Regulation of the phosphotransferase system (PTS)-mediated sugar uptake in *Corynebacterium glutamicum* in response to perturbations of the central metabolism

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Abstract

Corynebacterium glutamicum is a Gram-positive bacterium used in the biotechnological production of amino acids. It co-metabolizes most substrates, such as glucose and sucrose. The uptake and concomitant phosphorylation of those two substrates is mediated by the phosphoenolpyruvate (PEP)-dependent phosphotransferase system (PTS), consisting of the common proteins HPr and EI, and an array of substrate-specific EII permeases. The PTS plays a central role in the regulation of nutrient uptake and metabolism in bacteria. However, the regulatory functions of the PTS in *C. glutamicum* are not understood.

As the availability of NADPH is a limiting factor for the biosynthesis of amino acids, the deletion of *pgi,* encoding the enzyme phosphoglucoisomerase, is a promising approach for strain improvement. It blocks the first step of glycolysis and directs the glucose-derived carbon flux towards the NADPH-producing pentose phosphate pathway. However, despite that *C. glutamicum* Δ*pgi* grows well with sucrose as a sole carbon source, addition of glucose arrests growth by causing repression of *ptsS*, encoding the sucrose-specific EII^{suc}, and a drastic sucrose uptake inhibition. The regulatory mechanism behind these phenomena was unknown and has been investigated here.

It was shown that the glucose addition inhibits sucrose uptake in *C. glutamicum* Δ*pgi* prior to *ptsS*-repression and this fast process is not prevented by transcriptional or translational inhibitors. Analysis of the phosphorylation state of HPr - the last common component of the PTS phosphorylation cascade - indicated that the uptake inhibition is caused by a rapid depletion of HPr~P. The addition of non-PTS substrates which generate carbon flux towards glucose-6-P like *e.g.* maltose or glucose-6-P, uptake of which was enabled by the heterologously expressed transporter UhpT, led to similar growth and sucrose uptake inhibition as the addition of glucose. Unlike glucose, those substrates do not consume PEP for their uptake, so that the HP~P depletion is not caused by a decrease of the PEP/pyruvate ratio but by a glucose-6-P stress response mechanism. Perception of the glucose-6-P stress and the following response initiation requires the glucose-specific EII^{glc} as in EII^{glc}-deficient *pgi* mutants sucrose uptake was not inhibited by glucose, glucose-6-P or maltose addition. Further, it was shown that the low *ptsS*-mRNA levels observed in *C. glutamicum* Δ*pgi* after glucose addition are a consequence of transcriptional repression by the regulator SugR. EMSA studies indicated fructose-1-P and to a lesser extent fructose-6-P as inhibitors of the SugR binding to the *ptsS*-promoter region.

Taken together, this work shows that EII^{glc} is part of a novel mechanism for the perception of sugar-P stress which leads to instantaneous inhibition of the PTS phosphorylation cascade and consequently PTS activity in *C. glutamicum*. Additionally, this rapid uptake inhibition leads to low fructose-1-P formation and thus by an inducer exclusion mechanism, to SugR-dependent reduction of *ptsS*-expression. A suppressor mutation in the gene *cg0790* (*lpdA*) was found to improve significantly the growth, sucrose uptake and *ptsS*-expression of *C. glutamicum* Δ*pgi* during cultivation in the presence of glucose. The role of the novel regulatory mechanism for PTS regulation in *C. glutamicum* is also discussed.

Kurzzusammenfassung

Corynebacterium glutamicum ist ein Gram-positives Bakterium, das für die biotechnologische Herstellung von Aminosäuren verwendet wird. Es co-verstoffwechselt die meisten Substrate, wie z. B. Glucose und Saccharose. Die Aufnahme und gleichzeitige Phosphorylierung dieser zwei Substrate wird vom Phosphoenolpyruvat (PEP)-abhängigen Phosphotransferase System (PTS) vermittelt, bestehend aus den allgemeinen Komponenten HPr und EI und mehreren substratspezifischen EII Permeasen. Das PTS spielt eine zentrale Rolle bei der Regulation der Nahrungsaufnahme und Metabolismus in Bakterien. Jedoch sind die regulatorischen Funktionen des PTS in *C. glutamicum* nicht verstanden.

Die Verfügbarkeit an NADPH ist ein limitierender Faktor für die Biosynthese von Aminosäuren, sodass die Deletion von *pgi*, welches das Enzym Phosphoglucoisomerase kodiert, ein vielversprechender Ansatz zur Stammverbesserung ist. Dabei wird der erste Schritt der Glykolyse blockiert und der Glucose-abgeleitete Kohlenstoffffluss wird in Richtung des NADPH-erzeugenden Pentosephosphatwegs gelenkt. *C. glutamicum* Δ*pgi* wächst gut mit Saccharose als einziger Kohlenstoffquelle, doch Zugabe von Glukose führt zur Wachstumshemmung, indem die Expression von *ptsS*, das die Saccharose-spezifische EII^{Suc} kodiert, und die Saccharoseaufnahme gehemmt werden. Der regulatorische Mechanismus hinter diesen Phänomenen war unbekannt und wurde hier untersucht.

Es wurde gezeigt, dass die Saccharoseaufnahme in *C. glutamicum* Δ*pgi* durch Glucosezugabe sofort und vor der *ptsS*-Repression inhibiert wird und dass dieser Prozess transkriptions- und translationsunabhängig ist. Die Analyse des Phosphorylierungszustandes von HPr - die letzte gemeinsame Komponente der PTS Phosphorylierungskaskade - zeigte, dass die Aufnahmehemmung durch eine schnelle Erschöpfung von HPr~P verursacht wird. Die Zugabe von nicht-PTS Substraten die zur Bildung von Glucose-6-P führen, wie z. B. Maltose oder Glucose-6-P, dessen Aufnahme durch heterologe Expression vom Transporter UhpT ermöglicht wurde, führte zur ähnlichen Wachstums- und Saccharoseaufnahmehemmung wie die Zugabe von Glucose. Im Gegensatz zu Glukose verbrauchen diese Substrate kein PEP für ihre Aufnahme, so dass die Erschöpfung von HP~P nicht durch Verringerung des PEP/Pyruvat-Verhältnisses, sondern von einem Glucose-6-P Stressantwort Mechanismus verursacht wird. Die Wahrnehmung des Glucose-6-P Stresses und die folgende Initiation der Stressantwort erfordert die glukosespezifische EII^{Glc} als in EII^{Glc}-defizienten *pgi* Mutanten die Saccharoseaufnahme durch Glucose, Glucose-6-P oder Maltose nicht inhibiert wurde.

Weiterhin wurde gezeigt, dass das die Abnahme der *ptsS*-mRNA Mengen in *C. glutamicum* Δ*pgi* nach Glucosezugabe eine Folge der transkriptionellen Repression von *ptsS* durch den Reglulator SugR ist. Des Weiteren zeigten EMSA-Studien Fruktose-1-P und Fructose-6-P als Inhibitoren der SugR-Bindung an die *ptsS*-Promotorregion.

Zusammengenommen zeigt diese Arbeit, dass EII^{Glc} Teil eines neuen Mechanismus für die Wahrnehmung von Zucker-P Stress ist, der zur sofortigen Hemmung der PTS Phosphorylierungskaskade und damit der PTS-Aktivität in *C. glutamicum* führt. Darüber hinaus führt diese schnelle Aufnahmehemmung zur verringerten Fructose-1-P Bildung und damit nach einem inducer exclusion Mechanismus zur SugR-abhängigen *ptsS*-Repression. Schließlich wurde hier auch eine Suppressormutation im Gen *cg0790* (*lpdA*) gefunden, die das Wachstum, die Saccharose-Aufnahme und die *ptsS*-Expression von *C. glutamicum* Δ*pgi* während einer Kultivierung in Gegenwart von Glucose deutlich verbessert. Die Rolle des neuen Regulationsmechanismus für die PTS Regulierung in *C. glutamicum* wird diskutiert.

Contents:

1. Introduction

During their adaptation to the dynamic environment organisms have to deal not only with fluctuating abiotic factors such as pH, temperature or osmolarity but should also ensure the maintenance of energetic and metabolic balance inside their cells – a process depending on the availability and selective uptake of proper nutrients. Accordingly, the crossing point of transport, catabolism and anabolism is coordinated by a complex sensing and regulatory network. The precise understanding of this network is therefore crucial for the targeted usage or abatement of particular organisms and our ability to predict or influence their behaviour under different conditions.

1.1. *Corynebacterium glutamicum*

Discovered over 50 years ago as a natural producer of glutamate[1], the immotile, non-sporulating soil bacterium *Corynebacterium glutamicum* has become one of the central organisms of modern day microbiology and biotechnology. Phylogenetically it counts to the Gram-positive suborder of rod-shaped *Corynebacterianeae*. Typical for the members of this suborder is the high GC-content of the genomic DNA as well as the specific mycolic acid layer in the cell wall, representing, similarly to the outer membrane of Gram-negative bacteria, an additional permeability barrier for substances outside the cell[2, 3]. *C. glutamicum* is non-pathogenic and therefore has been used as a model organism for closely related pathogens like *C. diphteriae, Mycobacterium tuberculosis* and *M. leprae*. Facilitated by the good amenability for genetic manipulations of this species and its fully sequenced 3,3 Mbp genome[4, 5], works with *C. glutamicum* have contributed to the better understanding of growth, division and resistance mechanisms of the *Corynebacterianeae* [6-8].

C. glutamicum has gained its greatest significance, however, as one of the main organisms for the biotechnological production of amino acids. Nowadays, over 2.2 million tons of glutamate and 1.7 million tons of lysine are gained annually through the industrial utilisation of *C. glutamicum*[9], which consequently determines the increased economic interest in this

organism. Metabolic engineering strategies enabled the production of further amino acids like lysine, threonine, isoleucine, phenylalanine, arginine, cysteine, and cysteine derivatives[10]. In recent years the application and establishment of *C. glutamicum* for the production of further chemicals such as vitamins, ethanol, isobutanol, lactate and succinate has been an object of intensive investigations[11-16].

As a consequence of the increased biotechnological interest the metabolism of *C. glutamicum* has been intensively studied[17]. However, the regulation of substrate uptake during growth on mixed nutrients and its interrelation with the central metabolism has not been in the focus of research so far and remains in wide areas unclear. This is surprising as molasses and starch hydrolysates, used as the main, cheap feedstock in most of the established industrial fermentation processes, contain a broad and often varying spectrum of simple carbohydrates (sugars) - mostly glucose, fructose and their disaccharide-product sucrose [18, 19].

1.2. Carbohydrate metabolism of *C. glutamicum*

Carbohydrates are one of the three main macronutrients and the most abundant biological compound in living organisms[20]. They consist of carbon, hydrogen, and oxygen atoms and range from simple monosaccharides, structured either as hexoses (glucose, fructose, galactose) or pentoses (ribose, xylose), to complex polysaccharides (starch, glucogen, cellulose), consisting of long chains of glycosidically bound monosaccharides.

C. glutamicum is a facultative anaerobic chemoheterotroph and as such utilises carbohydrates as primary source of both carbon and energy. It possesses two metabolic pathways for the utilisation of the carbohydrates – the glycolytic and the pentose phosphate pathways.

1.2.1. Glycolysis

Glycolysis is the preferential and most effective catabolic pathway of most organisms for the utilisation of carbohydrates. It represents the process of conversion of glucose to pyruvate, in which a high number of basic precursors are made available to other metabolic pathways and the released energy is used for the generation of ATP and reduction equivalents in the form of NADH.

The first step of glycolysis involves activation of glucose via phosphorylation to glucose-6-phosphate. This process is catalysed by a hexokinase or in the majority of bacteria this phosphorylation often occurs during the uptake of the sugar into the cell, as it will be described more explicitly below (1.3). Next, glucose-6-phosphate is transformed to fructose-6-phosphate (F6P) by the enzyme phosphoglucoisomerase (encoded by *pgi*) (Fig. 1). F6P is phosphorylated by phosphofructokinase (*pfkA*) to fructose-1,6-bisphosphate, which is then split into glyceraldehyde-3-phosphate (GAP) and dihydroxyacetone phosphate, which is further isomerised to a second GAP molecule. Several transformations of the obtained triose molecules follow in the energy generating phase of the glycolysis. First, the glyceraldehyde-3-P dehydrogenase (*gapA, gapB*) catalyses the phosphorylation of GAP to 1,3-bisphosphoglycerate under the reduction of NAD^+. The highly energetic triose product carries over one of its phosphate groups to ADP thus resulting in ATP and 3-phosphoglycerate, which is consequently transformed to 2-phosphoglycerate by the phosphoglycerate mutase. The 2-phosphoglycerate is reversibly converted by enolase (*eno*) to the important intermediate phosphoenolpyruvate (PEP). In the final step of glycolysis PEP is irreversibly dephosphorylated to pyruvate by the pyruvate kinase (*pyk*) under the formation of ATP. Thus, the final result of the utilization of 1 mol glucose via glycolysis is the formation of to 2 mol pyruvate under the net conversion of 2 mol ADP to ATP and two mol NAD^+ to $NADH+H^+$. Pyruvate could then be oxidised by the pyruvate dehydrogenase complex to acetyl-CoA and thus enter the tricarboxylic acid cycle (TCA), where it is completely catabolised to CO_2 and energetic compounds.

The reverse process of glucose synthesis from pyruvate - the gluconeogenesis - shares many of the glycolytic metabolic steps except for the exergonic, irreversible reactions catalysed by the phosphofructokinase and the pyruvate kinase. The formation of PEP from pyruvate is managed by the enzymes pyruvate carboxylase, using ATP, and PEP carboxykinase, using GTP, through the generation of oxaloacetate as intermediate. Noteworthy, considerable PEP carboxykinase activity is present in *C. glutamicum* even during growth in glucose minimal medium[21]. The second differing reaction is the conversion of F-1,6-BP to F6P by the enzyme fructose-1,6-bisphosphatase under the release of pyrophosphate. Gluconeogenesis is an energy consuming, anabolic pathway and is therefore activated by increased ATP levels in the cell. Gluconeogenesis is important for the energy storage and cell wall synthesis.

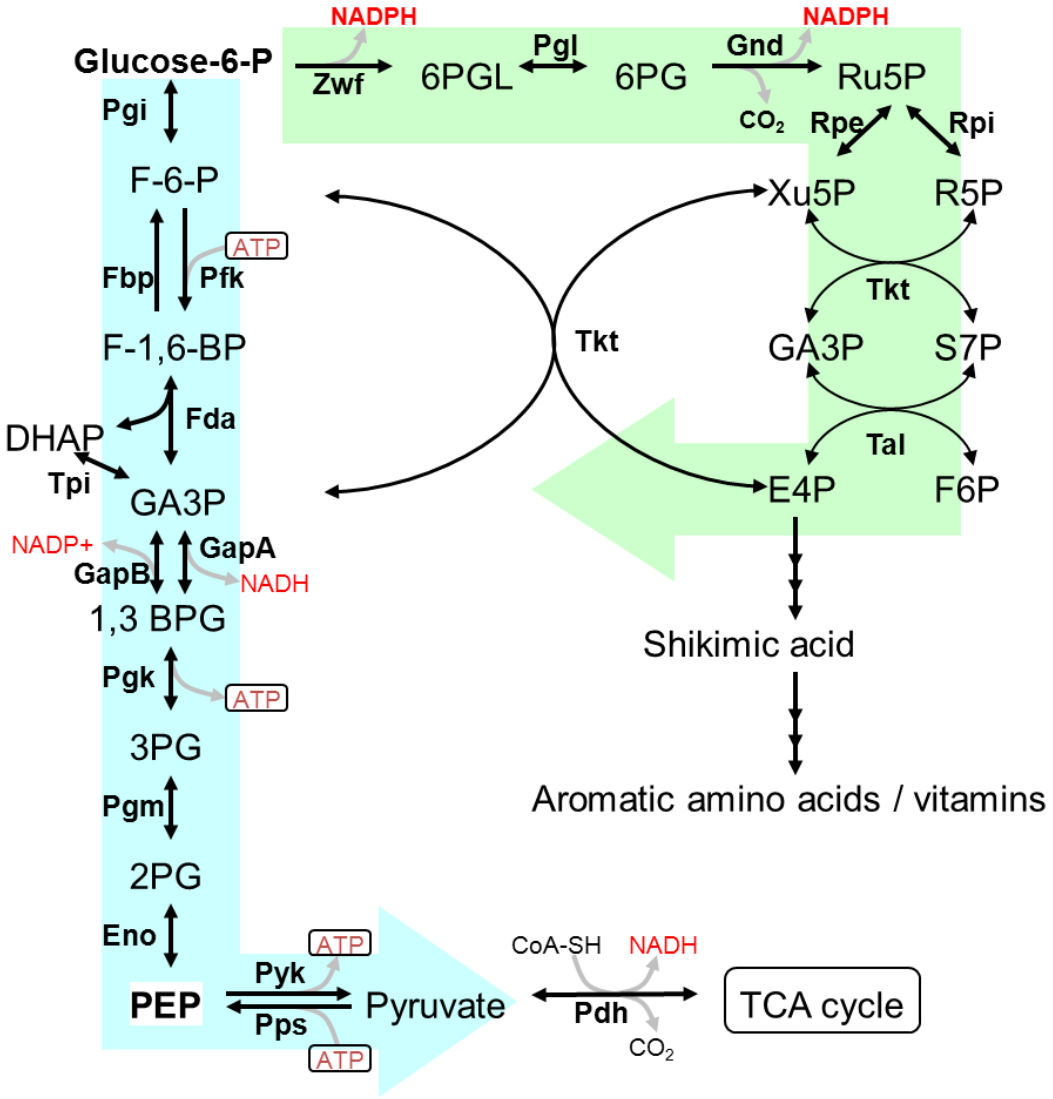


Figure 1: Glycolysis, gluconeogenesis and the pentose phosphate pathway in *C. glutamicum*. Abbreviations - substrates: fructose-6-phosphate (F6P); fructose-1,6-bisphosphate (F-1,6-BP); dihydroxyacetone phosphate (DHAP); glyceraldehyde-3-phosphate (GA3P); 1,3-bisphosphoglycerate (1,3 BPG); 3-phosphoglycerate (3PG); 2-phosphoglycerate (2PG); phosphoenolpyruvate (PEP); 6-phosphoglucono-δ-lactone (6PGL); 6-phosphogluconate (6PG); ribulose-5-phosphate (Ru5P); ribose-5-phosphate (R5P); xylose-5-phosphate (Xu5P); seduheptulose-7-Phosphate (S7P); erythrose-4-phosphate (E4P) - enzymes: phosphoglucoisomerase (Pgi); phosphofructokinase (Pfk); phosphotrioseisomerase (Tpi); glyceraldehyde-3-P dehydrogenase (GapA, GapB); phosphoglycerate mutase (Pgm); enolase (Eno); pyruvate kinase (Pyk); pyruvate dehydrogenase complex (Pdh); fructose-1,6-bisphosphatase (Fbp); pyruvate carboxylase (Pyc); PEP carboxykinase (Pck); glucose 6-phosphate dehydrogenase (Zwf); 6-phosphogluconate dehydrogenase (Gnd); glucanolactonase (Pgl); transketolase (Tkt); transaldolase (Tal); ribulose-5-hosphate 3-epimerase (Rpe); ribulose-5-phosphate isomerase (Rpi)

1.2.2. The pentose phosphate pathway

The pentose phosphate pathway (PPP) fulfils two primary purposes in cells - the generation of reducing equivalents in the form of NADPH, necessary for reductive biosynthetic reactions, and the formation of ribose-5-phosphate, an essential component of nucleotides and nucleic acids. The pathway could be divided in two distinct phases. The first oxidative

part is responsible for NADPH generation, using energy from the conversion of glucose-6-phosphate into ribulose-5-phosphate. It involves three consecutive reactions. First, glucose-6-phosphate is transformed to 6-phosphoglucono-δ-lactone by the enzyme glucose 6-phosphate dehydrogenase (*zwf*), driving the first reduction of one molecule $NADP^+$ to $NADPH+H^+$. Then, hydrolysis of the product results 6-phosphogluconate, which is finally decarboxylated by the 6-phosphogluconate dehydrogenase (*gnd*) to the pentose ribulose-5-phosphate and CO_2, generating a second molecule of NADPH. In the second non-oxidative part of the PPP the ribulose-5-phosphates are transformed over several isomerisations to three-, four-, five-, six- and seven-carbon sugar-phosphates to fructose-6-phosphate and GA3P, which then can be further utilised in the glycolysis (Fig. 1). The rearrangements of the carbon skeleton of the substrates in this part offer the cell a broad spectrum of intermediates, most significantly of which ribose-5-phosphate – a precursor for the synthesis of histidine, purines and pyrimidines - as well as erythrose-4-phosphate, which together with PEP is the precursor for the synthesis of shikimic acid, aromatic amino acids and aromatic vitamins.

Due to the loss of carbon atoms in the form of CO_2 during the oxidative part, the PPP is the less effective pathway for carbohydrate utilisation compared to glycolysis. Therefore, the partitioning of carbon flux between the PPP and glycolysis depends on the intracellular NADPH demand[22]. During production of glutamate, requiring one molecule NADPH for its synthesis, the carbon flow through the PPP was shown to be lower than during lysine production, when four molecules of NADPH are needed. Even though other enzymes like the malic enzyme or the isocitrate dehydrogenase are also able to catalyse the reduction of NADP to NADPH, the oxidative part of the PPP remains the main generator of NADPH in the cell, which also determines the increased interest on that pathway[23].

1.3. The phosphotransferase system (PTS)

The phosphoenolpyruvate-dependent phosphotransferase system (PTS) catalyses the uptake and phosphorylation of a high number of sugars and sugar derivatives. In contrast to the majority of other transport systems, the PTS is found exclusively in bacteria and has a unique mechanism. Unlike primary active transporters, like the ATP-binding cassette (ABC)

transporters, which utilize the free energy of ATP hydrolysis or secondary active transporters which use the energy of an existing electrochemical gradient, the PTS uses the energy of phosphoryl group translocation from the glycolytic intermediate phosphoenolpyruvate (PEP) to the substrate, triggering thereby not only its uptake but also concomitant phosphorylation. Thus, the PTS substrate is directly activated and could enter the central metabolism, whereas the substrates transported by primary or secondary active transporters require additional phosphorylation by a kinase, which makes the PTS-mediated uptake in general more effective. Indeed, the most common sugars like glucose, fructose or sucrose are transported in the majority of studied bacteria by the PTS[24].

The composition of the PTS is similar in all studied bacteria (Fig. 2). It consists of two common cytoplasmic proteins, enzyme I (EI) and HPr, forming the substrate-unspecific part of the phosphorylation cascade and an array of substrate-specific enzyme II complexes (EIIs). The EII complex composition is variable but in general consists of three fused or separate proteins – the cytosolic EIIA and EIIB mediating phosphorylation of the substrate and the membrane spanning EIIC forming the substrate binding site and translocation channel [24].

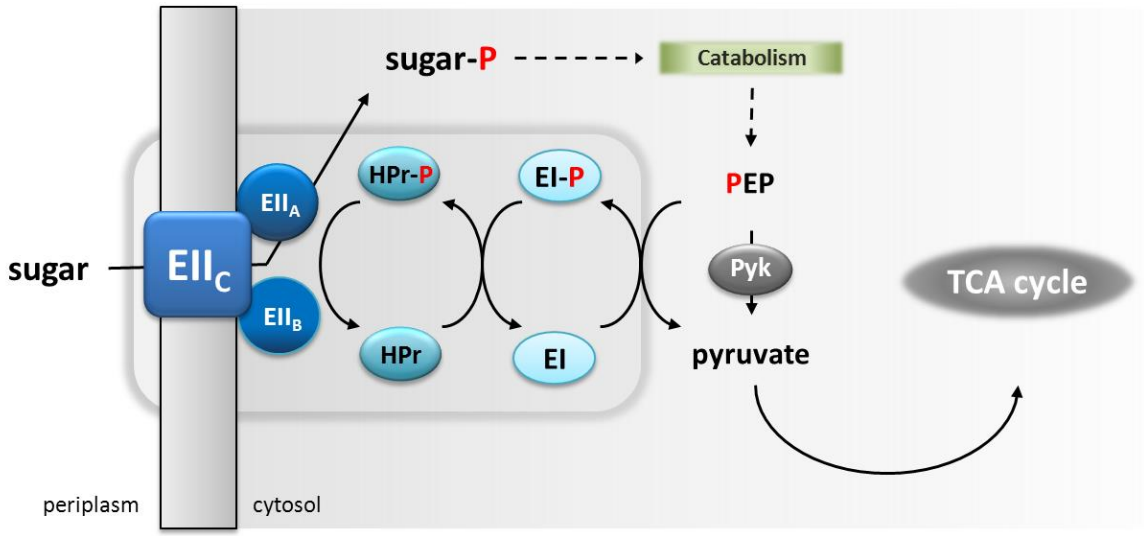


Figure 2: Schematic view of the sugar uptake mediated by the phosphoenolpyruvate-dependent phosphotransferase system (PTS).

The phosphorylation cascade required for transport and phosphorylation of substrates starts with autophosphorylation of the EI dimer at a conserved histidyl residue converting PEP to pyruvate [25]. The resulting EI~P dimer dissociates and passes the phosphoryl group to the highly conserved histidyl residue (His-15) of HPr [26]. Subsequently HPr~P phosphorylates a

histidyl-residue of the EIIA domains of the substrate-specific permeases [27]. Inside the EII complex the phosphoryl group is then transferred from EIIA~P to a histidyl or cysteyl residue of EIIB, which finally phosphorylates the EIIC-bound substrate, which is subsequently released into the cytoplasm[24]. All reactions within the PTS are reversible with the exception of the final step of substrate phosphorylation. Hence, the phosphorylation state of PTS components reflects beside carbohydrate availability also the physiological state of the cell[28]. This fact enables the PTS to fulfill not only catalytic purposes but also to coordinate diverse cellular processes like carbon and nitrogen metabolism, chemotaxis, biofilm formation, and virulence[24, 29].

1.3.1. The phosphotransferase system (PTS) of *C. glutamicum*

In *C. glutamicum*, the common PTS proteins EI and HPr, encoded by *ptsI* and *ptsH*, as well as a glucose-, fructose- and sucrose-specific PTS permease, encoded by *ptsG*, *ptsF* and *ptsS*, have been described[30, 31]. An EII permease belonging to the ascorbate PTS family is also encoded in the genome, but its substrate or function is unknown[32]. Nonetheless, the substrate spectrum of the PTS in *C. glutamicum* is limited as for example *E. coli* contains at least 15 different, thoroughly described EII complexes, and a similar number of PTS permeases have been found in *B. subtilis*[24].

After being transferred into the cytosol and simultaneously phosphorylated to G6P, glucose transported by the PTS can enter the central metabolism as described above (1.2.1; 1.2.2). Other PTS substrates are also utilized by the glycolytic and pentose phosphate pathways. Fructose for example, is transported into the cell via the PTS in the form of fructose-1-phosphate but after an additional phosphorylation by the 1-phosphofructokinase enters the glycolysis as fructose-1,6-phosphate and is further metabolised (Fig. 3).

Slightly more complex is the introduction to the metabolism of the third PTS substrate in *C. glutamicum* – sucrose. Sucrose is a disaccharide, consisting of glycosidically bound glucose and fructose unit. After entering the cell through EII^{suc} in the form of sucrose-6-phosphate, this carbohydrate is hydrolysed by the sucrose-6-phosphate hydrolase ScrB to G6P and fructose[33]. Due to the lack of a fructokinase in *C. glutamicum* however, the internally liberated fructose cannot be metabolised and is therefore exported through an unknown transporter in order to be re-imported through EII^{fru} in the accessible for the metabolism fructose-1-phosphate form[34] (Fig. 3).

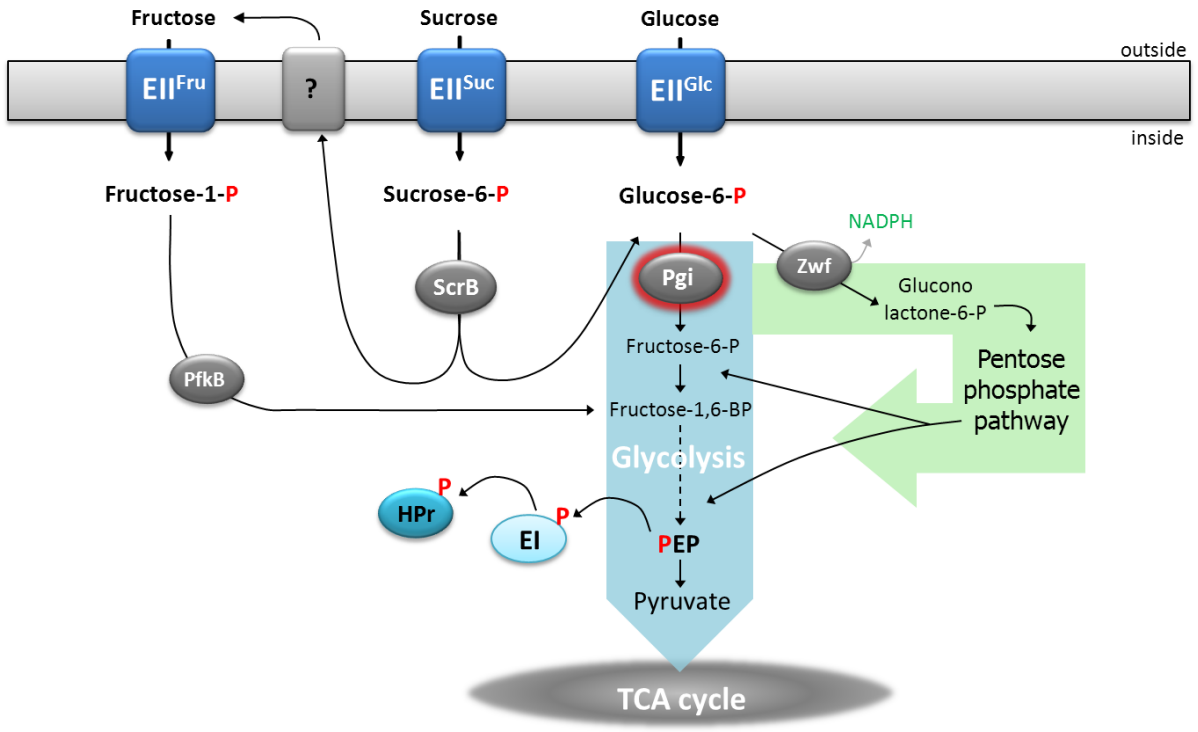


Figure 3: Schematic overview of the PTS mediated glucose and sucrose uptake in *C. glutamicum* and the following metabolic pathways for their utilization.

