)]	Growth rate [h^{-1}]
C. glutamicum ATCC 13032	22.7 ± 3.4	0.37 ± 0.0
C. glutamicum Δmus	n.d.	n.d.
C. glutamicum IM*cg2701*	n.d.	n.d.
C. glutamicum IM*musG*	n.d.	n.d.
C. glutamicum IM*musE*	n.d.	n.d.
C. glutamicum IM*cg2707*	27.5 ± 2.8	0.36 ± 0.0
C. glutamicum IM*musK*	2.5 ± 0.5	0.07 ± 0.0

Notably, the inactivation of *musI* had an effect on maltose uptake indicating that this gene is involved in maltose utilization in *C. glutamicum*, whereas the second hypothetical protein encoded by *cg2707* does not seem to be involved in the maltose utilization, as the inactivation had no effect on growth with maltose and maltose uptake.

Together these data present that the maltose uptake system MusIFGK_2-E is encoded by *musG*, *musE*, *musK*, in addition *musI*, which encodes a protein with unknown function, and most probably *musF*.

3.1.6 MusI: A novel component of an ACB transporter for maltose uptake

Data from Northern Blot analyses, RT-PCRs and growth experiments revealed that maltose uptake in *C. glutamicum* is not only catalyzed by the two permease subunits MusF and MusG, the maltose binding protein MusE and the ATPase subunit MusK, there is also another Protein MusI important for the uptake and/or utilization of maltose.

Northern Blot analyses and RT-PCR data showed that *musI* forms an operon together with *musG* and probably *musF*, indicating that this gene is a crucial part of the maltose uptake system. So far no function was assigned to this protein. It consists of 213 amino acids forming five predicted transmembranen domains (TMHMM v. 2.0; Krogh *et. al*., 2001). So it is possible that this protein is part of the pore formed by the permeases MusF and MusG or it might be important for the correct assembly of the different subunits, working like a chaperon. BLAST data bank analyses showed that the protein has no putative conserved domains.

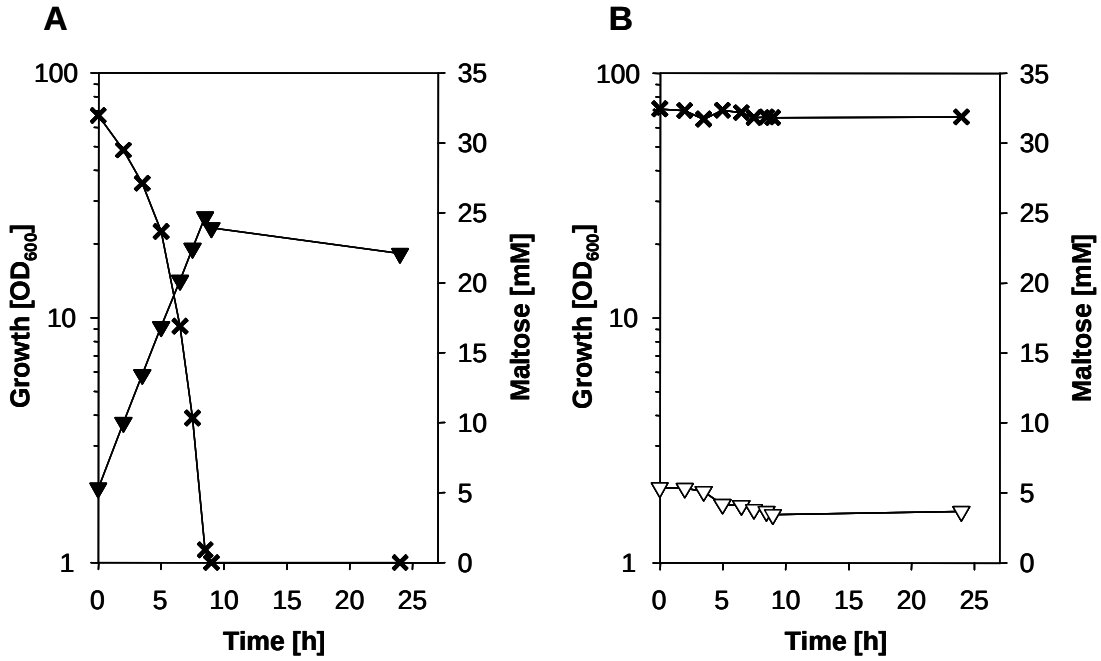

Figure 20: Growth of *C. glutamicum* IM*musI* (pXMJ19_*musI*) (A) and *C. glutamicum* IM*musI* (B) in minimal medium with 1% maltose. Growth of: (▼) *C. glutamicum* IM*musI* (pXMJ19_*musI*), (▽) *C. glutamicum* IM*musI*. Consumption of: (✕) maltose.

To further analyze MusI the expression plasmid pXMJ19_*musI* was constructed and used for complementation of *C. glutamicum* IM*musI*. Growth experiments in minimal medium with maltose showed that *C. glutamicum* IM*musI* (pXMJ19_*musI*) regained the ability to utilize for maltose growth with a rate of 0.30 ± 0.01 h^{-1} (Fig. 20, A). The integration mutant IM*musI* did not grow at all leaving the maltose unused (Fig. 20, B). These results were supported by radioactive transport measurements showing that *C. glutamicum* IM*musI* (pXMJ19_*musI*) takes up maltose with a rate of 16.7 ± 2.3 nmol/(min*mg cdm), whereas maltose uptake in *C. glutamicum* IM*musI* was not detected.

The strain *C. glutamicum* IM*musI* and the function of MusI were further analyzed by Nora Kuhlmann during her Bachelor thesis.

3.1.7 Expression analyses of the maltose uptake system

Growth experiments and radioactive transport measurements with strains deleted in transcriptional regulators already indicated that RamA and SugR might be involved in the regulation of the maltose uptake system. To reveal if there are putative binding sites for those regulators present, the upstream regions of *musK*, *musE* and *musFGI* were analyzed with 5'-RACE. The transcription start sites for *musK* and *musFGI* were identified being located 88 bp and 62 bp upstream of the start of the respective sequence (Fig. 21); the transcriptional start site for *musE* was not identified. Further analyses revealed two putative RamA binding sites in the upstream regions of *musE* and *musFGI* located 16 bp and 43 bp, respectively, upstream of the start of the sequences (Fig. 21). There were no putative RamA binding sites found in the upstream region of *musK*. Further, no putative binding sites for SugR were detected in the analyzed sequences.

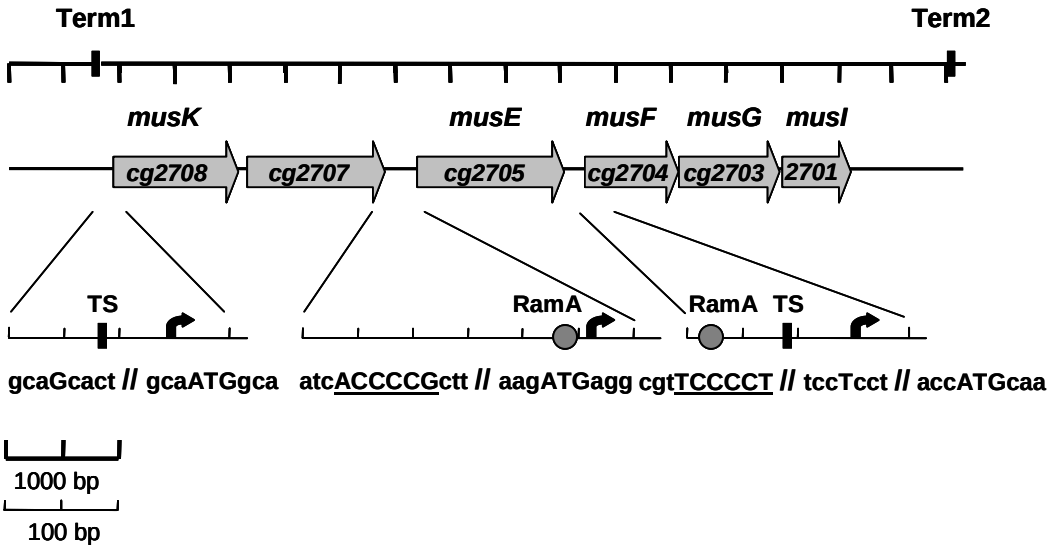

Figure 21: Genomic locus of MusIFGK$_2$-E, sequence and location of putative RamA binding sites and transcription start sites. Putative RamA binding sites: capitalized and underlined; transcription start site: one capitalized letter.

To test whether the reduced uptake and growth rates of *C. glutamicum* RG2 (Δ*ramA*) and *C. glutamicum* Δ*sugR* were caused by the transcriptional regulation of the maltose uptake system, RNA hybridization analyses were performed after cultivation of the cells with glucose, maltose and acetate, respectively. *C. glutamicum* Δ*sugR* was used for expression analyses, although there were no putative SugR binding sites found in the upstream regions of *musK*, *musE* and *musFGI*, because this strain showed decreased growth in minimal medium with maltose and reduced maltose uptake rates like *C. glutamicum* RG2 (see 3.1.3).

The results of the transcriptional analyses show for the WT cells that *musE* and *musFGI* expression was high on glucose and lower on maltose (Fig. 22). The expression was lowest in cells that were cultivated with acetate, supporting the observations of the radioactive transport measurements (see 3.1.3). The ATPase subunit encoded by *musK* in contrast did not seem to be differentially expressed under all growth conditions tested (Fig. 22).

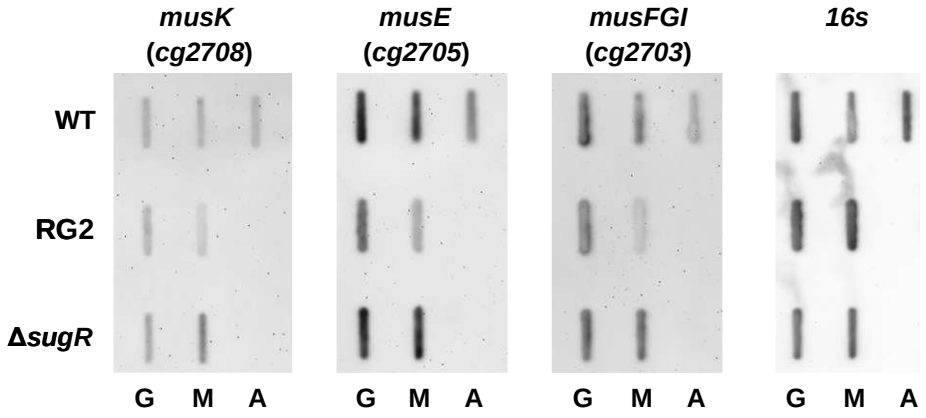

Figure 22: RNA hybridization experiments showing the transcriptional regulation of *musIFGK-E* by RamA and SugR in dependence of the carbon source. Cultivation with G = glucose, M = maltose, A = acetate, WT = *C. glutamicum* WT, RG2 = *C. glutamicum* RG2 (ΔramA), Δ*sugR* = *C. glutamicum* Δ*sugR*.

In the RamA deficient strain *C. glutamicum* RG2, *musE* and the operon *musFGI* were downregulated at the transcriptional level on glucose and on maltose compared to the WT. In cells that were cultivated with maltose the expression of *musE* and *musFGI* was even lower than in WT cells cultivated with acetate (Fig. 22). Interestingly, the *musK* expression level in RG2 cells was similar to the one in WT cells, corresponding to the observation that no putative RamA binding sites are present in the upstream region of *musK*. The expression level of *musE*, *musFGI*, and *musK* in *C. glutamicum* Δ*sugR* were similar to those found in the WT under all growth conditions tested (Fig. 22). These findings are supported by the missing SugR binding sites in the analyzed upstream regions of *musK*, *musE* and *musFGI*, but contradict the observed growth phenotype and the reduced transport rate of *C. glutamicum* Δ*sugR.*

3.1.8 Substrate specificity of MusIFGK$_2$-E

To test whether MusIFGK$_2$-E is specific for maltose or has a broader substrate spectrum such as e.g. MalFGK$_2$-E of *E. coli*, which in addition to maltose transports maltodextrins (Boos & Shumann, 1998), radioactive transport assays were performed with the following

unlabeled substrates in 100-fold excess: maltose glucose, trehalose, isomaltose, maltodextrins with increasing length from maltotriose to maltoheptaose and acarbose.

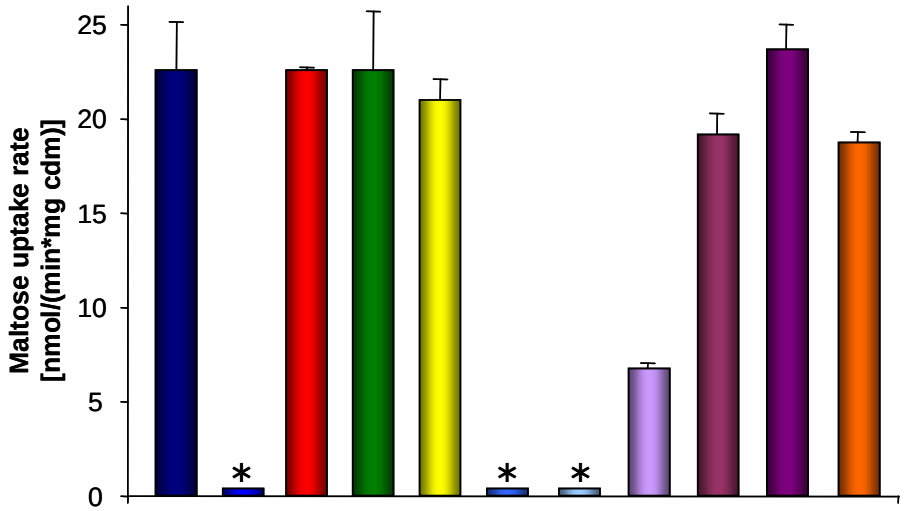

Figure 23: Effect of competitors on maltose uptake rates of *C. glutamicum* WT. Cells were cultivated in minimal medium with 1.5% glucose. The reaction was started by the addition of 50 µM [^{14}C]-maltose and 5 mM of the competitor. No competitor (dark blue), maltose (blue), glucose (red), trehalose (green), isomaltose (yellow), maltotriose (light blue), maltotetraose (pale blue), maltopentaose (lavender), maltohexaose (plum), maltoheptaose (violet), acarbose (orange), (∗) uptake of label only.

Besides the addition of maltose only maltotriose and maltotetraose quenched the uptake of labeled maltose (Fig. 23; light blue, pale blue). The added maltopentaose had a similar effect, reducing the detected uptake rate to 32% [6.8 ± 0.3 nmol/(min*mg cdm)] of the initial one (Fig. 23, lavender). Glucose, trehalose, acarbose and maltodextrins with more than five glucose moieties had no effect on maltose uptake in *C. glutamicum* (Fig. 23; red, green, plum, violet, orange).

These results indicate that the maltose uptake system is not specific for the uptake of maltose, it might also facilitate the uptake of maltotriose, maltotetraose and probably, with a lower affinity, maltopentaose. It is also possible that these substances only block the uptake of maltose which can be tested in growth experiments.

To analyze if MusIFGK$_2$-E is also capable of transporting maltodextrins, growth experiments with *C. glutamicum* WT and *C. glutamicum* Δmus in minimal medium with maltotriose as substrate were performed. The data show that the WT grew on maltotriose with a growth rate of 0.40 ± 0.00 h^{-1}, reaching a final optical density of 14.0 ± 0.2 after 24 h (Fig. 24, A). *C. glutamicum* Δmus on the contrary did neither grow nor utilize maltotriose (Fig. 24, B). These results show that MusIFGK$_2$-E is not only the maltose but also the maltodextrin uptake system of *C. glutamicum*. It is very likely that maltotetraose and

maltopentaose are also transported by the maltose uptake system of *C. glutamicum* which has already been shown for MalFGK$_2$-E of *E. coli* that is capable of transporting maltodextrins up to seven to eight glucose units (Boos & Shumann, 1998).

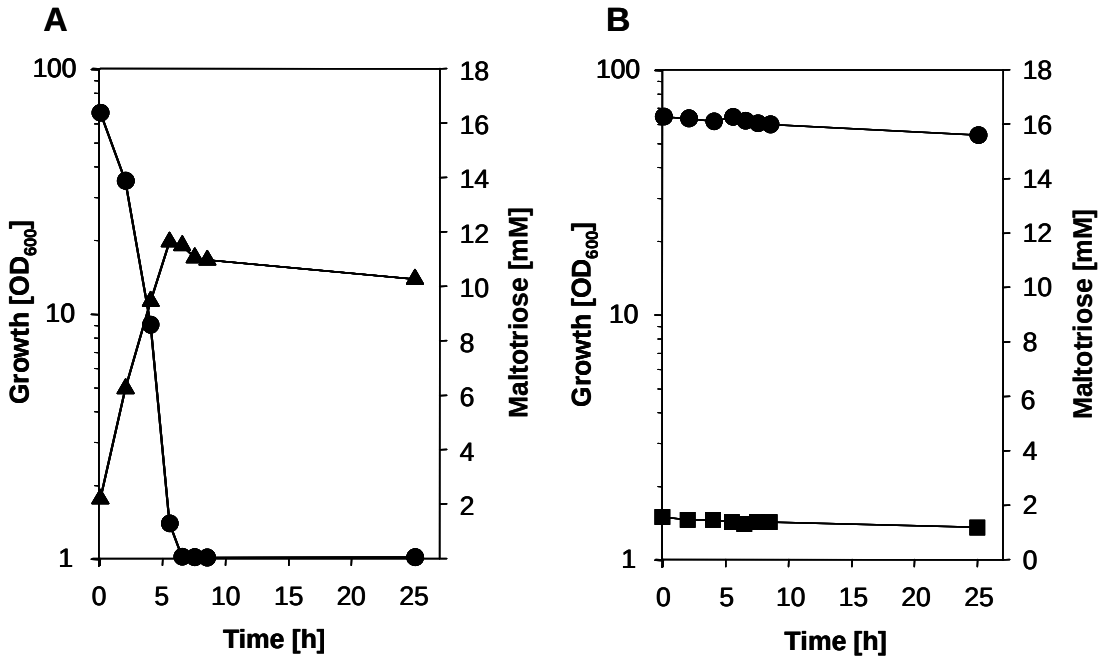

Figure 24: Growth of *C. glutamicum* WT (A) and *C. glutamicum* Δmus (B) in minimal medium with 1% maltotriose. Growth of: (▲) *C. glutamicum* WT, (■) *C. glutamicum* Δmus. Consumption of: (●) maltotriose.