While EII^{suc} is essential for growth of *C. glutamicum* on sucrose as a sole carbon source, the organism is still capable of slow residual glucose or fructose utilisation in the absence of EII^{glc} or EII^{fru} respectively[32]. The secondary active inositol transporters IolT1 and IolT2 are responsible for the slow, additional uptake of glucose under excess concentrations of this carbohydrate[35], whereas an unspecific transport of fructose through the EII^{glc} has been suggested as a reason for the second phenomenon[36]. Phosphorylation and utilisation of unspecifically transported glucose through the IolT1 and IolT2 permeases is enabled by the presence of the ATP dependent glucokinase Glk and the polyphosphate/ATP dependent glucokinase Ppgk [35]. The product of unspecific fructose uptake by the EII^{glc} is expected to be fructose-6-P [36].

1.4. Regulatory mechanisms in the mixed substrate utilization in Gram-positive and low-GC Gram-negative bacteria

In an environment of mixed nutrients most bacteria selectively adjust their uptake to a preferred compound, most dominantly glucose, which fits best to their metabolic capacity

and requirements. As long as the preferred carbon source is available transport and utilization of the less favourable substrate is prevented – a process known as carbon catabolite repression (CCR). Catalysing the uptake of many primary carbon sources, the PTS indispensably plays a central sensing and regulatory role in the CCR. Depending on their phosphorylation state - being predominately phosphorylated in their inactive and unphosphorylated in their active state - components of the PTS affect the activity and/or transcription of other enzymes mediating sugar uptake and utilisation. The broad range of mechanisms behind those regulatory functions have been thoroughly described for several Gram-negative and low-GC Gram-positive model organisms[24, 29].

In Gram-negative bacteria the phosphorylation state of EII plays a central regulatory role. This is well exemplified by the *lac* operon of *Escherichia coli* which is one of the paradigms for CCR and transcriptional regulation in general. The transcription of the *lac* operon, comprising genes for the uptake and utilisation of lactose, is down-regulated by the repressor LacI when no lactose is present in the cell[84]. Uptake of lactose releases the binding of LacI at the *lac* operator site and thus enables the transcription of the operon. However, in a process called inducer exclusion, the unphosphorylated form of the soluble $EIIA^{glc}$ component of the glucose-specific PTS is able to bind and thus inhibit the lactose permease in the presence of glucose thus limiting the formation of the intracellular inducer allolactose[37].

On the other hand, even without LacI repression, binding of the cAMP-dependent global transcriptional activator Crp to the *lac* promoter is also necessary for the effective expression of the *lac* operon. The level of cAMP in the cell, though, is dependent on the phosphorylation state of $EIIA^{glc}$. Phosphorylated $EIIA^{glc}$ activates the adenylyl cyclase thus raising cAMP synthesis. In turn, the global regulator Crp becomes active and affects the transcription of many metabolic and transporter genes, inclusively the *lac* operon and genes of the PTS[38]. In that manner the cell ensures additionally that even when the inducer lactose is available, the lactose metabolism gets active only if the preferred carbon source glucose is not transported into the cell.

A direct control of transcriptional regulators by the PTS has also been shown[39]. The Mlc protein is a repressor of the *ptsHIcrr* operon in *E. coli*, encoding for the general PTS components HPr and EI and the glucose specific $EIIA^{glc}$. Unlike LacI, however, Mlc binding to DNA is controlled not by an effector molecule but directly by the PTS itself. When glucose is

not available the membrane integrated EIIBC^{glc} component of the glucose-specific PTS is in its phosphorylated, inactive form and the *ptsHIcrr* operon is repressed by Mlc. However, dephosphorylation of EIIBC^{glc} as a consequence of a glucose uptake sequesters Mlc to the cell membrane, which leads to its release from the *ptsHIcrr* promoter[39].

Another regulatory mechanism responding to the phosphorylation state and thus presence of specific substrate of a PTS transporter in *E. coli* has been described for the *bgl* operon, encoding genes for the utilisation of β-glucosides. In this case substrate-specific expression of the operon is regulated by controlled transcriptional antitermination by the protein BglG, encoded by the first gene of the operon, *bglG* [40]. When no substrate is present the permease EII^{bgl}, encoded by the second gene of the operon, *bglF*, phosphorylates BglG which could no longer inhibit the premature termination of *bgl*-transcription[41, 42]. Upon addition of β-glucosides, EII^{bgl} dephosphorylates BglG, thus relieving its inhibition. Interestingly, phosphorylation of BglG on a distinct residue by HPr is also required for BglG activation, thereby connecting the transcription of the *bgl* operon to the general state of the PTS [43].

The PTS determines the CCR of low-GC Gram-positive bacteria like *Bacillus subtilis*, as well. A central role thereby has the phosphorylation state of the common component HPr. In contrast to enteric bacteria, this group of organisms possesses an HPr kinase [44]. In conditions of high intracellular concentrations of fructose-1,6-phosphate and ATP or pyrophosphate the HPr kinase catalyses the phosphorylation of HPr at a Ser-46 residue. This is a regulatory site different from the catalytic His-15 phosphorylated by EI during sugar uptake. The product HPr-Ser~P has a lower affinity to EI than unphosphorylated HPr thus resulting in the inhibition of the phosphorylation cascade and PTS activity[24]. Additionally, similarly to the cAMP-Crp mechanism, HPr~Ser-P activates a global transcriptional regulator – the carbon catabolite protein CcpA – playing a central role in the control of carbon catabolism[45].

Furthermore, in Gram-positive bacteria also the His15-phosphorylated form of HPr controls the activity of various proteins. A high number of transcriptional regulators are controlled by an HPr-His~P-mediated phosphorylation at a histidyl residue of a specific PTS regulation domain (PRD)[46]. The glycerol kinase GlkP is also regulated by His15-phosphorylated HPr so that the glycerol uptake and metabolism is controlled in response to availability of PTS substrates[47]. Thus, the ratio of serine-, histidine- or unphosphorylated HPr is able to ensure a precise tuning of the energetic and metabolic state of the low-GC Gram-positive bacteria.

1.5. Regulation of the carbohydrate utilization in *C. glutamicum*

In contrast to well-studied organisms like *Escherichia coli* or *Bacillus subtilis* showing distinct CCR, *C. glutamicum* is known for the simultaneous metabolisation of most carbon sources. Glucose for example is co-metabolised with acetate[48], lactate[49], propionate[50], pyruvate and serine[41], protocatechuate and vanillate[52], gluconate[53], maltose[54], and fructose[55]. Just a few exceptions are the sequential consumption of glucose before glutamate[56] or ethanol[57]. The simultaneous utilization of carbon sources in *C. glutamicum*, however, still appears to be regulated. During cultivation on glucose plus acetate, both the glucose and acetate uptake rate are reduced by 50% compared to the rates during cultivation on either of the substrates alone, resulting in similar growth and total carbon uptake rates [48]. Similar effects have been observed during glucose plus fructose co-utilization [55]. Yet, the understanding of the regulatory mechanisms responsible for those uptake adjustments is limited.

A few transcriptional regulators have been found to play a role in the regulation of mixed substrate utilization in *C. glutamicum*. The DeoR-type transcriptional regulator SugR is involved in the glucose uptake adjustment during growth on glucose-acetate mixtures as the deletion of *sugR* resulted in increased glucose uptake[58]. SugR is the master regulator of all PTS genes in *C. glutamicum* and is also involved in the regulation of further genes of glycolysis, PPP or lactate metabolism[59]. During growth on gluconeogenetic carbon sources such as acetate and pyruvate the regulator protein SugR inhibits expression of *ptsG, ptsH, ptsI* and the fructose-PTS gene cluster[58]. However, the binding of SugR to the *ptsG* promoter region is repressed by the glycolytic intermediate fructose-6-P [58]. On the other hand, fructose-1-phosphate but not fructose-6-P, as well as in much higher concentrations glycolytic intermediates such as G6P and fructose-1,6-bisphosphate, has been discussed as negative effector of SugR-binding to the *ptsI*- and *gapA*-promoters [60]. Hence, PTS genes are transcribed when metabolites as intracellular indicators for the presence of sugars de-repress their inhibition by SugR. Thus, it remains unclear why during glucose-acetate cultivation SugR reduces *ptsG*-expression.

Binding motifs for the transcriptional regulators RamA and RamB, which control genes for the utilization of acetate and ethanol, has been found in the promoter regions of *ptsG* and *ptsS.* Their function in the preferential utilization of glucose before ethanol or in the uptake adjustment during glucose-acetate co-utilization, however, has not been clarified[61].

Similarly to glucose-acetate co-utilization, *C. glutamicum* consumes glucose at a reduced rate when gluconate is present. The functionally equivalent GntR1 and GntR2 transcriptional regulators have been shown to activate the expression of *ptsG* in the absence of gluconate, thus leading to lowered expression during co-utilization[53].

The role of the PTS in the sugar uptake regulation in *C. glutamicum* is also poorly understood. *C. glutamicum* possesses an adenylate cyclase and CRP-proteins have been described in closely related bacteria, such as *Streptomyces coelicolor*[62] or *M. tuberculosis*[63], so that a cAMP signal transduction system involved in the control of the carbon uptake and metabolism similar to the one described in Gram-negative bacteria could be expected. Indeed, a homologue of *E. coli*'s global regulator Crp has been identified - the cAMP-dependent regulator GlxR[64]. It has been suggested that GlxR is partially responsible for the CCR of glutamate uptake in the presence of glucose but disruption of the *glxR* gene resulted in severe growth defects on all tested carbon sources so that the exact function of GlxR remains unclear[64]. Nonetheless, apart from a cAMP signal transduction system, no HPr kinase, required for the CCR in *B. subtilis*, or enzymes with similar properties have been found in *C. glutamicum* [30]. No separate EIIA protein, which is the central component of CCR in Gram-negative bacteria, is present, either. Hence, the regulatory functions of the PTS shown for most bacteria might not be relying on the same regulatory mechanisms for the control of the carbohydrate utilization in *C. glutamicum*.

1.6. The *pgi* mutant of *C. glutamicum*

A variety of metabolic engineering strategies have been applied in order to optimize the efficiency of substrate utilization and productivity of different biotechnologically important organisms[65, 66]. One of them is the redirection of the carbon flux form glycolysis towards the PPP which would increase the generation of NADPH and thus improve precursor supply for many biotechnologically relevant anabolic processes. One possibility to achieve that is the abolishment of the first step of glycolysis through chromosomal inactivation of *pgi*, encoding the first glycolysis-specific enzyme phosphoglucoisomerase (Pgi). However, such a drastic interference in the metabolism of an organism represents a risk of severe changes in its physiological properties which might overshadow the aimed positive effects.

Indeed, despite the presence of alternative pathways for the utilization of glucose-6-P the blockage of glycolysis by deletion of the *pgi* gene resulted in severe inhibition of the growth on glucose in various microorganisms like *e.g. E.coli*[67] or *B. subtilis*[68]. The growth deficits of *E. coli* Δ*pgi* were attributed to imbalances of the NADPH metabolism and accumulation of glucose-6-P. The PPP generated NADPH over-shoot seems deleterious due to the limited capacity for re-oxidation of NADPH in the Pgi-deficient *E. coli* mutant [69]. Deficits in NADPH regeneration could be alleviated in *E. coli* by the action of the soluble transhydrogenase UdhA, which catalyses the reversible transfer of reducing equivalents between NAD and NADP pools[69]. The absence of functional PntA, a transhydrogenase catalysing the reduction of NADP with NADH, also seemed to improve the growth of *E. coli* Δ*pgi* with glucose [70].
Further, during cultivation of *E. coli* Δ*pgi* on glucose a drastic *ptsG*-repression and reduced substrate consumption rate have been observed, as well[71]. The lowered transcript amounts are caused by a post-transcriptional regulation by the small RNA (sRNA) SgrS, functioning as a rescue mechanism against glucose-phosphate-stress[72]. A base-pairing between *SgrS* and the *ptsG*-mRNA stabilised by the chaperon Hfq introduces the accelerated degradation of the *ptsG*-transcript by RNase E. The sRNA *sgrS* was shown also to stabilize the dicistronic *pldB-yigL* mRNA, encoding a sugar-P phosphatase[73]. Additionally, *scrrS* encodes a short peptide SgrT, which is described as a direct inhibitor of PTS-mediated glucose uptake[74, 75].

The sufficient supply of NADPH is one of the critical factors also for the production of amino acids by *C. glutamicum* so that the redirection of the carbon flux towards the PPP by deletion of *pgi* is again a promising approach for strain improvement[76]. Similarly to *E. coli* Δ*pgi*, however, growth of *C. glutamicum* Δ*pgi* with glucose as a sole carbon source is drastically inhibited[77]. Moreover, despite that growth with sucrose as sole carbon source is not affected, addition of glucose to sucrose-cultivated *C. glutamicum* Δ*pgi* cells arrests their growth and leads to strong reduction of the *ptsS*-mRNA amounts and sucrose uptake [78]. Additionally to being biotechnologically interesting due to the fact that glucose and sucrose are very abundant building blocks of many raw materials used as a feedstock for industrial processes, this CCR-like phenomenon indicates the presence of a so far unknown regulatory network for the control of PTS-mediated sugar uptake in *C. glutamicum*. However, the sugar-P stress recovery mechanisms by *sgrS* demonstrated for *E. coli* does not seem to be responsible for the effects in *C. glutamicum*. Despite the presence of an RNase E, no Hfq or

sgrS homologues have been identified so far in this organism. Also, the heterologous overexpression of *udhA* in *C. glutamicum* Δ*pgi* has resulted only in a weak growth improvement during cultivation on glucose[77].

1.7. Objectives of the thesis

An initial investigation of the growth inhibition of *C. glutamicum* Δ*pgi* during cultivation on sucrose plus glucose suggested the involvement of the PTS-repressor SugR. Additional deletion of *sugR* in *C. glutamicum* Δ*pgi* abolished the negative effect of glucose on *ptsS*-expression in the resulting double mutant. Consequently, a regulatory model was proposed in which the presence of glucose induces a SugR-dependent repression of *ptsS* which in turn leads to the inhibition of the sucrose uptake in the cell which results in the poor growth of the pgi mutant with sucrose plus glucose.

However, the derepression of *ptsS* was not sufficient to fully recover the growth or sucrose uptake of *C. glutamicum* Δ*pgi* Δ*sugR* clearly indicating the involvement of additional regulatory mechanisms[92]. Hence, this work aims to elucidate the regulatory mechanism determining the glucose-triggered inhibition processes in sucrose plus glucose cultivated *C. glutamicum Δpgi* cells. Thereby, the role of the transcriptional repression by SugR should be investigated more precisely. Accordingly, the reason and mechanism of sucrose uptake inhibition after addition of glucose should be investigated as it seemed that this process might be uncoupled from the detected *ptsS*-mRNA amounts in the cell.

Furthermore, the mechanism of activation of the SugR-mediated *ptsS*-repression should be clarified. Even for the well-studied sugar-P stress response mechanism of *E. coli* the signal and respective signal transduction mechanism leading to the activation of the *sgrS*-mediated regulation is still unknown. In that aspect, it should be investigated whether changes in the balance of the intracellular metabolite concentrations or changes in the state of the PTS components as a direct consequence of the activity of the transporter are initial triggers for the observed regulatory effects in the sucrose plus glucose cultivated *C. glutamicum Δpgi* cells.

2. Materials and methods

2.1. Bacterial strains, plasmids und oligonucleotides

The bacterial strains used in this work are listed in Table 1.

Table 1: Bacterial strains used during this work:

strain	genotype	reference
Escherichia coli		
DH5α *mcr*	*endA1, supE44, thi-1, recA1, hsdR17, gyrA96, relA1, lacZα*	Grant *et al*. [79]
K-12 (substr. MG 1655)	F-, *ilvG, rph1*	Bachmann [80]
BL21 (DE3)	F- *dcm ompT hsdS*(rB- mB-) gal λ(DE3)	Studier & Moffatt [81]
Corynebacterium glutamicum		
ATCC 13032	wild-type	Kinoshita *et al*., [1]
Δ*pgi*	ATCC 13032 with a chromosomal deletion of *pgi*	Hagmann *et al*. [82]
Δ*sugR*	ATCC 13032 with a chromosomal deletion of *sugR*	Engels & Wendisch [58]
Δ*pgi* Δ*sugR*	Δ*pgi* with a chromosomal deletion of *sugR*	Petrov [83]
Δ*ptsG*	ATCC 13032 with a chromosomal deletion of *ptsG*	Henrich [84]
Δ*pgi* IM*ptsG*	Δ*pgi* with insert inactivation of *ptsG*	Petrov [83]
Δ*pgi* Δ*ptsG*	Δ*pgi* with a chromosomal deletion of *ptsG*	this work
Δ*scrB*	ATCC 13032 with a chromosomal deletion of *scrB*	Engels *et al*. [33]
Δ*scrB* Δ*ptsS*	Δ*scrB* with a chromosomal deletion of *scrB* and *ptsS*	this work
Δ*ptsS*	ATCC 13032 with a chromosomal deletion of *ptsS*	this work
Δ*lpdA*	ATCC 13032 with a chromosomal deletion of *lpdA*	this work
Δ*pgi* SM-K6	Δ*pgi* with point mutation in *lpdA*	this work
Δ*ptsH*	ATCC 13032 with a chromosomal deletion of *ptsH*	Lindner *et al*. [35]
Δ*ptsI*	ATCC 13032 with a chromosomal deletion of *ptsI*	Kuhlman *et al.* [85]

The plasmids used in this work and their main properties are listed in Table 2.

Table 2: plasmids used during this work and their relevant properties:

plasmid	relevant proparties	reference
pEKEx2	Kan^R, *lacI,*	Eikmanns *et al.* [63]
pEKEx2–*sugR*	pEKEx2 with *sugR*-Insert	Petrov [78]

pEKEx2-*ptsG_HIS*	pEKEx2 with ptsG with C-terminal poly-glycine linker His-tag sequence	Petrov [78]
pEKEx2-*ptsS*	pEKEx2 with inducible *ptsS*	this work
pEKEx2-*ptsS_Rho*	pEKEx2 with inducible *ptsS* with C-terminal Rho tag	this work
pEKEx2-*lpdA*	pEKEx2 with inducible *lpdA*	this work
pEKEx2-*lpdA*^{K6}	pEKEx2 with inducible *lpdA*^{K6}	this work
pEKEx2-*ptsH_FLAG*	pEKEx2 with inducible *ptsH* with C-terminal FLAG tag	this work
pEKEx2-*Strep_ptsI*	pEKEx2 with *cg1048- ptsI* with N-terminal Strep tag	this work
pEKEx2-*uhpT*	pEKEx2 with *uhpT* from *E. coli*	this work
pK19mobsacB	deletion vector, KanR, *oriVE.c.*, *oriT*, *mob*, *sacB*	Schäfer *et al*. [86]
pK19mobsacB-*ptsG*	plasmid for chromosomal deletion of *ptsG*	Henrich [84]
pK19mobsacB-*ptsS*	plasmid for chromosomal deletion of *ptsS*	this work
pK19mobsacB-*lpdA*	plasmid for chromosomal deletion of *lpdA*	this work
pBB1	Chl^r, *C. glutamicum*/*E. coli* shuttle vector (P_{tac} *lacI*^{q}; pBL1, $OriV_{C.g.}$, $OriV_{E.c.}$)	Krause *et al*. [87]
pBB1-*ptsG*	Derived from pBB1, for constitutive expression of *ptsG*	Krause *et al*. [87]
pET2	KanR, *oriVE,c*, *oriV*	Vasicova *et al*. [88]
pET2-PR*ptsS*	Derived from pET2, carrying the ptsS promoter in front of the cat reporter gene	Vasicova *et al*. [88]
pET28	Overexpression vector with T7 promoter	Novagen
pET28-*sugR*	Expression vector for SugR_HIS production in BL21 cells	this work

All oligonucleotides used in this study have been obtained from Eurofins MWG Operon (Ebersberg, Germany) and are listed in table 3.

Table 3: Oligonucletides used in this work - restriction sites are underlined:

oligonucleotide	**sequence (5' to 3')**	**purpose**
16S-sonde-T7-rev	GGTACCGAACCAGTGTGGCACATC	slot-blot
16S-sonde-for	GAATTCGATGCACCGAGTGGAAGT	slot-blot / RT-PCR
ptsG-Sonde-T7-rev	GGGCCTAATACGACTCACTATAGGGTGGC AGGAAGTAGAAGAC	slot-blot
ptsG-Sonde-for	ATGCATTCTAGATAACGAGGGCA	slot-blot
ptsF-Sonde-rev	GGGCCCTAATACGACTCACTATAGGGACG CTCGCGGTCTTTAACTC	slot-blot
ptsF-Sonde-for	GCGATGCCAACTTGGTGTTC	slot-blot
ptsS-Sonde-T7-rev	GGGCCCTAATACGACTCACTATAGGGAC TGGCAAGAACGCGAACG	slot-blot
ptsS-Sonde-for	ACATTGGCGGCGAAGACAAC	slot-blot / RT-PCR

RT_ptsS_f_rev	CGACGATGATCTGGAACATACC	RT-PCR
RT_16S_rev	TTCACAGACGACGCGACAA	RT-PCR
BS-*ptsS*_for	AAGGTCCTCTAGCGTGCCGTTATTG	*ptsS* probe EMSA
BS-*ptsS*_rev	TTGTCTTCGCCGCCAATGTC	*ptsS* probe EMSA
BS-*ptsG*_for	GACAGTGAACCCTCCCAAAG	*ptsG* probe EMSA
BS-*ptsG*_rev	TCATGATAAGCCGGAAACCC	*ptsG* probe EMSA
BS-*cg2228*_for	GTTCGCTACGTCCGAGTGATCACC	*control* probe EMSA
BS-*cg2228*_rev	GGGATTCCCTAGTGCATAAG	*control* probe EMSA
sugR_BamHI_for	CGGGATCCATGTACGCAGAGGAGCG	PCR-sugR
sugR_EcoRI_rev	GCGAATTCTCATTCTGCAATCACAAC	PCR-sugR
ptsG_chk_for	ACACATTAAGTGGTAGGCGCTGAGG	Δ*ptsG* verification
ptsG_chk_rev	AAGGTCCTCTAGCGTGCCGTTATTG	Δ*ptsG* verification
ptsG_for_kpnI	GGTACCGGGTGGGTTTCCGGCTTATC	PCR-*ptsG*_His
ptsG_rev__His_sacI	GAGCTCTTAGTGGTGATGGTGATGATGTCC ACCTCCCTCGTTCTTGCCGTTGACC	PCR-*ptsG*_His
ptsH_for_kpnI	GGTACCGATTAACGGCGTAGCAACAC	PCR *ptsH*_FLAG
ptsH_rev_Flag	GAGCTCTTACTTATCGTCGTCATCCTTGTAGTCTCCACCTCCCT CAGCGTCAAGGTCCTGTG	PCR *ptsH*_FLAG
ptsI_for_Strep_SacI	GTCGACAGGAGAGTATCTATGTGGAGCCACCCGCAGTTCGA AAAGGGAAGCGGAGCTACTGTGGCTGATGTG	PCR Strep*_ptsI*
ptsI_rev_BamHI	GGATCCGAGCACGTGGTCATCAAATC	PCR Strep*_ptsI*
ptsS_for_Rho_SacI	GTCGACGTGATCGCGGACGATAATAC	PCR-*ptsS*_Rho
ptsS_rev_Rho_SalI	GGGGAGCTCTCATTAAGCTGGCGCCACCTGGGAAGTCTCGG TGCCGGAGGAGCCTGGTGTTGCTGGCACCGCTT	PCR-*ptsS*_Rho
ptsS_del_chkA	AGCCCTGATGGTGATGGTT	Δ*ptsS* verification
ptsS_del_chkB	GCGCTCTCGGAATACTCAAG	Δ*ptsS* verification
ptsS_del_A_for	GGATCCCGCAACCACGCGATCTATCA	deletion of *ptsS*
ptsS_del_A_rev	CCAACTTCCATACTCCATTCCGTTGTCTTCGCCGCCAATGT	deletion of *ptsS*
ptsS_del_B_for	GGAATGGAGTATGGAAGTTGGAGCGTCAGCGATGCCATGTT	deletion of *ptsS*
ptsS_del_B_rev	TCTAGACCGAGGCATGACCTGGTTGA	deletion of *ptsS*
uhpT_for_BamHI	GGATCCAGGAGAGTATCTAGTAACCCATGCTGGCTTTC	PCR *uhpT*
uhpT_rev_EcoRI	GAATTCTTCACTACGCTGGAAGTCAC	PCR *uhpT*
lpdA_for	AACCGCTTACCAGTGGTTTC	PCR *lpdA*
lpdA_rev	CAGGAGTGCACAAAGCAATC	PCR *lpdA*
lpdA_del_A_EcoRI	GAATTCATCTACAATCCGGGTGTTCC	deletion of *lpdA*
lpdA_del_B	CCAACTTCCATACTCCATTCCACCGCCGATAATTACGATCC	deletion of *lpdA*
lpdA_del_C	GGAATGGAGTATGGAAGTTGGCTGTTCTGCCGCCGTAACTC	deletion of *lpdA*
lpdA_del_D_XbaI	TCTAGATGACCTGCACGACTTCAAGC	deletion of *lpdA*
lpdA_chk_for	GACATGCGTCCGCTTATCTC	Δ*lpdA* verification
lpdA_chk_rev	GTCACTGCGTCCTAGTATCG	Δ*lpdA* verification

2.2. Media and cultivation

For growth experiments *E. coli* and *C. glutamicum* cells and their derivatives were pre-grown over night aerobically in LB, BHI or TY complex medium at 37 °C and 30 °C, respectively. For the main-culture the cells of

the pre-culture were washed twice with 0.9% (w/v) NaCl before inoculating at the desired starting optical density (OD_{600}). The main main-cultures were further cultivated under aerobic conditions on a rotary shaker as 50 ml cultures in 500 ml baffled Erlenmeyer flasks at 125 rpm in complex or CgC minimal medium, supplemented with the respective carbon source and trace salts. The growth of the bacterial cultures was monitored photometrically at 600 nm (OD_{600}).
Plasmid-carrying strains were selectively incubated with appropriate antibiotics (50 µg/ml kanamycin, 6 µg/ml chloramphenicol, and 100 µg/ml carbenicillin)

LB-Medium:
(Sambrook & Russel, 2001 [89]):

10g/l	Bacto-Trypton
5g/l	Bacto-Yeast Extract
10g/l	NaCl

TY-medium:

15g/l	Bacto-Trypton
10g/l	Bacto-Yeast Extract
5g/l	NaCl

Minimalmedium CgC:
(Eikmanns *et al.*, [90])

5 g/l	Amoniumsulfat
5 g/l	Harnstoff
21 g/l	MOPS
1 g/l	Dikaliumhydrogensulfat
1 g/l	Kaliumhydrogensulfat
0.25 g/l	Magnesiumsulfate
10 mg/l	Calciumchloride
0.2g/l	Biotin
1ml/l	trace salts stock solution

Trace salts stock solution:

16.4 g/l	$FeSO_4 \times 7H_2O$
10 g/l	$MnSO_4 \times H_2O$
0.2 g/l	$CuSO_4 \times 5H_2O$
1 g/l	$ZnSO_4 \times 7H_2O$
0.02 g/l	$NiCl_2 \times H_2O$

2.3. Molecular biological methods

2.3.1. Preparation of chemo-competent *E. coli* DH5α cells

The used chemo-competent *E.coli* DH5α cells were prepared according to the method described previously by Inoue *et al.*[91]. For that purpose, 20 ml of LB medium were inoculated with a colony of the desired strain and incubated shaking overnight at 37 °C. The next day 250 ml SOB medium were inoculated to an OD_{600} of 0.05 and further incubated at room temperature until an optical density of 0.2 - 0.3 was reached. The cells were then centrifuged (2000 x g, 4 °C, 10 min) and the pellet was resuspended in 80 ml ice-cold TB buffer. After 10 min incubation on ice, the cells were centrifuged again and the pellet was taken in 20 ml of ice-cold TB buffer. Finally, DMSO (7%) was added drop-wise to the buffer, the cell suspension was aliquoted, frozen in liquid nitrogen and stored at -80 °C.

TB-Puffer:

PIPES (pH 6.7)	10 mM
KCl	250 mM
CaCl2	15 mM
MnCl2	55 mM
Sterile filtered	

SOB-Medium (pH 7.0):

Trypton	20 g/L
Hefeextrakt	5 g/L
NaCl	10 mM
KCl	2.5 mM
$MgSO_4$ / $MgCl_2$	10mM each

2.3.2. Transformation of *E. coli* DH5α cells

The transformation of chemo-competent *Esherichia coli* DH5α cells was carried out through heath shock. The cells were thawed on ice and after the addition of 1-5 µl of the chosen plasmid further incubated for 30 min on ice. Subsequently, the cells were given a heat shock for 45 sec. at 42 °C and after the immediate addition of 800 µl pre-warmed LB-medium let to regenerate for 1 hour at 37 °C. At the end the cells were plated with the help of sterile glas perls on LB complex medium agar-plates containing the respective antibiotic for selection and grown at 37 °C.

2.3.3. Preparation of chemo-competent *C. glutamicum* cells

Competent *C. glutamicum* cells were prepared as described by Liebl *et al*.[92] with small modifications. For the pre-culture we used 50 ml of BHIS complex medium (BHI medium containing 0.5 M sorbitol) in a 500 ml baffled Erlenmeyer flask, which was incubated over-night on a rotary shaker at 30 °C and 125 rpm. From that culture a fresh main culture (100 ml BHIS medium in 500 ml baffled Erlenmeyer flask) was inoculated at an OD_{600} of 0.25-0.3. This main culture was incubated further on a rotary shaker at 30 °C and 125 rpm to an OD_{600} of 1.5 to 1.75. After reaching the desired cell density, the cultures were cooled on ice (10 min), transferred in 50 ml Falcon tubes and centrifuged (10 min, 4000 rpm, 4 °C). The cells were then washed three times with 20 ml ice-cold TG buffer (1 mM Tris, 10% (v/v) glycerol, pH 7.5) and subsequently twice with 50 ml ice-cold 10% (v/v) glycerol. Finally, the pellets were resuspended in 1 ml ice-cold 10% (v / v) glycerol, distributed in aliquots of 100 µl, frozen in liquid nitrogen and stored at -80 °C.

2.3.4. Transformation of *C. glutamicum* cells

Competent *C. glutamicum* cells were thawed on ice and transformed by electroporation for 5ms (2.5 kV, 200Ω, 25µF) with a Micro PULSER™ apparatus (Bio-Rad, Munich). Directly after electroporation 0.8 ml sterile BHI complex medium (37 g/L brain hearth infusion; Difco, Detroit, USA) was added to the cells, which were incubated for 6 min at 43 °C in order to prevent the digestion of the foregin DNA. After that heat shock the cells were regenerated for 1 hour at 30 °C on a rotary shaker. Finally, the cells were plated with the help of sterile glass perls on complex medium agar-plates containing the respective antibiotic for selection and incubated overnight at 30 °C.

2.3.5. Plasmid construction and analysis

For the construction of plasmids or for analytical restriction digests the high fidelity restriction enzymes from the company Fermentas (St. Leon-Rot, Germany) were used according to the conditions recommended in the manufacturer protocols. The following electrophoretic separation of the digest products was performed via ethidium bromide-stained 1 % agarose gels in 1x TAE-buffer (0.04 M Tris, 0.5 mM EDTA, pH 7.5 adjusted with acetic acid).

DNA fragments were ligated with T4 DNA ligase as recommended in the producer manual (Fermentas, St. Leon-Rot).The recirculation of plasmids before ligation with inserts was prevented trough a dephosphorylation of the vectors with Antarctic phosphatase (Fermentas, St. Leon-Rot) for 30 min at 37 °C. The inactivation of the phosphatase was performed by heating the sample at 70 °C for 20 min.

2.3.6. Cloning strategies

The method of allelic replacement via heterologous recombination described by Schäfer *et al.*[68] was used for the construction of chromosomal modifications of *C. glutamicum* strains. Plasmids carrying an antibiotic-selection marker and lacking an origin of replication for *C. glutamicum* were extended at their cloning sites with a respective fragment (~500 bp) complementary to the chosen gene, which should be modulated. For the generation of insertion mutants the fragment sequence was chosen to be corresponding to a middle part of the targeted gene in order to limit the possibility of truncated but still active proteins resulting from the modified gene. The sequences for the respective His- and FLAG-tags could be seen in table 3 and are also reviewed by Arnau *et al.*[93]

2.3.7. DNA amplification via polymerase chain reaction (PCR)

For the amplification of the different DNA-fragments used during our studies we used the proofreading Pfu-polymerase (Fermentas, St. Leon-Rot) for whole genes and the Taq-polymerase (New England Biolabs, Schwalbach) or the EconoTaq® Plus Green Master Mix (BioCat, Heidelberg) for analytical DNA amplifications such as colony PCR tests.

2.3.8. DNA purification

The isolation of plasmids from cell cultures or of DNA fragments after PCR or gel electrophoresis was performed with the Nucleo-Spin Plasmid Quick Pure or Nucleo-Spin Extract II kits of the company Macherey und Nagel (Düren). All steps were performed as recommended in the applied protocols.

2.3.9. Isolation of genomic DNA from *C. glutamicum*

10 ml of culture were harvested and resuspended in 1 ml of TE buffer (10 mM Tris, 1 mM EDTA pH 8.0 with HCl). After the addition of 70 µl lysozyme (200 mg / ml) and 100 µl RNase A (2 mg / ml) the cells were incubated for 2 h at 37 ° C. After further addition of 200 µl 10% SDS, 150 µl proteinase K (20 mg / ml) and 3 ml of TE buffer, the cells were incubated at 37 °C for at least 3 h. 2 ml of NaCl (6 M) was then added to the suspension. After centrifugation (35 min, 4000 rpm, RT, Centrifuge 5810 R, Eppendorf, Hamburg, Germany), the supernatant was mixed with 2.5 times volume of ethanol. The precipitate was washed with 70% ethanol and resuspended in 1 ml dd water. To remove the remaining proteins phenol-chloroform purification (Roti phenol / chloroform, Roth, Karlsruhe, Germany) was performed. The solution was washed in 1ml phenol:chloroform:isoamyl alcohol (25:24:1), the upper aqueous phase was washed again with 0.8 ml chloroform and the supernatant was resuspended in 2.5x volumes of ethanol. After 30 min incubation at – 20 °C the solution and centrifugation for 20 min at 4000 rpm the pellet was washed with 70% ethanol and the DNA was finally dissolved in 250 µl dd water and stored at – 20 °C.

2.3.10. Electro mobility shift assay (EMSA) analysis of SugR binding to DNA

Gel shift assays with SugR were performed as described previously[58] with slight modifications. Various concentrations of purified SugR were mixed with promoter DNA of *ptsS*, *ptsG* or *pyk* in Binding Buffer (50 mM Tris-HCl; 10% [v/v] glycerol; 50 mM KCl; 10 mM MgCl2; 1 mM EDTA; 2.5 mM CaCl; 50 mM NaCl; 2 mM DTT; pH 7.5). The required for the EMSA DNA fragments were generated by PCR and purified after gel extraction. A non-target promoter fragment of the coding sequence of *cg2228* [52] was added as a negative control. To test for possible effectors, the protein was incubated with glucose-6- phosphate, fructose-6-P, sucrose-6-P, fructose-1,6-BP or fructose-1-P (2 mM or 20 mM each) in the binding buffer for 15 min before addition of the protein. Each after addition of SugR each sample was incubated for 30 min at 30 °C. Finally, DNA loading dye was added and the samples were analyzed via 2% agarose gels in TA buffer (40mM Tris, 20mM acetic acid).

2.3.11. Sequencing

2.3.10.1. Whole genome re-sequencing

For the genome re-sequencing of the generated suppressor mutants a chromosomal DNA purified to $OD_{260/280}$ > 1.8 and $OD_{260/230}$ > 1.9 was used. The raw data for the whole genome sequencing of the generated suppressor mutants was performed via 50 bp paired end sequencing on a Genome Sequencer Illumina HiSeq2000 by GATC-Biotech (Konstanz, Germany). The quality evaluation of the obtained raw-data was performed with the software FastQC and Solexa. The mapping was performed with the BowTie and BWA programmes, allowing a higher or lower SNP/InDel sensitivity, respectively. The following analysis and search for SNP's and InDels was performed via the software Samtools, Vcftools, Picard, Tablet and partially GATK.

2.3.11.2. Plasmids and PCR products

The plasmids and chromosomal modifications created in this work were eventually verified via plasmid or PCR-product sequencing performed by GATC-Biotech (Konstanz, Germany) according the Sanger chain-termination method. The chromatograms were analysed with the programme Chromas (Version, 1.45, Southport, Queensland, Australia) and the results were later transmitted to sequences via SE-Central Clone Manager5 software.

2.3.12. RNA-analysis

RNA hybridization experiments were performed similarly as described in Möker *et al*.[94]. To avoid RNase contamination, the used equipment and solutions were, if possible, autoclaved for 40 min at 121 ° C to eliminate RNase contamination. DNA contamination was tested via PCR.

2.3.12.1. Total-RNA isolation from *C. glutamicum*

For the RNA-analysis *C. glutamicum* cells were harvested in the exponential growth phase (OD_{600} 6-7) from the ongoing growth experiments. The samples were centrifuged (30 sec, 13000 rpm) and the pellets were immediately frozen in liquid nitrogen in order to avoid shock reactions and associated changes in RNA composition. The samples were then stored until usage at -80 °C.

For cell disruption and RNA isolation, the samples were thawed on ice and resuspended in a mixture of 350µl RA1 buffer (NucleoSpin RNA II kit; Macherey-Nagel, Düren) and 3.5µl mercaptoethanol. The resuspended samples were then transferred into 2-ml screw cap tubes with 250µg glass beads and mechanically disrupted (3 x 30s, 6.5 rps) in a Ribolyser apparatus (Thermo Hybaid GmbH, Garching). After subsequent centrifugation (1 min, 13000 rpm) 300µl of the supernatant were mixed in fresh reaction tubes with 300µl 70% ethanol by vortexing and transferred into a NucleoSpin RNA II purification column. From that point the further RNA purification steps were performed according to the manufacturer's manual.

2.3.12.2. Measurement of the RNA-concentration

The quantification of RNA concentration of the isolated RNA was determined photometrically in quartz cuvettes with a layer thickness of 1 cm in Ullrospec 2000 spectrophotometer (GE Healthcare) at a wavelength of 260 nm.

2.3.12.3. Preparation of DIG-labelled RNA-probes

For the specific detection of specific transcripts in the total RNA preparations digoxigenin (DIG) labelled antisense RNA probes 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.

Transcription mix:
14 µl PCR product
2 µl 10x NEB buffer for RNA-polymerases
2 µl DIG RNA Labeling Mix (Roche Diagnostics Gmbh, Mannheim)
2 µl T7-polymerase (New Engalnd Biolabs, Schwalbach)

The reaction mix was incubated for 2 hours at 37 °C and then, after addition of 1 µl RNase-free DNase for the removal of unwanted DNA templates, for further 25min at the same temperature. The probes were then stored until use at -80 °C.

2.3.12.4. RNA-Analysis: Northern blot

The analysis of the RNA samples was performed by Northern blot hybridisation in the form of slot blots. A total RNA amount of 2 µg per slot was applied. Diluted in 10x SSC with bromophenol blue (1 µl bromophenol blue solution to 10 ml 10 X SSC) the RNA samples were applied to an equilibrated in water nylon membrane (Biobond ™ Nylon membrane, Sigma) using a slot-blot apparatus (S & S Minifold I, Schleicher & Schuell, Dassel) under a gentle pump generated pressure of 20 mbar. For an increased efficiency a same sized Whatman paper (Carl Roth GmbH, Karlsruhe) equilibrated in 10x SSC was added under the nylon membrane. After the samples were applied to the membrane, the RNA was fixed by UV irradiation (125 mJ/cm^2) in a cross-linker (Bio-Link, LTF-Labortechnik, Wasserburg).

For the hybridisation the samples were grouped in hybridisation tubes according to the DIG-labelled antisense RNA probes they should be detected with. The tubes were then blocked for 1 h at 50 °C in 15 ml hybridisation solution. Then the corresponding DIG-labeled RNA probes were added and incubated at 65 °C overnight.
Subsequently the blots were washed twice in wash solution 1 at room temperature for 15 min and twice in wash solution 2 at 65 °C for 25 min. After a brief washing in washing buffer (maleic acid buffer with 0.3% (v/v) Tween 20) the membranes were blocked for 30 min at room temperature (rolling) in 20 ml of 1x blocking reagent (Roche Diagnostics GmbH, Mannheim). Thereupon, anti-DIG-alkaline phosphatase conjugate (Roche Diagnostics GmbH, Mannheim) was added according to manufacturer's protocol and incubated for further 30 min at room temperature. For the detection of the RNA the membranes were washed three times in washing buffer for 20 min, equilibrated briefly for 5 min in detection buffer and incubated with diluted 1:100 in detection buffer CSPD reagent (Roche Diagnostics GmbH, Mannheim, Germany) in darkness for 15 min at 37 °C. The chemiluminescence of the CSPD reagent, emitted during its reaction with the anti-DIG-alkaline phosphatase conjugate bound to the DIG-labelled RNA probes, was detected with the CCD camera of the LAS 1000 CH system (Fuji, Sraubenhardt).

Hybridisation solution:	**20 X SSC:**	**Maleic acid buffer**
Formamid, 20x SSC 10 X Blocking-reagent 10% (w/v) Na-Lauroylsarkosinat 10 % (w/v) SDS	3 M NaCl 0,3 M tri-Natriumcitrat pH (HCl) = 7,0	0,1 M Maleinsäure 0,15 M NaCl, pH (NaOH) = 7,5

Detection buffer:	**Washing solution 1:**	**Washing solution 2**
0,1 M Tris 0,1 M NaCl pH (NaOH) = 9,0	2 x SSC 0,1% SDS	0,2 X SSC 0,1% SDS

2.3.12.5. Real-time PCR analysis:
For the real time PCR 1 µg of the isolated mRNA samples were converted to cDNA with the ReveseAid Kit (Fermentas) according to producers protocols. Pooled cDNA samples were used as standards or respectively as calibration curve. Accordingly diluted samples and standards were run in triplicates on a 96 well PCR plate using a Bio-Rad CFX real-time cycler. The reaction was performed in a final volume of 20 µl, containing the appropriate concentrations cDNA, gene-specific primers and KAPA SYBR FAST Master Mix Universal (Peqlab, Germany). The following thermal programme was applied: a single cycle of DNA polymerase activation for 10 min at 95 °C followed by 40 amplification cycles of 15 s at 95 °C (denaturing step) and 15 sec at 58 °C followed by and 15 sec at 72 °C (annealing & extension step). Subsequently, melting temperature analysis of the amplification products was performed by gradually increasing the temperature from 50 to 95 °C in steps of 0.5 °C per 5 sec. The melting curves of the samples were controlled for secondary peaks in the –d(RFU)/dT chart. Calculation of the threshold cycle values (C_T) for every sample was performed using analysed using the CFX Manager Software.

2.4. Protein biochemical and analytical methods

2.4.1. Protein analysis via polyacrylamide gel electrophoresis (PAGE)

All performed electrophoretic protein separations were performed with 12%- or 15%-polyacrylamide gels. The samples were diluted if needed in order to achieve equal protein amounts, mixed with gel loading dye and for the denaturating conditions boiled for 5 min at 95 °C.

Gel loading dye (4x):	
250 mM	Tris-HCl, pH 6,8
8 % [w/v]	SDS (absent for native gels)
40 %	glycerol
0,2 M	Dithiothreitol (DTT)
0,04 g	bromphenol blue

Electrophoresis buffer (10x):	
1.92 M	glycine
0.25 M	Tris
1 % [w/v]	SDS
in aqua dest.	

The analysis of the HPr phosphorylation state was performed similarly as described previously with small modifications[95]. The separation of proteins was performed in 15% SDS-gels as described elsewhere for the analysis of EIIA phosphorylation state[96]. Samples were prepared as described below (2.4.5). Samples were not boiled (unless when used as a control), were kept on ice and the gel running was performed at 4 °C, in order to prevent heat dephosphorylation of the samples.

2.4.2. Western blot analysis of tagged proteins

For the transfer of the separated proteins from the SDS-gel we prepared a PVDF membrane and Whatman papers, corresponding to the size of our gel. The membrane was made hydrophilic by short dipping in methanol and then both Whatman papers and the membrane were wetted with Blotting buffer (0.01 mM CAPS, 10% [v/v] methanol), respectively.

The transfer was performed for 90 min at 0.8 mA per cm^2 of gel. After the transfer the membrane was placed for 1 hour into a blocking solution (50 mM Tris, 0.9% NaCl with 5% non-fat milk, pH 7.4) at room temperature as a preparation for the later protein detections with specific antibodies. The detection of the bands was performed with freshly mixed NBT+BCIP staining solution.

2.4.3. Preparation of crude cell extracts

For the crude cell extracts preparation of the studied *E. coli* or *C. glutamicum* strains cells were harvested after at least 4 hours of incubation in liquid medium at the according optimal temperature. After being washed three times in 0.9% (w/v) saline and once in extract buffer (25 mM MES, 10 mM $MgCl_2$, pH 5.4) the pellets were resuspended in 2 ml extract buffer, transferred into 2-ml screw cap tubes with 250µg glass beads and mechanically disrupted (4 x 30 sec, 6.5 m/sec) in a Ribolyser apparatus (Thermo Hybaid GmbH, Garching). In order to prevent overheating of the samples between each of the four cycles in the Ribolyser the samples were cooled down on ice for 5 min. Finally, the samples were centrifuged (4 °C, 14000 rpm, 20 min) the supernatant crude cell extract was collected in a new tube and stored on ice until its appliance in the following enzymatic measurements. For the analysis of the EII^{glc}-His membrane proteins we used TBS buffer (50 mM Tris, 0.9% NaCl, ph 7.4) and the extracts were additionally ultra-centrifuged for 30 min at 80000 rpm.

2.4.4. Determination of protein concentration

The protein concentrations of the crude cell extracts were determined according to Bradford[97]. Therefore the Roti-NanoQuant reagent (Carl Roth GmbH, Karlsruhe) was used according to the manufacturer's recommendations. Bovine serum albumin (BSA) was used for calibration.

The concentration of membrane integrated proteins was measured by amido black staining according to Schaffner and Weissmann[98] with BSA solutions of different concentration as reference.

2.4.5. Sample preparation for quantification of intracellular glucose-6-P concentrations

For the measurement of intracellular glucose-6-P cencentrations the cells were separated from their suspending medium by silicone oil centrifugation. Eppendorf tubes containing 90 µl perchloric acid 20% (v/v), covered with 200 µl silicone oil (d = 10.4, AR 200, Wacker, Miinchen) and 600 µl of cell culture were centrifuged for 6 sec (Microfuge E, Beckman). The supernatant was separated and the tubes were cut through the silicone oil layer, 135 µl water and 100 µl silicone oil (d = 1.07) were added and the sediments were resuspended and placed in an ultrasonic bath for 10 min. The samples were neutralized with 5 M KOH / 1M triethanolamine and

incubated for 30 min at 4 °C. Finally, the samples were centrifuged for 20 min at 4 °C and the aqueous phase was collected.

2.4.6. Enzymatic quantification of glucose-6-P

The concentration of intracellular glucose-6-P was determined enzymatically. The assay contained 2mM NADP and cell extract in a final volume of 240 µl, adjusted with G6Buffer (0.15 m TEA, 10 mM $MgSO_4$, 0.1 M KCl). The mearuremetn was started with the addition of 10 µl glucose-6-P dehydrogenase (0.1 U). The absorbance of the samples was measured photometrically in Ultrospec 2000 spectrophotometer (GE Healthcare) at a wavelength of 340 nm at constant temperature of 37°C.

For the measurement of the glucsose-6-P consumption when glucose-6-P was used as a carbon source, the same method was used, however, supernatants instead of cell extracts were measured.

2.4.7. Chloramphenicol acetyl-CoA transferase (CAT) assay

The chloramphenicol acetyl-CoA transferase assay was used to determine the promoter strength of the *ptsS* gene in various strains. The *ptsS*-promoter was placed in the vector pET2 in front of the *cat* gene encoding the chloramphenicol acetyl-CoA transferase. The enzyme catalyses the transfer of the acetyl group from acetyl-CoA to chloramphenicol. The free CoA-group converts the reagent 5,5-dithiobis (2-nitrobenzoic acid) (DTNB) to yellowish 2-nitro-5-thiobenzoeic acid (TNB) which can be quantified at 412 nm (ε=13.6 $mM^{-1}*cm^{-1}$). The amount of hydrolyzed acetyl-CoA is proportional to the generated TNB. The assay contained 195 µl DTNB [0.4 g/l dissolved in 50 mM Tris-HCl (pH=7.6)] and crude extract in a final volume of 250 µl, adjusted with 50 mM Tris-HCl puffer. Absorption was measured spectrophotometrically and the reaction was started after addition of 15 µl 5 mM acetyl-CoA. The reaction was performed at 37 °C.

2.4.8. Phosphoglucoisomerase (Pgi) activity assay

The generated pgi derivative strains and suppressor mutants were controlled also by Pgi activity measurements. The Pgi activity was measured spectrophotometrically in a coupled assay, following NADPH formation at 340 nm at 30°C in final volume of 1 ml. The assay contained 50 mM Tris (pH 7.2), 0.02 M $MgCl_2$, cell extract, 0.5mM NADP, 1mM fructose 6-phosphate, and 1 U glucose6-P dehydrogenase.

2.4.9. Metabolite analysis via TLC

Thin-layer-chromatography (TLC) was applied for the qualitative analysis of carbohydrates in culture supernatants and of intracellular carbohydrates. ADAMANT silica gel 60 TLC plates (20 × 20 cm, Carl Roth, Karlsruhe) were used as stationary phase. If necessary, samples were concentrated by evaporation. For the analysis of intracellular carbohydrates, cells were separated from the medium by filtration through a glass fibre filter and two washing steps of 3ml minimal medium. The intracellular metabolites were obtained by incubation of the filters in 70% isopropanol and following evaporation of the isopropanol and exchange against water. Finally, the samples were analysed via TLC.

TLC plates were run up to four times in pyridine/1-butanol/H2O (30/70/10 (v/v/v)). Non-labelled carbohydrates were detected by spraying with 4% (v/v) H_2SO_4 in methanol followed by heating to 110°C. ^{14}C-labelled carbohydrates were analyzed in a BAS-1800 imager (Fujifilm, Japan) via BAS-MP imager plates (Fujifilm, Japan) after over-night exposure.

2.4.10. Overproduction and purification of the SugR protein:

For the in vitro analysis of SugR a heterologous *sugR* expression in *E. coli* BL21 was carried out in shaker flask cultures. A 25ml pre-culture was inoculated in LB medium with the appropriate antibiotic and incubated overday at 37°C and 110 rpm. From those cultures a main culture was inoculated at OD_{600} of 0.2 in LB medium with the respective antibiotic. After the cells reached an OD_{600} of 1 the expression of the SugR protein was induced by addition of 0.1 mM IPTG. Expression was then carried out by further cultivation of the cells for 5 h. These cultures were harvested by centrifugation 4500 rpm at 4°C (Avanti Centrifuge J25, Beckman,Rotor JLA 10.500). After washing with cold TBS buffer (20mM Tris/HCl, 0.3 M NaCl, pH 7.8) the cells were frozen at -20°C.

For the purification of SugR the cell pellets were resuspended on ice in Buffer A (50 mM Tris pH 7.5; 500 mM NaCl;, 5 mM Imidazol 0.5 mM DTT) and cell extracts were prepared with the aid of Frech pressure cell (SLM Amino, Spectronic Instruments, Rochester, NY). After each passage through the press cell the cell extracts were cooled down on ice for 5 min. Finally, the cell debris was removed via centrifugation for 20 min at 4000 rpm and 4 °C followed by 1h at 80000 rpm and 4 °C.
The SugR protein carrying a metal affinity tag composed of repetitive Histidin (NH2-HHHHHHHQHH-COOH) sequence was purified from the cell extracts by Nickel chelate affinity chromatography on Ni-IDA-sepharose (HiTrap) chelate column 1 ml/5 ml (GE Healthcare, Freiburg) by an AEKTA FPLC system(GE-Healthcare, Freiburg). After loading diluted extracts the contaminating proteins were washed away by washing the column with Buffer A and subsequently eluted by increasing the percentage of Buffer B (50 mM Tris pH 7.5; 500 mM NaCl, 500 mM Imidazol 0.5 mM DTT) to 100%. Fractions with the highest protein content were pooled, and the elution buffer was exchanged against BS buffer (100 mM Tris-HCl, 30% [v/v] glycerol, 100 mM KCl, 20 mM MgCl2, 1 mM EDTA, pH 7.5) using PD10 columns. The resulting protein pools were aliquoted and stored at -20°C until usage.

2.4.11. Radiochemical uptake measurements

The determination of uptake rates was performed with ^{14}C (U) radioactively labelled glucose, fructose (Moravek Biochemicals, Brea, USA), maltose, glucose-6-P or sucrose (American Radiolabeled Chemicals Inc, Saint Louis, USA). The radioactive compounds were added in identical proportions to defined concentrations of equivalent unlabelled substrate, so that the final concentration of unlabelled sugars for each single measurement was corresponding to a defined total amount of the radio-labelled sugar.
The cells used for the measurement were taken from exponentially growing in liquid medium cultures at an approximate OD_{600} = 7 and washed three times with at least 10 ml CgC minimal medium at 4 °C. Finally, the cells were resuspended in cold CgC medium to the desired optical density (for most measurements OD_{600} = 1.8).
Before the start of the uptake measurement through the addition of a substrate the cells were pre-tempered for 3 min at 30 °C in a water bath. The total reaction volume per single measurement was 2.01 ml. At given time intervals (resolution of 15 sec) after addition of the radio-labelled substrate, 200 µl samples were filtered through glass fibre filters (Type F; Millipore, Eschborn) and washed twice with 2.5 ml of 0.1 M LiCl. The radioactivity of the sample was determined using scintillation fluid (Rotiszinth; Roth) and a scintillation counter (LS 6500; Beckmann, Krefeld).
If not indicated otherwise, the substances tested in the rapid uptake inhibition studies of the respective transport processes were added to the setup 45 or 60 sec after the measurement was started and were in 10-fold excess compared to the concentration of the labelled substrate.
For the exclusion of transcriptional and translational processes the cells were treated before measurement with rifampicin or chloramphenicol, as described elsewhere [99, 100].

3. Results

3.1. Temporal development of the regulatory processes

Deletion of *pgi* in *C. glutamicum* blocks the first step of glycolysis and redirects the glucose-derived carbon flux towards the PP pathway. So far it has been shown that the addition of glucose to sucrose cultivated *C. glutamicum* Δ*pgi* cells leads to heavily impaired growth by causing *ptsS*-repression and sucrose uptake inhibition[83]. The temporal development and dependences between these two processes, however, remained unclear. The initial model assumed that the presence of glucose activates the transcriptional repressor SugR, which reduces the *ptsS*-expression and as a consequence inhibits the uptake of sucrose. However, even though the deletion of *sugR* in the *C. glutamicum* Δ*pgi* Δ*sugR* double mutant derepressed *ptsS* and improved the long term growth of the cells cultivated with sucrose plus glucose, it still did not recover the growth rate to wild-type levels (Fig. 4, A). Additionally, in those cells the sucrose uptake was still impaired, indicating that the lack of *ptsS*-mRNA might not be the cause of uptake inhibition as originally assumed [83].

To clarify the relations between the *ptsS*-repression and sucrose uptake inhibition after glucose addition in *C. glutamicum* Δ*pgi* the dynamics of the two processes for a time interval of 240 min after glucose addition were compared. Unlike in the wild-type or *C. glutamicum* Δ*pgi* Δ*sugR* strain, in the Δ*pgi* mutant the *ptsS*-mRNA levels were significantly reduced after 1h of incubation with sucrose plus glucose and remained at low levels. However, inside the first 26 min of incubation none of the three strains showed signs of *ptsS*-repression (Fig. 4, B). In contrast, the sucrose uptake rate in both *C. glutamicum* Δ*pgi* and *C. glutamicum* Δ*pgi* Δ*sugR* was drastically decreased already 1 min after glucose addition. The initial rates of 19.2 ± 0.9 and 19.0 ± 1.3 dropped to 4.4 ± 0.6 and 6.5 ± 0.8 nmol/(min*mg dw), respectively, and remained at similar low levels of less than 3.5 nmol/(min*mg dw) (Fig. 4, C).

In comparison to the initial model, these results demonstrate a reversed order of the two main processes of the regulatory mechanism determining the *C. glutamicum* Δ*pgi* growth inhibition after glucose addition: (i) at the beginning glucose causes a fast initial inhibition of the sucrose uptake, which is then followed by (ii) a SugR-mediated *ptsS*-repression, strengthening the sucrose uptake inhibition by reducing the *de novo* synthesis of EII^{suc}. This

new constellation also shifted the central focus for the understanding of the regulatory phenomena on the unknown mechanism of the initial sucrose uptake inhibition, which now could no longer be explained with the *ptsS*-repression by SugR and was potentially initiating step for the following processes. The uptake inhibition and *ptsS*-repression still might be coupled but the connections between them had to be clarified from a new perspective.

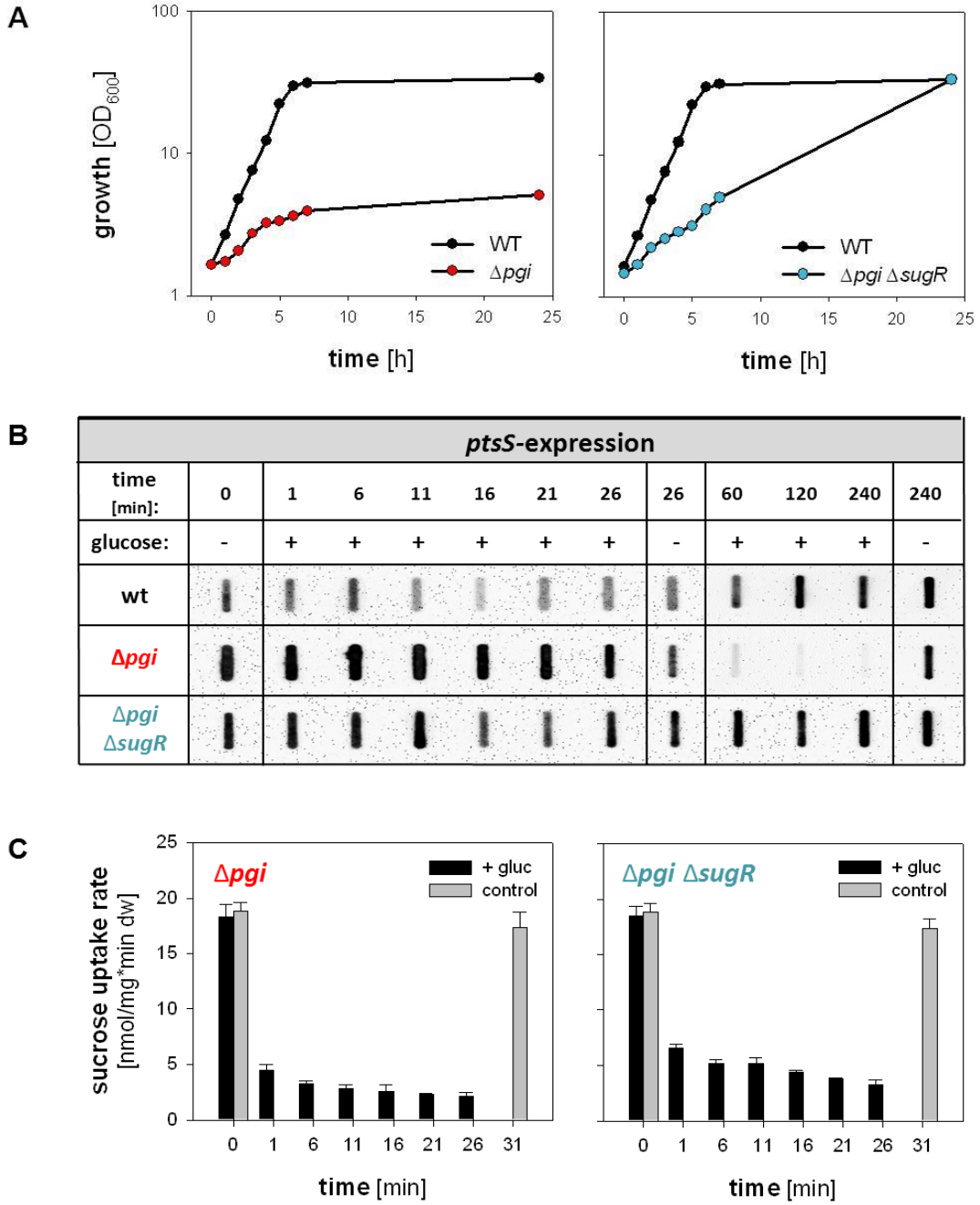


Figure 4: Temporal development of the sucrose uptake and *ptsS*-repression after glucose addition. A: Growth of *C. glutamicum* (wt; μ= 0.42 h^{-1}), *C. glutamicum* Δ*pgi* (μ= 0.10 h^{-1}) and *C. glutamicum* Δ*pgi* Δ*sugR* (μ= 0.21 h^{-1}) cells cultivated with sucrose plus glucose. **B:** Dot blot analysis of the *ptsS*-mRNA of the three strains cultivated with sucrose after or without addition of glucose measured inside 240 min. **C:** Sucrose uptake rates of the Δ*pgi* and Δ*pgi* Δ*sugR* mutants after or without glucose addition. As a control no glucose was added.