3.1.9 Connections between maltose and glucose metabolism

The previously presented results that maltose is taken up via the binding protein dependent ABC transport system MusIFGK$_2$-E and the perfect co-metabolization of maltose and glucose, does not point to an obvious connection between those two metabolic pathways. Interestingly, it has been published that two strains inactivated in EI and HPr, respectively, two proteins of the PTS phosphorylation cascade, show reduced growth when cultivated with maltose (Parche *et al*., 2001).

In order to have a closer look at this observation, the mutant *C. glutamicum* Δ*hpr* was used for growth experiments in minimal medium with maltose as sole carbon source. Indeed, the mutant showed a dramatically reduced growth with a growth rate of 0.12 h^{-1} compared to 0.39 h^{-1} of the WT, with both strains reaching comparable optical densities of 19 and 17, respectively (Fig. 25).

To test whether the growth phenotype was due to reduced maltose uptake rates radioactive transport measurements were conducted with *C. glutamicum* Δ*hpr* after different cultivation conditions. The transport data show that the maltose uptake rate was

reduced when Δ*hpr* cells were cultivated in TY rich medium before they were cultivated in minimal medium with maltose as sole carbon source, compared to the WT [10.2 ± 1.2 nmol/(min*mg cdm) and 17.5 ± 0.2 nmol/(min*mg cdm), respectively] (Tab. 7). Notably, the uptake rate of 33.5 ± 2.2 nmol/(min*mg cdm) in Δ*hpr* cells adapted to maltose was even higher than in WT cells [17.0 ± 6.4 nmol/(min*mg cdm), Tab. 7]. The maltose uptake rates of both strains were similar when cells were cultivated exclusively on TY medium.

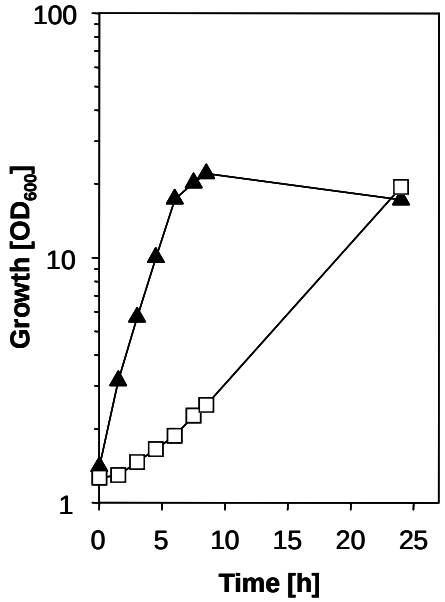

Figure 25: Growth of *C. glutamicum* WT and *C. glutamicum* Δ*hpr* in minimal medium with 1% maltose. Growth of: (▲) *C. glutamicum* WT, (□) *C. glutamicum* Δ*hpr*.

Table 7: Maltose uptake rates in *C. glutamicum* WT and *C. glutamicum* Δ*hpr* in dependence of the cultivation conditions. Cells were cultivated in TY rich medium (TY) overnight before they were cultivated in minimal medium with 1.5% glucose (Glu) and 1.5% maltose (Mal), respectively or they were exclusively cultivated in the respective medium. (n.d.) not determined.

	Maltose uptake rate [nmol/(min*mg cdm)]				
pre / main culture	TY / Glu	TY / Mal	Glu / Glu	Mal / Mal	TY / TY
C. glutamicum WT	30.3 ± 2.4	17.5 ± 0.2	22.7 ± 3.4	17.0 ± 6.4	29.4 ± 2.0
C. glutamicum Δ*hpr*	n.d.	10.2 ± 1.2	n.d.	33.5 ± 2.2	29.1 ± 0.7

3.2 Trehalose metabolism of *C. glutamicum*

In *C. glutamicum* three trehalose biosynthesis pathways are present (Wolf *et al*., 2002), underlining the physiological importance of this carbohydrate for the bacterium. Trehalose serves as a compatible solute in *C. glutamicum* under special conditions such as nitrogen- and carbon-limitation (Gebhardt, 2005). Even more important is the function of trehalose as a building block for the synthesis of mycolates (Tropis *et al*., 2005). In this context it was hypothesized that *C. glutamicum* is equipped with a trehalose export system which has to be identified (Tropis *et al*., 2005). Further it has been published that *C. glutamicum* is neither able to grow with trehalose as sole carbon source nor is equipped with an uptake system for trehalose (Wolf, 2002; Gebhardt, 2005; Tropis *et al*., 2005).

3.2.1 *C. glutamicum* utilizes trehalose for growth

Trehalose has been shown to be an important building block for the synthesis of mycolates in *C. glutamicum* (Tropis *et al*., 2005); to analyze effects of trehalose on cell wall synthesis *C. glutamicum* was cultivated in minimal medium with glucose plus trehalose. As depicted in Fig. 26 growth rates were similar with 0.40 ± 0.02 h^{-1}, but interestingly the final optical density was significantly higher (31) in cultivations with added trehalose than without (18) (Fig. 26; A, B).

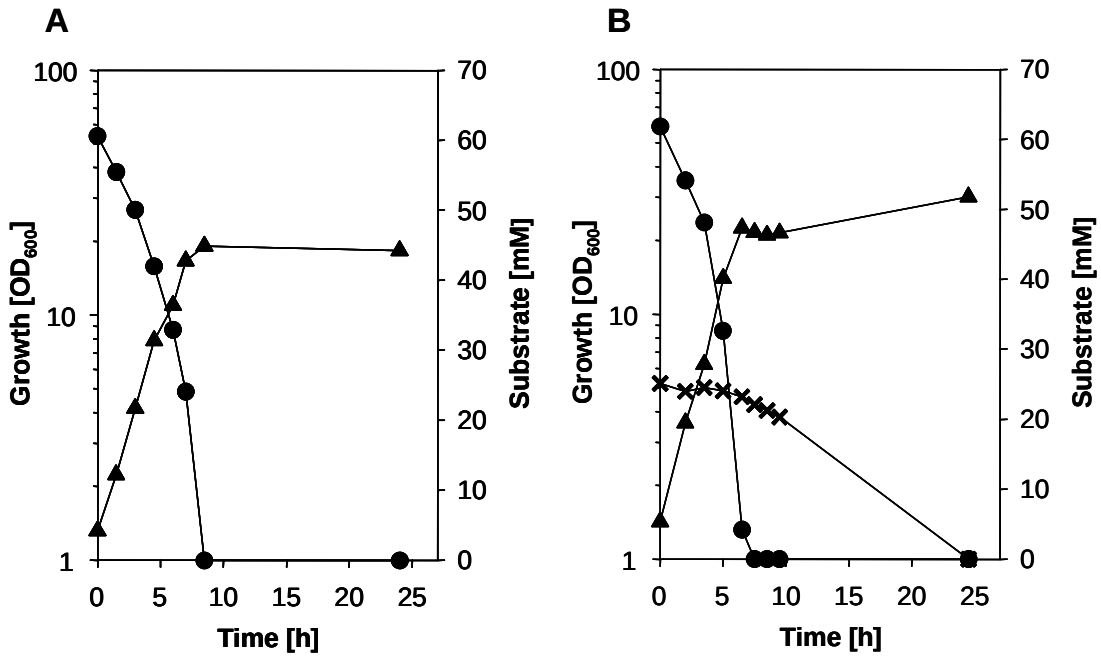

Figure 26: Growth of *C. glutamicum* WT cultivated in minimal medium with 1% glucose (A) and 1% glucose plus 1% trehalose (B). Growth of: (▲) *C. glutamicum* WT. Consumption of: (●) glucose; (✕) trehalose.

The substrate consumption data underlined that trehalose was utilized by *C. glutamicum* for growth as it was completely consumed (Fig. 26, B). Trehalose consumption was detectable from the beginning of the cultivation but seemed to speed up after glucose was consumed (Fig. 26, B).

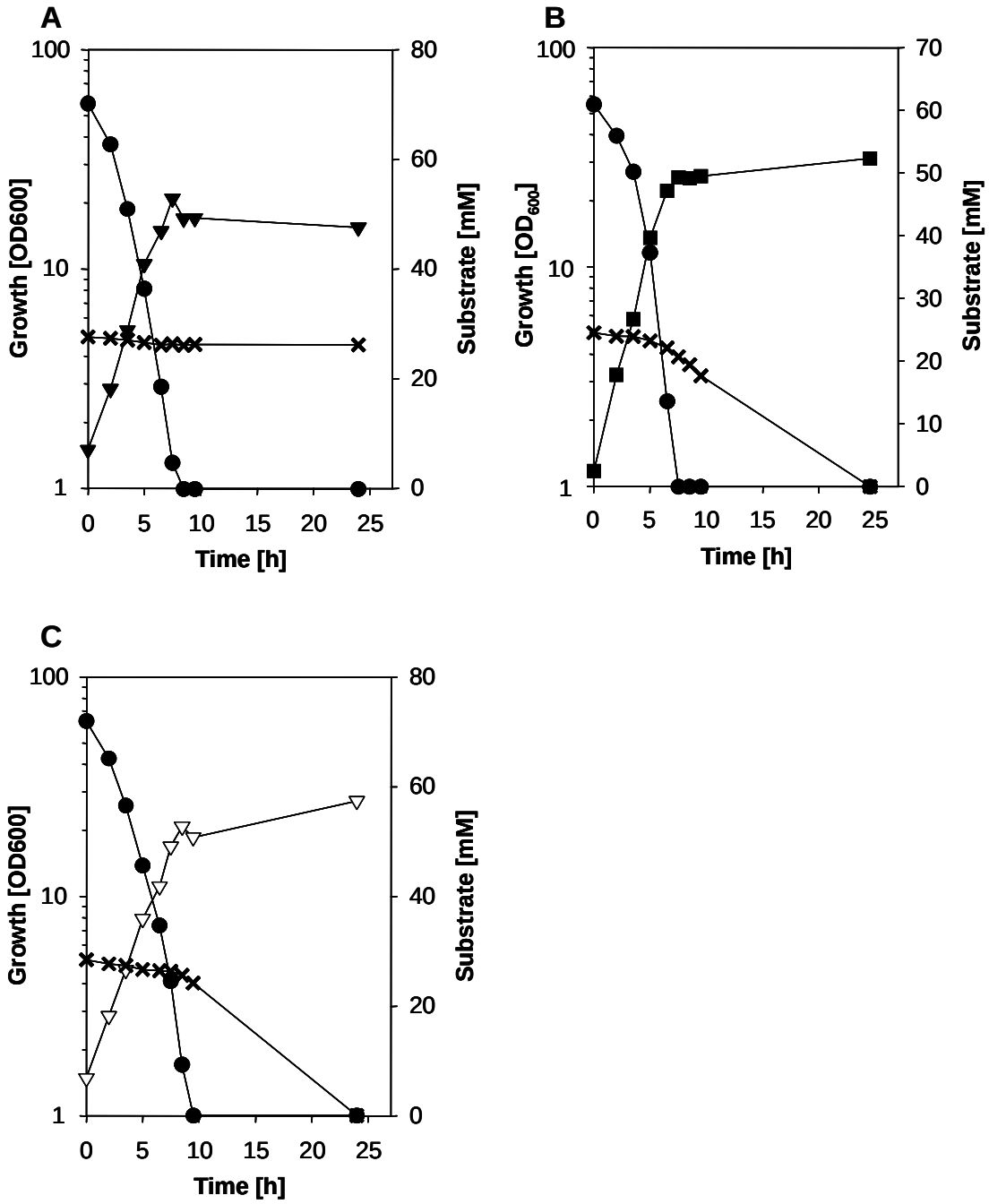

Figure 27: Growth of *C. glutamicum* Δ*treS* (pXMJ19) (A), *C. glutamicum* Δmus (B) and *C glutamicum* Δ*treS* (*pXMJ19_treS*) (C) in minimal medium with 1% glucose plus 1% trehalose. Growth of: (▼) *C. glutamicum* Δ*treS* (pXMJ19), (■) *C. glutamicum* Δmus, (▽) *C glutamicum* Δ*treS* (pXMJ19_*treS*). Consumption of: (●) glucose, (×) trehalose.

It was considered that trehalose is not directly imported by *C. glutamicum* and utilized as carbon source. It was arguable if trehalose in the external medium is converted to maltose by excreted TreS, which catalyzes the interconversion of trehalose and maltose, and subsequently taken up by the maltose uptake system.

To rule out this hypothesis growth experiments with *C. glutamicum* Δ*treS* and *C. glutamicum* Δ*mus* in minimal medium with glucose plus trehalose were performed. The growth data show that *C. glutamicum* Δ*treS* and *C. glutamicum* Δ*mus* were growing with a similar growth rate of 0.40 ± 0.03 h^{-1} utilizing the given glucose (Fig. 27; A, B).

The Δ*mus* mutant reached a significantly higher optical density of 31 and was able to utilize the added trehalose for growth (Fig. 27, B). The Δ*treS* mutant on the contrary was not able to utilize the added trehalose, reaching a lower optical density of 16 and not consuming trehalose (Fig. 27, A). The strain *C glutamicum* Δ*treS* (pXMJ19_*treS*), carrying the *treS* expression plasmid pXMJ19_*treS* regained the ability to utilize trehalose indicated by an optical density of 27 and the total consumption of trehalose (Fig. 27, C). These results revealed that trehalose is not taken up by the maltose uptake system MusIFGK_2-E after external conversion to maltose by TreS. At the same time it was shown that TreS mediated conversion from trehalose to maltose is an important step for trehalose utilization in *C. glutamicum*.

3.2.2 Biochemical characterization of trehalose uptake in *C. glutamicum*

As growth experiments with glucose plus trehalose as additional substrate showed that *C. glutamicum* indeed is able to utilize trehalose for growth, the uptake of trehalose was characterized biochemically applying radioactive transport assays with [^{14}C]-trehalose. *C. glutamicum* WT cells were cultivated in TY medium to mid-exponential growth phase, harvested and washed twice with ice cold CGC minimal medium, before they were used for the measurements.

To reveal the biochemical parameters K_m and V_{max} for the trehalose uptake, transport assays with substrate concentrations ranging from 0.1 µM to 20 µM were performed. Plotting the data of *C. glutamicum* WT according to the Michaelis-Menten equation revealed a saturation kinetic (Fig. 28, A). Furthermore, the data were plotted according to Lineweaver-Burk in a double reciprocal plot (Fig. 28, B). Kinetic parameters were derived by using the Sigma Plot 10.0 software, revealing a K_m of 0.16 ± 0.02 µM and a V_{max} of 2.5 ± 0.1 nmol/(min*mg cdm) for trehalose uptake. There was no indication for a second system, such as biphasic dependence of the uptake rate on substrate concentration, showing that trehalose uptake is facilitated by a single slow high affinity transport system.

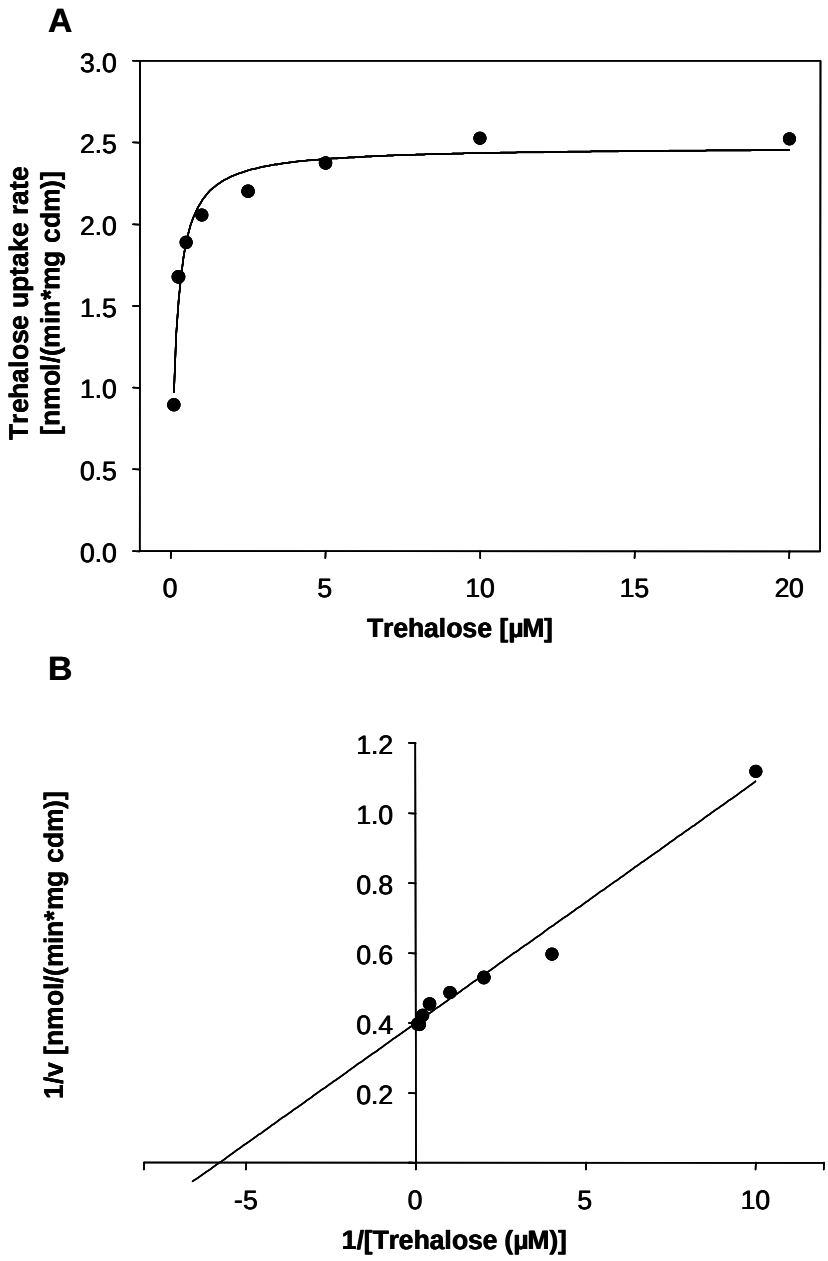

Figure 28: Biochemical characterization of trehalose uptake in *C. glutamicum* WT. [^{14}C]-trehalose uptake was measured with different concentrations (0.1 - 20 µM). (A) Data plotted according to Michaelis-Menten equation, (B) Data plotted according to Lineweaver-Burk.

To analyze the substrate spectrum of the transport system trehalose uptake was measured with the following unlabeled substrates in 100-fold excess: trehalose, maltose, isomaltose, acarbose, sucrose, galactose and maltotriose were used. Only unlabeled trehalose added to the experiment led to the transport rate not being detectable radioactively, since the high excess of unlabeled trehalose quenched the labeled one (Fig.

29). None of the other substances tested were competing with trehalose for uptake, indicating that they are no substrates for the trehalose import system (Fig. 29).

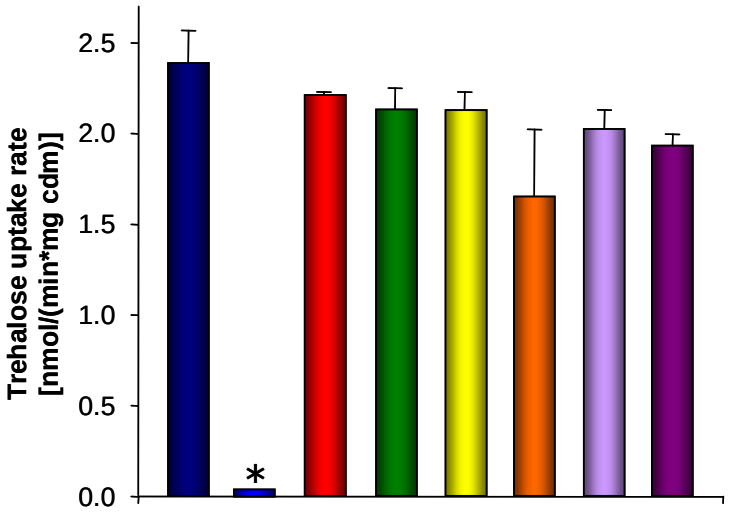

Figure 29: Effect of competitors on trehalose uptake rates of *C. glutamicum* WT. Cells were cultivated in TY medium. The reaction was started by the addition of 50 µM [^{14}C]-trehalose and 5 mM of the competitor. No competitor (dark blue), trehalose (blue), maltose (red), isomaltose (green), acarbose (yellow), sucrose (orange), galactose (lavender), maltotriose (plum), (∗) uptake of label only.

3.2.3 Identification of the trehalose uptake system of *C. glutamicum*

So far it has been published that *C. glutamicum* is not able to grow with trehalose as sole carbon source and that there is no trehalose uptake system present (Tropis *et al*., 2005, Wolf, 2002). But recent findings showed that *C. glutamicum* is indeed able to utilize trehalose in mixed carbon source cultivations (see 3.2.1) and is able to take up trehalose via a high affinity transport system (see 3.2.2). Little was known about trehalose uptake systems in related bacteria but in *Streptomyces reticuli* MsiK-dependent uptake of trehalose was mentioned (Schlösser, 2000). The data suggested that the MsiK protein is an ATP-binding protein that assists a trehalose ABC transporter (Schlösser, 2000). Sequence comparison of MsiK of *S. reticuli* and *C. glutamicum* revealed a homologous protein with an identity of 59% to MsiK of *S. reticuli* (e-value 7e-125), annotated as MsiK2 (*cg0835*), being the ATPase component of an ABC transport system. A second homologous protein with similar identity was identified being the ATPase component MusK of the maltose uptake system MusIFGK$_2$-E. This system has already been shown not to be involved in trehalose utilization (see 3.2.1). Therefore, the only candidate for the trehalose uptake system of *C. glutamicum* was the ABC transporter encoded by *cg0831*-

cg0835. To test if the genes *cg0831-cg0835* indeed encode the trehalose uptake system a deletion mutant was constructed in which the genes from *cg0831* to *cg0835* were deleted by homologous recombination using the plasmid pK19mobsacB_tusDel. The deletion was verified by PCR and the so constructed mutant named *C. glutamicum* Δtus.

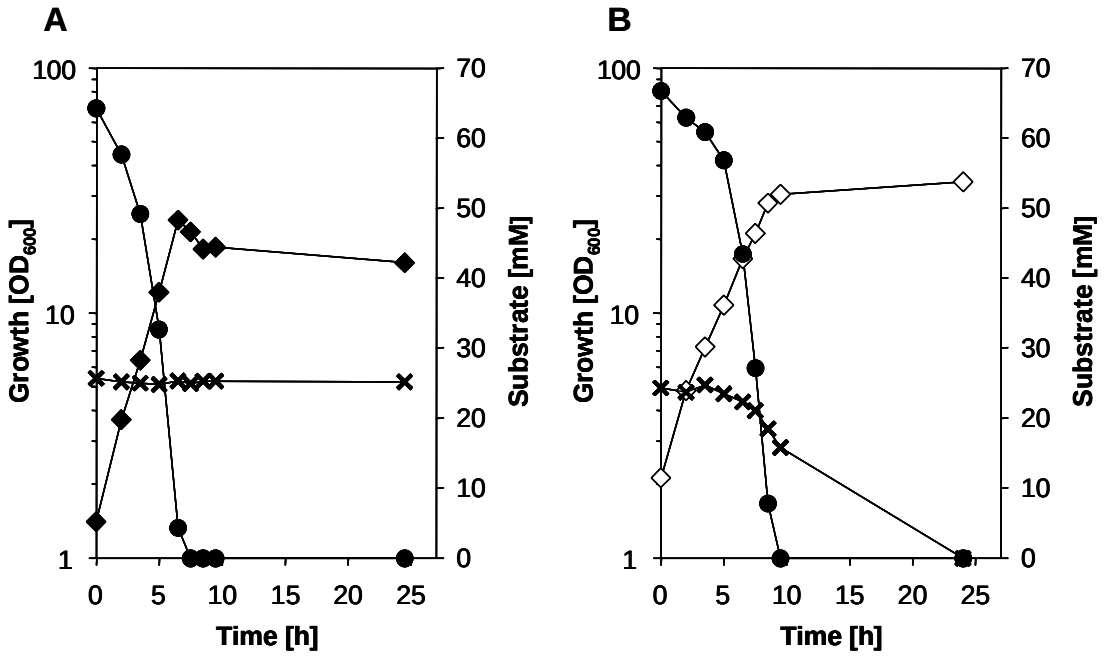

Figure 30: Growth of *C. glutamicum* Δtus (A) and *C. glutamicum* Δtus (pXMJ19_*tusFGK-E*) (B) in minimal medium with 1% glucose plus 1% trehalose. Growth of: (◆) *C. glutamicum* Δtus, (◇) *C. glutamicum* Δtus (pXMJ19_*tusFGK-E*). Consumption of: (●) glucose, (✕) trehalose.

The mutant was used for growth experiments in minimal medium with glucose plus trehalose. The deletion strain *C. glutamicum* Δtus was growing on glucose with a growth rate of 0.39 ± 0.02 h^{-1}, but was not able to utilize trehalose as carbon source, resulting in an optical density 16 (Fig. 30, A). Subsequent, radioactive transport measurements showed that *C. glutamicum* Δtus was indeed not able to take up trehalose (data not shown).

To proof that the genes *cg0831-cg0835* encode the trehalose uptake system of *C. glutamicum* the expression plasmid pXMJ19_*tusFGK-E* was constructed carrying the genes *cg0831*, *cg0832*, *cg0833*, *cg0834* and *cg0835*. Growth experiments with *C. glutamicum* Δtus (pXMJ19_*tusFGK-E*) in minimal medium with glucose plus trehalose showed that this strain regained the ability to take up trehalose, reaching a final optical density of 34, although with a reduced growth rate of 0.28 ± 0.01 h^{-1} compared to the deletion mutant (Fig. 30; A, B). The given results reveal that the trehalose uptake system of *C. glutamicum* is encoded by the genes *cg0831-cg0835*. Therefore, the system was named trehalose uptake system $TusFGK_2$-E, according to the maltose uptake system of

E. coli MalFGK$_2$-E, comprising two permease subunits *tusF* (*cg0831*), *tusG* (*cg0832*), the trehalose binding protein *tusE* (*cg0834*) and the ATPase subunit *tusK* (*cg0835*).

3.2.4 Transcriptional organization of *tusFGK-E*

The transcriptional organization of the trehalose uptake system TusFGK$_2$-E was analyzed by Northern Blot and RNA hybridization experiments. For DIG-labeled RNA probe synthesis one permease component (*tusG*) was chosen to represent an essential part of the uptake system (Fig. 31, C)

The Northern Blot analysis showed a signal at about 1800 bases (Fig. 31, A) indicating that the genes of the trehalose uptake system do not form an operon. It is more likely that *tusG* (*cg0832*) forms an operon with *tusF* (*cg0831*) the second permease component, since the length of both genes adds to 1873 bp.

As shown by RNA hybridization analysis the expression of *tusG* (Fig. 31, B) was independent of the carbon source used for cultivation (glucose, maltose and acetate, respectively). This indicated that the genes for the trehalose uptake system of *C. glutamicum* are expressed during the exponential growth phase independent of the carbon source.

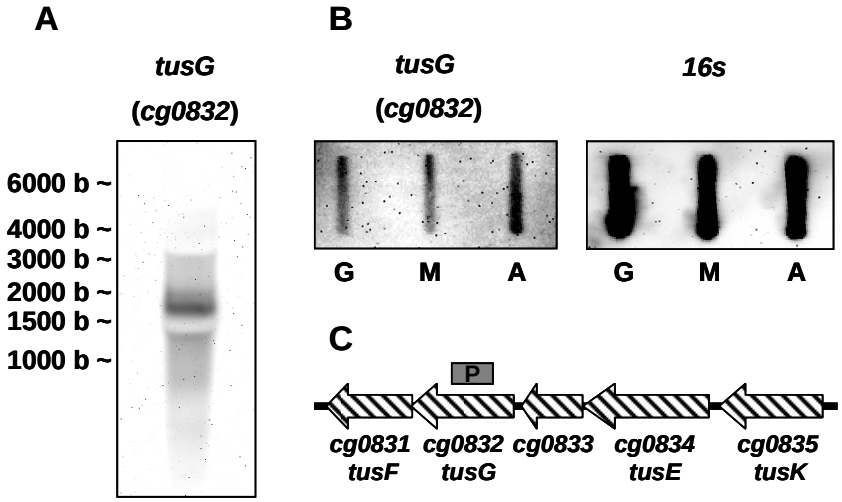

Figure 31: Northern Blot (A) and RNA hybridization experiment (B) of *tusG* (*cg0832*) of *C. glutamicum* cultivated with different carbon sources and genes encoding the trehalose uptake system (C). RNA levels of *tusG* and *16s* were monitored with DIG-labeled antisense-RNA probes. G = glucose, M = maltose, A = acetate, *tusF* (*cg0831*) = permease, *tusG* (*cg0832*) = permease, *cg0833* = hypothetical protein, *tusE* (*cg0834*) = binding protein, *tusK* (*cg0835*) = ATPase component, P = probe *tusG* . All annotations are predicted, according to Kalinowski *et al*., 2003..

3.2.5 Direct evolution of *C. glutamicum* for the utilization of trehalose as sole carbon source

As *C. glutamicum* possesses a trehalose uptake system it was challenging to see whether *C. glutamicum* can be evolved to utilize trehalose as sole carbon source. Therefore, *C. glutamicum* WT was cultivated in minimal medium with 4% trehalose. After several days of incubation at 30℃ the culture started to g row very slowly. Cells from this slow growing culture were used for inoculating fresh medium with trehalose as carbon source. This step was repeated until a *C. glutamicum* culture was obtained that was definitely growing faster with trehalose. The so gained *C. glutamicum* spontaneous-mutant (TM) was used for growth experiments in minimal medium with trehalose. *C. glutamicum* WT was used as reference strain. *C. glutamicum* TM grew on trehalose with a growth rate 0.12 ± 0.1 h^{-1} to an optical density of 22 after 30 h (Fig. 32, ●), the WT instead did not grow higher than to an optical density of 2.6 in this period (Fig. 32, ▲). These results clearly show that *C. glutamicum* evolved to grow with trehalose as sole carbon source.
Subsequent, *C. glutamicum* TM was tested for the ability to produce amino acids from trehalose. For L-glutamate production the TM was cultivated with 2% trehalose as sole carbon source before the production was triggered by the addition of penicillin.

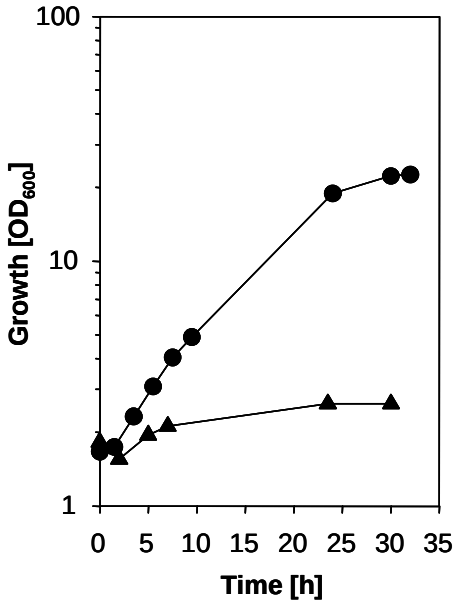

Figure 32: Growth of *C. glutamicum* WT and *C. glutamicum* TM in minimal medium with 1.5% trehalose as sole carbon source. Growth of: (▲) *C. glutamicum* WT, (●) *C. glutamicum* TM.

As control the WT and the TM were cultivated on 2% glucose and treated alike. L-glutamate production was monitored via HPLC, 2 h, 24 h and 48 h after penicillin was

added. The data show that the *C. glutamicum* TM was able to produce L-glutamate from trehalose (Fig. 33, green). Although the amount of produced L-glutamate of 3 ± 0.3 mM after 24 h and 9 ± 0.7 mM after 48 h was lower than in the control cultivation with glucose (9 ± 1.2 mM and 10 ± 3.0 mM, respectively, Fig. 33, red) and the WT control (11 ± 0.5 mM and 12 ± 1.3 mM, respectively, Fig. 33, blue).

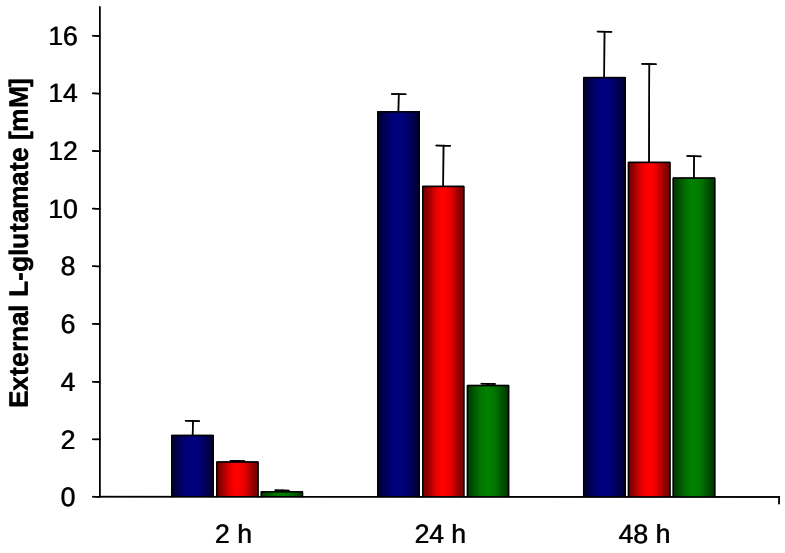

Figure 33: L-glutamate production of *C. glutamicum* WT and *C. glutamicum* TM. *C. glutamicum* WT, 2% glucose (blue); *C. glutamicum* TM, 2% glucose (red); *C. glutamicum* TM, 2% trehalose (green).

3.3 The trehalose export hypothesis

One hypothesis for the mycolate synthesis in *C. glutamicum* and corynebacteria in general (Tropis *et al*., 2005), states that mycolic acids and trehalose are synthesized in the cytoplasm and subsequently transported separately across the plasma membrane via unknown transport mechanisms (Fig. 34). In the cell wall one molecule of mycolic acid and one molecule of trehalose are esterified to form trehalose monomycolate (TMM) (Tropis *et al*., 2005). TMM is the precursor for the synthesis of trehalose dimycolate (TDM) and arabinogalactan mycolate (AGM) (Fig. 34). These three mycolates are major components of the unique cell wall of corynebacteria, causing the high resistance of these bacteria to common antibiotics.

This hypothesis was based on the observations that *C. glutamicum* is not able to grow with trehalose as sole source of carbon and that a strain deficient in all three trehalose synthesis pathways (*C. glutamicum* Δ*otsA* Δ*treS* Δ*treY*) was not able to synthesize mycolates when cultivated with sucrose as carbon source (Wolf *et al*., 2003; Tropis *et al*., 2005). When this triple-mutant was supplemented with external trehalose during growth, the cells were able to synthesize trehalose monomycolate, showing that external

trehalose is important for mycolate synthesis in *C. glutamicum* (Gebhardt, 2005). From these results it was concluded that there is an export system for trehalose present in *C. glutamicum* that facilitates the transport of trehalose across the plasma membrane.