3.2. Step 1: Rapid uptake inhibition

3.2.1. Characterization of the inhibition:

Optimized assay for uptake inhibition studies – The initial studies of the sucrose uptake inhibition in *C. glutamicum* Δ*pgi* after glucose addition demonstrated that this process takes place prior to *ptsS*-repression and thus might play a determining role in the entire regulatory cascade. Furthermore, such a fast PTS response to the addition of a second carbon source has not been described so far for this organism and the mechanistic background was unknown. An unspecific inhibition of the sucrose uptake by glucose was excluded as the addition of glucose had no effect on the uptake of sucrose in EII^{glc}-deficient *C. glutamicum* strains[78]. Hence, in order to understand the phenomena observed after addition of glucose to sucrose cultivated *C. glutamicum* Δ*pgi* cells a new, optimized uptake assay was required. As the main part of uptake reduction had been taking place within 1 min after glucose addition, the new assay focused on the time period of 145 sec with the uptake being measured every 15 sec. The inhibiting substrate is added during the ongoing uptake measurement, so that the inhibiting effects are measured as directly as possible (see M&M). Indeed, using this assay I could show that the addition of glucose to sucrose cultivated *C. glutamicum* Δ*pgi* cells caused a switch of the initial sucrose uptake rate from 19.2 ± 1.3 nmol/(min*mg dw) to a stable rate of 4.5 ± 0.6 within less than 15 sec (Fig. 5).

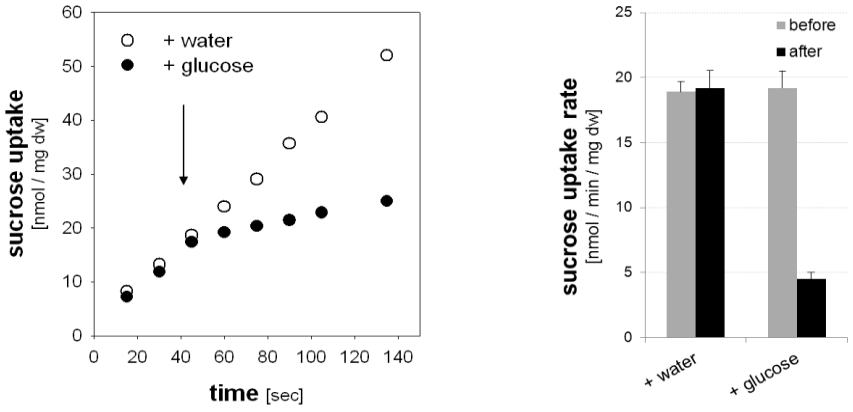


Figure 5: Rapid sucrose uptake inhibition in *C. glutamicum* Δ*pgi:* after 45 sec the tested substances- water as a control and glucose has been added to the sucrose cultivated cells. The left panel displays the uptake rates before and after addition of the substances. Shown are results of at least three independent measurements.

In fact, with the resolution of 15 sec no transitional stage between the inhibited and non-inhibited rates could be detected. Extrapolation of the rates before and after addition of glucose indicated reaction times of even less than 2-3 sec, which could not be measured without automated equipment. Nonetheless, this assay presented a fast and direct method to study the rapid uptake inhibition observed in *C. glutamicum* strains under different conditions and showed that the studied here uptake inhibition is a rapid process.

Role of transcriptional and translational regulation – The high velocity of sucrose uptake inhibition observed in *C. glutamicum* Δ*pgi* cells made the involvement of transcriptional and translational processes unlikely. Additionally, the sucrose uptake inhibition by glucose in the *C. glutamicum* Δ*pgi* Δ*sugR* mutant was also taking place within 15 sec, confirming that SugR is not required for the rapid sucrose uptake inhibition by glucose (SupFig. 1). Nonetheless, in *E. coli*, for example, the sRNA *sgrS* is able to produce a short peptide SgrT, causing a fast and direct inhibition of glucose uptake upon sugar-P stress[74]. To exclude the possibility of *e.g.* SgrT-like mechanism involved in the process, I performed the rapid (145 sec) and fast (30 min) uptake experiments with cells treated with transcriptional (rifampicin) or translational (chloramphenicol) inhibitor. Indeed, the results confirmed that no transcriptional or translational processes are required for the observed rapid sucrose uptake reduction after glucose addition in *C. glutamicum* Δ*pgi* as no differences in the responses between the treated and non-treated cells were observed (Fig. 6, SupFig. 2). Hence, the mechanism of uptake inhibition studied here is a rapid and entirely biochemical – *i. e.* acting directly at the level of protein activity - process.

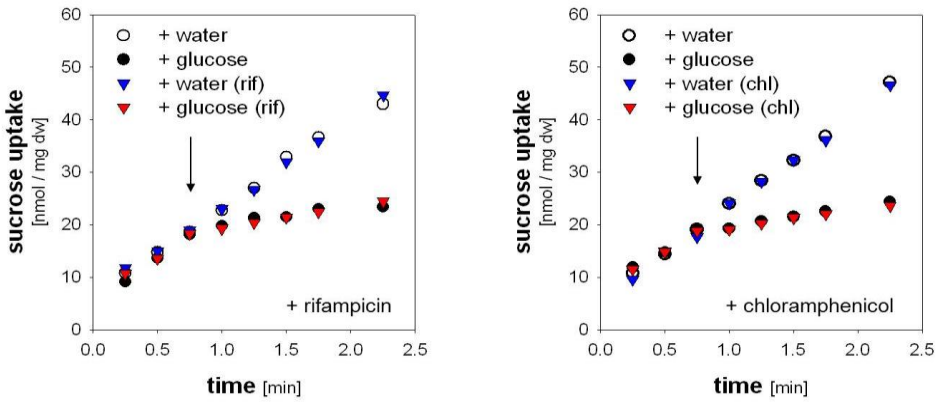


Figure 6: Effect of transcriptional and translational inhibitors on the rapid uptake inhibition in *C. glutamicum* Δ*pgi*: Rapid sucrose uptake inhibition assays performed with sucrose cultivated cells with or without treatment with transcriptional inhibitor rifampicin or translational inhibitor chloramphenicol. Shown are representative results of at least three independent measurements.

Two potential scenarios could explain why glucose causes a drastic, biochemical sucrose uptake inhibition in *C. glutamicum* Δ*pgi* within few seconds:

A: *a PEP-regeneration problem* – The PTS-mediated uptake of glucose or sucrose requires a constant consumption of PEP by the PEP-EI-HPr phosphorylation cascade. Normally this is not a problem as this consumption is compensated by the following metabolisation of glucose or sucrose, which even leads to a net gain for the cell of approximately 1 molecule PEP per transported monosaccharide. However, the PEP-regeneration in *C. glutamicum* Δ*pgi* might be insufficient as deletion of *pgi* blocks glycolysis and limits the glucose-driven carbon flux just to the ineffective and via feedback inhibition limited pentose phosphate pathway. In this case, addition of glucose would activate the EII^{glc} permeases but as the PEP "invested" for the uptake of glucose would not be effectively regenerated in *C. glutamicum* Δ*pgi*, the total PEP-regeneration rate in the mutant would decrease. As a result, the rate of the PTS phosphorylation cascade would start to drop and with it the sucrose uptake rate would become reduced. This process would continue until a new, lower steady state between PEP-consumption and PEP-regeneration is achieved (decreased growth rates) or until complete collapse of the system (growth inhibition).

B: *a sugar-P stress response* – the second possibility why *C. glutamicum* Δ*pgi* might react dramatically to glucose addition was the presence of a sugar-P stress response, which prevents the further accumulation of glucose-6-P by inhibiting the glucose and sucrose uptake in the cell. Complex mechanisms inhibiting the PTS activity or expression of PTS-genes as a response of sugar-P accumulation have been described in other organisms (see Introduction). However, homologues of the players involved in these mechanisms have not been identified for *C. glutamicum* and they also require transcriptional of translational processes, which do not play a role in the studied here regulation. Hence, the potential targets for direct biochemical inhibition of the sucrose uptake due to glucose-6-P accumulation should be different from those known form other bacteria. Of course, the involvement of a sugar-P stress response in the severe growth and uptake inhibition in *C. glutamicum* Δ*pgi* would not exclude also the presence of the described above PEP-regeneration problem. A mixture of two mechanisms was possible.

3.2.2. Trigger of the inhibition

3.2.2.1. Signal initiating the inhibition

Effects of maltose addition on the PTS-mediated uptake in *C. glutamicum* Δ*pgi* – In order to analyze if a PEP-regeneration problem or a sugar-P stress is the reason for the observed regulatory phenomena in *C. glutamicum* Δ*pgi* after addition of glucose it was important to uncouple the EII^{glc} activity from the generation of glucose-6-P after addition of a second substrate. The absence of EII^{glc} abolished completely the growth and rapid sucrose uptake inhibition after glucose addition in *C. glutamicum* Δ*pgi* Δ*ptsG* (Fig. 8, A). This result confirmed that glucose does not act as unspecific inhibitor of the sucrose uptake. However, in this case both the additional PEP-consumption by the EII^{glc} activity as well as the potential accumulation of glucose-6-P was avoided.

Maltose is a disaccharide consisting of α(1-4) linked glucose molecules. Similarly to other organisms, *C. glutamicum* metabolizes maltose by a complex pathway, involving the formation of maltodextrin and glucose, which are both degraded to glucose 6-phosphate via the maltodextrinphosphorylase MalP and the α-phosphoglucomutase Pgm [101], and the ATP- and PP_i-dependent glucose kinases Glk and PPgk[102, 103], respectively. Though, in contrast to glucose, maltose is taken up in this organism by the specific ABC-transporter $MusEFGK_2I$ and not by the PTS [104]. Hence, addition of maltose would lead to glucose-6-P formation without EII^{glc} activation and PEP-consumption via the PTS phosphorylation cascade (Fig. 7).

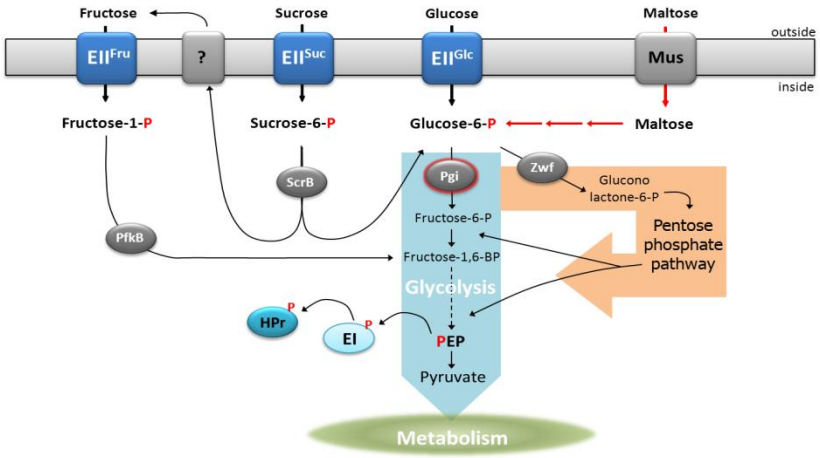


Figure 7: Simplified scheme of the metabolism of maltose, glucose and sucrose in *C. glutamicum*

As shown on figure 8 B, the growth rate of the *C. glutamicum* Δ*pgi* cells cultivated with sucrose plus maltose (0.29 ± 0.01 h^{-1}) was significantly reduced compared to cultivations with sucrose as the sole carbon source (0.42 ± 0.01 h^{-1}) (Fig. 8, A). Furthermore, the growth rate of *C. glutamicum* Δ*pgi* with maltose as a sole carbon source (0.09 ± 0.01 h^{-1}) was also drastically reduced compared to the wild-type (0.41 ± 0.02 h^{-1}) and similar to the growth rate of the *pgi* mutant during cultivation with glucose as the sole carbon source (0.09 ± 0.01 h^{-1}). Nonetheless, the maltose plus sucrose cultivated *C. glutamicum* Δ*pgi* cells were still able to reach wild-type-like final OD_{600} after 24 h.

Similarly as glucose, maltose also caused rapid sucrose uptake inhibition in *C. glutamicum* Δ*pgi* (Fig. 8, B). Within 15 sec after maltose addition the initial sucrose uptake rate of the mutant dropped from 18.1 ± 1.7 nmol/(min*mg dw) to 8.6 ± 0.6 nmol/(min*mg dw). Uptake measurements with radiolabelled maltose confirmed that maltose was taken up in those cells (14.6 ± 0.4 nmol/(min*mg dw)). Interestingly, in the *C. glutamicum* Δ*pgi* cells pre-cultivated with maltose the maltose uptake rates were lower (6.3 ± 0.1 nmol/(min*mg dw) and thus comparable to the glucose uptake rates in the *pgi*-mutant, which was consistent with the similar, reduced growth rates with those substrates.

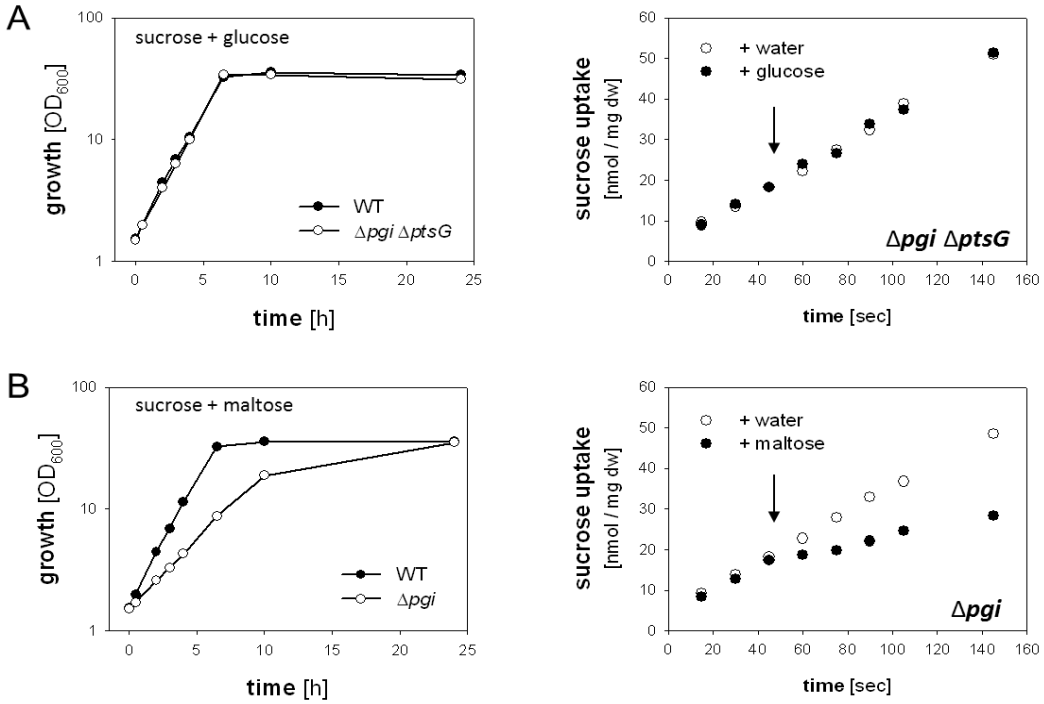


Figure 8: Growth and rapid sucrose uptake inhibition by addition of maltose in *C. glutamicum Δpgi* **(A)** and *C. glutamicum* **(B)** cells. Sucrose and sucrose plus maltose were the substrates used for the growth experiments and the cells used for the uptake inhibition assays were pre-cultivated on sucrose as a sole carbon source. The maltose uptake rate of the used cells was controlled in order to ensure that maltose was taken up by the cells.

Taken together, these results demonstrated that in *C. glutamicum* Δ*pgi* the PTS-mediated sucrose uptake is rapidly reduced by the addition of a second carbon source leading to the intracellular formation of glucose-6-P even when its uptake is not mediated by the PTS. This observation could not be explained with a mechanism of non-compensated PEP consumption and indicated the presence of a sugar-P stress response in *C. glutamicum*.

Enzymatic measurement of intracellular glucose-6-P concentrations – The presence of a sugar-P stress response in other *pgi*-deficient bacteria has been shown to correlate with increased intracellular glucose-6-P concentrations [105]. Indeed, when glucose was present in the medium *C. glutamicum* Δ*pgi* accumulated 4 to 6-fold higher amounts of glucose-6-P compared to the wild-type (Fig. 9, A). The measured concentrations were comparable with previous measurements for *pgi*-deficient *E. coli* strains as well as with the reported intracellular concentrations for the *C. glutamicum* wild-type [105-107].

Further, I showed that the presence of maltose leads to increased glucose-6-P accumulation in *C. glutamicum* Δ*pgi,* as well (Fig. 9, B). This result was consistent with the previous observations that similarly to glucose the addition of maltose also leads to rapid growth and sucrose uptake inhibition in *C. glutamicum* Δ*pgi*.

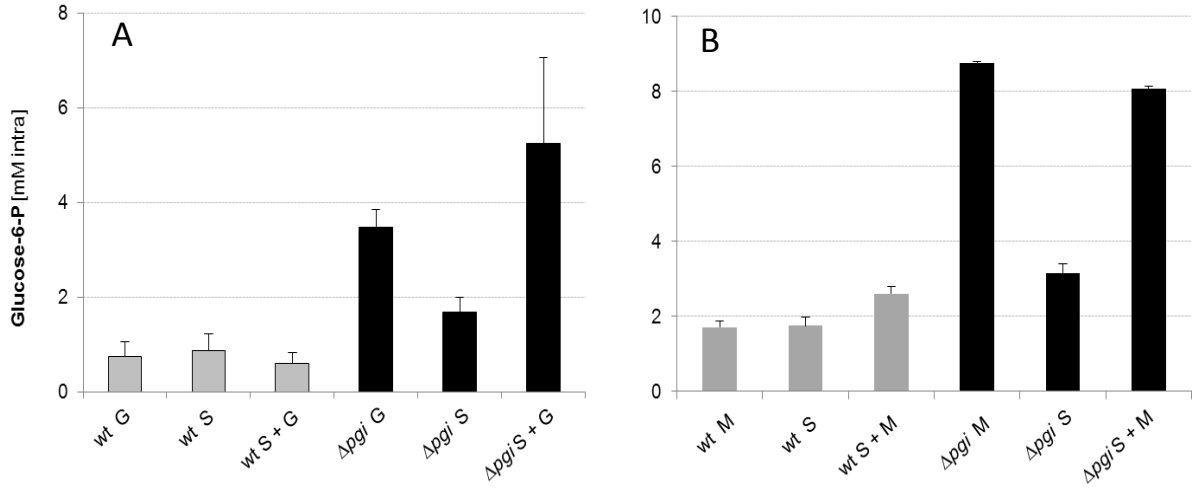


Figure 9: Intracellular Glucose-6-P concentrations in *C. glutamicum* and *C. glutamicum Δpgi*: cells were cultivated in minimal medium with glucose (G), sucrose (S), glucose plus sucrose (S+ G), maltose (M) or sucrose plus maltose (S + M). The concentrations were measured 30 min after inoculation. The results are the mean of at least three technical and two biological replicates. The left and right panels represent the average results of different sets of biological replicates.

Ribose as a non-PTS substrate affecting the carbon flux and PEP / pyruvate pool – The monosaccharide ribose is another non-PTS sugar in *C. glutamicum* transported by the ABC-transporter RbsACBD[108]. However, unlike maltose, the utilization of ribose bypasses the initial step of glycolysis catalysed by Pgi and after a phosphorylation by the ribokinase RbsK directly enters the non-oxidative part of the pentose phosphate pathway. To test if the rapid uptake response observed in *C. glutamicum* Δ*pgi* after glucose or maltose addition is a general response to the addition of any glycolytic substrate, I studied the effects caused by addition of ribose. The presence of ribose should not increase the amount of active EII permeases nor lead to direct formation of glucose-6-P and could even be expected to increase the generation of PEP in the cell.

Indeed, the presence of ribose did not reduce the sucrose uptake of *C. glutamicum* Δ*pgi* (Fig. 10, B & C). A ribose addition had no positive effect on the rapid sucrose uptake inhibition triggered by glucose, either, thus indicating again that a simple PEP-limitation is not the reason for the studied here drastic uptake reduction after glucose addition.

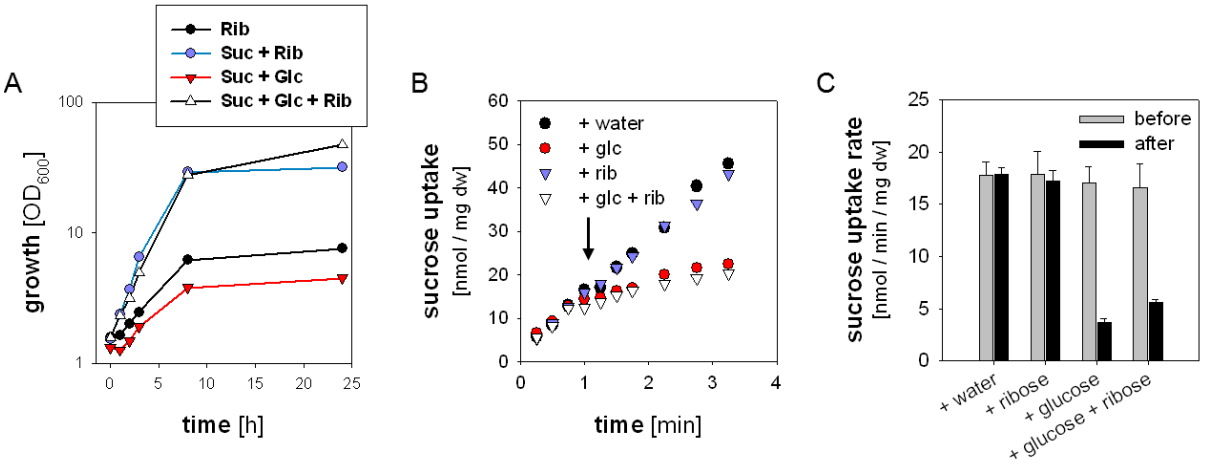


Figure 10: Growth (A) and rapid sucrose uptake inhibition (B & C) by addition of ribose in *C. glutamicum Δpgi*. For the growth experiments the cells were cultivated with 1% ribose (μ = 0.19 h^{-1}), 1% sucrose + 1% ribose (μ = 0.33 h^{-1}), 1% sucrose + 1% glucose (μ = 0.10 h^{-1}) and 1% sucrose + 1% glucose + 1% ribose (μ = 0.35 h^{-1}). The cells for the uptake measurements were pre-cultivated on sucrose plus ribose.

Even though the addition of ribose did not alleviate the sucrose uptake inhibition by glucose in *C. glutamicum* Δ*pgi,* ribose was utilized by the mutant as demonstrated by the improved growth of the cells when all three sugars were available (Fig. 10, A). Hence, the neutral behaviour of ribose regarding the rapid sucrose uptake inhibition was not caused by the lack of ribose import. Moreover, this observation showed that the presence of glucose is not *per se* toxic for *C. glutamicum* Δ*pgi* and the reason for the drastic growth reduction after glucose

addition is the blocked uptake of the otherwise effectively utilized sucrose rather than general inhibition of the growth ability of the cell.

Connection between the PTS and maltose utilization – The experiments with maltose or ribose addition to sucrose cultivated *C. glutamicum* Δ*pgi* cells indicated that the rapid sugar uptake inhibition studied here is a novel stress response to the accumulation of glucose-6-P rather than a PEP-regeneration problem. However, a recent work in our group suggested a link between the phosphorylation state of HPr and the regulation of the maltose utilisation[85]. In cells lacking H15 phosphorylated HPr the growth on maltose was heavily impaired. Furthermore, by studying the growth of the strains *C. glutamicum* Δ*ptsI* and *C. glutamicum* Δ*ptsH*, lacking the EI and HPr components of the PTS phosphorylation cascade, respectively, I could show that the absence of phosphorylated HPr was inhibiting the maltose utilization specifically. Both mutants had no growth problems when ribose was added to maltose or was the only carbon source available (table 4).

Table 4: Growth rates of *C. glutamicum ΔptsH* and *C. glutamicum ΔptsI* cells cultivated with 1% ribose (G), 1% maltose (M), 1% glucose (G) and 1% ribose + 1% maltose (RM).

	Δ*ptsH* R	Δ*ptsH* M	Δ*ptsH* G	Δ*ptsH* RM	Δ*ptsI* R	Δ*ptsI* M	Δ*ptsI* G	Δ*ptsI* RM
growth rate (h^{-1})	0.33	0.10	0.06	0.33	0.27	0.10	0.06	0.23

Further studies showed that the uptake of maltose was not affected but the activity of the maltodextrin phosphorylase MalP was inhibited [85]. As it is still not known how HPr~P would stimulate the activity of MalP, these results created an uncertainty about the usage of maltose as a non-PTS equivalent of glucose for the rapid sucrose uptake inhibition assays. It was now potentially possible that in the *pgi* mutant the utilization of maltose affects the PTS phosphorylation cascade and this rather than the observed glucose-6-P accumulation is the reason for the rapid sucrose uptake inhibition.

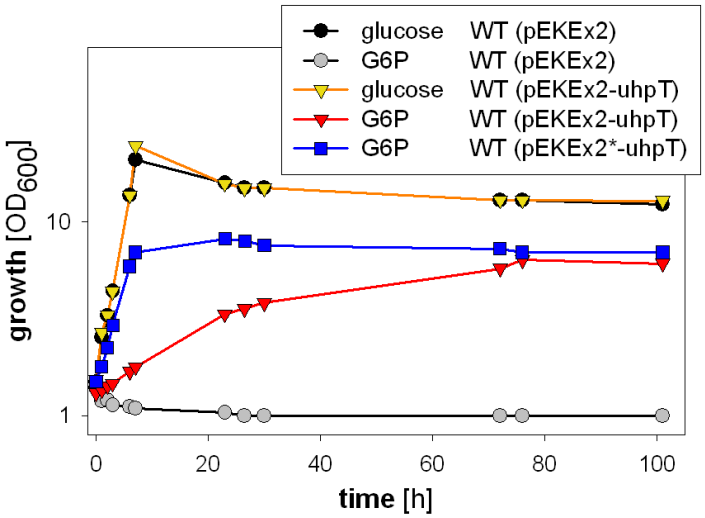
Heterologous expression of the glucose-6-P transporter UhpT – The formation of intracellular glucose-6-P after addition of maltose to the culture broth requires several metabolic steps (see above, p.32; Fig. 7). One way to confirm that the rapid sucrose uptake inhibition after maltose addition is mediated specifically by the glucose-6-P accumulation

and not by intermediates or processes connected with the few steps of maltose utilization prior to glucose-6-P formation was the direct, non-PTS-mediated uptake of glucose-6-P.

C. glutamicum is not able to utilize extracellular glucose-6-P as a carbon source (Fig. 11). However, a glucose-6-P uptake system has been identified in *E. coli* [109]. The member of the Major Facilitator Superfamily (MFS) transporters UhpT mediates the uptake of glucose-6-P in exchange for inorganic phosphate. Furthermore, UhpT has been shown to function as a monomer, which is an advantage for the reconstitution of its activity in other organisms[110]. Hence, a heterologous expression of *uhpT* seemed a promising tool to study whether a glucose-6-P stress response or an unbalanced PEP-consumption is the cause of growth and rapid sucrose uptake inhibition in *C. glutamicum* Δ*pgi.*

- Plasmid optimization, growth and G6P uptake characterization -

C. glutamicum was transformed with a pEKEx2 plasmid carrying the *uhpT* gene under a strong, IPTG-inducible promoter. Indeed, the resulting *C. glutamicum* (pEKEx2-*uhpT*) strain was able to grow with external glucose-6-P as a sole carbon source, in contrast to the empty vector control strain (Fig. 11). Enzymatic studies of the supernatant proved that glucose-6-P was completely depleted from the medium when the cells reached the stationary phase.