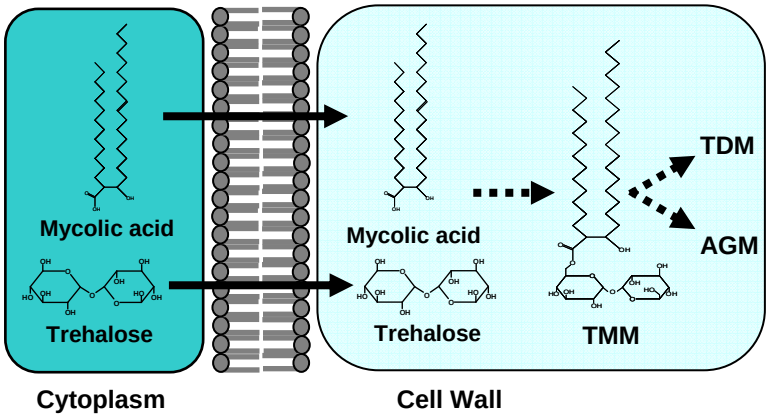

Figure 34: Model of mycolate synthesis in *C. glutamicum*, according to Tropis *et al*., 2005. (TMM) trehalose monomycolate, (TDM) trehalose dimycolate, (AGM) arabinogalactan mycolate, (→) unknown transport mechanisms, (⇢) catalyzed by several enzymatic steps.

This hypothesis was only valid as long as there was no import system detected for trehalose. Afore presented results revealed that *C. glutamicum* indeed is equipped with an uptake system for trehalose so that the trehalose export hypothesis had to be reassessed, because that means that trehalose can be taken up into the cell where it subsequently might be esterified with mycolic acids in the cytoplasm. Afterwards the trehalose monomycolate (TMM) can be exported by an unknown transport system as proposed for TMM synthesis in *M. tuberculosis* by Takayama and co-workers (2005).

To reassess the trehalose export hypothesis the genes from *cg0831* to *cg0835*, encoding the trehalose uptake system, were deleted in the triple-mutant *C. glutamicum* Δ*otsA* Δ*treS* Δ*treY* by homologous recombination using the plasmid pK19mobsacB_tusDel. The correct deletion was verified by PCR and the so constructed mutant named *C. glutamicum* Δ*otsA* Δ*treS* Δ*treY* Δtus. This strain should neither be able to synthesize nor able to import trehalose. *C. glutamicum* Δ*otsA* Δ*treS* Δ*treY* Δtus was used for growth experiments in minimal medium on sucrose with and without the addition of trehalose. Sucrose was used as carbon source because it has been shown that this carbohydrate can not be used for mycolate synthesis by *C. glutamicum* Δ*otsA* Δ*treS* Δ*treY* (Wolf *et al*., 2003; Tropis *et al*., 2005). As shown in Fig. 35, A, growth of *C. glutamicum* Δ*otsA* Δ*treS* Δ*treY* Δtus and *C. glutamicum* Δ*otsA* Δ*treS* Δ*treY* was slowed down compared to the WT with growth

rates of 0.27 ± 0.0 h^{-1}, 0.26 ± 0.05 $h^{-1}$and 0.39 ± 0.03 h^{-1}, respectively. Growth of the mutants even slowed down after six hours of cultivation to 0.12 ± 0.0 h^{-1} and 0.18 ± 0.01 h^{-1}, respectively (Fig. 35, A). When trehalose was additionally present, growth of *C. glutamicum* Δ*otsA* Δ*treS* Δ*treY* Δtus and *C. glutamicum* Δ*otsA* Δ*treS* Δ*treY* became faster and constant with growth rates of 0.27 ± 0.0 $h^{-1}$and 0.30 ± 0.03 h^{-1}, respectively (Fig. 35, B). This shows that the addition of trehalose did not only have a positive effect on the triple-mutant but also on the quadruple-mutant that was deleted in the genes encoding the trehalose uptake system.

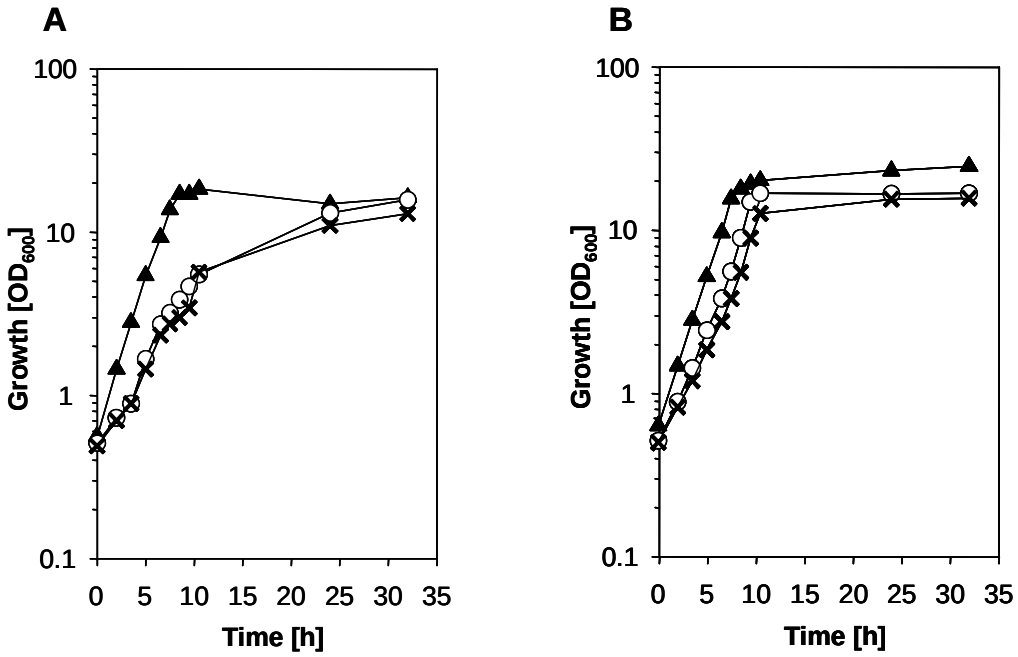

Figure 35: Growth of *C. glutamicum* strains in minimal medium with 1% sucrose (A) and 1% sucrose plus 1% trehalose (B). Growth of: (▲) *C. glutamicum* WT, (○) *C. glutamicum* Δ*otsA* Δ*treS* Δ*treY*, (×) *C. glutamicum* Δ*otsA* Δ*treS* Δ*treY* Δtus.

To proof that trehalose is still incorporated in trehalose mycolates in *C. glutamicum* Δ*otsA* Δ*treS* Δ*treY* Δtus a detailed mycolate analysis was done. For the qualitative analysis of mycolates *C. glutamicum* WT and *C. glutamicum* Δ*otsA* Δ*treS* Δ*treY* Δtus were cultivated in minimal medium on glucose with and without addition of trehalose, before the mycolates were extracted. The thin layer chromatography (TLC) of the lipid extracts from *C. glutamicum* WT and *C. glutamicum* Δ*otsA* Δ*treS* Δ*treY* Δtus show that the quadruple-mutant was not able to produce trehalose mycolates on glucose, whereas TDMs were detectable in the WT lipid preparation (Fig. 36). Another mycolate that was found is glucose mycolate which is produced when glucose is the only carbon source (Tropis *et al*., 2005). When trehalose was added to the cultivations TMMs and TDMs were detected in the WT and in *C. glutamicum* Δ*otsA* Δ*treS* Δ*treY* Δtus (Fig. 36). This result shows that the

quadruple-mutant is indeed able to synthesize trehalose mycolates from external trehalose, underlining that the trehalose export hypothesis is still valid. Further indicating that trehalose can be externally esterified with the mycolate moiety.

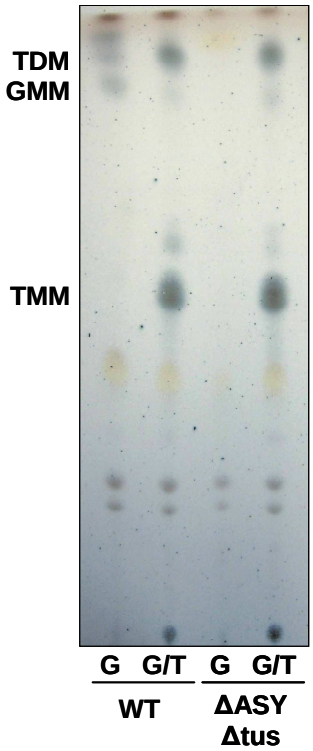

Figure 36: TLC of comparative mycolate analysis of *C. glutamicum* strains. Cells were cultivated in minimal medium with 3% glucose (G) and 3% glucose plus 2% trehalose (G/T). (WT) *C. glutamicum* WT, (ΔASY Δtus) *C. glutamicum* Δ*otsA* Δ*treS* Δ*treY* Δtus, (TMM) trehalose monomycolate, (TDM) trehalose dimycolate, (GMM) glucose monomycolate.

3.3.1 Screening system for mutants defective in trehalose export

It has been shown that an imperfect or missing mycolate layer, caused by deletions in the trehalose synthesis pathways leads to a higher susceptibility of *C. glutamicum* to the antibiotics penicillin, erythromycin and ethambutol (Gebhardt, 2005). These results were used to establish a screening system to identify a potential trehalose export system. As control strains *C. glutamicum* Δ*otsA* Δ*treS* Δ*treY* (pEKEx2), defective in trehalose synthesis, *C. glutamicum* WT (pEKEx2) and *C. glutamicum* RES 167 (pEKEx2) were used. For the screening the cells were cultivated in minimal medium with fructose, since fructose can not be used for building up mycolates (Gebhardt, 2005). For the assay ethambutol was chosen as antibiotic which proofed to be more stable than erythromycin (Griffith *et al.*, 1992). Ethambutol disrupts arabinogalactan synthesis by inhibiting the

enzyme arabinosyl transferase. Several concentrations of this antibiotic were tested and finally 1 µg/ml ethambutol was the concentration to discriminate best between *C. glutamicum* Δ*otsA* Δ*treS* Δ*treY* (pEKEx2), *C. glutamicum* WT (pEKEx2) and *C. glutamicum* RES 167 (pEKEx2) (Fig. 37).

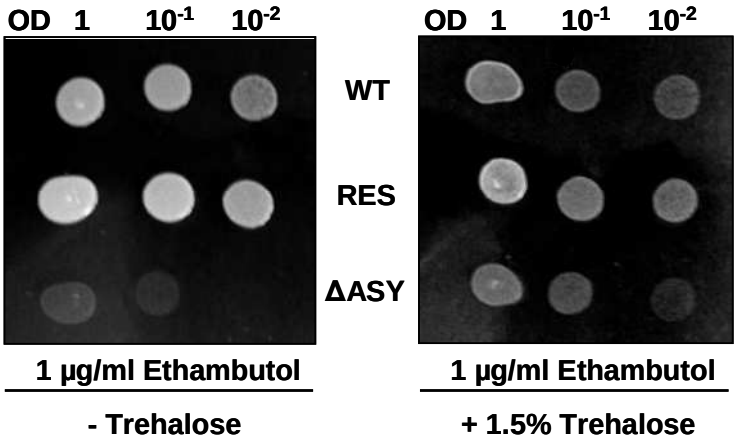

Figure 37: Screening assay for the identification of the trehalose export system of *C. glutamicum*. Cells were cultivated on CGC-agar with 1.5% fructose, 2 µg/ml kanamycin, 1 µ/ml ethambutol and + / - 1.5% trehalose. 3 µl of the cultures with indicated optical densities were spotted for each dot and incubated at 30℃ for 48 h. WT = *C. glutamicum* WT (pEKEx2), RES = *C. glutamicum* RES 167 (pEKEx2), ΔASY = *C. glutamicum* Δ*otsA* Δ*treS* Δ*treY*

For the experiment cells were cultivated in microtiter plates; after overnight cultivation the optical density was adjusted to 0.1 and 3 µl of the cultures were spotted on agar plates that contained 1.5% fructose, 25 µg/ml kanamycin and 1 µg/ml ethambutol. Afterwards the plates were incubated at 30℃ for 48 hours. Screeni ng of three transporter mutant libraries (data not shown), identified three mutants that showed the same susceptibility to 1 µg/ml ethambutol as the control strain *C. glutamicum* Δ*otsA* Δ*treS* Δ*treY* (pEKEx2). These mutants were inactivated in *cg0486* (encoding an ABC transporter permease protein), *cg3128* (encoding an ATPase component) and *cg0284* (encoding a RND superfamily drug efflux protein), respectively. Analyses, including growth experiment, of the candidates revealed that trehalose had no positive effect on growth of these mutants (data not shown). Surprisingly, Boltres (2009) identified the same mutants as candidates for the fructose export system with a different approach. Taken together this indicates that these mutants have a general defect which has nothing to do with trehalose export.

3.3.2 Approach for the biochemical characterization of trehalose export

The screening for the identification of the trehalose export system did not result in the identification of the trehalose exporter, requiring another approach to characterize and finally identify this system of *C. glutamicum*. At first the export of trehalose should have been characterized biochemically. The experimental system based on the assumption that imported maltose will be completely used for trehalose synthesis when the maltose metabolic pathway is inactivated by the deletion of MalQ, the key enzyme of maltose metabolism (Fig. 38). In the experiment *C. glutamicum* Δ*malQ* was thought to produce [^{14}C]-trehalose from [^{14}C]-maltose catalyzed by the trehalose synthase TreS. In theory this would promote [^{14}C]-trehalose export enabling the biochemical characterization of the export process (Fig. 38).

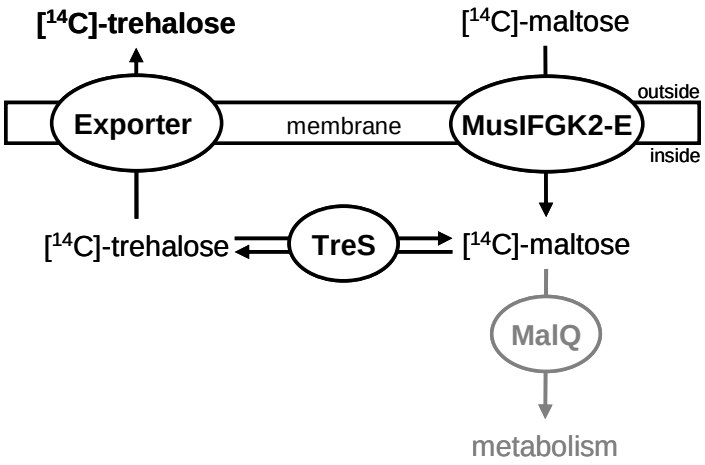

Figure 38: Diagram of an experimental setup to measure [^{14}C]-trehalose export after conversion of [^{14}C]-maltose by TreS. *C. glutamicum* Δ*malQ* (inactivated MalQ shown in grey) should direct all imported [^{14}C]-maltose to [^{14}C]-trehalose by TreS; working hypothesis.

Interestingly, the MalQ deficient strain, *C. glutamicum* Δ*malQ*, still showed residual growth with maltose and utilized all maltose under mixed carbon source cultivations, e.g. with glucose plus maltose, for growth (Kempkes, 2009). These data indicated that another pathway for maltose utilization must be present in *C. glutamicum* (Kempkes, 2009). As reference strain *C. glutamicum* Δ*malQ* Δ*treS* was constructed, this strain should not to be able to utilize maltose, both for growth and trehalose synthesis. *C. glutamicum* Δ*malQ* Δ*treS*, in contrast to *C. glutamicum* Δ*malQ*, was not able to grow with maltose and in co-cultivation experiments with glucose plus maltose the double mutant just utilized the given glucose (data not shown and Fig. 39, A).

To reveal if the observed growth phenotype of the double mutant *C. glutamicum* Δ*malQ* Δ*treS* was due to the inactivation of TreS the strain *C. glutamicum* Δ*malQ* Δ*treS*

(pXMJ19_*treS*) was constructed. Growth experiments with *C. glutamicum* Δ*malQ* Δ*treS* and *C. glutamicum* Δ*malQ* Δ*treS* (pXMJ19_*treS*) were performed in minimal medium with glucose plus maltose to test whether the strain expressing plasmid encoded *treS* regains the ability to utilize maltose for growth. The data show that both strains were growing with comparable growth rates of 0.34 h^{-1} for *C. glutamicum* Δ*malQ* Δ*treS* and 0.36 h^{-1} for *C. glutamicum* Δ*malQ* Δ*treS* (pXMJ19_*treS*) (Fig. 39; A, B). Interestingly, neither of the mutants was able to utilize maltose for growth, shown by identical optical densities of 18 and unutilized maltose after 24 h (Fig. 39; A, B).

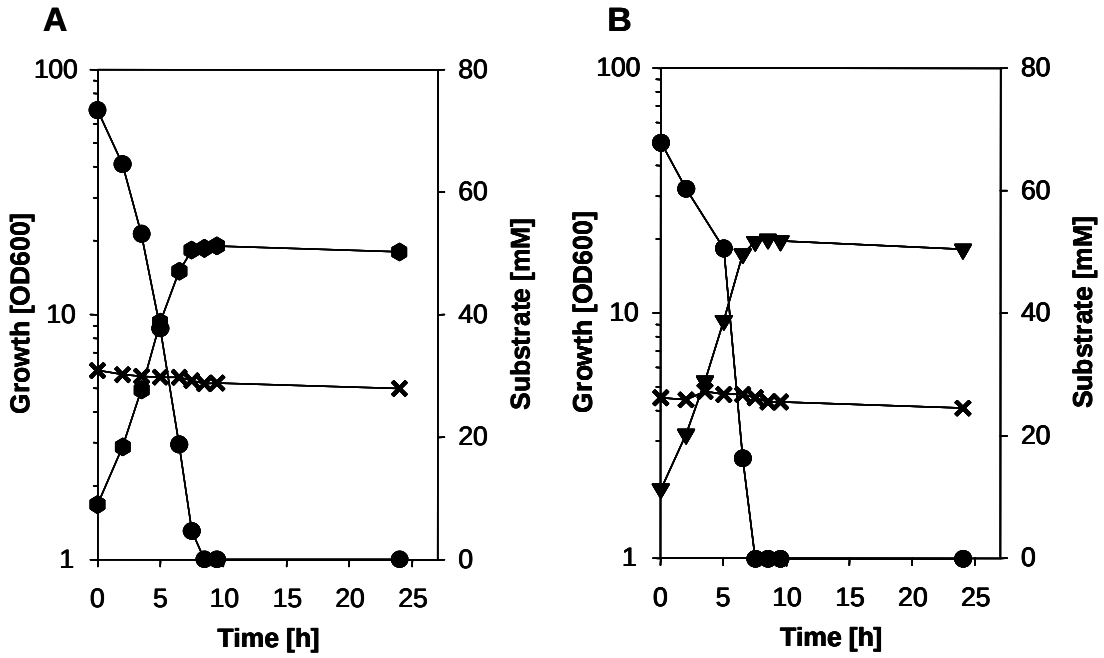

Figure 39: Growth of *C. glutamicum* Δ*malQ* Δ*treS* (A) and *C. glutamicum* Δ*malQ* Δ*treS* (pXMJ19_*treS*) (B) in minimal medium with 1% glucose plus 1% maltose. Growth of: (⬢) *C. glutamicum* Δ*malQ* Δ*treS*, (▼) *C. glutamicum* Δ*malQ* Δ*treS* (pXMJ19_*treS*). Consumption of: (●) glucose, (✕) maltose.

There might be two reasons for this surprising result; the first one is that the complementation plasmid was defect and the second one is that the deletion of *treS* inactivated another important factor for utilizing maltose.

3.3.3 TreX: A new player in trehalose/maltose metabolism

While identifying factors that can be affected by treS deletion it was interesting to discover the gene *treX* (*cg2530*) downstream adjacent to the *treS* gene (*cg2529*). Sequence comparison of *treX* revealed homologies to *pep2* (*rv0127*) of *M. tuberculosis* (37% sequence identity, e-value 3e-50), being downstream of *treS* (*rv0126*). Pep2 is homologous to the maltokinase Mak1 from related actinomycetes (*Actinoplanes*

missouriensis and *Streptomyces coelicolor*), that phosphorylates maltose to maltose-1-phosphate using ATP (Kalscheuer *et al*., 2010a). Further it was published that this enzyme is a part of a novel pathway from trehalose to α-glucan in *M. tuberculosis*, comprising TreS mediated conversion of trehalose to maltose, Pep2 mediated maltose-1-phosphat formation and GlgE, which has been shown to be a maltosyltransferase that incorporates maltose-1-phosphate into α-glucan (Kalscheuer *et al*., 2010a).

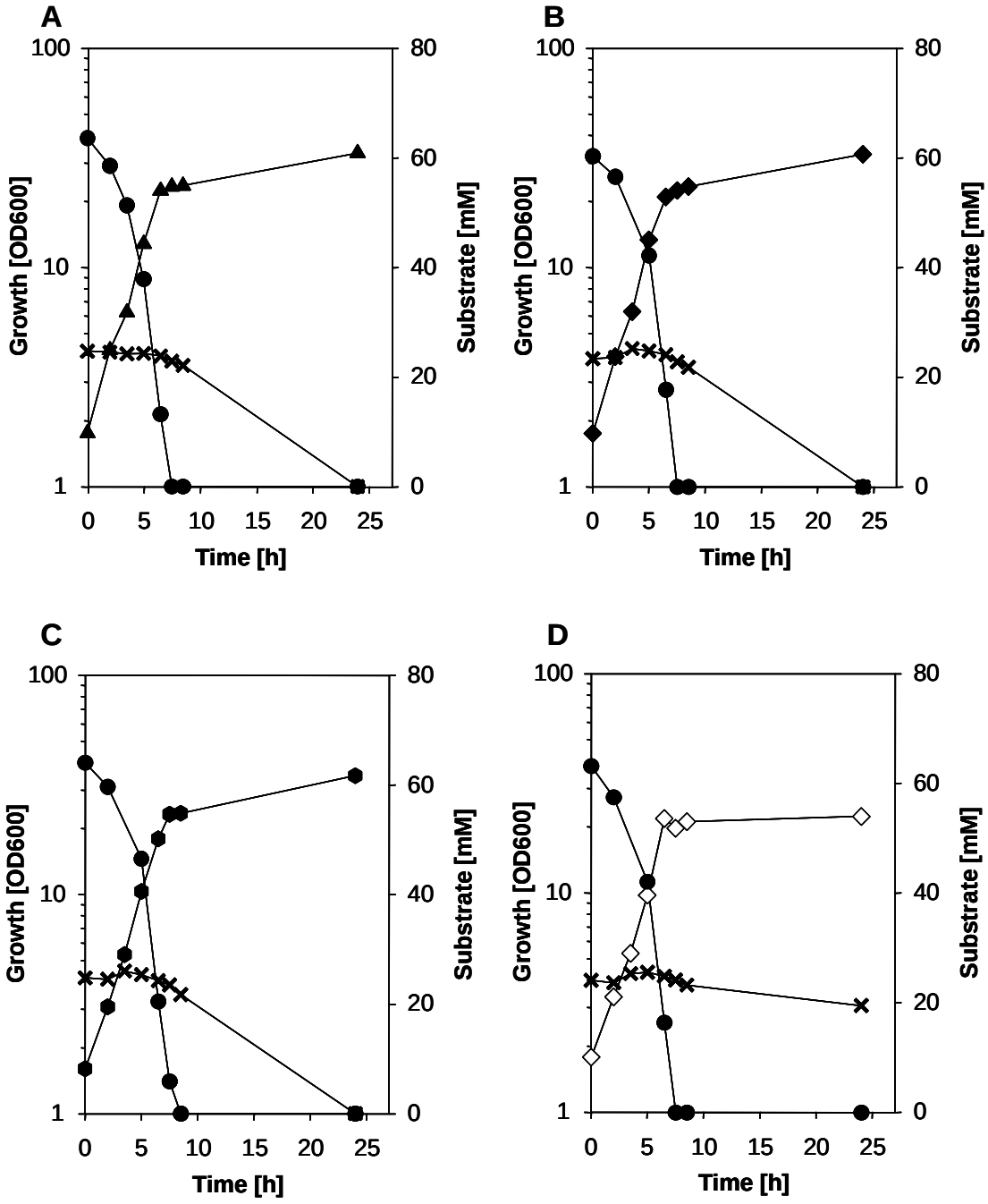

Figure 40: Growth of *C. glutamicum* strains in minimal medium with 1% glucose plus 1% trehalose. Growth of: (A) (▲) *C. glutamicum* WT, (B). (◆) *C. glutamicum* IM*treX*, (C) (⬢) *C. glutamicum* Δ*malQ,* (D) (◇) *C glutamicum* Δ*malQ* IM*treX*. Consumption of: (●) glucose, (✕) trehalose.

To test if this pathway is also present in *C. glutamicum treX* was knocked out via pDrive integration in the *C. glutamicum* WT and the *C. glutamicum* Δ*malQ* mutant. *C. glutamicum* IM*treX* and *C. glutamicum* Δ*malQ* IM*treX* were used for growth experiments in minimal medium with glucose plus trehalose. Trehalose was used as additional substrate to make sure that TreS mediated conversion of trehalose and maltose was not influenced by *treX* inactivation. As control *C. glutamicum* WT and *C. glutamicum* Δ*malQ* were used. As depicted in Fig. 40, all strains grew with identical growth rates of 0.40 ± 0.2 h^{-1}. The WT, Δ*malQ* and the IM*treX* mutant reached final optical densities of 33 ± 1 after 24 h of cultivation, thereby utilizing all glucose and trehalose (Fig. 40; A, B, C). This showed that the inactivation of *treX* had no influence on the ability of *C. glutamicum* to utilize trehalose for growth. Interestingly, *C. glutamicum* Δ*malQ* IM*treX* did not seem to be able to utilize trehalose for growth, although the trehalose concentration was lower after 24 h (Fig. 40, D). TreS seemed not to be influenced by *treX* inactivation as shown by *C. glutamicum* IM*treX* that was able to utilize trehalose for growth. Since *C. glutamicum* Δ*malQ* IM*treX* was not able to utilize trehalose for growth, comparable to *C. glutamicum* Δ*malQ* Δ*treS* that was not able to utilize maltose, it was concluded that the deletion of *treS* exerts an influence on *treX* leading to its inactivation. This explains why the plasmid pXMJ19_*treS* did not restore growth of *C. glutamicum* Δ*malQ* Δ*treS* with glucose plus maltose. At the same time these results show that a second pathway for the utilization of trehalose/maltose is present in *C. glutamicum*.

3.3.4 A new approach to characterize the trehalose export biochemically

The first approach to characterize the export of trehalose biochemically using the strain *C. glutamicum* Δ*malQ* failed, since this strain was still able to utilize maltose in mixed carbon source cultivations for growth (Kempkes, 2009), indicating a second pathway for maltose utilization. This pathway was identified channeling trehalose/maltose into metabolism via maltose-1-phosphate and α-glucan (see 3.3.3). For the second approach to characterize the export of trehalose biochemically, the strain *C. glutamicum* Δ*malQ* IM*treX* was used. This strain should not be able to utilize maltose for growth but for trehalose synthesis, as the gene *treX* was inactivated in *C. glutamicum* Δ*malQ*, without the inactivation of *treS*. *C. glutamicum* Δ*malQ* IM*treX* was used for comparative growth experiments with the WT in minimal medium with glucose plus maltose. As shown in Fig. 41, *C. glutamicum* Δ*malQ* IM*treX* did not seem to utilize maltose for growth in mixed carbon source cultivations, indicated by a lower final optical density of 22 compared to 36 of the WT after 24 h. Interestingly, the mutant strain started to grow with a growth rate of 0.33 h^{-1} which slowed down after 4 h to 0.06 h^{-1}, compared to constant growth of the WT (0.40 ± 0.01 h^{-1}) (Fig. 41).

To increase the productivity of *C. glutamicum* Δ*malQ* IM*treX* for trehalose the strain was transformed with the TreS expression plasmid pXMJ19_*treS*. The strain *C. glutamicum* Δ*malQ* IM*treX* (pXMJ19_*treS*) should produce higher amounts of trehalose from maltose via the trehalose synthase TreS than its parental strain *C. glutamicum* Δ*malQ* IM*treX* because of the overexpression of TreS.

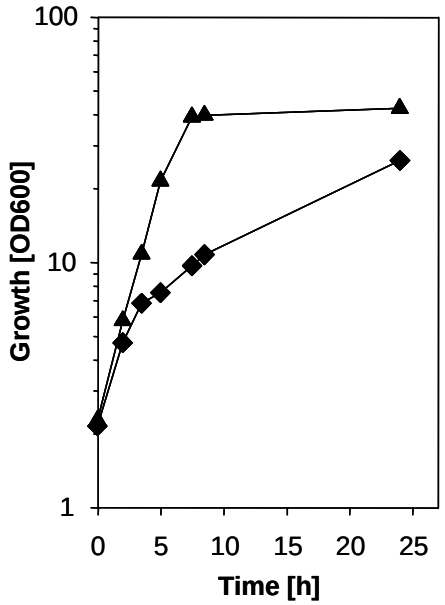

Figure 41: Growth of *C. glutamicum* WT and *C. glutamicum* Δ*malQ* IM*treX* in minimal medium with 1% glucose plus 1% maltose. Growth of: (▲) *C. glutamicum* WT, (◆) *C glutamicum* Δ*malQ* IM*treX*.

Before this strain was used for trehalose export measurements using labeled substrate, *C. glutamicum* Δ*malQ* IM*treX* (pXMJ19_*treS*) was cultivated in minimal medium with glucose plus maltose in comparison to the WT. Growth of *C. glutamicum* Δ*malQ* IM*treX* (pXMJ19_*treS*) started with an initial growth rate of 0.31 ± 0.01 h^{-1}, but after 4 h the growth of the mutant slowed down to 0.08 ± 0.01 h^{-1}, compared to constant growth of the WT (0.40 ± 0.01 h^{-1}) (Fig. 42; A, B). Interestingly, both strains reached comparable optical densities of 34 for the WT and 31 ± 1 for the mutant strain after 24 h (Fig. 42; A, B). This indicated that *C. glutamicum* Δ*malQ* IM*treX* (pXMJ19_*treS*) utilized maltose for growth, and indeed when looking at the substrate consumption it can be seen that the mutant utilized maltose (Fig. 42, B), although much slower than the WT control (Fig. 42, A). At the same time the utilization of glucose was substantially slowed down in *C. glutamicum* Δ*malQ* IM*treX* (pXMJ19_*treS*) (Fig. 42, B). This result was rather surprising, as the strain *C. glutamicum* Δ*malQ* IM*treX* seemed not to be able to utilize maltose for growth (Fig. 41).

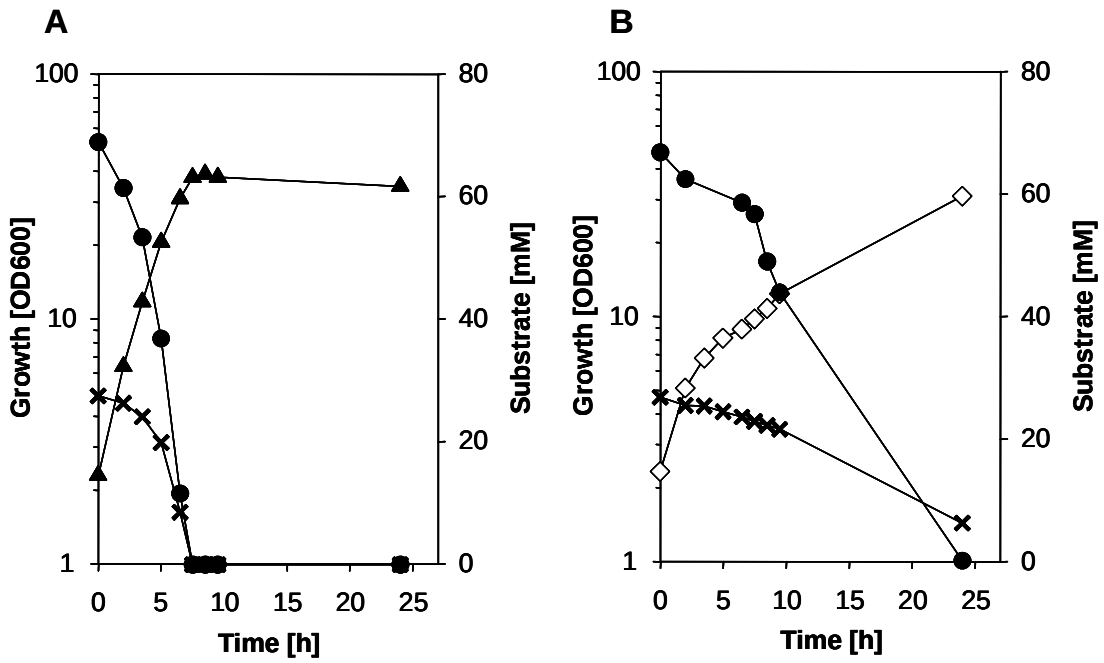

Figure 42: Growth of *C. glutamicum* WT (pXMJ19) (A) and *C. glutamicum* Δ*malQ* IM*treX* (pXMJ19_*treS*) (B) in minimal medium with 1% glucose plus 1% maltose. Growth of: (▲) *C. glutamicum* WT (pXMJ19), (◇) *C glutamicum* Δ*malQ* IM*treX* (pXMJ19_*treS*). Consumption of: (●) glucose, (✕) maltose.

To test whether extracellular trehalose was accumulated by the two strains *C. glutamicum* Δ*malQ* IM*treX* and *C. glutamicum* Δ*malQ* IM*treX* (pXMJ19_*treS*) comparative TLC analyses were performed. Samples were taken at the beginning of the cultivation (0 h) and at the end (24 h).

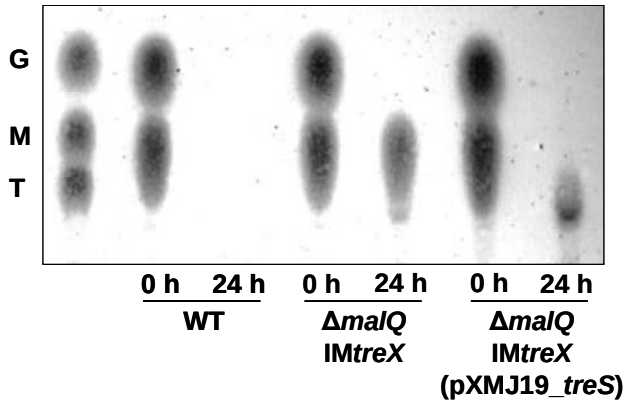

Figure 43: Comparative TLC analysis of culture supernatants of *C. glutamicum* strains cultivated in minimal medium with 1% glucose plus 1% maltose. WT = *C. glutamicum* WT, Δ*malQ* IM*treX* = *C. glutamicum* Δ*malQ* IM*treX*, Δ*malQ* IM*treX* (pXMJ19_*treS*) = *C. glutamicum* Δ*malQ* IM*treX* (pXMJ19_*treS*), G = glucose, M = maltose, T = trehalose.

The TLC data clearly show that the WT utilized all glucose and maltose present in the medium, without producing and excreting detectable amounts of trehalose after 24 h (Fig. 43). *C. glutamicum* Δ*malQ* IM*treX* only utilized the glucose whereas most of the maltose was left and only minor amounts of trehalose were detectable after 24 h (Fig. 43.). Strikingly, the data for *C. glutamicum* Δ*malQ* IM*treX* (pXMJ19_*treS*) show that this strain utilized all given glucose and maltose after 24 h, producing and exporting trehalose into the medium (Fig. 43). Notably, the spot detected for trehalose after 24 h was much smaller than the initial maltose one indicating that this strain not only used the given maltose for trehalose production but also for growth. HPLC analyses of culture supernatants revealed that only 6 ± 0.2 mM trehalose was excreted into the medium, whereas the major proportion (77%) of the initial 27 mM maltose, which was added to the cultivation, must have been used for growth.

To make sure that the spot visible in the *C. glutamicum* Δ*malQ* IM*treX* (pXMJ19_*treS*) cultivation after 24 h (Fig. 43) was trehalose, an enzymatic test using trehalase and maltase was performed and the samples analyzed via TLC. The data showed that the trehalose spot detected after 24 h in the *C. glutamicum* Δ*malQ* IM*treX* (pXMJ19_treS) culture supernatant was indeed trehalose, since the incubation with trehalase cleaved the product into glucose. The incubation with maltase on the contrary did not release any glucose (Fig. 44).