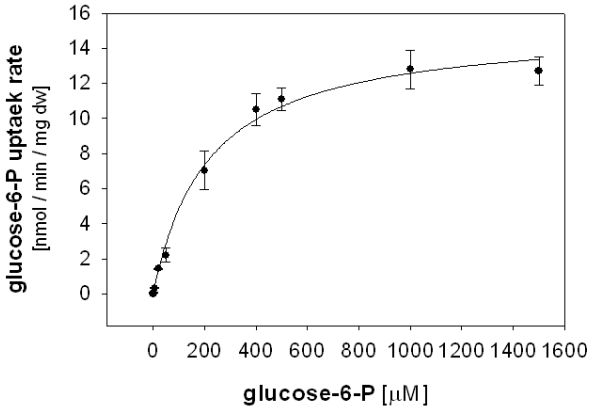
growth rate on: [h^{-1}]	Glucose	Glc6P
C. glutamicum (pEKEx2)	0.35 ± 0.02	0
C. glutamicum (pEKEx2-*uhpT*)	0.35 ± 0.01	0.04
C. glutamicum (pEKEx2*-*uhpT*)	0.35 ± 0.02	0.22 ± 0.01

Figure 11: Growth of *C. glutamicum* (pEKEx2) and *C. glutamicum* (pEKEx2-*uhpT*) with glucose-6-P or glucose as carbon sources. The cells were incubated in presence of 0.1 mM IPTG.

Nonetheless, the growth rate of *C. glutamicum* (pEKEx2-*uhpT*) on glucose-6-P was just 11.4 % of the growth rate observed when glucose was used as a carbon source (Fig. 11, table). This indicated a very poor activity of the heterologously expressed UhpT transporter

in *C. glutamicum*. Variations of the IPTG concentrations and induction times did not lead to significant improvement. Unfortunately, as the utilization of glucose-6-P is not completely inhibited in *C. glutamicum* Δ*pgi* due to the presence of the pentose phosphate pathway, a much slower glucose-6-P uptake rate compared to the glucose and sucrose uptake rates would not allow the usage of the UhpT transporter as a tool to test whether gluose-6-P is the trigger of rapid uptake inhibition.

In order to improve the efficiency of the glucose-6-P uptake a mutagenesis approach was used. The strain *C. glutamicum* (pEKEx2-*uhpT*) was inoculated at low OD_{600} in minimal medium with glucose-6-P as a sole carbon source and after 32 h (early stationary phase) inoculated in fresh medium again at low starting OD_{600}. Indeed, after four re-inoculation steps the growth rate of the strain with glucose-6-P was improved significantly. To confirm that the growth improvement was not caused by a strain mutation, the plasmid pEKEx2-*uhpT* was isolated and transferred in fresh *C. glutamicum* cells. The newly transformed strain *C. glutamicum* (pEKEx2*-*uhpT*) retained the improved ability to utilize glucose-6-P (μ= 0.22 ± 0.01 h^{-1}) (Fig. 11). Interestingly, sequencing analysis revealed that the promoter region or the coding sequence of *uhpT* was not mutated in the new plasmid pEKEx2*-*uhpT*. The size of the plasmid was not changed, either. The exact reason for the improved glucsose-6-P utilization remains to be clarified.



growth rate on: [h^{-1}]	Glc	G6P	Suc	Suc+Glc
C. glutamicum Δ*pgi* (pEKEx2)	0.11 ±0.02	0	0.42 ±0.02	0.12 ±0.02
C. glutamicum Δ*pgi* (pEKEx2*-*uhpT*)	0.11 ±0.02	0.20 ±0.02	0.41 ±0.02	0.12 ±0.01
C. glutamicum Δ*pgi* Δ*ptsG* (pEKEx2*-*uhpT*)	0.02	0.20 ±0.01	0.42 ±0.02	0.41 ±0.01

Figure 12: Characterization of the glucose-6-P uptake in *C. glutamicum* (pEKEx2*-*uhpT*) and growth rates of *C. glutamicum* Δ*pgi* (pEKEx2*-*uhpT*) and *C. glutamicum* Δ*pgi* (pEKEx2*-*uhpT*) on 1% [w/v] glucose-6-P, glucose, sucrose or sucrose plus glucose and. Results are the mean of three measurements. Cells were incubated in presence of 0.1 mM IPTG.

Studies with radioactively labeled glucose-6-P confirmed the glucose-6-P uptake ability of the new *C. glutamicum* (pEKEx2*-*uhpT*) strain, whereas no labelled glucose-6-P was imported in the control strain *C. glutamicum* (pEKEx2). Characterisation of the uptake in the

uhpT-expression strain indicated an V_{max} of 15.2 ± 0.5 nmol/(min*mg dw) and a K_m of 215 ± 26 µM, which was consistent with the observed growth rates (Fig. 12). These values were comparable with measured UhpT activities in *E. coli* [111].

Next, the pEKEx2*-*uhpT* plasmid was transferred in the mutants *C. glutamicum* Δ*pgi* and *C. glutamicum* Δ*pgi* Δ*ptsG.* Despite the absence of Pgi, the resulting strains had no significant growth disadvantage during cultivation with glucose-6-P compared to the wild-type derived *C. glutamicum* (pEKEx2*-*uhpT*) and both grew with similar growth rates of 0.20 ± 0.02 h^{-1} until complete depletion of the substrate (Fig. 12 table; Fig. 14, A). Further, the growth rate of *C. glutamicum* Δ*pgi* (pEKEx2*-*uhpT*) with glucose-6-P was higher than the growth rate with glucose (0.11 ± 0.02 h^{-1}), indicating again that the inhibited substrate uptake rather than the metabolic capacity of the cell is the reason for the drastic growth reduction of the *pgi*-mutant with glucose as a sole carbon source.

Characterization of the glucose-6-P uptake in sucrose cultivated *C. glutamicum* Δ*pgi* (pEKEx2*-*uhpT*) cells determined V_{max} of 19.4 ± 1.1 nmol/(min*mg dw) and K_m of 150 ± 27 µM (SupFig. 3). Interestingly, these values were slightly higher than in the wild-type strain carrying the vector. However, if the cells were pre-cultivated in presence of glucose-6-P the uptake of glucose-6-P was reduced to lower rates of 8 - 10 nmol/(min*mg dw), indicating a feedback adjustment of the UhpT transporter to the accumulation of glucose-6-P (SupFig. 3). Nonetheless, these glucose-6-P uptake rates were similar and even slightly higher to the glucose and maltose uptake rates measured in *C. glutamicum* Δ*pgi* and therefore should be sufficient to simulate the addition of glucose or maltose in the *pgi*-deficient mutant, which was the final aim of the plasmid construction.

- Effects of the addition of glucose-6-P to C. glutamicum strains carrying pEKEx2-uhpT -*

The addition of glucose-6-P to sucrose cultivated *C. glutamicum* Δ*pgi* (pEKEx2*-*uhpT*) cells caused rapid sucrose uptake inhibition (4.8 ± 0.9 nmol/(min*mg dw)), which was very similar to the inhibition observed after glucose addition (Fig.13, D). The growth of the strain with sucrose plus glucose-6-P was also inhibited and similar to the growth when glucose-6-P was the only substrate available – growth rates of 0.21 ± 0.02 h^{-1} and 0.20 ± 0.02 h^{-1}, respectively (Fig. 13, C). In the control strain *C. glutamicum* Δ*pgi* (pEKEx2) the presence of glucose-6-P had no effect on the growth with or uptake of sucrose (Fig. 13, A&B).

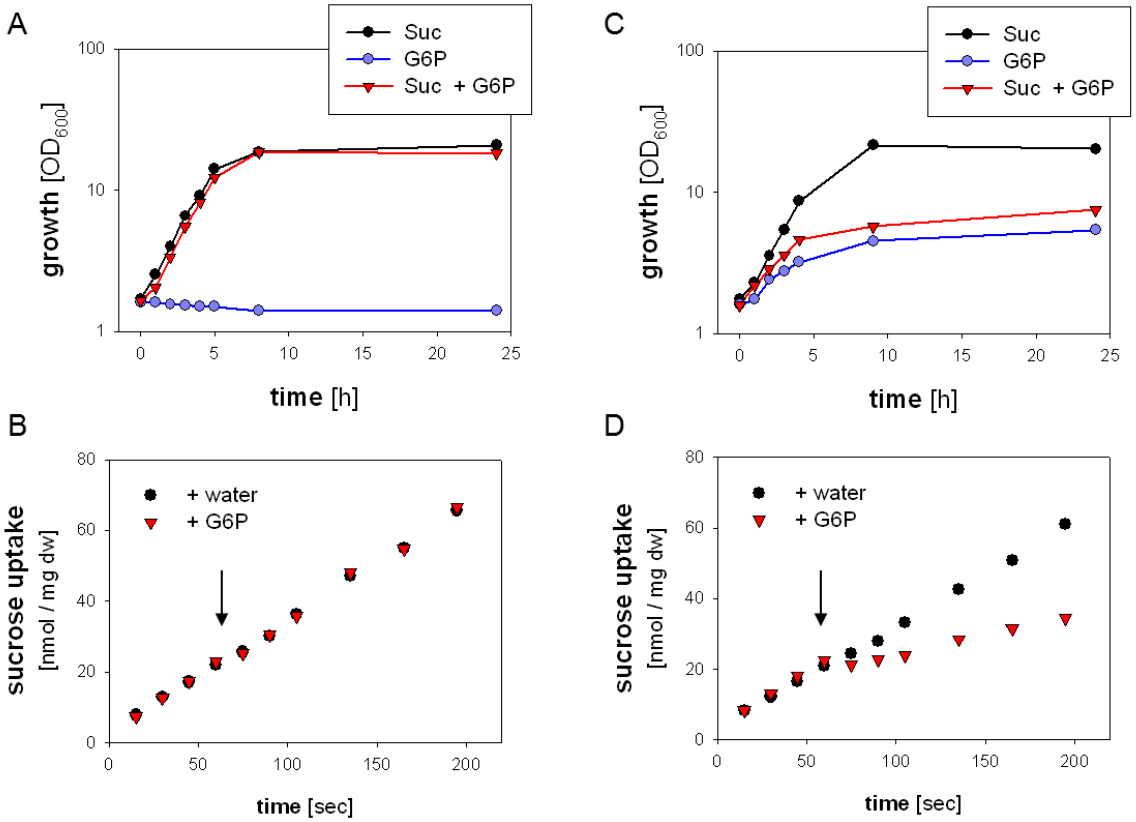


Figure 13: Effects of the addition of glucose-6-P *C. glutamicum* Δ*pgi* (pEKEx2*-*uhpT*): Growth on sucrose, glucose-6-P or sucrose plus glucose-6-P of *C. glutamicum* Δ*pgi* (pEKEx2) **(A)** and *C. glutamicum* Δ*pgi* (pEKEx2*-*uhpT*) **(B).** Sucrose uptake after glucose-6-P addition in *C. glutamicum* Δ*pgi* (pEKEx2) **(D)** and *C. glutamicum* Δ*pgi* (pEKEx2*-*uhpT*) **(E).** Glucose uptake after glucose-6-P addition in *C. glutamicum* Δ*pgi* (pEKEx2*-*uhpT*) **(C)**. Growth of *C. glutamicum* Δ*pgi* (pEKEx2*-*uhpT*) with glucose or glucose plus glucose-6-P (F). All cultures were incubated in presence of 0.1 mM IPTG and 1% [wt/vol] of the respective substrates. Shown are representative results of at least three independent measurements.

To ensure that the observed effects after glucose-6-P addition were caused specifically by UhpT-mediated uptake of glucose-6-P control experiments were conducted. The absence of ^{14}C labelled glucose-6-P uptake in the control strain *C. glutamicum* Δ*pgi* (pEKEx2) confirmed that no unspecific uptake was taking place (Fig. 13, B). An unspecific glucose-transport by UhpT was also excluded as addition of excess glucose in *C. glutamicum* Δ*pgi* Δ*ptsG* (pEKEx2*-*uhpT*) had no negative effect on the uptake of radiolabelled glucose-6-P (Fig. 14, C). *Vice versa*, addition of excess glucose-6-P had no effect on the uptake of radiolabeled glucose or sucrose, as well (Fig. 13, B). Hence, a significant glucose contamination of the used glucose-6-P stock could not be expected. Further, the glucose-6-P uptake rate of the *C. glutamicum* Δ*pgi* Δ*ptsG* (pEKEx2*-*uhpT*) cells was not lower compared to the uptake rate of *C. glutamicum* Δ*pgi* (pEKEx2*-*uhpT*) and both strains grew with similar growth rates with glucose-6-P as a sole carbon source (Fig. 12 & 14, A). This suggested that the plasmid

pEKEx2*-*uhpT* does not lead to extracellular glucose-6-P dephosphorylation followed by glucose uptake. Moreover, the addition of excess glucose did not cause a reduction of the uptake of ^{14}C labelled glucose-6-P in *C. glutamicum* Δ*pgi* (pEKEx2*-*uhpT*) (Fig. 14, B). This showed additionally that a production of intracellular glucose, which is exported from the cell and consequently stimulating activity of EII^{glc} for its reuptake is not taking place.

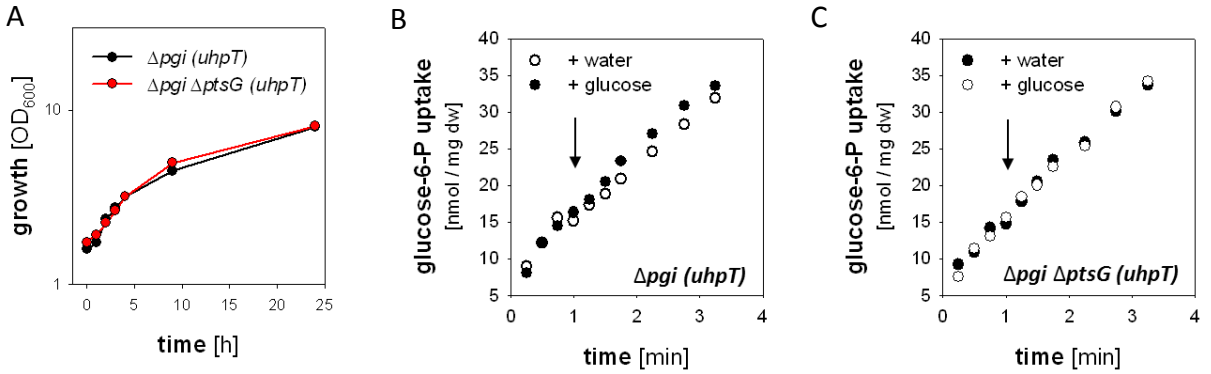


Figure 14: Specificity of UhpT-mediated glucose-6-P uptake: A: growth of *C. glutamicum* Δ*pgi* (pEKEx2*-*uhpT*) and *C. glutamicum* Δ*pgi* Δ*ptsG* (pEKEx2*-*uhpT*) on glucose-6-P as a sole carbon source. **B:** Glucose-6-P uptake after addition of excess 50mM glucose in *C. glutamicum* Δ*pgi* (pEKEx2*-*uhpT*). **C:** Glucose-6-P uptake in *C. glutamicum* Δ*pgi* Δ*ptsG* (pEKEx2*-*uhpT*) after addition of 50mM glucose. All cultures were incubated in presence of 0.1 mM IPTG. Shown are representative results of three independent measurements.

Taken together, the heterologous expression of *uhpT* enabled the specific and PTS-independent uptake of glucose-6-P in *C. glutamicum* strains. The addition of glucose-6-P caused growth and rapid sucrose uptake inhibition in *C. glutamicum* Δ*pgi* (pEKEx2*-*uhpT*). These results were consistent with the previous observations made after glucose or maltose addition as a second substrate to sucrose and thereby confirmed the previous conclusions that intracellular accumulation of glucose-6-P and not uncompensated PEP-consumption is the signal for the rapid sucrose uptake inhibition in *C. glutamicum* Δ*pgi*.

3.2.2.2. Sensing of the signal

Involvement of EII^{glc} in the rapid uptake regulation in *C. glutamicum* Δ*pgi* – As the addition of non-PTS substrates leading to the intracellular formation of glucose-6-P triggered a rapid sucrose uptake inhibition in *C. glutamicum* Δ*pgi* independently whether EII^{glc} was active or not, it could be concluded that in *C. glutamicum* Δ*pgi* Δ*ptsG* the lack of response to the addition of glucose was due to the abolished glucose-6-P accumulation. However, when I

studied specifically the function of EII^{glc} in the inhibitory effects caused by maltose addition a more sophisticated mechanism was revealed.

The rapid sucrose uptake inhibition after maltose addition in *C. glutamicum* Δ*pgi* was abolished in the EII^{glc}-deficient strain *C. glutamicum* Δ*pgi* Δ*ptsG* (Fig. 15, A). The growth of the double mutant with sucrose plus maltose was not reduced, either. As previous experiments in our lab have shown that the glucose-specific PTS is not able to transport maltose [84], these results suggested that the response of *C. glutamicum* Δ*pgi* to glucose-6-P accumulation requires the presence of EII^{glc} even when the permease is inactive.

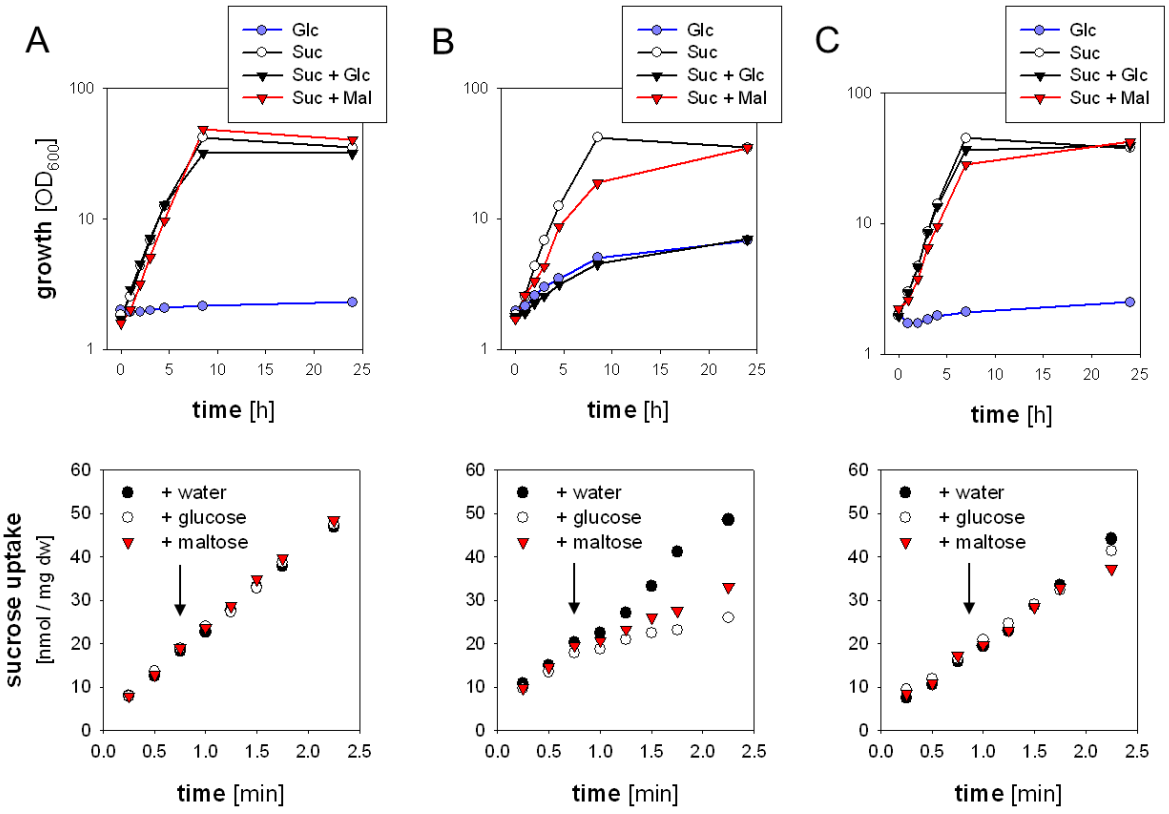


Figure 15: Abolishment of the growth and sucrose uptake inhibition in *C. glutamicum Δpgi ΔptsG* (A) and complementation experiments in *C. glutamicum Δpgi ΔptsG* (pMM-*ptsG*) (B) and the control strain *C. glutamicum Δpgi ΔptsG* (pMM) (C). For the growth experiments the cells were cultivated with 2% glucose, 2% sucrose, 1% sucrose + 1% glucose and 1% sucrose + 1% maltose. Shown are representative results of at least three independent measurements. The cells for the uptake measurements were pre-cultivated on sucrose and the presence of maltose uptake was controlled.

To confirm that those observations were caused specifically by the absence of EII^{glc} *ptsG* was ectopically expressed in *C. glutamicum* Δ*pgi* Δ*ptsG*. The constitutive expression of *ptsG* from the previously described pMM-*ptsG* plasmid[77] in the insertion mutant *C. glutamicum* Δ*pgi* IM*ptsG* (pMM-*ptsG*) restored the negative effect on growth of glucose on sucrose cultivated

cells (Fig. 15, B). Similarly as in *C. glutamicum Δpgi*, the strain *C. glutamicum Δpgi* IM*ptsG* (pMM-*ptsG*) showed again rapid sucrose uptake reduction after addition of maltose or glucose, which was not taking place in the control strain *C. glutamicum Δpgi* IM*ptsG* (pMM) (Fig. 15, C).

In addition, the response to glucose-6-P addition in the *C. glutamicum* Δ*pgi* Δ*ptsG* (pEKEx2*-*uhpT*) strain was tested, as well. Indeed, in absence of EII^{glc} the addition of glucose-6-P had no negative effects on the growth with sucrose or on the sucrose uptake ability of the strain (Fig. 16). The strain was still importing and utilizing gluose-6-P, as shown by uptake and enzymatic glucose-6-P substrate consumption measurements, so that glucose-6-P accumulation was taking place in those cells but the absence of EII^{glc} prevented the induction of the inhibitory sugar-P stress response. Furthermore, this result showed that if the sucrose uptake was not rapidly inhibited by the regulatory process studied here the cells would have been able to grow despite the accumulation of glucose-6-P.

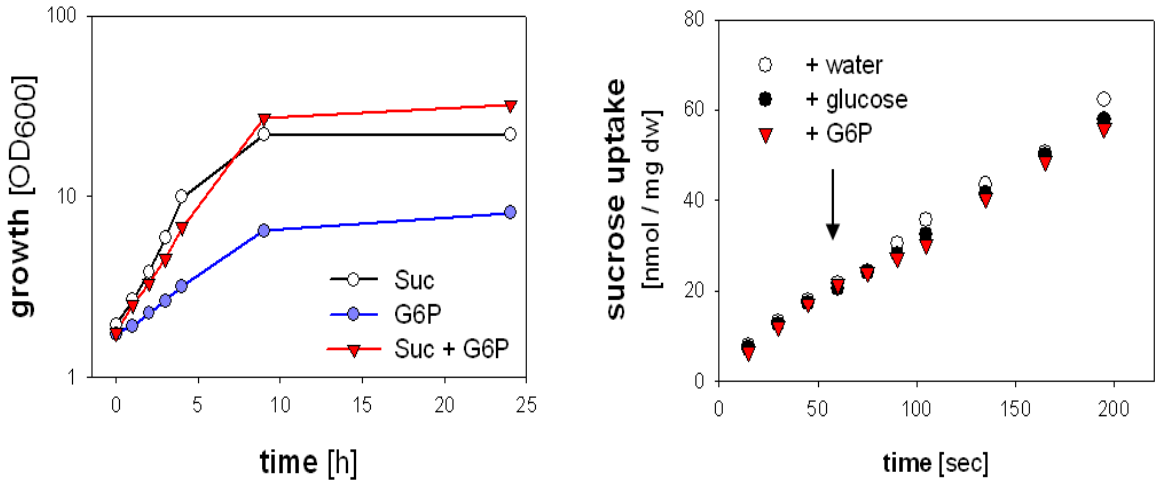


Figure 16: Abolishment of the growth and sucrose uptake inhibition by addition of glucose-6-P in *C. glutamicum Δpgi ΔptsG* (pEKEx2*-*uhpT*). For the growth experiments the cells were cultivated with 1% glucose-6-P, 1% sucrose, 1% sucrose + 1% glucose-6-P. The cells for the sucrose uptake measurements were pre-cultivated on sucrose and the presence of glucose-6-P uptake was controlled.

Taken together, the results demonstrated that the rapid sucrose uptake inhibition in *C. glutamicum* Δ*pgi* after glucose addition is a sugar-P stress response, which could be triggered also by the addition of non-PTS substrates (*e.g.* maltose). However, even if not actively transporting the glucose specific permease EII^{glc} is required for the signal perception of the glucose-6-P accumulation and activation of the mechanism of rapid uptake reduction.

3.2.3. How is the uptake inhibition achieved

3.2.3.1. The fructose uptake as a target of rapid inhibition:

Fructose as substrate of the third EII in *C. glutamicum* – A rapid reduction of the PTS-mediated uptake might be achieved by two scenarios – fast blockage of the EII functionality (via a direct interaction with an effector molecule and/or degradation of the permease) or by blockage of the PEP-EI-HPr-EII phosphorylation cascade, which would restrict the energetic supply for the EII activity. If addition of glucose causes rapid sucrose uptake reduction by inhibition of the PEP-EI-HPr phosphorylation cascade, it would be expected that the uptake of all PTS substrates and respectively the activity of all PTS permeases would be inhibited, as well. Besides the glucose- and sucrose-specific permeases, the PTS of *C. glutamicum* includes one more permease – the fructose-specific EII^{fru}.

Indeed, addition of glucose rapidly reduced the fructose uptake rate in *C. glutamicum* Δ*pgi* - from 28.7 ± 1.9 to 12.9 ± 1.5 nmol/(min*mg dw) (Fig. 17, B). Addition of maltose in the *pgi* mutant or addition of glucose-6-P in *C. glutamicum* Δ*pgi* (pEKEx2*-*uhpT*) had similar effects, reducing the fructose uptake to 12.8 ± 1.2 and 16.9 ± 1.9 nmol/(min*mg dw), respectively. Furthermore, as shown above for the sucrose uptake, the absence of EII^{glc} abolished the fructose uptake inhibition after glucose, maltose or glucose-6-P addition (Fig. 17, C).

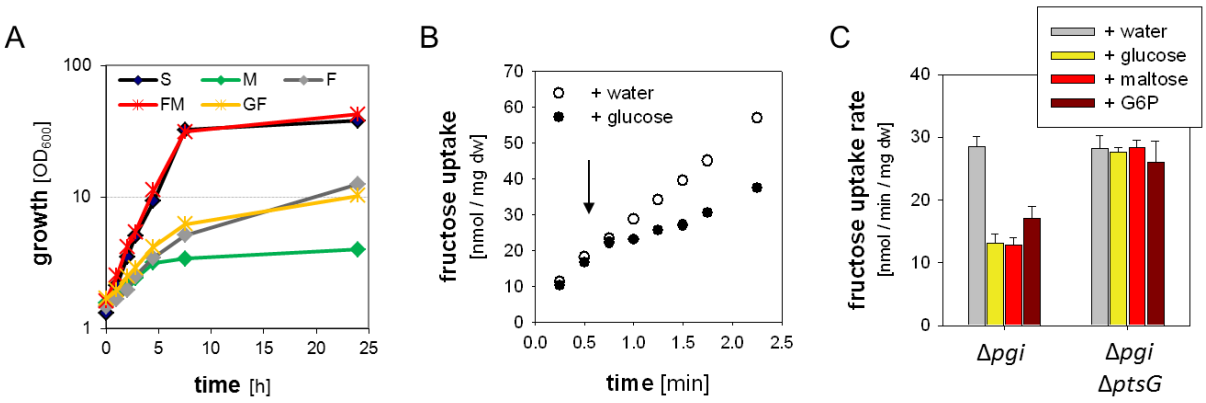


Figure 17: Rapid fructose uptake inhibition by glucose in *C. glutamicum Δpgi*: (A): growth of *C. glutamicum* Δ*pgi* with sucrose (2% [w/v]), maltose (2% [w/v]), fructose (2% [w/v]), fructose plus maltose (1% [w/v] each), and fructose plus glucose (1% [w/v] each). Cells were cultivated in minimal medium with sucrose, in order to ensure high expression of both *ptsF* and *ptsG*[78]; **(B):** Fructose uptake inhibition after addition of glucose; **(C):** Fructose uptake rates after addition of glucose, maltose of glucose-6-P in *C. glutamicum* Δ*pgi* or *C. glutamicum* Δ*pgi* (pEKEx2*-*uhpT*), respectively.

C. glutamicum Δ*pgi* has growth deficits with fructose as a sole carbon source [83]. The growth rate is reduced to 0.17 ± 0.02 h^{-1} and the substrate is not completely consumed after 24 h of cultivation. As explanation for that has been suggested the inability of the Pgi-deficient strain to produce glucose-6-P via gluconeogenesis, which is required for cell wall synthesis. As a further evidence for that I showed that the absence of PfkA, which is a glycolysis-specific enzyme which is not involved in gluconeogenesis, abolished growth on glucose but did not have a negative effect on the growth with fructose in *C. glutamicum* Δ*pfkA* (SupFig 4). Similarly to the inhibited growth with glucose, the growth of the *pgi* mutant with maltose as a sole carbon source was also poor (growth rates of 0.09 ± 0.01 h^{-1} and 0.13 ± 0.01 h^{-1}, respectively). However, the growth of *C. glutamicum* Δ*pgi* with maltose plus fructose was normal (0.42 ± 0.02 h^{-1}) and comparable with the growth of the *pgi* mutant on sucrose (Fig. 17, A). The reached final OD_{600} indicated that both substrates were completely consumed. Apparently the two substrates could complement each other. The strain *C. glutamicum* Δ*pgi* (pEKEx2*-*uhpT*) had also a normal growth with fructose plus glucose-6-P and was able to consume completely the available substrates (data not shown). However, the growth of the *pgi* mutant with glucose plus fructose was significantly reduced compared to the growth with maltose plus fructose or glucose-6-P plus fructose and similar to the growth of the strain with fructose as a sole carbon source (Fig. 17, A). Hence, the combination glucose plus fructose was not able to complement each other for normal growth of the *pgi* mutant as it was in case of maltose-fructose or glucose-6-P-fructose co-utilization. As the addition of glucose, maltose or glucose-6-P caused similar fructose uptake reduction, the mechanistic reason for the difference between the growth behaviour during cultivation with maltose plus fructose and glucose plus fructose is unclear.

Nevertheless, the addition of glucose appeared to cause rapid reduction of the general PTS-activity in *C. glutamicum* Δ*pgi*. Hence, a direct inhibition of each of the PTS permeases as well as inhibition of the PTS phosphorylation cascade was possible mechanisms for the rapid regulation of the PTS activity.In order to elucidate which one of those two possibilities is responsible for the rapid inhibition of PTS-mediated uptake in *C. glutamicum* Δ*pgi* after glucose addition *in vitro* analysis of the PTS components and their activity was conducted.