These results show that *C. glutamicum* seems to be able to utilize maltose for growth in the Δ*malQ* IM*treX* (pXMJ19_treS) strain via TreS, suggesting an additional function of this protein. This strain was not used for the biochemical characterization of trehalose export in *C. glutamicum* so that this system remains uncharacterized.

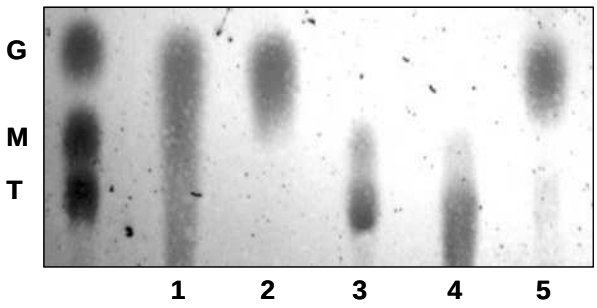

Figure 44: Comparative TLC of an enzymatic test using maltase and trehalase.

Lane 1: maltose + maltase, Lane 2: trehalose + trehalase, Lane 3: sample Δ*malQ* IM*treX* (pXMJ19_*treS*), Lane 4: sample Δ*malQ* IM*treX* (pXMJ19_*treS*) + maltase, Lane 5: sample Δ*malQ* IM*treX* (pXMJ19_*treS*) + trehalase; ΔmalQ IMtreX (pXMJ19_treS) = *C. glutamicum* Δ*malQ* IM*treX* (pXMJ19_*treS*), sample = culture supernatant after 24 h of cultivation, G = glucose, M = maltose, T = trehalose.

4. Discussion

4.1 The maltose uptake system MusIFGK$_2$-E of *C. glutamicum*

One aim of the present work was to characterize and identify the uptake system for maltose of *C. glutamicum* with the help of biochemical and molecular biological methods to broaden the understanding of maltose metabolism in *C. glutamicum*. The biochemical characterization of this system with the help of radioactively labeled substrate, revealed that maltose is taken up by a single high affinity transport system with a K_m of 1.2 ± 0.2 µM and a V_{max} of 26.2 ± 1.0 nmol/(min*mg cdm). The application of different inhibitors indicated that the import is mediated by an ABC transport system. Transport measurements with cells cultivated with glucose, maltose and acetate revealed that maltose uptake rates were higher in cells cultivated with glucose than with maltose and especially acetate. With the help of these biochemical data and published transcriptome data dealing with differentially expressed genes in cells cultivated with glucose and acetate, respectively (Cramer, 2006; Auchter *et al*., 2010), a candidate was identified (*cg2703-cg2708*) encoding an ABC transport system. And indeed the mutant *C. glutamicum* Δmus, deleted in *cg2703-cg2708* was not able to utilize maltose as carbon source indicating that the deleted genes encode the maltose uptake system. With the help of an expression plasmid (pXMJ19_*musFGK-E*) encoding the deleted genes it was shown that the deletion mutant regained the ability to grow with and utilize maltose. These results were supported by radioactive transport measurements showing that the deletion mutant was not able to take up maltose, whereas *C. glutamicum* Δmus (pXMJ19_*musFGK-E*) was able to.

Looking at the genes encoding the maltose uptake system, it is interesting to see that there are more genes present than needed for forming an ABC transport system. All important components (permease subunits, ATPase subunit and substrate binding protein) are encoded by the ORFs of the maltose uptake system, but in addition there are two more genes present (*cg2707*, *cg2701*) annotated as hypothetical proteins, with unknown functions. Growth experiments with single knockout mutants with maltose revealed, that *cg2707* is not essential for the maltose uptake system in contrast to *cg2701*, named *musI*, which showed to be important for maltose uptake. Further, Northern Blot analyses and RT-PCRs addressing the genetically organization showed that *musI* and *musG* most probably together with *musF* form an operon, whereas *musK* and *musE* are transcribed monocistronically, this is unlike to the well studied ABC transporter MalFGK$_2$-E of *E. coli* in which *malEFG* form one operon and *malK lamB malM* form a second one (Boos & Shuman, 1998).

Competition experiments showed that the uptake of maltose is not influenced by the addition of glucose, trehalose, acarbose and maltodextrins with more than five glucose moieties but the addition of maltotriose, maltotetraose and maltopentaose had an effect on maltose uptake, indicating that they are also substrates for the maltose uptake system. Growth experiment on maltotriose with the *C. glutamicum* WT and *C. glutamicum* Δmus revealed that indeed *C. glutamicum* takes up maltotriose via the maltose uptake system MusIFGK$_2$-E. It is very likely that maltotetraose and maltopentaose are also utilized by *C. glutamicum* via the maltose uptake system as it has been shown that the maltose uptake system MalFGK$_2$-E of *E. coli* is capable of transporting maltodextrins up to seven to eight glucose units (Boos & Shumann, 1998).

The gene *musI* encodes a hypothetical protein with five predicted transmembrane domains that shares no similarities to so far characterized proteins. Additional complementation experiments with plasmid encoded MusI showed that *C. glutamicum* IM*musI* regained the ability to take up maltose. In Western Blot experiments a tagged MusI protein was detected exclusively in the membrane fraction of *C. glutamicum* showing that MusI is a membrane protein or at least associated to the membrane (Kuhlmann, 2010). Further, bacterial two-hybrid assays trying to identify possible interactions between MusI and other proteins of the maltose uptake system, indicated that MusI might not interact with the MusFGK$_2$ complex, so that it is possible that MusI is not part of the substrate pathway (Kuhlmann, 2010). The data further showed that MusI might interact with itself. In this point it could resemble YidC of *E. coli*, a membrane protein important for the stability of MalF during oligomerization of the MalFGK$_2$-complex (Wagner *et al*., 2008). It might be possible that MusI is also important for the insertion of MusF and MusG into the membrane (Kuhlmann, 2010). Although BLAST data bank analyses revealed no homologies to LamB, a specific outer membrane porin important for the uptake of maltose into the periplasm in *E. coli*, or other known porins it might also be possible that the novel component MusI is involved in facilitating the transport of maltose across the mycolate layer, rather than being involved in the oligomerization of the MusFGK$_2$-complex. But so far this is speculation and further research has to be done to finally reveal the function of this novel essential component MusI in maltose uptake.

Taking together these data show that the maltose and maltodextrin uptake system of *C. glutamicum* is the high affinity ABC transport system MusIFGK$_2$-E consisting of two permease subunits MusG and MusF, encoded by *musG* and *musF* (*cg2703*, *cg2704*), one maltose-binding protein MusE, encoded by *musE* (*cg2705*), an ATPase subunit MusK, encoded by *musK* (*cg2708*) and a novel essential component MusI, encoded by *musI*

(*cg2701*). This system resembles the well studied maltose/maltodextrin uptake system MalFGK$_2$-E of *E. coli*, which serves as a model for ABC transporters in general (Bordignon *et al*., 2010). It also consists of two permeases MalG and MalF, the maltose binding protein MalE, and MalK, the ATP-hydrolyzing subunit of the transporter. Further, both systems have a comparable K_m of 1µM for maltose (Dippel & Boos, 2005). The identification of the ABC transporter MusIFGK$_2$-E underlines that the maltose metabolism in *C. glutamicum* is very similar to the well studied maltose and maltodextrin metabolism of the Gram-negative bacterium *E. coli* (Boos & Shuman, 1998). It is rather different to other Gram-positive bacteria such as *B. subtilis* which takes up maltose via a specific phosphotransferase system (Schönert *et al*., 2006) hydrolyzing the cytoplasmic maltose-phosphate by an α-glucosidase (MalA), resulting in glucose and glucose-6-phosphate which is then channeled into metabolism (Schönert *et al*., 2006) or *L. sanfrancisco* which takes up maltose by an H^+-symporter cleaving it afterwards via a maltose phosphorylase into glucose and β-glucose-1-phosphate (Neubauer *et al*., 1994).

4.2 Regulation of MusIFGK$_2$-E

Results from radioactive transport measurements showed that the maltose uptake rate was always lower in cells cultivated with acetate compared to cells cultivated with glucose or maltose, indicating that the maltose uptake system is regulated at the level of gene expression. To further elucidate this regulation phenomenon three putative candidates, RamA, RamB and SugR, were tested to be involved in the regulation. They were chosen because they have been described to be involved in the regulation of the central metabolism of *C. glutamicum*, including sugar uptake, glycolysis, gluconeogenesis, acetate and ethanol metabolism (Gaigalat *et al*., 2007; Engels & Wendisch 2007; Auchter *et al*., 2010).

Growth experiments with *C. glutamicum* RG2 (Δ*ramA*) revealed that this strain is impaired for growth with maltose. Transcriptome data dealing with differentially expressed genes in cells cultivated with glucose and acetate, respectively, showed lower mRNA levels of the genes encoding the maltose uptake system (*cg2701*, *cg2703*, *cg2704*, *cg2705*, *cg2708*) in the *ramA* deletion mutant (*C. glutamicum* RG2) compared to the WT (Auchter *et al*., 2010). These data indicated an activating function of RamA on the expression of the genes encoding the maltose uptake system. This hypothesis was confirmed by the fact that putative RamA binding sites were identified in the upstream region of *musE* and the operon *musFGI*. RNA hybridization experiments with RNA from cells cultivated with glucose and maltose, respectively, showed that *musE* and the operon *musFGI* are down regulated at the transcriptional level in the RamA deficient strain RG2, whereas the

ATPase subunit encoded by *musK* does not seem to be regulated. Further, radioactive transport measurements in the *ramA* deletion strain revealed that the transport rates for maltose were lower than in the WT, which was especially striking in glucose adapted cells where the rate was as low as 1.8 ± 0.9 nmol/(min*mg cdm) compared to 22.7 ± 3.4 nmol/(min*mg cdm) in WT cells. These results indicated that RamA is involved in the transcriptional regulation of the maltose uptake system in *C. glutamicum*. Although, this regulation does not seem to be important when cells are cultivated in rich medium, as shown by high maltose uptake rates in *C. glutamicum* RG2 cells cultivated exclusively in TY medium.

The deletion of *ramB* (*C. glutamicum* RG1) had no effect on growth with maltose. Interestingly, data from radioactive transport measurements showed lower maltose uptake rates in the Δ*ramB* mutant compared to the WT. As the transcriptional data from Auchter *et al*., 2010 showed that the genes of the maltose uptake system were not differentially expressed in *C. glutamicum* RG1 the effect on the maltose uptake rate is most probably a secondary one.

Growth of *C. glutamicum* Δ*sugR* in minimal medium with maltose as sole carbon source was also decreased comparable to *C. glutamicum* RG2. Data from radioactive assays further revealed that the transport rates were always lower in the Δ*sugR* strain than in the WT. Interestingly, the expression level of *musE*, *musFGI*, and *musK* did not differ from those detected in the WT. This result is verified by the absence of putative SugR binding sites in the upstream regions of *musE*, *musFGI*, and *musK* but does not support the results from growth and radioactive experiments. Therefore, it is possible that SugR does not directly influence the expression of the *mus* genes but it is possible that this transcriptional repressor affects other proteins regulating maltose uptake and metabolism in *C. glutamicum*.

Interestingly, the obvious differences in the maltose uptake rates detected were completely abolished in cells cultivated exclusively in TY medium, indicating that at least another regulatory factor is involved in the expression of the *mus* genes. This goes in line with the finding that e.g. RamA can posses a dual function as repressor in complex medium and as activator when cells were grown in minimal with glucose (Auchter *et al*., 2010). These contrary effects were explained to be due to interaction or competition of RamA with an effector or with a further (regulatory) protein specific for the one or the other condition (Auchter *et al*., 2010).

Taking all the results into account it can be hypothesized that the global regulator RamA seems to be involved in the regulation of some genes of the maltose uptake system (*musE*, *musFGI*) of *C. glutamicum*. On the contrary RamB does not seem to regulate the system but it can not be excluded that it controls the function of other regulators. SugR also does not seem to influence the maltose uptake system directly at the expression level most probably controlling the function of other unknown regulators having an indirect effect on maltose uptake and/or utilization.

The best studied maltose/maltodextrin uptake system, the ABC transporter $MalFGK_2$-E of *E. coli*, shows a surprisingly complex regulation. The regulation of this system is specifically controlled by MalT, an inducer-dependent *mal* gene activator (Boos & Böhm, 2000; Dippel *et al*., 2005). Transcriptional activation of MalT requires ATP and maltotriose, two effectors of the protein (Boos & Böhm, 2000). Further, the maltose system of *E. coli* is subject to catabolite repression as well as inducer exclusion. The expression of *malT* is controlled by the cyclic AMP / catabolite gene activator protein as well as Mlc, a global repressor of sugar metabolism, which are controlled by the PTS-mediated transport of glucose itself (Postma *et al*., 1996; Plumbridge, 2002). The target of inducer exclusion is MalK, in this process unphosphorylated $EIIA^{Glc}$ of the glucose-specific PTS is thought to interact with the regulatory domain of MalK to curb transport activity (Böhm *et al*., 2002). The regulatory domain of MalK also interacts with MalT, leading to the inhibition of this transcriptional activator (Joly *et al*., 2004).
This shows that the regulation of maltose uptake and utilization in *C. glutamicum* seems to be completely different and not as strictly regulated compared to the established system in *E. coli*, as *C. glutamicum* co-metabolizes maltose with glucose and the addition of glucose does not influence maltose transport rates, not showing catabolite repression or inducer exclusion.

4.3 Nonclassical regulatory phenomena

C. glutamicum is known to be able to utilize a variety of different carbon sources such as sugars, organic acids and alcohols for growth. Most of these carbon sources are utilized simultaneously; sequential utilization of substrates by *C. glutamicum* causing biphasic growth has only been observed for the mixtures of glucose plus glutamate, glucose plus ethanol, and acetate plus ethanol (Krämer *et al*., 1990; Arndt & Eikmanns, 2007; Arndt *et al*., 2008). This is special since most bacteria cultivated in media containing two or more carbon sources adapt their metabolism to the utilization of their preferred substrate. This successive utilization of substrates is mediated by regulatory mechanisms summarized as carbon catabolite repression (CCR) and is often represented by a biphasic growth

behavior (Deutscher, 2008; Görke & Stülke, 2008). Such diauxic growth can be observed e.g. for *E. coli* cultivated with glucose plus lactose or maltose.
Although numerous studies of metabolism and its regulation have been conducted, the molecular mechanism of global carbon regulation is still not clearly understood in *C. glutamicum* (Moon *et al*., 2007; Arndt & Eikmanns, 2008). Major players of CCR in other organisms are components of the sugar phosphotransferase system (PTS) such as the enzyme $EIIA^{Glc}$ in *E. coli* and HPr in *B. subtilis*, which have besides their catalytic function an important regulatory role (Deutscher, 2008). *C. glutamicum* also possesses these components but their regulatory role is not well studied. Further, *C. glutamicum* possesses the cAMP receptor protein (CRP) homologue GlxR, which plays a global role in the transcriptional network of *C. glutamicum* (Park *et al*., 2010).

In this work it was shown that maltose, additionally to the effect on *ptsG* expression, positively affects mechanisms contributing to glucose utilization in *C. glutamicum*. To a certain extent, this positive influence of maltose on the utilization of glucose represents a phenomenon so far unknown in bacteria (Krause *et al*., 2010). Interestingly, it has been shown for *C. glutamicum* R that the PTS sugars sucrose and fructose also have a positive effect on *ptsG* expression (Tanaka *et al*., 2008), probably exerting a comparable influence on glucose utilization as maltose. But the molecular mechanism of this positive regulation remains elusive.
Further, it was demonstrated in the present work that cultivation with glucose has a positive effect on maltose uptake rates in *C. glutamicum* being contrary to other bacteria such as *E. coli* and *B. subtilis*. Additionally RNA expression experiments revealed that the genes encoding the ABC transport system $MusIFGK_2$-E were highly expressed when cells were cultivated with glucose. Interestingly, it has already been published that strains inactivated in EI and HPr, respectively, two proteins of the PTS phosphorylation cascade show reduced growth when cultivated with maltose (Parche *et al*., 2001). These observations indicate a regulatory connection between glucose and maltose metabolism.

To reassess the effect of *hpr* inactivation growth experiments with *C. glutamicum* Δ*hpr* in minimal medium with maltose were performed showing that this strain indeed exhibits a severe growth defect. Determination of maltose transport rates in the Δ*hpr* mutant revealed lower uptake rates in cells that were cultivated with maltose for only six hours, whereas the uptake rates of cells adapted to maltose were even higher compared to the WT under the same conditions. As it has been published that *C. glutamicum* does not posses an HPr kinase/phosphatase (Parche *et al*., 2001), important for the phosphorylation of Hpr at Ser-46 during CCR in low-GC Gram-positive bacteria such as

B. subtilis (Deutscher, 2008), it seems likely that the observed phenotype of the Δ*hpr* mutant is due to missing Hpr phosporylated at the catalytic His-15. This is further supported by the observation that a strain with an inactivated EI protein, important for phosphorylation of Hpr at His-15, also showed reduced growth with maltose (Parche *et al*., 2001). It has been published that P-His-Hpr can transfer its phosporyl group also to non-PTS proteins such as glycerol kinase (Charrier *et al.*, 1997) or antiterminators and transcriptional activators possessing the PTS regulation domain (Viana *et al*., 2000). Therefore, it might be possible that P-His-Hpr in *C. glutamicum* has a dual function in catalyzing the transfer of phosphate to the EII subunit of the PTS and a regulatory one by activating unknown factors influencing maltose transport and/or utilization. This regulation seems only important for the transition from one carbon source to another because *C. glutamicum* Δ*hpr* cells exclusively cultivated with maltose and complex medium, respectively, did not show reduced maltose transport rates. In future this hypothesis can be tested in growth experiments with *C. glutamicum* Δ*hpr* adapted to maltose and with an Hpr version in which phosphorylation of His-15 is either prevented or diminished. Until the function of HPr in carbon regulation in *C. glutamicum* is not elucidated the connection between maltose and glucose metabolism remains elusive.

4.4 The trehalose uptake system TusFGK$_2$-E of *C. glutamicum*

Although *C. glutamicum* is known to be able to utilize a wide variety of different carbon sources for growth and the production of amino acids, it has been published that *C. glutamicum* is neither able to grow with trehalose as sole carbon source nor equipped with an uptake system for trehalose (Wolf, 2002; Gebhardt, 2005; Tropis *et al*., 2005). This has been concluded by the observations that *C. glutamicum* is not able to grow with trehalose as sole carbon source (Wolf, 2002); further, [^{14}C]-trehalose uptake measurements which were performed to reveal the localization of the synthesis of trehalose monomycolate showed that radioactivity was only detected in the cell wall of *C. glutamicum* and not in the cytoplasm, also speaking against the presence of an trehalose uptake system in *C. glutamicum* (Gebhardt, 2005). In fact, trehalose in *C. glutamicum* serves as a compatible solute after an osmotic up-shift, under special conditions such as nitrogen- and carbon-limitation (Gebhardt, 2005). Even more important is the function of trehalose in the general physiology of *C. glutamicum* and its relatives, since trehalose is an important building block for the synthesis of mycolates, major and structurally important components of the cell envelope of *Corynebacterineae* (Tropis *et al*., 2005).

To analyze effects of trehalose on cell wall synthesis *C. glutamicum* was cultivated in minimal medium with glucose plus trehalose. Interestingly, *C. glutamicum* WT showed

higher optical densities after 24 h in cultivations with the addition of trehalose compared to cultivations without, indicating that *C. glutamicum* is able to utilize trehalose for growth in mixed carbon source cultivations. This was supported by substrate consumption data, showing that at the end of the cultivations all trehalose was consumed by the cells. Additional growth experiments with *C. glutamicum* Δmus and *C. glutamicum* Δ*treS* revealed that trehalose is not taken up by the maltose uptake system MusIFGK$_2$-E after external conversion to maltose by TreS. The results from these growth experiments revealed that *C. glutamicum* is indeed able to take up trehalose so it was concluded that a corresponding uptake system must be present as well. To characterize the uptake system biochemically, radioactive uptake measurements using [^{14}C]-labeled trehalose were established. The results of these experiments revealed that trehalose is taken up via a high affinity transport system with an apparent K_m of 0.16 ± 0.02 µM and a V_{max} of 2.5 ± 0.1 nmol/(min*mg cdm). Competition experiments with trehalose, maltose, isomaltose, acarbose, sucrose, galactose and maltotriose as possible competitors for trehalose uptake showed that the system is specific for the uptake of trehalose.

This result raises the question why the transport system was not identified before by Gebhardt (2005). In her work [^{14}C]-trehalose was used to localize the synthesis of trehalose monomycolate in *C. glutamicum*. Gebhardt thoroughly determined the accumulation of [^{14}C]-trehalose in isolated cell envelopes, obtained by permeabilization of WT cells, compared to the accumulation of [^{14}C]-trehalose in intact cells. During these experiments a very low but significant uptake rate was detected in the WT cells, but since equal amounts of [^{14}C]-trehalose were measured in isolated cell envelopes, but nothing in the cytosol, and in intact cells it was concluded that *C. glutamicum* can not take up trehalose into the cytoplasm but it was integrated into the cell envelope (Gebhardt, 2005).

But why has the uptake not been detected into the cytoplasm although there is an uptake system present? One reason might be that the measurements were performed in a KPi buffer with pH 7.5. While the radioactive transport assay was established for the present study for the uptake of maltose, several buffers and different pH values were tested showing that maltose transport rates were five-fold higher when measured in CGC minimal medium (pH 6.8) compared to KPi (pH 7.5) (data not shown). This is probably also true for the uptake rate of trehalose which is as low as 2.5 ± 0.1 nmol/(min*mg cdm) even under optimized conditions. This hypothesis is underlined by the result of [^{14}C]-glucose uptake measurements performed by Gebhardt which were conducted to rule out that the energy state of the cells used was insufficient. In these experiments a glucose uptake rate of 9 ± 0.7 nmol/(min*mg cdm) was determined (Gebhardt, 2005), being only

26% of the glucose uptake rate of 35 ± 3 nmol/(min*mg cdm) under optimized conditions that were applied during the present work (see 3.1.2). Although glucose is taken up by PTS and maltose and trehalose are taken up by ABC transport systems this argument indicates that it is likely that trehalose uptake was not detected in the work of Gebhardt (2005) because of unfavorable experimental conditions for sugar transport measurements.

After characterizing trehalose uptake it was the aim to identify the trehalose uptake system in *C. glutamicum*. At that time little was known about trehalose uptake systems in closely related bacteria but in *Streptomyces reticuli* an MsiK-dependent uptake system for trehalose was hypothesized (Schlösser, 2000). The data suggested that the MsiK protein is an ATP-binding protein that assists a trehalose ABC transporter (Schlösser, 2000). Sequence comparison of MsiK of *S. reticuli* and *C. glutamicum* revealed a homolog protein being the ATPase component of an ABC transport system (*cg0831-cg0835*). The constructed deletion mutant *C. glutamicum* Δtus, in which all the addressed genes were deleted, was used for growth experiments with glucose plus trehalose. The gained results showed that the deletion strain was not able to utilize trehalose for growth. Following radioactive transport measurements further gave proof that this strain lost its ability to transport trehalose. The so identified system was named trehalose uptake system $TusFGK_2$-E. To support that the genes deleted in *C. glutamicum* Δtus encode the uptake system for trehalose, the expression plasmid pXMJ19_*tusFGK-E* was constructed for complementation. The so constructed strain *C. glutamicum* Δtus (pXMJ19_*tusFGK-E*) regained the ability to take up and utilize trehalose for growth, further giving proof that *tusF* (*cg0831*), *tusG* (*cg0832*), *tusE* (*cg0834*) and *tusK* (*cg0835*) encode the trehalose uptake system of *C. glutamicum*. Northern Blot analyses showed that the genes encoding the system are not transcribed in an operon; only *tusF* and *tusG* seem to form an operon. Further experiments by Gerd Seibold revealed that *tusE* and *tusK* are transcribed monocistronically (Gerd Seibold, preliminary data).

Another reason why the trehalose uptake system was not revealed before by the work of Gebhardt (2005) might be that the system is only expressed under special conditions. To answer the question, whether this system is constitutively expressed in *C. glutamicum* RNA hybridization experiments were performed with RNA from cells cultivated in minimal medium with glucose, maltose and acetate, respectively. Concerning the expression of *tusG* the data showed that this gene is expressed during the exponential growth phase independent of the carbon source (glucose, maltose and acetate, respectively). This indicates that the trehalose uptake system $TusFGK_2$-E must have been expressed during

the experiments in the work of Gebhardt (2005). Further, it seems to be involved in the general physiology of *C. glutamicum* rather than being important under special conditions.

But what is the physiological role of the trehalose uptake system TusFGK$_2$-E in *C. glutamicum*? Since growth experiments showed that *C. glutamicum* is not able to grow with trehalose as sole carbon source the role of the uptake system does not seem to be to take up trehalose as source of energy. This assumption is further underlined by the fact that the trehalose uptake rate is very low and it seems to be not sufficient to promote exponential growth when trehalose is the sole carbon source. Although the appearance of a *C. glutamicum* spontaneous-mutant on trehalose as sole carbon source showed that cells can evolve for growth and even amino acid production from trehalose, the function of TusFGK$_2$-E seems not to be to supply the cell with carbon for growth.
In *E. coli* trehalose serves as a good carbon source for growth at both high and low osmolality (Boos *et al*., 1990). The uptake of trehalose is facilitated by a phosphotransferase system (PTS) leading to trehalose-6-phosphate. It continues with hydrolysis to trehalose and proceeds by splitting trehalose by an amylotrehalase, releasing one glucose residue with the simultaneous transfer of the other to a polysaccharide acceptor (Boos *et al*., 1990). This indicates that not only the transport of trehalose is mediated by different uptake systems in *C. glutamicum* and *E. coli* but also the physiological role of both systems seems to be different. In *E. coli* trehalose can serve as energy source and osmoprotectant whereas in *C. glutamicum* trehalose rather serves as an important part of the cell wall and osmoprotectant than carbon source.
Very recently Kalscheuer and co-workers identified the trehalose uptake system LpqY-SugA-SugB-SugC of *M. tuberculosis* and *M. smegmatis*, two close relatives of *C. glutamicum* (Kalscheuer *et al*., 2010b). The identification of a trehalose uptake system was very surprising because *M. tuberculosis* predominantly grows intracellularly within phagosomes of host phagocytes, indicating that host lipids provide the main carbon and energy source, with carbohydrates being largely inaccessible (Kalscheuer *et al*., 2010b). They were able to show that the ABC transporter LpqY-SugA-SugB-SugC is a high affinity transport system that efficiently acquires trehalose at low nanomolar concentrations and that it is constitutively expressed (Kalscheuer *et al*., 2010b). Further they proved with the help of mouse infection studies that the LpqY-SugA-SugB-SugC transporter-mediated uptake of trehalose is of crucial importance for the virulence of *M. tuberculosis* (Kalscheuer *et al*., 2010b). It was assumed that LpqY-SugA-SugB-SugC is important for recycling of trehalose during mycolic acid processing, which evolved as an adaptation to a nutrient-restricted intracellular lifestyle by maintaining energy efficiency of cell wall biosynthesis (Kalscheuer *et al*., 2010b). These assumptions open a new and interesting

aspect of sugar transporters and it is likely that the trehalose uptake system TusFGK$_2$-E has a similar function in the physiology of *C. glutamicum*. As the system also has a very high affinity for its substrate but a low transport rate it is possible that TusFGK$_2$-E might be used for the recycling of trehalose that has been liberated during mycolate synthesis in the cell envelope.

4.5 TreS: An important enzyme in trehalose metabolism

During the first growth experiments of *C. glutamicum* with glucose plus trehalose it was arguable if trehalose is directly imported or if trehalose in the external medium is converted to maltose by excreted TreS, which catalyzes the interconversion of trehalose and maltose, so that maltose might be subsequently taken up by the maltose uptake system MusIFGK$_2$-E. Growth of *C. glutamicum* Δmus and *C. glutamicum* Δ*treS* in minimal medium with glucose plus trehalose revealed that the maltose uptake system is not important for trehalose utilization but TreS is essential. In earlier studies this enzyme has already been considered to be a substitute for a trehalase (Wolf *et al*., 2003). This was assumed because of several observations, including that *C. glutamicum* misses a homolog to known trehalase genes, that the deletion of *treS* led to increased cytoplasmic trehalose levels and that the *treS* transcript can be found independent of whether TreS-mediated trehalose synthesis is detectable or not (Wolf *et al*., 2003). Further, *in vitro* assays showed that TreS not only catalyzes trehalose synthesis from maltose but also maltose synthesis from trehalose. For the *in vivo* situation it was hypothesized that the preferred conversion is trehalose to maltose, since trehalose was found in 1000-fold higher concentrations in *C. glutamicum* than maltose (Wolf *et al*., 2003). For the physiological role of TreS it was concluded that it mediates the trehalose recycling through transformation to maltose after an osmotic up-shift (Wolf *et al*., 2003).

To verify that the observed growth defect of the *treS* deletion mutant was due to missing TreS activity an expression plasmid encoding TreS (pXMJ19_*treS*) was constructed and used for complementation. And indeed, the strain *C. glutamicum* Δ*treS* (pXMJ19_*treS*) regained the ability to utilize trehalose for growth in mixed carbon source cultivations. This result finally gave proof that TreS indeed is the substitute for a missing trehalase in *C. glutamicum*, important for channeling trehalose into metabolism.

4.6 Trehalose export in *C. glutamicum*

In *C. glutamicum* and related bacteria trehalose plays a major role in the general physiology as a crucial part in cell wall architecture and synthesis. The mycolates derived from the esterification of trehalose to mycolic acids are functionally important cell wall

constituents that cause the high resistance of these organisms to common antibiotic treatment (Tropis *et al*., 2005).

For *C. glutam*icum it has been demonstrated that a strain that was constitutively unable to synthesize trehalose (*C. glutamicum* Δ*otsA* Δ*treS* Δ*treY*) showed distinct changes in the cell surface properties such as clumping (Wolf, 2002) and was devoid of the cell wall fracture plane as well as trehalose mycolates (Tropis *et al*., 2005). When the respective triple-mutant was supplemented with external trehalose, the cells gained viability and were able to synthesize trehalose monomycolate, showing that external trehalose is important for mycolate synthesis in *C. glutamicum* (Tropis *et al*., 2005). This observation was the first hint that the biosynthesis of mycolates does not depend on activated trehalose from the cytoplasm. Further, it was shown that [^{14}C]-trehalose can not be taken up by *C. glutamicum* cells (Gebhardt, 2005). From these data it was suggested that the biosynthesis of mycolates takes place in the cell envelope of *C. glutamicum*. This hypothesis can be supported by the identification of a membrane transporter gene locus that preferably seems to move short-chain corynemycolic acids from the cytoplasm across the membrane (Wang *et al*., 2006). The just explained hypothesis stands in contrast to the model of mycolate synthesis in *M. tuberculosis* of Takayama and co-workers (2005) in which the production of trehalose monomycolate is proposed to occur in the cytosol, followed by the export via an unknown transport system (Takayama *et al*., 2005). This model was recently supported by the observation that *M. tuberculosis* mutants deficient in trehalose import were not able to incorporate external [^{14}C]-trehalose into cell wall mycolates (Kalscheuer *et al*., 2010b).

One aim of this work was to identify and characterize the trehalose export system of *C. glutamicum*. Unexpectedly and in contradiction to literature data for *C. glutamicum* the present study revealed the presence of a trehalose import system. This challenged the hypothesis drawn by Tropis and co-workers (2005) that trehalose is exported and that trehalose mycolate synthesis takes place in the cell wall. With a trehalose uptake system present the situation changes because external trehalose can be taken up into the cytoplasm where it might be esterified to mycolic acids and afterwards the produced trehalose mycolate can be exported via an unknown transport system, resembling the situation in mycobacteria (Takayama *et al*., 2005).

To reassess whether external trehalose is important for mycolate biosynthesis the trehalose uptake system was deleted in the triple-mutant *C. glutamicum* Δ*otsA* Δ*treS* Δ*treY* leading to a strain that is neither able to synthesize nor to import trehalose. The so gained strain *C. glutamicum* Δ*otsA* Δ*treS* Δ*treY* Δtus was used for growth experiments on sucrose with and without the addition of trehalose showing that external trehalose was also beneficial for growth of the quadruple-mutant. To demonstrate that the positive effect

of trehalose on growth of *C. glutamicum* Δ*otsA* Δ*treS* Δ*treY* Δtus was due to synthesized trehalose mycolates a detailed mycolate analysis was conducted. These data showed that the quadruple-mutant was not able to produce trehalose mycolates when cultivated with glucose, but when trehalose was added to the cultivations TMMs and TDMs were detected, giving proof that *C. glutamicum* Δ*otsA* Δ*treS* Δ*treY* Δtus is indeed able to synthesize trehalose mycolates from external trehalose. These data revealed that the trehalose export hypothesis is still valid and that there is an export system for trehalose present in *C. glutamicum*.

To identify a potential trehalose export system a screening system based on the results of Gebhardt (2005) was established. In this work it was shown that an imperfect or missing mycolate layer, caused by deletions in the trehalose synthesis pathways, leads to a higher susceptibility to the antibiotics penicillin, erythromycin and ethambutol (Gebhardt, 2005). This effect was overcome by the addition of external trehalose. Screening of three transporter mutant libraries revealed three candidates. The subsequent analyses of the candidates showed that they are not involved in trehalose export.

To gain insight into trehalose export in *C. glutamicum* the transport should have been characterized biochemically. The first approach was to construct a *C. glutamicum* strain that is not able to utilize maltose for growth but trehalose synthesis via TreS. In such a strain the export of trehalose should be characterized using radioactively labeled substrates e.g. in experiments with transport inhibitors. Initial setups using the strain *C. glutamicum* Δ*malQ* failed, since this strain was still able to utilize maltose in mixed carbon source cultivations for growth, indicating a second pathway for maltose utilization (Kempkes, 2009). This pathway was identified channeling maltose into metabolism via maltose-1-phosphate and α-glucan (see 4.7). The inactivation of this pathway by *treX* integration led to a strain that is not able to utilize maltose for growth but for trehalose synthesis via TreS. The so constructed strain *C. glutamicum* Δ*malQ* IM*treX* seemed not to be able to utilize maltose for growth even in mixed carbon source cultivations. To increase the productivity of *C. glutamicum* Δ*malQ* IM*treX* for trehalose the strain was transformed with the TreS expression plasmid pXMJ19_*treS*. However, instead of producing higher amounts of trehalose leading to measurable trehalose export across the plasma membrane this strain gained the ability to utilize maltose for growth which was also underlined by substrate consumption data. To monitor trehalose formation in *C. glutamicum* Δ*malQ* IM*treX* (pXMJ19_*treS*) in comparison to *C. glutamicum* Δ*malQ* IM*treX* and the *C. glutamicum* WT, comparative TLC analyses with culture supernatants were performed. These data revealed that the WT did not excrete detectable amounts of

trehalose under the conditions tested and the double mutant *C. glutamicum* Δ*malQ* IM*treX* only produced minor amounts of trehalose leaving most maltose unutilized. The expression strain *C. glutamicum* Δ*malQ* IM*treX* (pXMJ19_*treS*) in contrast utilized all the given maltose, producing and exporting trehalose into the medium. HPLC analyses of culture supernatants revealed that only 6 ± 0.2 mM trehalose was excreted into the medium, whereas the major proportion (77%) of the initial 27 mM maltose was utilized for growth.