3.2.3.2. Analysis of the common PTS components: HPr

Generation of tagged HPr – A C-terminally FLAG-tagged HPr protein was created. The protein was functional as shown by complementation experiments in *C. glutamicum* Δ*ptsH* (pEKEx2-*ptsH_FLAG*). The growth of the strain on PTS substrates as well as its glucose and sucrose uptake ability was restored to wild-type levels. A distinct band of apparent size of 15 kDa was detected in cell extracts of *C. glutamicum* Δ*ptsH* (pEKEx2-*ptsH_FLAG*) but not in the control strain *C. glutamicum* Δ*ptsH* (pEKEx2) (Fig. 18, B). As expected, the HPr protein was found predominately in the cytosolic fractions. Stronger induction of *ptsH-FLAG* increased the amount of HPr-FLAG in the cell but did not lead to further improvement of the growth or glucose and sucrose uptake rates (Fig. 18).

A

IPTG [mM]	0	0.01	0.1	1
Δ*ptsH* (pEKEx2)	0	0	0	0
Δ*ptsH* (pEKEx2-*ptsH*)	0.41 ±0.02	0.41 ±0.02	0.41 ±0.02	0.41 ±0.01

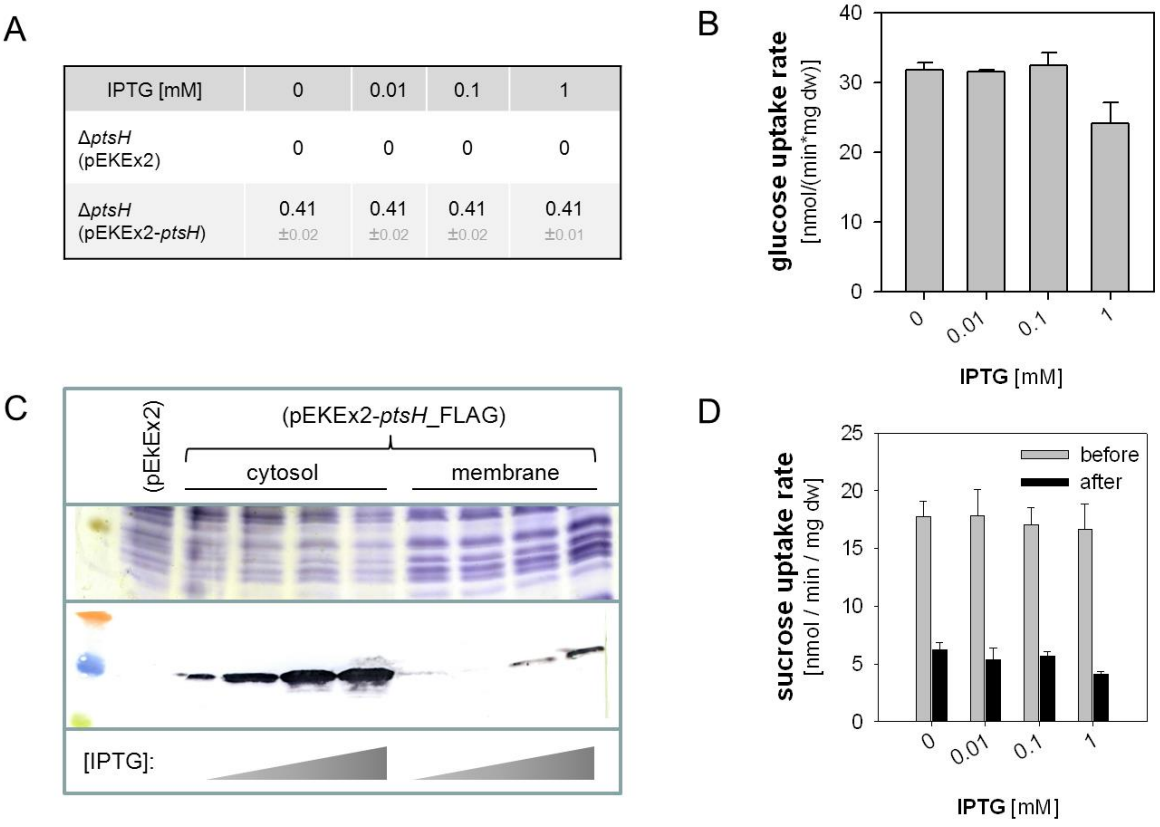


Figure 18: Analysis of the functionality of FLAG-tagged HPr. A: Growth rates of *C. glutamicum* Δ*ptsH* (pEKEx2) and *C. glutamicum* Δ*ptsH* (pEKEx2-*ptsH_FLAG*) cultivated with sucrose. The expression of ptsH was induced with different concentrations of IPTG – form 0 to 1 mM. **B:** Glucose uptake rates of *C. glutamicum* Δ*ptsH* (pEKEx2-*ptsH_FLAG*) cells cultivated with sucrose and different concentrations of IPTG – from 0 to 1mM. **C:** Western blot analysis of HPr_FLAG found in the membrate and cytosolic fractions from the cells from point B. **D:** Sucrose uptake rate reduction after addition of glucose in sucrose pre-cultivated *C. glutamicum* Δ*pgi* (pEKEx2-*ptsH_FLAG*) cells induced with different IPTG concentrations from 0 to 1 mM.

As it was not clear if the physiological amounts of HPr were exceeded in the Δ*ptsH* mutant the strains *C. glutamicum* (pEKEx2-*ptsH_FLAG*) and *C. glutamicum* Δ*pgi* (pEKEx2-*ptsH_FLAG*) were also tested. Again, the overexpression of *ptsH_FLAG* did not change the growth phenotype or the sucrose uptake rates of the cells (SupFig. 5). Furthermore, in *C. glutamicum* Δ*pgi* (pEKEx2-*ptsH_FLAG*) the rapid sucrose uptake inhibition after glucose addition was not alleviated with increasing expression of *ptsH_FLAG* (Fig. 18, D). These results indicated that an increased amount of HPr is not affecting the PTS-mediated uptake. A limitation of the PEP-EI-HPr phosphorylation cascade ratio as a reason for the rapid regulation of the PTS activity was still not excluded, though, the amount of HPr did not seem to be the bottleneck in this process.

Analysis of the phosphorylation state of HPr – To distinguish whether EII^{glc} mediates the rapid sucrose and fructose inhibition by (A) direct blockage of EII permeases or by (B) inhibition of the PEP-EI-HPr phosphorylation cascade, I studied the phosphorylation state of HPr, which is the last common component of the PTS phosphorylation cascade. The analysis of the HPr~P/HPr ratio during different cultivation conditions was performed via Western blot analysis of the FLAG-tagged HPr protein, as described in 2.4.1.

Two main HPr bands could be detected in the *C. glutamicum* (pEKEx2-*ptsH_FLAG*) cells cultivated on glucose, sucrose or sucrose plus glucose: the previously observed band of 15 kDa and a lower band of apparent size of 14 kDa (Fig. 19). Upon heat treatment of the samples the lower band disappeared and the 15 kDa band became more intensive. Similar observation was made after treatment of the samples with alkaline phosphatase, as well, indicating that the lower 14 kDA band represents phosphorylated and the upper 15 kDa band unphosphorylated HPr (SupFig. 6, B). As no HPr kinase has been found in *C. glutamicum* a non-catalytic phosphorylation of HPr was not expected [30, 112].

The HPr~P / HPr ratio in the wild-type was similar during cultivation with glucose, sucrose or glucose plus sucrose and both the 15 and 14 kDa bands were detectable and comparably intense. In contrast, in the *C. glutamicum* Δ*pgi* (pEKEx2-*ptsH_FLAG*) cells cultivated with glucose or sucrose plus glucose the 14 kDa band was very weak, whereas the 15 kDa band remained intense (Fig. 18). This result suggested that the presence of glucose shifts the HPr~P/HPr balance in *C. glutamicum* Δ*pgi* significantly towards unphosphorylated HPr. That shift seemed to proceed rapidly, as well. The intense lower band in the sucrose cultivated

Δ*pgi* cells was dramatically reduced just 1 min after glucose addition to the culture broth (Fig. 19, Suc+Glc line). Hence, this result was consistent with the observed previously rapid sucrose uptake inhibition after glucose addition in the Pgi-deficient strain.

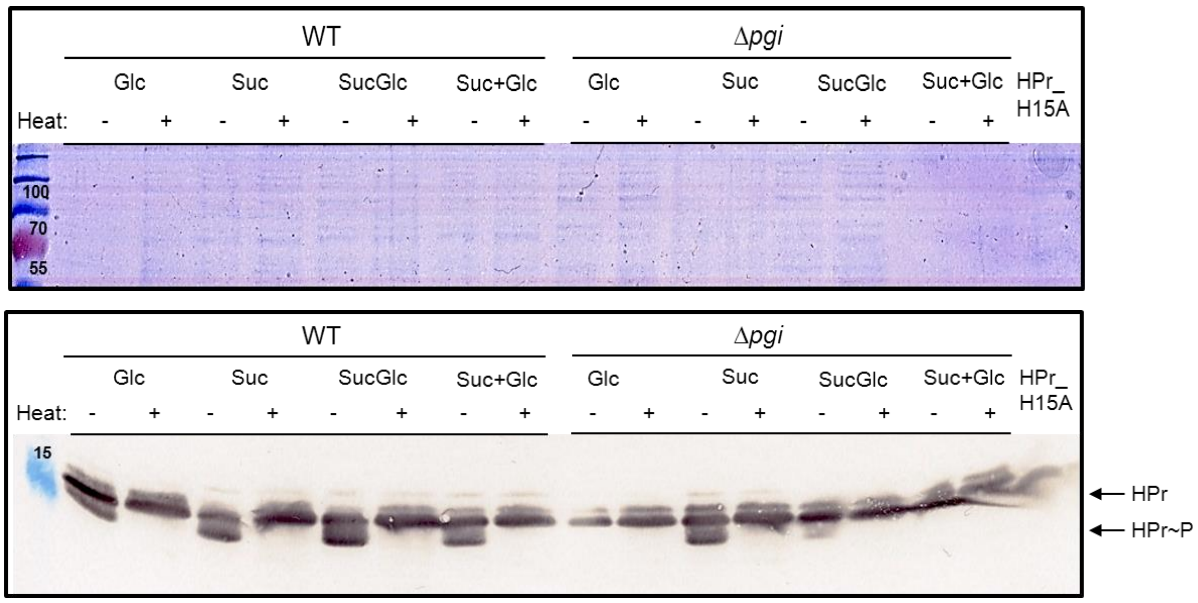


Figure 19: HPr phosphorylation state analysis of *C. glutamicum* (pEKEx2-*ptsH_FLAG*) and *C. glutamicum Δpgi* (pEKEx2-*ptsH_FLAG*) cells cultivated in minimal medium with glucose, sucrose or sucrose plus glucose as carbon source. Additionally, a sample of the sucrose cultivated cells was incubated for 1 min after addition of glucose (Suc + Glc samples). As a control the samples were dephosphorylated by boiling for 5 min and additionally the catalytically inactive mutated form HPr (HPr_H15A) was used as a negative control. Shown is one representative result of at least three independent experiments.

Similarly as glucose, addition of maltose also caused rapid sucrose uptake inhibition in *C. glutamicum Δpgi*. Indeed, the HPr~P / HPr ratio in the sucrose plus maltose cultivated Δ*pgi* mutant was reduced compared to the ratio in the cells cultivated with sucrose as a sole carbon source. This negative effect seems even stronger considering that in the wild-type strain the presence of maltose increased the HPr~P / HPr ratio of sucrose cultivated cells (Fig. 20, B).

Interestingly, during cultivation with sucrose plus maltose the *C. glutamicum Δpgi* (pEKEx2-*ptsH_FLAG*) mutant had stronger inhibited growth compared to the observed previously in the *C. glutamicum Δpgi* strain growth reduction by the presence of maltose. In fact, in *C. glutamicum* Δ*pgi* (pEKEx2-*ptsH_FLAG*) the growth reduction by maltose addition was comparable with the growth reduction by glucose addition and the cells did not reach a final OD_{600} above 4 (Fig. 20, A). The mechanism how an increased HPr amount would strengthen the inhibitory effect of maltose in the Δ*pgi* mutant might be connected with the regulatory

role of HPr in the maltose metabolism. However, the overexpression of *ptsH_FLAG* had no negative effects on the growth of the wild-type with sucrose plus maltose, indicating that the effect was specifically connected with the absence of Pgi (data not shown).

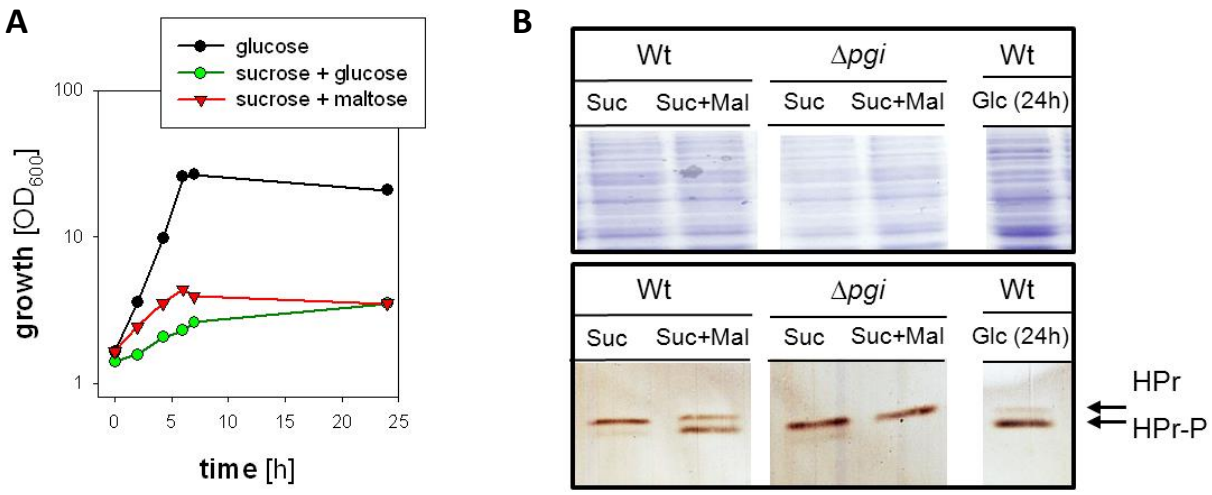


Figure 20: Effects of maltose on the phosphorylation state of HPr: A: Growth of *C. glutamicum Δpgi* (pEKEx2-*ptsH_FLAG*) with glucose (G; 2% [w/v]), sucrose plus glucose (1% [w/v] each), sucrose plus maltose (1% [w/v] each). Expression of *ptsH_FLAG* was induced with 0.1 mM IPTG; **B:** Western blot analysis of the phosphorylation state of FLAG-tagged HPr from *C. glutamicum* (pEKEx2-*ptsH_FLAG*) and *C. glutamicum Δpgi* (pEKEx2-*ptsH_FLAG*) cells cultivated with sucrose (Suc; 2% [w/v]), sucrose plus maltose (Suc+Mal; 1 + 1% [w/v]) and glucose (G; 1 + 1% [w/v]) as a substrates. Expression of *ptsH_FLAG* was induced with 0.2 mM IPTG; Shown is one representative result of at least three independent experiments.

The SugR-deficient *C. glutamicum* Δ*pgi* Δ*sugR* (pEKEx2-*ptsH_FLAG*) also had reduced HPr~P / HPr ratio if glucose or maltose were present in the culture broth, thus confirming that the rapid sucrose uptake reduction in *C. glutamicum Δpgi* by glucose and maltose addition is SugR-independent (SupFig. 6, A).

To determine the HPr~P / HPr ratio of the cell when the PTS is not active I also analyzed samples of the cultures after they have entered the stationary growth phase after the substrate have been consumed, during which no uptake is expected. Indeed, the majority of HPr in the cell was in its phosphorylated form (Fig. 19; SupFig. 6, B). Similar observation was made also for exponentially growing cells when no PTS substrates are available, indicating that this observation was not specific only for the stationary phase (SupFig. 6, C). Thus, the inactive state of the PTS corresponds to a high HPr-P / HPr ratio which slightly decreases when PTS-sugars become available.

3.2.3.3. Analysis of the common PTS components: EI

Tagged variants of PTS components: EI – An N-terminally Strep-tagged EI was created which efficiently complemented growth of *C. glutamicum* Δ*ptsI* with sucrose. The strain *C. glutamicum* Δ*ptsI* (pEKEx2-*Strep_ptsI*) reached growth rates comparable with the wild-type, whereas the control strain *C. glutamicum* Δ*ptsI* (pEKEx2) was unable to grow with sucrose (Fig. 21). Induction of the *Strep_ptsI* expression with different IPTG concentrations did not increase further the growth rate of *C. glutamicum* Δ*ptsI* (pEKEx2-*Strep_ptsI*) and the plasmid seemed to have a sufficient expression to complement the PTS activity even without IPTG addition (Fig. 21, A).

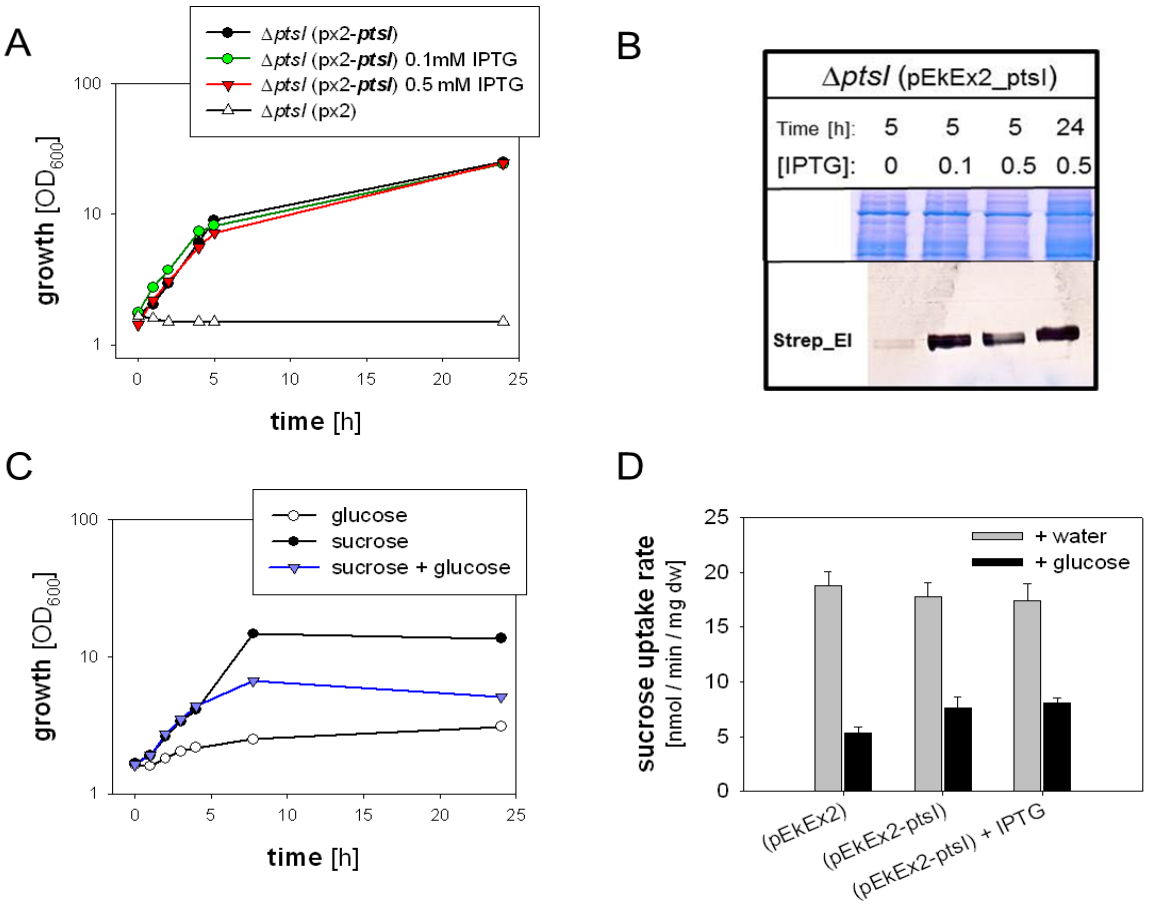


Figure 21: Analysis of the Strep-tagged EI construct. A: Growth of *C. glutamicum, C. glutamicum Δptsl* (pEKEx2) and *C. glutamicum Δptsl* (pEKEx2-*Strep_ptsI*) with sucrose (S; 2% [w/v]) as a sole carbon source. Expression of Strep_*ptsI* was induced with 0, 0.1 or 0.5 mM IPTG. **B:** SDS-PAGE and Western blot analysis of Strep_EI from cell extracts of *C. glutamicum Δptsl* (pEKEx2-*Strep_ptsI*) cells induced with 0, 0.1 or 0.5 mM IPTG. Samples were taken during the exponential growth phase (5 h) or the stationary phase (24h). **C & D**: Growth and rapid sucrose uptake inhibition after glucose addition in sucrose cultivated *C. glutamicum* Δ*pgi* (pEKEx2) and *C. glutamicum* Δ*pgi* (pEKEx2-*Strep_ptsI*).

Western blots analysis confirmed the presence of Strep-EI, detected as a single band of 65 kDa, and confirmed that the induction with higher concentrations of IPTG was resulting in increased production of EI in the *C. glutamicum* Δ*ptsI* (pEKEx2-*Strep_ptsI*) cells, even though the growth rates were similar (Fig. 21, B). The increase of the EI levels in the cell did not result in significant improvement of the sucrose uptake, either (data not shown).

The results of the HPr analysis showed that the rapid sucrose uptake reduction after glucose addition is caused by inhibition of the PTS phosphorylation cascade. Hence, an increased EI concentration in the cell could be able to avoid or at least reduce these negative effects, depending on how the limitation of the cascade was achieved. Indeed, the growth rate of *C. glutamicum* Δ*pgi* (pEKEx2-*Strep_ptsI*) with sucrose plus glucose was slightly increased (0.28 ± 0.01 h^{-1}) compared to the control strain *C. glutamicum* Δ*pgi* (pEkEx2) (0.09 ± 0.01 h^{-1}) (Fig. 21). Nonetheless, the reached final OD_{600} after 24 of incubation was only 30% of the wild-type values indicating that *C. glutamicum* Δ*pgi* (pEKEx2-*Strep_ptsI*) still had a significant growth deficit.

The sucrose uptake inhibition by glucose addition in the *C. glutamicum* Δ*pgi* (pEKEx2-*Strep_ptsI*) cells induced with 0.5 mM IPTG was slightly alleviated compared to the *C. glutamicum* Δ*pgi* (pEKEx2) cells: 8.1 ± 1.6 and 5.6 ± 1.3 nmol/(min*mg dw), respectively (Fig. 21, C).

Taken together, in addition to the FLAG-tagged HPr a functional C-terminally Strep-tagged EI was created and could be used for further *in vitro* PTS activity measurements. Further, it was shown that increased amount of EI in *C. glutamicum* Δ*pgi* (pEKEx2-*Strep_ptsI*) had a positive effect on the growth rate of the mutant with glucose plus sucrose and to a lower extent on the sucrose uptake reduction by the addition of glucose. However, both improvements were very weak and still did not lead to complete exhaustion of the available substrates.

3.2.4. Specificity of the rapid uptake inhibition

3.2.4.1. Sugar-P stress in *C. glutamicum* Δ*scrB*:

A rapid sugar-P stress response in *C. glutamicum* Δ*scrB* – Deletion of *scrB*, encoding the enzyme sucrose-6-phosphate hydrolase, prevents the first step of sucrose utilization – the hydrolysis of sucrose to glucose-6-phosphate and fructose. It has been demonstrated that *C. glutamicum* Δ*scrB* has a normal, wild-type-like growth with glucose but the growth on sucrose or on sucrose plus glucose as substrates is strongly inhibited[33]. These properties strongly resembled the situation in *C. glutamicum* Δ*pgi* during cultivation with those two sugars, with the difference that in the *scrB* mutant sucrose seemed to have inhibiting effect. Indeed, I showed that addition of sucrose caused drastic *ptsG*-repression as well as a rapid glucose uptake reduction within 15 sec in glucose cultivated *C. glutamicum* Δ*scrB* (Fig. 22). Furthermore, sucrose inhibited the fructose uptake in *C. glutamicum* Δ*scrB,* as well (SupFig. 7, C). As expected, the negative effects by sucrose addition were absent in the EII^{suc}-deficient strain *C. glutamicum* Δ*scrB* Δ*ptsS* (SupFig. 7, A & B). Hence, blockage of the sucrose utilization instead of the glucose utilization led to inverse effects to the observed earlier sugar-P stress response in *C. glutamicum* Δ*pgi*. These results showed that EII^{glc} could be both initiator and object of rapid regulation and indicated that the identified in *C. glutamicum* Δ*pgi* rapid stress response to glucose addition might be a more general reaction mechanism to sugar-P stress.

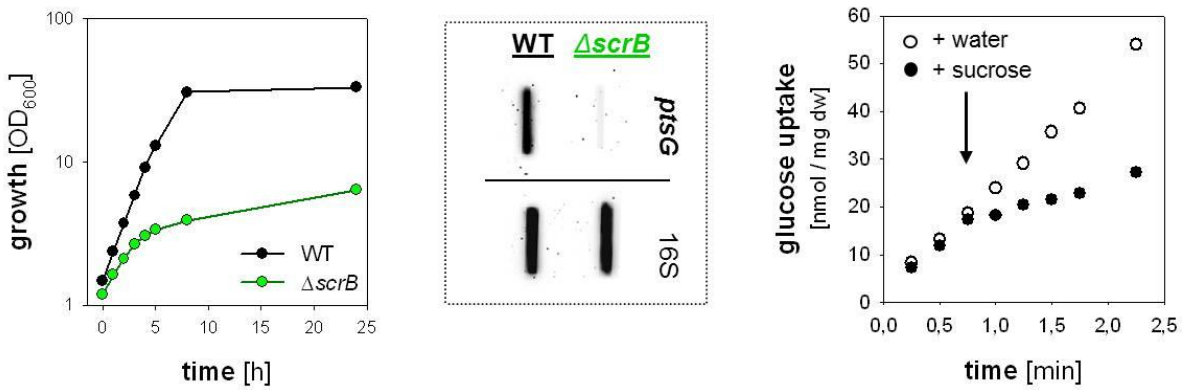


Figure 22: Rapid sucrose uptake inhibition by sucrose addition in *C. glutamicum ΔscrB*: growth and ptsG expression of wild-type and *ΔscrB* cells cultivated in minimal medium with sucrose plus glucose as well as glucose uptake inhibition by sucrose addition in glucose cultivated *C. glutamicum* Δ*scrB* cells. Shown are representative results of three measurements.

The PEP-regeneration problem in *C. glutamicum* Δ*scrB* – In contrast to *C. glutamicum* Δ*pgi* where the utilization of glucose-6-P is still possible because of the PPP, the deletion of *scrB* leads to a complete inhibition of the sucrose-6-P utilization. Hence, during cultivation on sucrose plus glucose in *C. glutamicum* Δ*scrB* the PEP-production rate by the PTS is in a delicate balance between the glucose and sucrose uptake rates ($\mathbf{V_{PEP} = V_{Glc} - V_{Suc}}$) as the uptake of 1 mol glucose consumes 1 mol PEP and regenerates through utilization of the sugar 2 mol of PEP (net +1) whereas the uptake of sucrose leads only to 1 mol PEP consumption per 1 mol of transported sucrose (net -1). To test if the rapid glucose uptake inhibition by sucrose in *C. glutamicum* Δ*scrB* is caused by a sugar-P stress response or by a PEP-regeneration problem, I followed the glucose and sucrose uptake for a time period of 26 min when both substrates were present during the measurement.

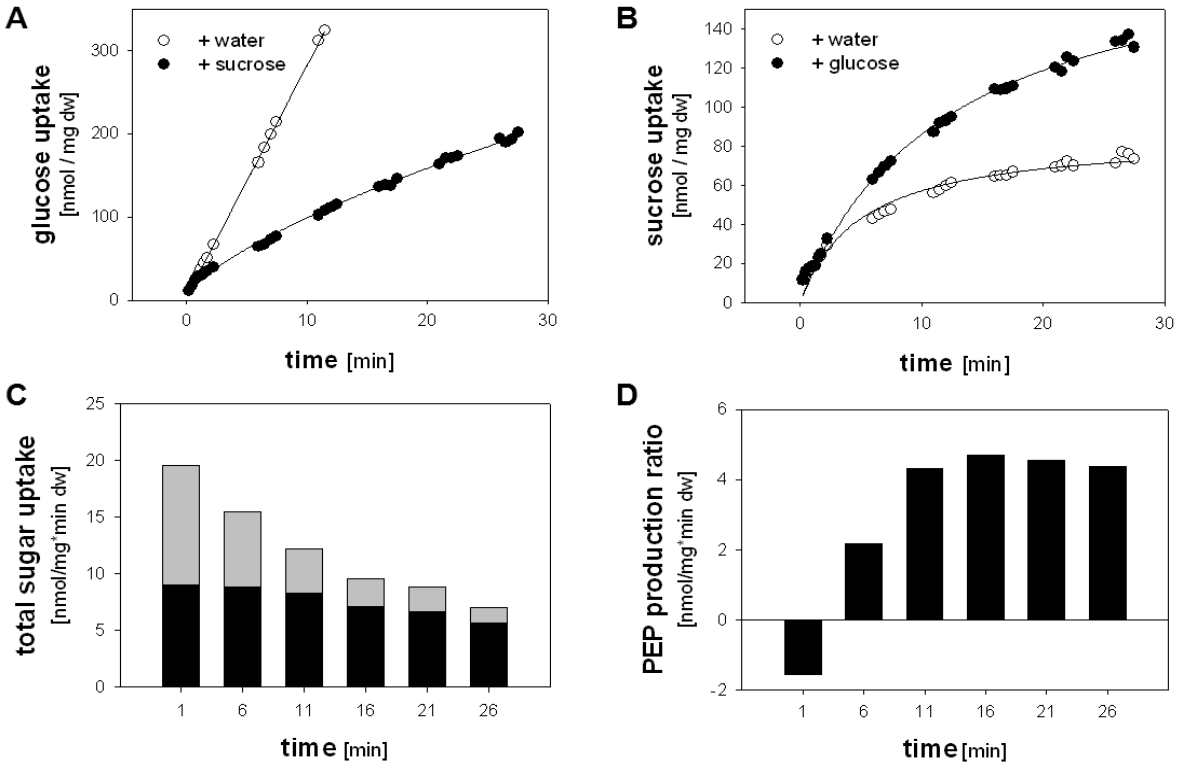


Figure 23: Prolonged studies of the glucose and sucrose uptake rates in *C. glutamicum* Δ*scrB* cells cultivated in minimal medium with glucose plus sucrose: Uptake of radiolabelled glucose **(A)** or sucrose **(B)** in presence or absence of sucrose or glucose, respectively. **C:** total sugar uptake rate as sum of the mean of measured sucrose (grey) and glucose (black) uptake rates. **D:** PEP production rate (according to $V_{PEP} = V_{Glc} - V_{Suc}$) corresponding to the measured uptake rates. Calculation of the glucose and sucrose uptake development after curve fitting (V_{Glc}= 443.3x/(x+35.1) and V_{Suc}= 193.5.3x/(x+12.5)) indicated that the PEP-production rate would become positive about 2.15 min after sucrose addition and would remain positive for t > 1h.