These data indicate that *C. glutamicum* Δ*malQ* IM*treX* (pXMJ19_treS) is able to utilize maltose for growth with the help of TreS, suggesting a third function of this protein. For TreS of *M. smegmatis* it has already been shown that this enzyme harbors in addition to its maltose-trehalose interconverting activity also an amylase activity, producing trehalose from glycogen (Pan *et al*., 2008). Interestingly, it has also been published that purified TreS form *M. smegmatis* and *Arthrobacter aurescens* produced 8%-13% free glucose during maltose-trehalose interconversion. From these results it was concluded that the catalytic mechanism may involve scission of the incoming disaccharide and transfer of glucose to an enzyme-bound glucose, so that glucose is an intermediate of the reaction (Pan *et al*., 2004; Xiuli *et al*., 2009). Therefore, it might be hypothesized that the overexpression of TreS enables *C. glutamicum* to use glucose that is liberated during maltose-trehalose interconversion for growth. To test this hypothesis it has to be revealed whether TreS of *C. glutamicum* is also able to produce free glucose during maltose-trehalose interconversion what is best studied with purified TreS enzyme in *in vitro* experiments.

Taking all the results into account it comes clear that an exporter for trehalose in *C. glutamicum* is present and that the current model for mycolate synthesis is still valid, although a direct proof of trehalose export under physiological conditions is still lacking and the corresponding export system remains undiscovered.

4.7 A second pathway for trehalose and maltose degradation

During the experimental setup to characterize trehalose export biochemically, which based on the assumption that imported maltose will be completely used for trehalose synthesis by TreS when the maltose metabolic pathway is inactivated by the deletion of MalQ, the key enzyme of maltose metabolism, it was revealed that *C. glutamicum* Δ*malQ* was still able to use maltose in mixed carbon source cultivations for growth, indicating the presence of a second pathway for the degradation of trehalose and maltose (Kempkes, 2009). Interestingly, the control strain deleted in *malQ* and *treS*, which should not be able to utilize maltose, both for growth and trehalose synthesis, was not able to utilize maltose for growth. To analyze whether this result was due to *treS* inactivation the double deletion

mutant *C. glutamicum* Δ*malQ* Δ*treS* was transformed with plasmid encoded *treS* (pXMJ19_*treS*). Data from the corresponding growth experiments with glucose plus maltose showed that *C. glutamicum* Δ*malQ* Δ*treS* (pXMJ19_*treS*) did not regain the ability to utilize maltose for growth. As the expression plasmid has been shown to complement *treS* deletion in *C. glutamicum* Δ*treS* (see 4.5) it was concluded that the deletion of *treS* must affect another factor that is important for trehalose and maltose degradation.

During the present work Kalscheuer and co-workers identified a novel pathway from trehalose to α-glucan in *M. tuberculosis* and *M. smegmatis* (Fig. 45). This pathway comprises four enzymatic steps; the first one is the reversible interconversion of trehalose and maltose via TreS, followed by Pep2 mediated phosphorylation of maltose to maltose-1-phosphate (Kalscheuer *et al*., 2010a). The so produced maltose-1-phosphate is afterwards incorporated into α-glucan via GlgE, which has been identified as a maltosyltransferase that uses maltose-1-phosphate; the linear α-glucan is further branched by GlgB (Kalscheuer *et al*., 2010a).

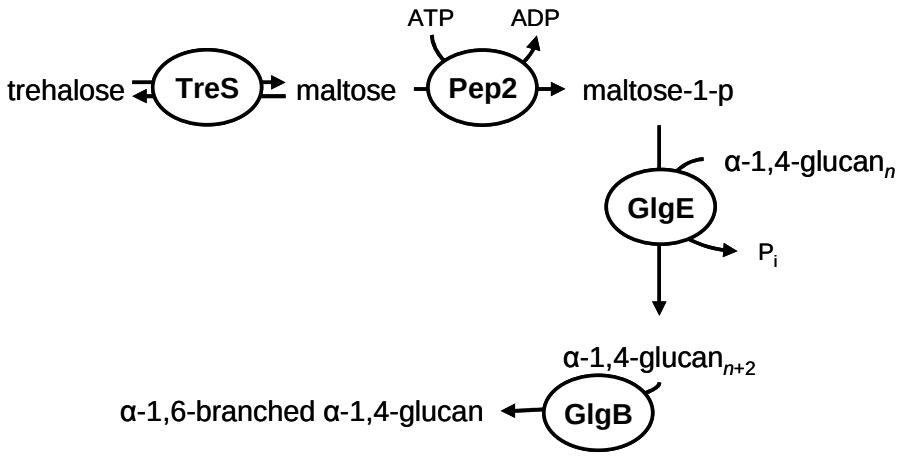

Figure 45: A novel pathway from trehalose to α-glucan, after Kalscheuer *et al*., 2010. TreS – maltose-trehalose interconverting activity, Pep2 – maltokinase, GlgE – maltosyltransferase, GlgB – branching enzyme. *n* = number of glucose units

The gene for the newly identified maltokinase Pep2 (*rv0127*) is located downstream of *treS* (*rv0126*) in *M. tuberculosis*. Interestingly, a homologue protein TreX can be found in *C. glutamicum* and its gene (*cg2530*) is located downstream adjacent to the *treS* (*cg2529*) gene. This led to the hypothesis that *treX* might be the key enzyme of a second pathway for trehalose and maltose utilization in *C. glutamicum*. The simultaneous inactivation of *treX* by *treS* deletion would explain the observed phenotype of *C. glutamicum* Δ*malQ* Δ*treS* in minimal medium with glucose plus maltose.

To test this hypothesis *treX* was knocked out via pDrive integration in the *C. glutamicum* WT and *C. glutamicum* Δ*malQ* mutant. Growth experiments with the so gained strains

C. glutamicum IM*treX* and *C. glutamicum* Δ*malQ* IM*treX* with glucose plus trehalose revealed that the *treX* inactivation mutant was still able to utilize trehalose for growth in mixed carbon source cultivations, showing that TreS-activity was not affected. The double mutant Δ*malQ* IM*treX* was not able to utilize trehalose for growth. Preliminary data from Gerd Seibold showed in addition that TreX of *C. glutamicum* also forms maltose-1-phospahte from maltose (Gerd Seibold, unpublished data) giving proof that TreX is the key enzyme of the second pathway for trehalose and maltose degradation in *C. glutamicum*. Growth experiments with the respective mutants were also performed in minimal medium with glucose plus maltose giving comparable results (Gerd Seibold, unpublished data). As the growth phenotype of *C. glutamicum* Δ*malQ* IM*treX* resembles the one of *C. glutamicum* Δ*malQ* Δ*treS* it can be concluded that the deletion of *treS* exerts an adverse effect on *treX* leading to its inactivation. This explains why the plasmid pXMJ19_*treS* did not complement the phenotype of *C. glutamicum* Δ*malQ* Δ*treS* in minimal medium with glucose plus maltose.

The physiological role of this pathway that has so far only been described for *M. tuberculosis* and *M. smegmatis* remains obscure (Kalscheuer *et al*., 2010a). For *M. tuberculosis* it was hypothesized that the function can be multifaceted, e.g. it can participate in the formation of the α-glucan capsule or it could be involved in the formation of intracellular glycogen for storage reasons (Kalscheuer *et al*., 2010a). Furthermore, because glycogen is a carbon storage compound the pathway might have a role in the persistence of *M. tuberculosis* within host microenvironments that exhibit restricted nutrient availability (Kalscheuer *et al*., 2010a).

Whether *C. glutamicum* also uses this pathway for the production of glycogen for carbon storage reasons or precursor supply for cell wall biosynthesis has to be revealed by further research. It has already been published that *C. glutamicum* catalyzes glycogen biosynthesis by GlgC, GlgA and GlgB, a common pathway in bacteria (Seibold *et al*., 2007). Further it has been shown that a strain deleted in the *glgC* gene was not able to form intracellular glycogen and that the absence of glycogen was not disadvantageous for *C. glutamicum* (Seibold *et al*., 2007). This indicates that the newly identified pathway does not seem to play a significant role in glycogen formation in the general physiology of *C. glutamicum*. Therefore, it might be possible that it is only active under special conditions but these needs to be analyzed further. The identification of this new pathway for trehalose and maltose degradation as well as the identification of similar trehalose uptake systems in *C. glutamicum*, *M. tuberculosis* and *M. smegmatis* again shows the close relation between these organisms.

4.8 Model of maltose and trehalose metabolism in *C. glutamicum*

The present study helps to draw an updated picture of maltose and trehalose metabolism in *C. glutamicum* and their interconnections. *C. glutamicum* takes up maltose by the here presented maltose / maltodextrin ABC transport system MusIFGK$_2$-E (Fig. 46), afterwards maltose can be channelled into metabolism by the 4-α-glucanotransferase (MalQ), which catalyzes the transfer from maltosyl and longer dextrinyl residues onto maltose, producing glucose and maltodextrins. The released glucose is then phosphorylated by the glucokinase (Glk), yielding glucose-6-phoshate which is then further metabolized (Seibold *et al*., 2009).

Notably, this study revealed a second pathway for maltose degradation channeling maltose into metabolism by TreX mediated phosphorylation of maltose to maltose-1-phosphate and subsequent incorporation into α-glucan via GlgE. The maltodextrins derived from both pathways are degraded by the maltodextrin phosphorylase (MalP), which forms glucose-1-phoshate by sequential phosphorolysis of the non-reducing end of larger dextrins (Seibold *et al*., 2009).

In addition to these results it must be highlighted that this study revealed the trehalose uptake system TusFGK$_2$-E of *C. glutamicum* (Fig. 46), a system that was thought not to be present (Tropis *et al*., 2005). This system is an ABC transporter that has a very high affinity for its substrate trehalose and a low V_{max}. It is most probably important for the recycling of trehalose that has been released during mycolate biosynthesis. The so imported trehalose can be converted by TreS to maltose, afterwards it is channelled into metabolism by the previously explained maltose degradation pathways.

The identification of a trehalose uptake system raised the question whether the trehalose export hypothesis is still valid. But it was shown that *C. glutamicum* is able to incorporate external trehalose into mycolates even when the uptake system is deleted indicating that a trehalose export system is present, but remains to be identified.

These data further raise the question how *C. glutamicum* prevents a trehalose futile cycle? Since *C. glutamicum* is able to produce, metabolize, export and import trehalose the system must be tightly regulated, e.g. to prevent that trehalose is exported or degraded during an osmotic up-shift when trehalose serves as a compatible solute. It might be possible that TreS serves as a sensor and/or regulator of the cellular trehalose level preventing a trehalose futile cycle.

But not only the regulation of the trehalose metabolism is left elusive; it remains to be revealed why *C. glutamicum* possesses two pathways for the degradation of trehalose / maltose and how these pathways are regulated.

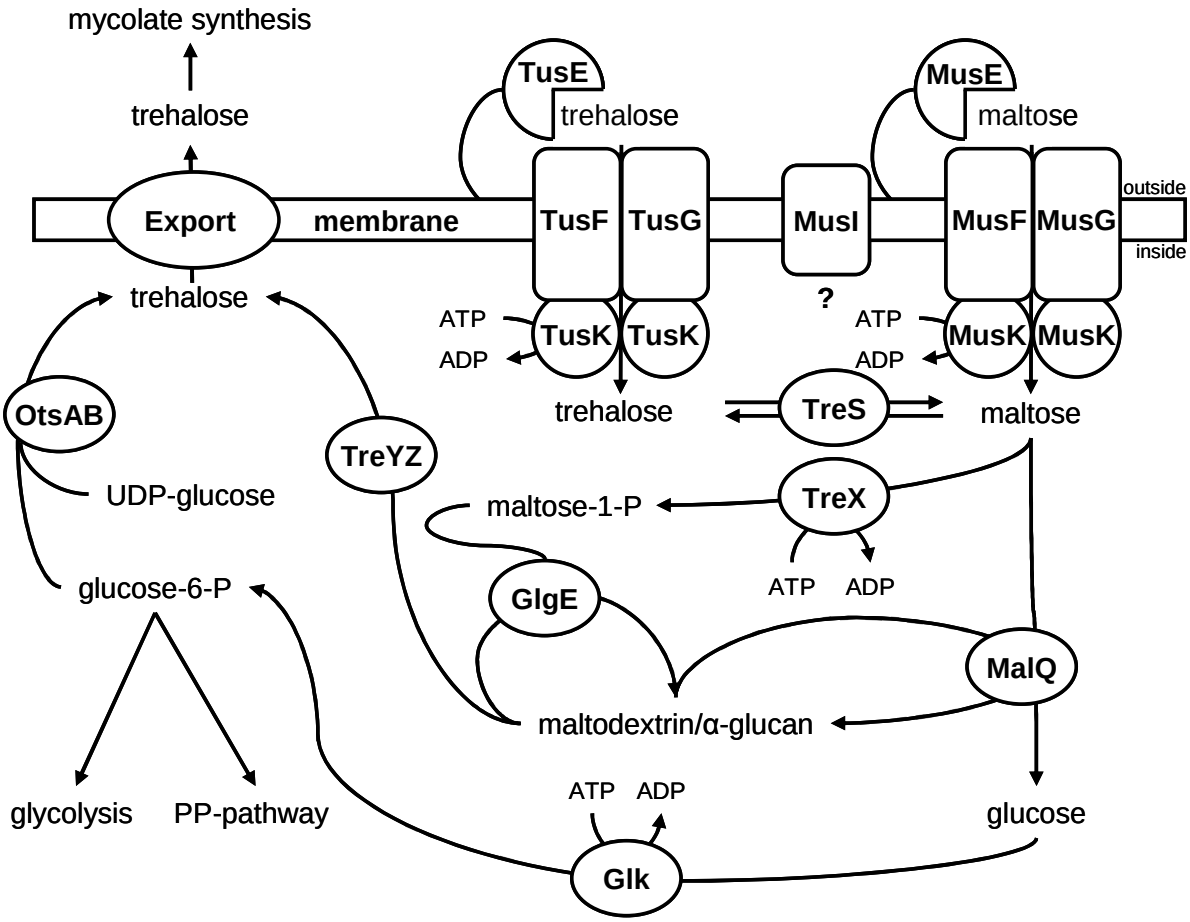

Figure 46: Model of the maltose and trehalose metabolic pathways and their interconnections in *C. glutamicum*. For the maltose metabolism MusIFGK$_2$-E with the permease components MusF and MusG, the ATPase component MusK, the maltose / maltodextrin binding-protein MusE and the protein MusI, MalQ – 4-α-glucanotransferase, and Glk – glucokinase are needed. The second maltose pathway involves TreX – maltokinase and GlgE – maltosyltransferase. For trehalose metabolism TusFGK$_2$-E with the permease components TusF and TusG, the ATPase component TusK, the trehalose binding-protein TusE as well as TreS – with maltose-trehalose interconverting activity are needed. The trehalose synthesis starting from maltodextrins involves the maltooligosyltrehalose synthase (TreY) and the maltooligosyltrehalose trehalohydrolase (TreZ). The second pathway is catalysed by the trehalose-6-phosphat synthase (OtsA) and the trehalose-6-phosphate phosphatase (OtsB), respectively, starting with UDP-glucose and glucose-6-phosphate. The export of trehalose for mycolate synthesis is facilitated by a so far unknown transport system. Maltodextrin degradation via MalP and Pgm is not shown in this model (compare Fig. 3).

5 Summary

The Gram-positive GC-rich bacterium *Corynebacterium glutamicum* is commercially used as a producer of amino acids, mainly L-glutamic acid and L-lysine. For the production of these amino acids maltose derived from starch hydrolysate can serve as a good and cheap carbon source. In the course of this project a very important step of maltose utilization was identified. It was revealed that maltose and further maltodextrins of a length of up to five glucose units are taken up via the ABC transport system MusIFGK$_2$-E. This system has a high affinity for its substrate (K_m of 1.2 ± 0.2 µM) and takes up maltose with a V_{max} of 26.2 ± 1.0 nmol/(min*mg cdm). It was also shown that the system is not transcribed in an operon. The genes *musK*, encoding the ATPase subunit, and *musE*, encoding the binding protein, are transcribed monocistronically, whereas *musG*, *musF*, encoding the permease subunits, and *musI*, encoding MusI form an operon. The system is regulated by the transcriptional regulator RamA and there seems to be a regulatory connection to phosphotransferase system mediated glucose utilization but this interconnection remains to be resolved. Further analyses showed that the maltose uptake system possesses an additional protein MusI that is important for the uptake of maltose into *C. glutamicum*. The function of this protein remains obscure but it was hypothesized that it might be involved in the assembly of the uptake system.

Besides being an important amino acid producer, *C. glutamicum* is a major model organism for the cell envelope and its synthesis of the suborder *Corynebacterineae*, including pathogenic members such as *M. tuberculosis*, *M leprae* and *C diphtheriae*. In this context trehalose plays an important role as component of mycolates, important building blocks of the cell wall of corynebacteria and mycobacteria. This study showed for the first time that *C. glutamicum*, in contradiction to already published data, is equipped with an uptake system for trehalose and is further capable to use trehalose for growth. The uptake of trehalose is mediated by the ABC transport system TusFGK$_2$-E, which showed to have a high affinity (K_m of 0.16 ± 0.02 µM) for trehalose and a low transport rate (V_{max} of 2.5 ± 0.1 nmol/(min*mg cdm)). The physiological role of the trehalose uptake system was hypothesized to be the recycling of released trehalose during mycolate biosynthesis.

These results challenged the hypothesis of Tropis *et al*., 2005 that for the synthesis of trehalose mycolates biosynthesis and export of trehalose is needed. But it was shown with the help of a quadruple-mutant that is neither able to synthesize nor to import trehalose, that the formation of trehalose mycolates is possible without the uptake of trehalose leaving the trehalose export hypothesis valid. Although numerous approaches for the identification and characterization of the trehalose export system were applied the export

system for trehalose of *C. glutamicum* remains to be discovered. After its uptake trehalose can be utilized for growth via TreS mediated conversion into maltose and further degradation by the known maltose pathway. Notably, this study revealed a second pathway for the utilization of trehalose / maltose involving TreS mediated conversion of trehalose to maltose, TreX, a newly identified maltokinase, mediated maltose-1-phosphat formation and α-glucan formation by GlgE, a pathway similar to the one found in *M. tuberculosis* (Kalscheuer *et al*., 2010a). The physiological role of this pathway remains to be further analyzed.

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