Within the first minute after sucrose was added to the glucose cultivated *C. glutamicum* Δ*scrB* cells the measured sucrose uptake rate was higher than the glucose uptake rate

resulting in a negative PEP-production rate (10.3 ± 0.9 compared to 8.5 ± 0.6 nmol/(min*mg dw), respectively). However, after the initial inhibition by the addition of sucrose the glucose uptake in *C. glutamicum* Δ*scrB* remained at relatively constant rates (Fig. 23, A), whereas the sucrose uptake had a hyperbolic behavior (Fig. 23, B). As a result, after 5 min the glucose uptake got higher than the sucrose uptake rate thereby resulting in a positive PEP-production rate (Fig. 23, C&D). Nonetheless, despite that the glucose uptake rate remained higher as the sucrose uptake for the rest of the measurement the total sugar uptake of the cells continued to decrease. Thus, the rapid glucose uptake inhibition was not caused by the previously described PEP-regeneration problem but by an independent regulatory mechanism. Otherwise, after a positive PEP-production rate had been reached the total sugar uptake rate in the cell should have risen again.

To test further if a PEP-limitation could be the reason for the rapid glucose inhibition by sucrose *C. glutamicum* Δ*scrB* I used the non-PTS substrate ribose, which should increase the carbon flux toward PEP synthesis without using PEP for its uptake. Similarly as in *C. glutamicum* Δ*pgi*, the presence of ribose improved the growth of *C. glutamicum* Δ*scrB* with sucrose plus glucose (0.24 h^{-1} ± 0.01) but was neutral in terms of uptake regulation (Fig. 24). The ribose addition did not cause glucose uptake inhibition and also did not alleviate the glucose uptake inhibition by sucrose (Fig. 24, B & C).

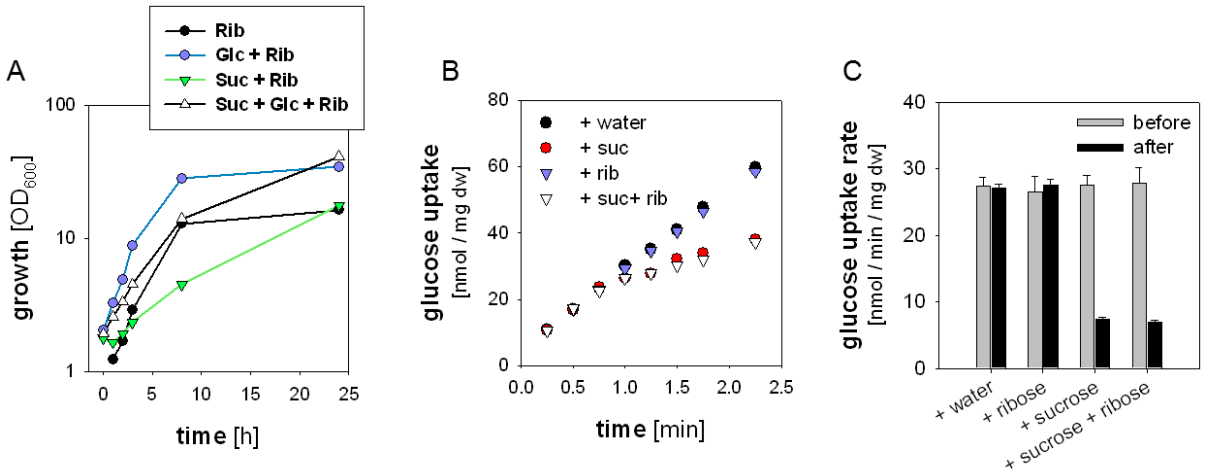


Figure 24: Growth (A) and rapid sucrose uptake inhibition (B & C) by addition of ribose in *C. glutamicum* *ΔscrB*. For the growth experiments the cells were cultivated with 1% ribose (μ = 0.33 h^{-1}), 1% glcose + 1% ribose (μ = 0.38 h^{-1}), 1% sucrose + 1% ribose (μ = 0.14 h^{-1}) and 1% sucrose + 1% glucose + 1% ribose (μ = 0.24 h^{-1}). The cells for the uptake measurements were pre-cultivated on glucose plus ribose.

Interestingly, the growth of *C. glutamicum* Δ*scrB* with sucrose plus ribose (0.14 h^{-1} ± 0.01) was reduced compared to the growth with ribose as a sole carbon source (0.33 h^{-1} ± 0.01). This indicated that the sucrose-6-P accumulation might be reducing, however, not abolishing, the ribose utilization or the general growth ability of the mutant.

The analysis of the HPr~P/HPr ratio in *C. glutamicum ΔscrB* (pEKEx2-*ptsH_FLAG*) indicated that analogically to *C. glutamicum* Δ*pgi* the presence of sucrose led to reduction of the HPr~P amounts in the cell (Fig. 25, B). However, the decrease in the HPr~P / HPr ratio during cultivation with glucose plus sucrose was not as dramatic as in the Δ*pgi* mutant. In fact, the overexpression of *ptsH* improved the growth of *C. glutamicum* Δ*scrB* with sucrose plus glucose (0.28 ± 0.01 h^{-1}), which was not the case in the Δ*pgi* mutant (Fig. 25, A).
Noteworthy, sucrose reduced also the growth of Δ*scrB* with maltose (0.15 ± 0.01 h^{-1}), which indicated a further regulatory function as sucrose-6-P is not expected to interfere directly with the utilization of maltose (unlike glucose-6-P) (Fig. 25, A). Interestingly the HPr~P amount in this case was again significantly reduced (Fig. 25, B).

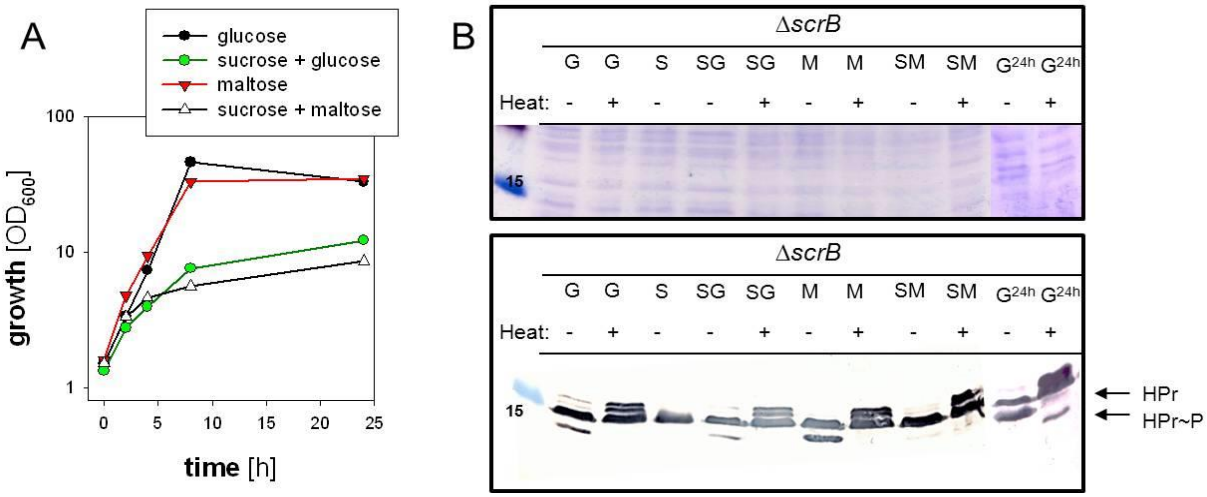


Figure 25: Growth (A) and HPr phosphorylation state analysis (B) of *C. glutamicum ΔscrB* (pEKEx2-*ptsH_FLAG*) cells cultivated in minimal medium with glucose, sucrose or sucrose plus glucose, maltose and sucrose plus maltose as carbon sources. As a control the samples were dephosphorylated by boiling for 5 min.

Free intracellular sugars as trigger of the inhibition mechanism – *In vitro* studies of the PTS of *E. coli* have shwon that PTS permeases are able to catalyse trans-phosphorylation between bound substrate and intracellular substrate-P but the physiological relevance of this phenomenon remained unclear as normally sugar-phosphates are rapidly utilized [61].

Though, in conditions of sugar-P stress this process might be taking place also *in vivo*. In fact, as it was not clear why the accumulation of glucose-6-P in absence of EII^{glc} does not lead to inhibitory effects in *C. glutamicum* Δ*pgi*, a model in which the accumulation of *e.g*. glucose-6-P leads to catalysed by EII^{glc} trans-phosphorylation and thereby formation of intracellular free glucose, which is the actual trigger of inhibition, seemed possible and could explain the phenomenon of trans-phosphorylation. To test this hypothesis and also analyse if sucrose-6-P accumulates in *C. glutamicum* Δ*scrB* the intracellular metabolites of *C. glutamicum* Δ*scrB* and *C. glutamicum* Δ*ptsH* cells were studied for different incubation periods of 0.5, 1, 2 and 5 min with ^{14}C sucrose. The extraction of intracellular metabolites was performed as described in 2.4.9 and the samples were analysed via TLC.

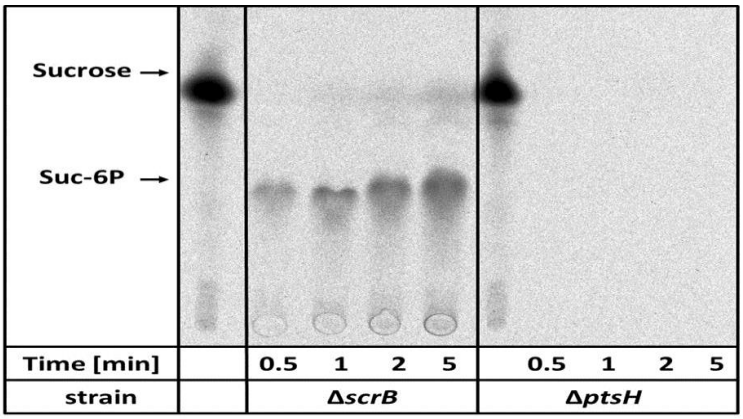


Figure 26: Analysis of the intracellular accumulation of sucrose-6-P and sucrose in *C. glutamicum* Δ*scrB* and *C. glutamicum* Δ*ptsH* cells inside 5 min incubation in presence of ^{14}C sucrose.

The results of the TLC analysis confirmed that sucrose-6-P accumulates in *C. glutamicum* Δ*scrB* shortly after [^{14}C]sucrose addition and this accumulation continued during the time of the measurement (Fig. 26). Interestingly, a weak second product corresponding to a spot of non-phosphorylated sucrose was detected in the cytosolic extracts of *C. glutamicum* Δ*scrB*, as well. The second spot was not a contamination by extracellular sucrose or PTS-independently transported sucrose as no ^{14}C labelled metabolites were detected in the control strain *C. glutamicum* Δ*ptsH*, in which the PTS–mediated uptake of sucrose is abolished. Hence, it is possible that during sugar-P stress a trans-phosphorylation activity of EII is taking place. However, as no second spot was detected during the first 30 sec of incubation, the potential intracellular accumulation of sucrose is unlikely to be involved in the mechanism of rapid glucose uptake inhibition but its relevance for the regulatory phenomena during sugar-P stress remains to be further investigated.

3.2.4.2. Rapid uptake regulation in the wild-type:

Response of *C. glutamicum* to addition of glycolytic substrates – In the wild-type *C. glutamicum* the addition of glucose during cultivation on sucrose did not inhibit growth or *ptsS*- and *ptsG*-expression[78]. However, the growth rate during cultivation on glucose plus sucrose (0.42 ± 0.02 h^{-1}) was not improved compared to cultivation on sucrose as a sole carbon source (0.42 ± 0.02 h^{-1}), either. This was surprising, as those sugars are co-utilized in this organism and a higher influx of carbon could be expected. Indeed, my previous studies have suggested that during cultivation on sucrose plus glucose the wild-type has slightly reduced sucrose and glucose uptake rates, thereby adjusting the total carbon uptake to similar levels as when just one of the two sugars is available[78]. Here I show that this is a rapid process, as well. Addition of glucose decreased the sucrose uptake rate of the wild-type from 19.1 ± 1.7 to 13.8 ± 0.8 nmol/(min*mg dw) within 15 sec (Fig. 27).

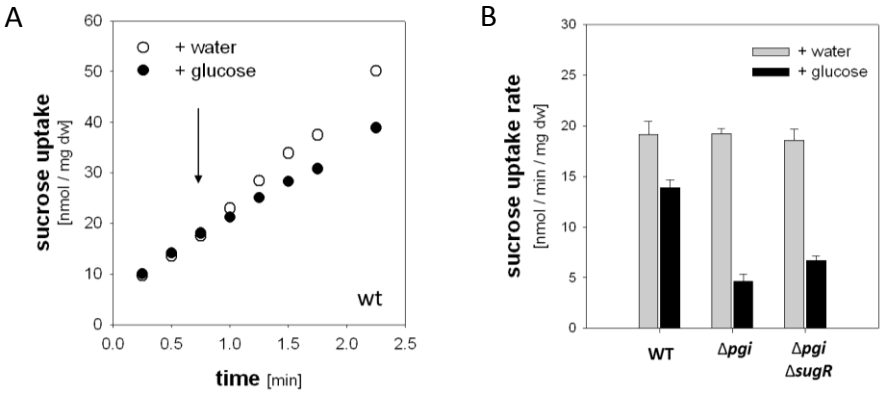


Figure 27: Rapid sucrose uptake inhibition in *C. glutamicum:* A: sucrose uptake inhibition after addition of glucose in wild-type *C. glutamicum*. **B:** Comparison of the uptake rates after addition of water (negative control, equal to the uptake rate with sucrose alone) or glucose in the wild-type and the Δ*pgi* and Δ*pgi* Δ*sugR* mutants. Shown is one representative result or the mean of at least three independent experiments.

This adjustment of the total carbon uptake rate to similar levels independently if one or more sugars are available could be determined by (i) limited metabolic capacity or (ii) limited PTS-uptake capacity of the cell. In the first case, the mechanism could be reacting similarly as in the Pgi-deficient mutant to the levels of a metabolic intermediate which is common for the two substrates, *e.g*. glucose-6-P, and which would start to accumulate if the uptake rate is higher than the rate of substrate utilization. In the second case, the rapid adjustment of the sugar uptake could be caused by a competition of the two EII permeases for a limited

rate of the common PEP-EI-HPr phosphorylation cascade - when a second PTS substrate becomes available, the EII^{suc} has to share the HPr~P required for its activity with, in this case, EII^{glc} and thus both permeases would work at slightly reduced rates.

The growth rate of the sucrose plus maltose cultivated wild-type cells (0.46 ± 0.02 h^{-1}) was increased when compared to cultivations with sucrose as the sole carbon source (0.42 ± 0.02 h^{-1}) (Fig. 28, A). Similarly, the addition of glucose-6-P had a positive effect on the growth of the wild-type-derived *C. glutamicum* (pEKEx2*-*uhpT*) with sucrose or glucose (Fig. 28, B). The growth rates reached during cultivation with glucose plus glucose-6-P (0.42 ± 0.02 h^{-1}) or with sucrose plus glucose-6-P (0.46 ± 0.02 h^{-1}) were significantly higher than the growth rates with either glucose or sucrose alone (0.34 ± 0.02 h^{-1} and 0.41 ± 0.02 h^{-1}, respectively). As all those substrates are metabolized by the same pathways, the growth rate improvements demonstrated that the factor limiting a faster growth of *C. glutamicum* with glucose plus sucrose is not the metabolic capacity of the cell but the substrate uptake rate.

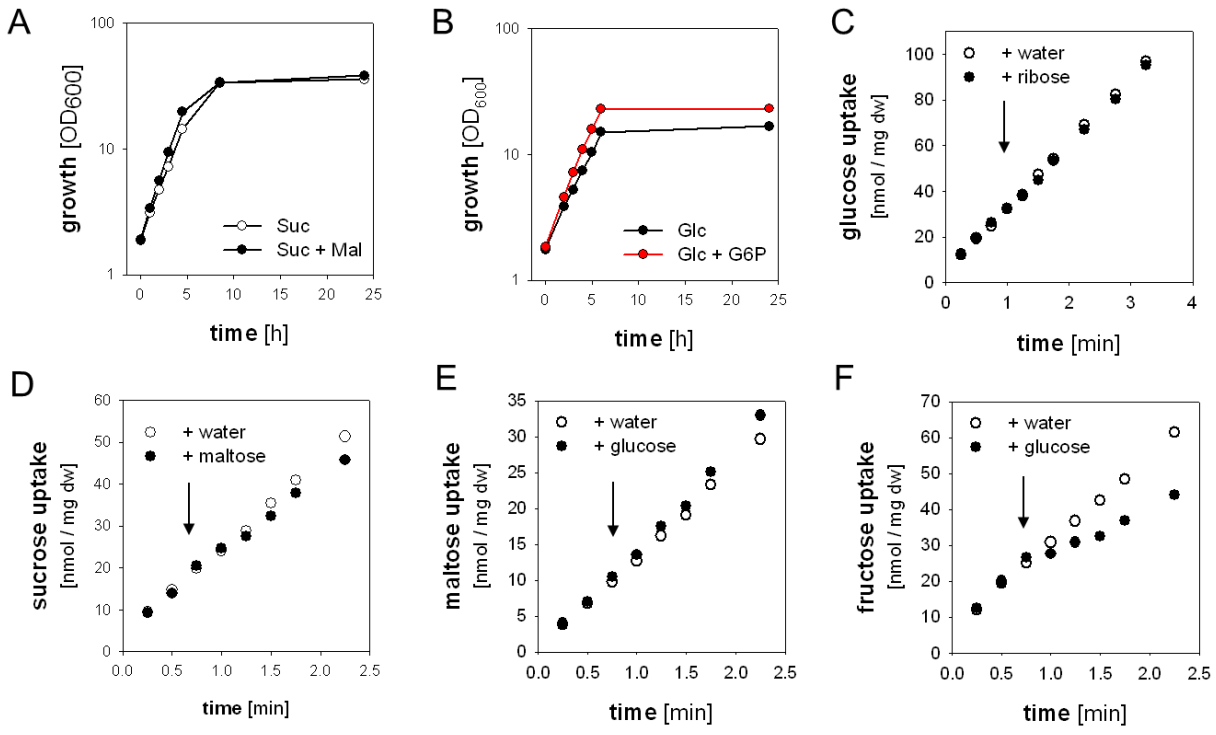


Figure 28: Co-utilization and uptake adjustment during cultivation on mixtures of PTS and of non-PTS substrates in *C. glutamicum:* growth of the wild-type on minimal medium with sucrose and sucrose plus maltose **(A)** or growth of *C. glutamicum* (pEKEx2*-*uhpT*) with glucose and glucose plus glucose-6-P **(B)**; effects of the addition of non-PTS substrates like ribose or on glucose **(C)** and sucrose **(D)** uptake in *C. glutamicum*. Effects of the addition of glucose on the non-PTS mediated uptake of maltose **(E)** or the PTS-mediated fructose uptake **(F)** in the wild-type. Shown is one representative result of three independent measurements. Arrows indicate the time point of addition of the second substrate.

Unlike the addition of sucrose, the addition of ribose had no effect on the glucose uptake in the wild-type, indicating that the cell was not responding to increased intermediates of the lower part of glycolysis (Fig. 28, D). The addition of maltose did not cause significant sucrose uptake inhibition in the wild-type, either (Fig. 28, C). Hence, the rapid sucrose uptake adjustment in the wild-type after glucose addition did not seem to respond to the increased flux towards glucose-6-P, either.

Further, the glucose addition reduced the PTS-mediated fructose uptake in the wild-type, as well: from 28.4 ± 1.5 to 13.8 ± 1.8 nmol/(min*mg dw) (Fig, 28). Though, addition of glucose had no effect on the uptake of the non-PTS substrate maltose (Fig. 28, D). These results were consistent with the hypothesis that the observed sugar uptake adjustment in the wild-type is caused by competition of the PTS permeases for a limited rate of the common phosphorylation cascade.

Taken together, a rapid uptake response to the addition of glucose is present not only in the *pgi*-deficient mutant but also in the wild-type. However, in contrast to *C. glutamicum* Δ*pgi*, the weaker effects in the wild-type did not correlate with accumulation of glycolytic intermediates but rather with the activation of a second PTS permease.

Response of *C. glutamicum* to addition of acetate – *C. glutamicum* can use acetate as a carbon source for growth. After uptake by the secondary transporter MctC, acetate is phosphorylated by the acetate kinase (Ack), converted by the phosphotransacetylase (Pta) to acetyl-CoA and can directly enter the TCA cycle. Though, similarly to what was shown for the cultivation on sucrose plus glucose, during co-utilisation of acetate and glucose the consumption rate for each of the two carbon sources in *C. glutamicum* is reduced to 50% of the rate observed during growth on either acetate or glucose alone, ensuring similar total carbon consumption rates in all three cases[48]. A *ptsG*-repression (*i. a.* by SugR) has been suggested as the reason for the glucose uptake reduction. However, here I have shown that the glucose uptake adjustment to acetate addition is again a rapid process and occurs in less than 15 sec (Fig. 29, A & B). In this time margin a transcriptional regulation seems unlikely. In fact, the rapid reduction of glucose uptake after acetate addition was still present in *C. glutamicum* Δ*sugR* (Fig. 29, C).

The glucose uptake adjustment was not observed in the Ack- or Pta-deficient mutants, either, indicating that the formation of acetyl-CoA is required for the observed regulatory response (Fig. 29, D&E). Furthermore, this result showed that the rapid glucose uptake reduction by acetate addition was not caused by pH-stress due to acidification of the cytoplasm.

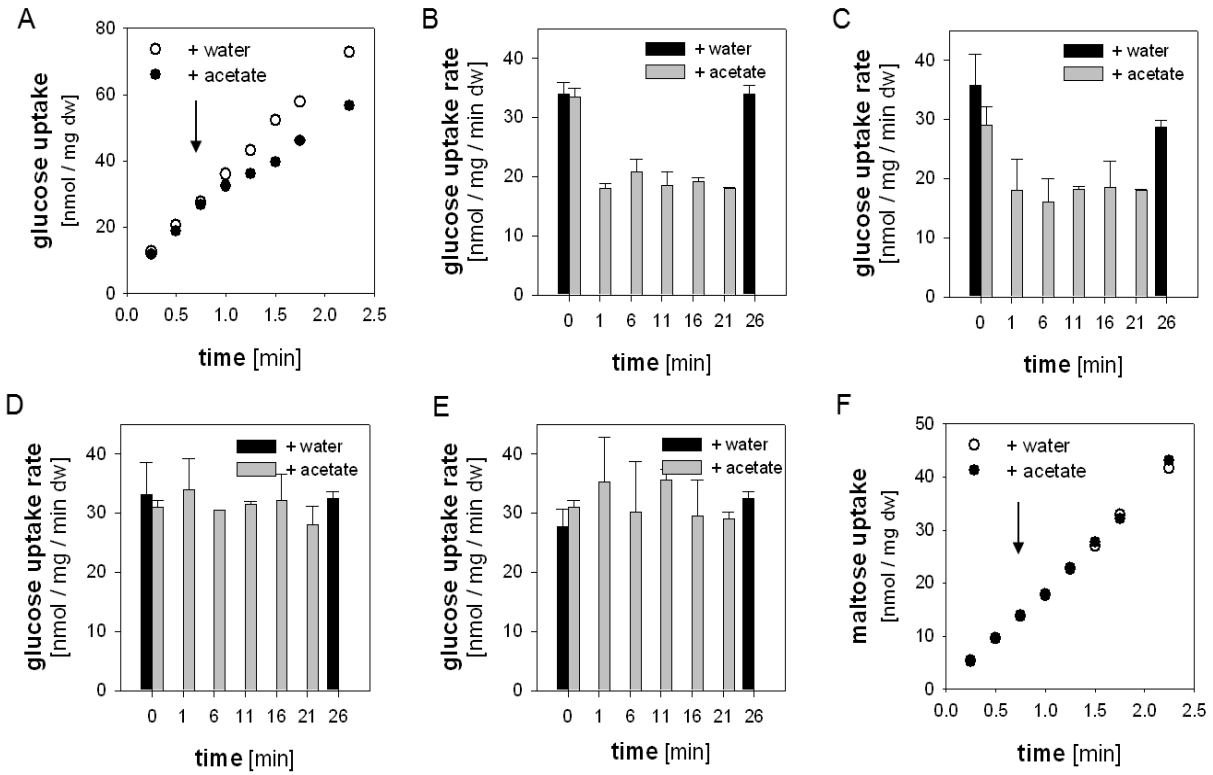


Figure 29: Effects by the addition of acetate on the glucose uptake of *C. glutamicum*: Glucose uptake after addition of 15mM acetate in glucose cultivated wild-type cells **(A & B)**, *C. glutamicum* Δ*sugR* **(C)**, *C. glutamicum* Δ*ack* **(D)**, *C. glutamicum* Δ*ptsG* **(E)**. Maltose uptake after or without addition of acetate in sucrose cultivated *C. glutamicum* and *C. glutamicum* Δ*pgi* cells **(F)**. The results are the mean of at least three independent measurements.

The sucrose uptake in *C. glutamicum* and *C. glutamicum* Δ*pgi* was rapidly reduced after addition of acetate, as well (Fig 30). However, in contrast to the drastic difference between the two strains after glucose addition, the reaction to acetate of the Pgi-deficient mutant was only marginally stronger compared to the wild-type: reduction from 19.9 ± 1.7 to 12.1 ± 0.5 and from 20.1 ± 1.2 to 13.2 ± 0.8 nmol/(min*mg dw), respectively. Furthermore, the absence of EII^{glc} did not abolish the sucrose uptake reduction after acetate addition in *C. glutamicum* Δ*pgi* Δ*ptsG*, indicating different regulatory mechanisms for the sucrose uptake reduction after glucose and after acetate addition in *C. glutamicum* Δ*pgi* (Fig. 30).

The addition of acetate had no effect on the maltose uptake in *C. glutamicum*, indicating that the target of the acetate inhibition was specifically the PTS-mediated glucose and sucrose uptake (Fig, 29, F).

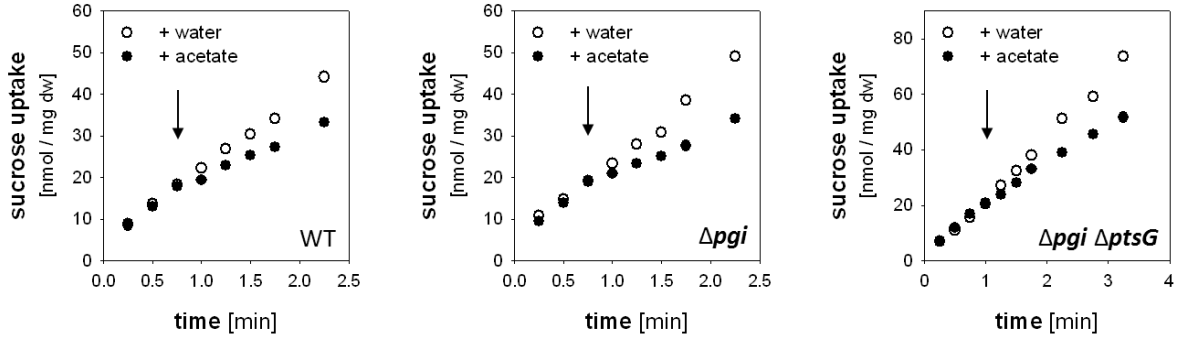


Figure 30: Effects by the addition of acetate on the sucrose uptake of *C. glutamicum, C. glutamicum* Δ*pgi and C. glutamicum* Δ*pgi* Δ*ptsG.* The cells were pre-cultivated on sucrose as a sole carbon source and measured at similar ODs after addition of 15mM acetate.

These results showed that the suggested previously transcriptional regulation of *ptsG* is not the only and probably not the primary reason for the reduced glucose uptake rates during cultivation of *C. glutamicum* with glucose plus acetate. Interestingly, the conclusion from my last chapter that the rapid glucose or sucrose uptake inhibition is dependent specifically on the activation of a second PTS permease did not seem in agreement with those observations. However, in contrast to the non-PTS substrates which did not cause glucose or sucrose uptake inhibition, acetate is a gluconeogenetic substrate entering the TCA cycle directly at the point where the pyruvate coming from glycolysis would be further utilized. Thus, it was possible that acetate competes with and thus reduces the pyruvate flux toward the TCA cycle and thereby changes the PEP/pyruvate flux ratio in the cell. If this was the case, further gluconeogenetic substrates, *e.g.* pyruvate itself, should result in similar uptake inhibition.

Effects of the addition of pyruvate on sugar uptake in *C. glutamicum* strains – Pyruvate is transported in *C. glutamicum* also by the monocarboxylic acid MFS transporter MctC [55]. Once in the cell, pyruvate directly enters the end of glycolysis and could be further metabolised via the TCA cycle or used for gluconeogenesis.

The wild-type growth rates during cultivation with pyruvate (0.40 ± 0.02 h^{-1}) or sucrose (0.42 ± 0.02 h^{-1}) were not lower compared to the mixed substrate cultivation with pyruvate plus

sucrose (0.40 ± 0.02 h^{-1}), indicating adjustment of the total carbon consumption rates (Fig. 31, A). Indeed, addition of pyruvate decreased the sucrose uptake of the wild-type cells within 15 sec (Fig. 31, C).

Pyruvate reduced the sucrose uptake in *C. glutamicum* Δ*pgi*, as well (Fig. 31, C). However, similarly as described above for acetate and unlike in the case of glucose addition, the extent of sucrose uptake reduction after pyruvate addition was similar in the wild-type and the Δ*pgi* mutant: from 19.6 ± 1.1 to 10.9 ± 0.8 and form 19.2 ± 1.2 to 10.5 ± 0.8 nmol/(min*mg dw). Further, the absence of EII^{glc} had no effect on the sucrose uptake inhibition by pyruvate (Fig. 31, C), so that this rapid sucrose uptake reduction is not caused by the response mechanism causing the rapid sucrose uptake inhibition after glucose addition in the *pgi* mutant.

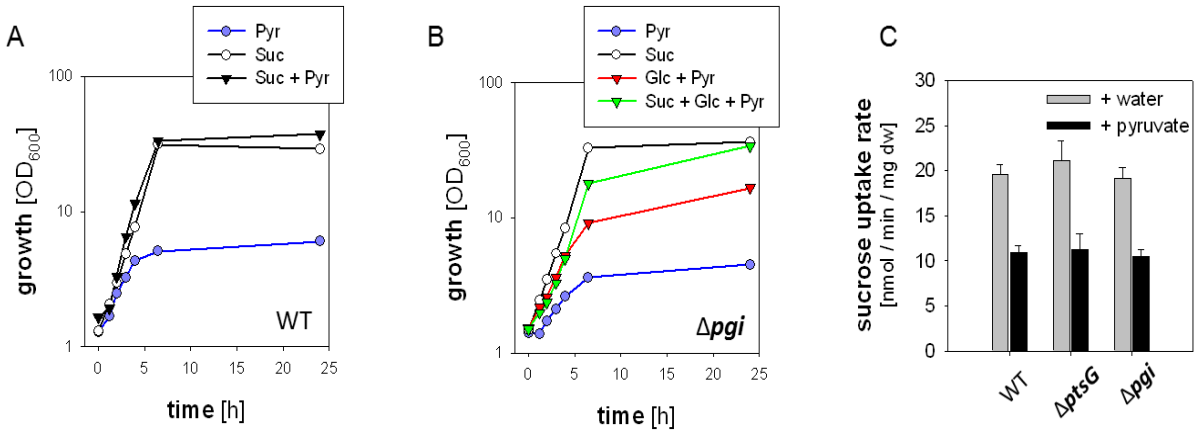


Figure 31: Effects by the addition of pyruvate in *C. glutamicum* and *C. glutamicum* Δ*pgi*. Growth of wild-type **(A)** and *C. glutamicum* Δ*pgi* **(B)** on pyruvate, sucrose, sucrose plus pyruvate, glucose plus pyruvate and sucrose plus glucose plus pyruvate 1% [w/v] each; **(C):** Rapid sucrose uptake inhibition in wild-type, *C. glutamicum* Δ*pgi* and *C. glutamicum* Δ*pgi* measured at similar OD_{600} after addition of 15mM pyruvate. The change of the sucrose uptake rate was within 15 sec. The results are one representative or the mean of three measurements.

It has been shown that during glucose-6-P stress in *E. coli* the addition of pyruvate has a lethal effect and causes cell lysis[114]. However, the presence of pyruvate as carbon source did not affect negatively and even improved the poor growth of *C. glutamicum* Δ*pgi* with glucose and glucose plus sucrose, respectively (Fig. 31, B).

In conclusion, the response to addition of pyruvate by the glucose and sucrose uptake in *C. glutamicum* was very similar to the effects caused by acetate addition, indicating that a decrease of the PEP/pyruvate ratio is able to cause rapid inhibition of the PTS activity.

3.2.4.3. Effects of the EII^{suc} / EII^{glc} ratio on the PTS activity:

The studies of the PTS-activity adjustment in the wild-type after addition of a second PTS substrate indicated that the effects could be caused by competition of the EII permeases for limited rate of the PEP-EI-HPr phosphorylation cascade. If this is the case, changes in the EII^{suc} / EII^{glc} ratio in the cell should affect the glucose and sucrose uptake adjustment after activation of the second permease.

Generation of tagged EII^{suc} – A C-terminally tagged version of EII^{suc} was created. The used Rho1D4 (short Rho) tag consists of the last 9 amino acids (T-E-T-S-Q-V-A-P-A) of the intracellular C-terminus of bovine rhodopsin and is reported to serve as a highly specific purification tag for membrane proteins[52]. The tag was connected to the C-terminus of EII^{suc} via a G-S-S-G linker. The gene encoding for the C-terminally tagged EII^{suc} variant was cloned in the vector pEKEx2, where it is under the control of the IPTG-inducible *lac* promoter.
As shown on figure 35, the tagged EII^{suc} version was functional and restored the growth of *C. glutamicum* Δ*ptsS* (pEKEx2-*ptsS_Rho*) with sucrose as a carbon source and this was not observed for the control strain *C. glutamicum* Δ*ptsS* (pEKEx2). The promoter of the pEKEx2-*ptsS_Rho* plasmid seemed to have high basal expression as complementation was observed even without IPTG induction. Increasing induction of *ptsS_Rho* by IPTG addition did not change significantly the growth or sucrose uptake ability of the strain, even though the EII^{suc} amount in the cell were increased as shown by Western blot analysis (Fig. 32, B & C). The band corresponding to the expected size of 70 kDa for EII^{suc}_Rho was more intense in the cells induced with higher IPTG concentrations and as expected was found predominately in the membrane fraction.
However, the level of IPTG induction had a significant effect on the rapid sucrose uptake reduction by glucose in *C. glutamicum* Δ*ptsS* (pEKEx2-*ptsS_Rho*). Even though the cells had similar growth and sucrose uptake rates during cultivation with sucrose as a sole carbon source (Fig 32, A), the sucrose uptake of the cells cultivated in absence of IPTG was reduced by the addition of glucose, whereas the cells which had induced *ptsS_Rho* expression by the presence of 0.5mM IPTG were less affected by the glucose addition (Fig 32, C).

To ensure that the positive effects by the *ptsS_Rho* overexpression were not caused by the added affinity tag, the native *ptsS* gene was also ectopically expressed in *C. glutamicum* Δ*ptsS* (pEKEx2-*ptsS*). No differences between the effects of a *ptsS* or *ptsS_Rho* overexpression could be observed both in terms of growth and sucrose uptake complementation or IPTG induction-dependent alleviation of the sucrose uptake reduction by glucose addition (SupFig. 8).

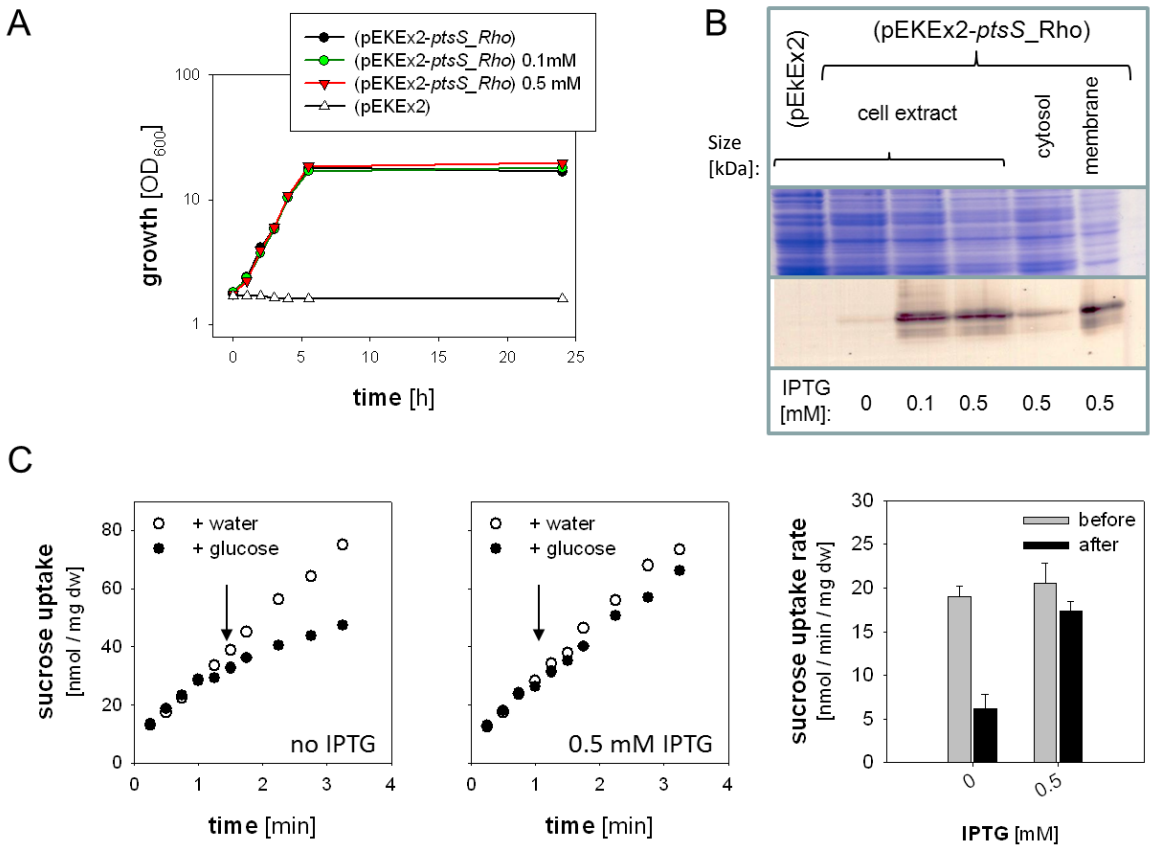


Figure 32: Analysis of the created Rho-tagged EII^{suc}: **(A):** Growth and **(B):** Western blot analysis of *C. glutamicum* Δ*ptsS* (pEKEx2) and *C. glutamicum* Δ*ptsS* (pEKEx2-*ptsS_Rho*) cells cultivated with sucrose (2% [w/v]) as a sole carbon source. Expression of *ptsS* and *ptsS_Rho* was induced with 0mM, 0.1 mM and 0.5mM IPTG. **(C):** Sucrose uptake of *C. glutamicum ΔptsS* (pEKEx2-*ptsS_Rho*) cells cultivated with sucrose (S; 1% [w/v]) in presence of no IPTG and 0.5mM IPTG after addition of glucose. Time point of sucrose addition is marked with an arrow.

These results indicated two important aspects: (i) the V_{max} value of the sucrose uptake is not affected by increased EII^{suc} amounts per cell, indicating that the HPr-P amount in the cell might be the factor limiting the V_{max} of the PTS permeases; (ii) increase in the amount of a EII permease which is object of negative regulation after the addition of second PTS substrate is able to reduce the level of uptake reduction.

Overexpression of *ptsS* in *C. glutamicum* Δ*pgi* – The rapid sucrose uptake inhibition by glucose in *C. glutamicum* Δ*pgi* correlated with drop of the HPr~P / HPr ratio. Hence, a competition of the PTS permeases for limited rate of the phosphorylation cascade was possible in this process, as well. Additionally, the analysis of EII^{suc} and EII^{glc} was interesting since a direct biochemical regulation of the EII permeases was not excluded. The effects of the overexpression of *ptsS* as well as *ptsS_Rho* in the *pgi* mutant were studied, as the C-terminal tag could obstruct potential regulatory interactions.

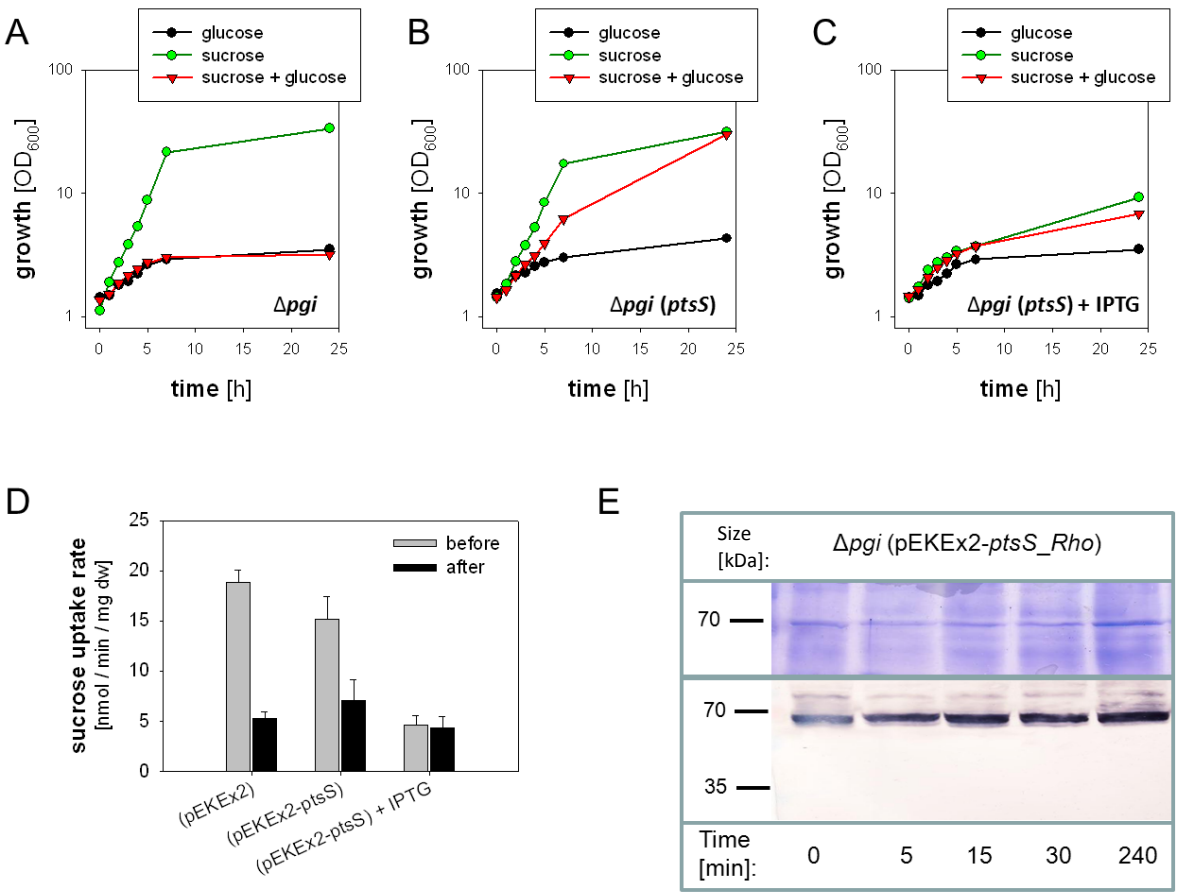


Figure 33: Effects of overexpression of *ptsS* in *C. glutamicum Δpgi*: Growth of *C. glutamicum* Δ*pgi* (pEKEx2) (A) and *C. glutamicum* Δ*pgi* (pEKEx2-*ptsS*) (non-induced (B) or induced with 0.2 mM IPTG (C)) with glucose (2% [w/v]), sucrose (2% [w/v]) or sucrose plus glucose (1 + 1% [w/v]); Glucose uptake inhibition by glucose addition (D) in *C. glutamicum* Δ*pgi* (pEKEx2) and *C. glutamicum* Δ*pgi* (pEKEx2-*ptsS*). Western blot analysis of EII^{suc} from sucrose cultivated *C. glutamicum* Δ*pgi* (pEKEx2-*ptsS_Rho*) 0, 5, 15, 30 and 240 min after glucose addition (E).

Surprisingly, the induced expression of *ptsS* in *C. glutamicum Δpgi* (pEKEx2-*ptsS*) led to poor growth of the strain with sucrose as a sole carbon source (Fig. 33, C). In fact, when expression of *ptsS* was induced with IPTG *C. glutamicum* Δ*pgi* (pEKEx2-*ptsS*) had similar growth rate on sucrose as on sucrose plus glucose (0.10 ± 0.01 and 0.11 ± 0.01 h^{-1},

respectively). This was not the case in the control strain *C. glutamicum Δpgi* (pEKEx2), which grew normally with sucrose (Fig. 33, A). The sucrose uptake rate of the sucrose + IPTG cultivated *C. glutamicum Δpgi* (pEKEx2-*ptsS*) cells was aslo drastically reduced (4.3 ± 0.4 nmol/(min*mg dw) (Fig. 33, D). This surprising phenotype could be reproduced with three independently generated *C. glutamicum Δpgi* (pEKEx2-*ptsS*) strains indicating that it was not caused by an unknown additional genome mutation. Furthermore, the *ptsS* overexpression had no negative effect in the wild-type. Thus, the combination of plasmid-encoded and chromosomal *ptsS* was not the reason for the negative effects, either.

Nonetheless, when the *ptsS* expression was not additionally induced by IPTG and relied on the observed previously low basal expression of pEKEx2-*ptsS* the growth of *C. glutamicum* Δ*pgi* (pEKEx2-*ptsS*) with sucrose was not negatively affected (Fig. 33, B). What is more, without IPTG induction the strain *C. glutamicum Δpgi* (pEKEx2-*ptsS*) had an improved growth with sucrose plus glucose compared to the control strain *C. glutamicum Δpgi* (pEKEx2) (0.2 ± 0.01 h^{-1} or 0.14 ± 0.01 h^{-1}, respectively). The rapid sucrose uptake inhibition after glucose addition in the non-induced sucrose cultivated *C. glutamicum Δpgi* (pEKEx2-*ptsS*) cells was indeed slightly alleviated compared to the *C. glutamicum Δpgi* (pEKEx2) cells (Fig. 33, D).

Overexpression of *ptsS_Rho* led to similar induction dependent inhibition of the growth of *C. glutamicum Δpgi* on sucrose like the expression of *ptsS*. Thus, to study if an enhanced EII^{suc} degradation might be involved in the rapid sucrose uptake inhibition I studied cell extracts of sucrose cultivated *C. glutamicum Δpgi* (pEKEx2-*ptsS_Rho*) cells after addition of glucose for a period of 240 min. The detected EII^{suc} bands did not indicate signs of increased degradation for the entire period of 240 min (Fig. 33, E).

Overexpression of *ptsS* or *ptsG* in *C. glutamicum* Δ*scrB* – An overexpression of *ptsS* in *C. glutamicum* Δ*scrB* might be expected to increase the glucose uptake inhibition after addition of sucrose. Indeed, the growth of *C. glutamicum ΔscrB* (pEKEx2-*ptsS*) on sucrose plus glucose was even more drastically slowed down when compared to growth of the control strain *C. glutamicum ΔscrB* (pEKEx2) (Fig. 34, A). Further, the sucrose addition led immediately to an almost complete block of the glucose uptake rate in *C. glutamicum ΔscrB* (pEKEx2-*ptsS*) (2.5 ± 0.7 nmol/(min*mg dw) whereas this effect was less dramatic in the control strain *C. glutamicum ΔscrB* (pEKEx2) (9.3 ± 0.6 nmol/(min*mg dw) (Fig. 34, C).

On the other hand, the overexpression of plasmid-encoded *ptsG* improved the growth rate of *C. glutamicum ΔscrB* (pEKEx2-*ptsG_HIS*) with sucrose plus glucose compared to the control strain *C. glutamicum ΔscrB* (pEKEx2): from 0.10 ± 0.01 to 0.21 ± 0.01 h^{-1}, respectively (Fig. 34, A). The results of the rapid glucose uptake inhibition by sucrose addition in glucose cultivated *C. glutamicum ΔscrB* (pEKEx2-*ptsG_HIS*) cells were consistent with the observed improved growth rate. Whereas sucrose inhibited rapidly the glucose uptake in *C. glutamicum ΔscrB* (pEKEx2), the negative effect of sucrose addition was almost abolished in *C. glutamicum ΔscrB* (pEKEx2-*ptsG_HIS*) (Fig. 34, C).

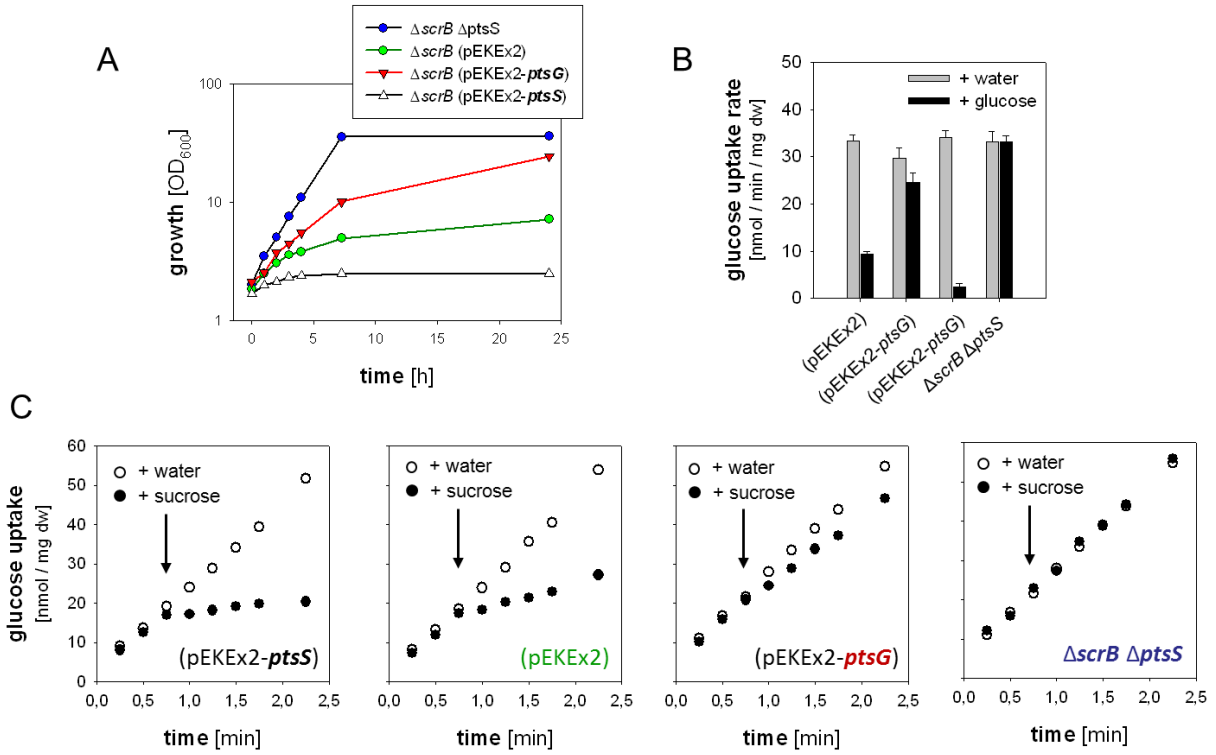


Figure 34: Effects of deregulated expression of *ptsS* or *ptsG* in *C. glutamicum ΔscrB*: Growth on sucrose plus glucose **(A)** and rapid glucose uptake inhibition after sucrose addition **(B & C)** of *C. glutamicum* Δ*scrB* (pEKEx2), *C. glutamicum* Δ*scrB* (pEKEx2-*ptsS*), *C. glutamicum* Δ*scrB* (pEKEx2-*ptsG_HIS*) and *C. glutamicum* Δ*scrB* Δ*ptsS*. Time point of sucrose addition is marked with an arrow.

Hence, the extent of glucose uptake reduction by sucrose addition in *C. glutamicum ΔscrB* is directly dependent on the EII^{glc}/EII^{suc} ratio in the cell. Overexpression of *ptsG,* which is the object of inhibition, diminished the negative effect of sucrose on both growth and rapid glucose uptake ability and the opposite, overexpression of *ptsS* in *C. glutamicum ΔscrB* (pEKEx2-*ptsS*) increased them.

3.3. Step 2: SugR-dependent transcriptional regulation

3.3.1. Mechanism of SugR activation:

***ptsS*-mRNA amounts after glucose addition** – The rapid sucrose uptake reduction after addition of glucose in *C. glutamicum* Δ*pgi* did not require transcriptional or translational processes. Though, the long-term adaptation of the Δ*pgi*-mutant to the presence of glucose was shown to include repression of *ptsS*-transcription by SugR. As shown previously and further confirmed here by Northern blot and real-time PCR analysis, the addition of glucose drastically reduced the *ptsS*-mRNA amounts in the Δ*pgi* mutant whereas no such effect was present in the wild-type (Fig. 37 & 38). This transcriptional regulation was dependent on the global PTS-repressor SugR as no changes after glucose addition were observed in the SugR-deficient strains *C. glutamicum* Δ*sugR* or *C. glutamicum* Δ*pgi* Δ*sugR*. As expected, the *ptsS*-mRNA amounts were not changed after glucose addition in the EII^{glc}-deficient strains *C. glutamicum* Δ*ptsG* and *C. glutamicum* Δ*pgi* Δ*ptsG,* either.

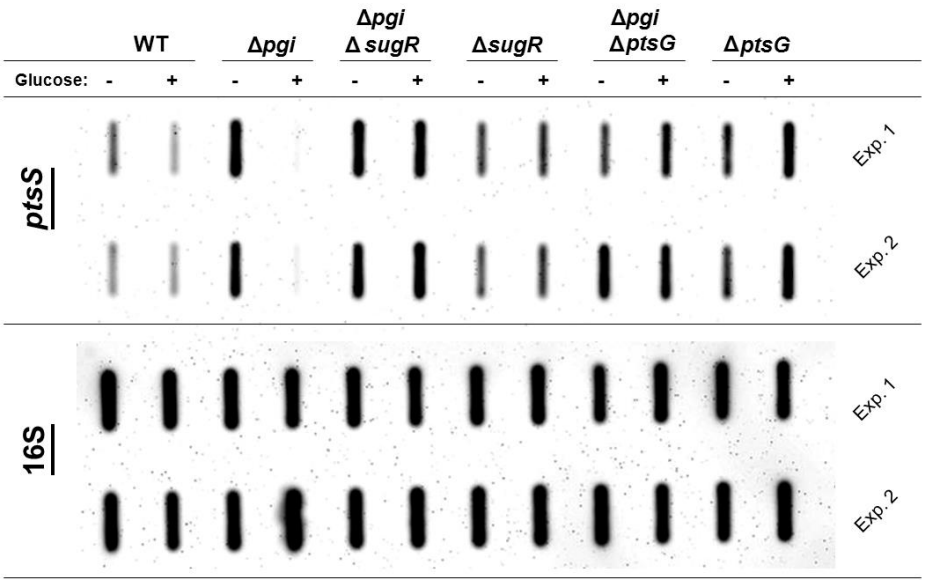


Figure 37: Slot blot analysis of the *ptsS*-mRNA amounts in *C. glutamicum, C. glutamicum Δpgi, C. glutamicum ΔsugR, C. glutamicum Δpgi ΔsugR, C. glutamicum ΔptsG* and *C. glutamicum Δpgi ΔptsG* cells cultivated in MM with sucrose (1 % [w/v]) or sucrose plus glucose (indicated with +; 1 % [w/v] each) as substrates. The probes were taken during the exponential growth phase, 4h after inoculation in the respective substrates. Shown are results of two of at least three independent experiments.

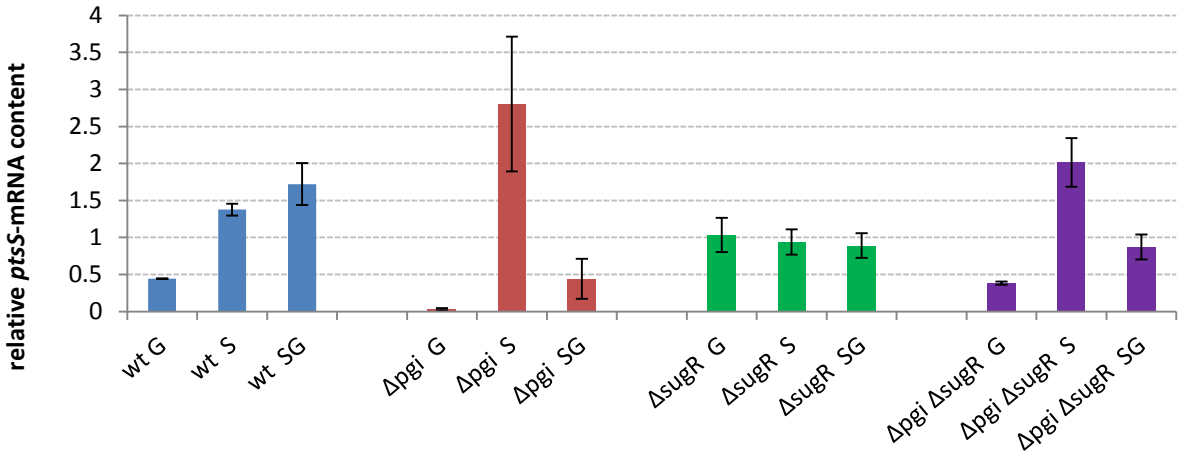


Figure 38: Real Time-PCR analysis of the *ptsS*-expression in *C. glutamicum*, *C. glutamicum Δpgi*, *C. glutamicum ΔsugR* and *C. glutamicum Δpgi ΔsugR* cells cultivated in MM with glucose (G; 2 % [w/v]), sucrose (S; 2 % [w/v]), sugrose plus glucose (SG; 1 % [w/v] each), maltose (M; 2 % [w/v]) or sucrose plus maltose (SM; 1 % [w/v] each) as a substrate. The values represent the relative content compared to a cDNA-pool of all used samples. As a control the amounts of 16S were measured, resulting in similar amounts.

CAT-assays for the *ptsS*-promoter activity in *C. glutamicum* strains – To investigate whether the lower *ptsS*-mRNA amounts were caused by inhibition of the transcriptional initiation or by induced mRNA degradation, as it is known for the sugar-P stress response in *E.coli* [74], the corresponding *ptsS*-promoter activity was determined using the chloramphenicol acetyltransferase (CAT) reporter-gene system.

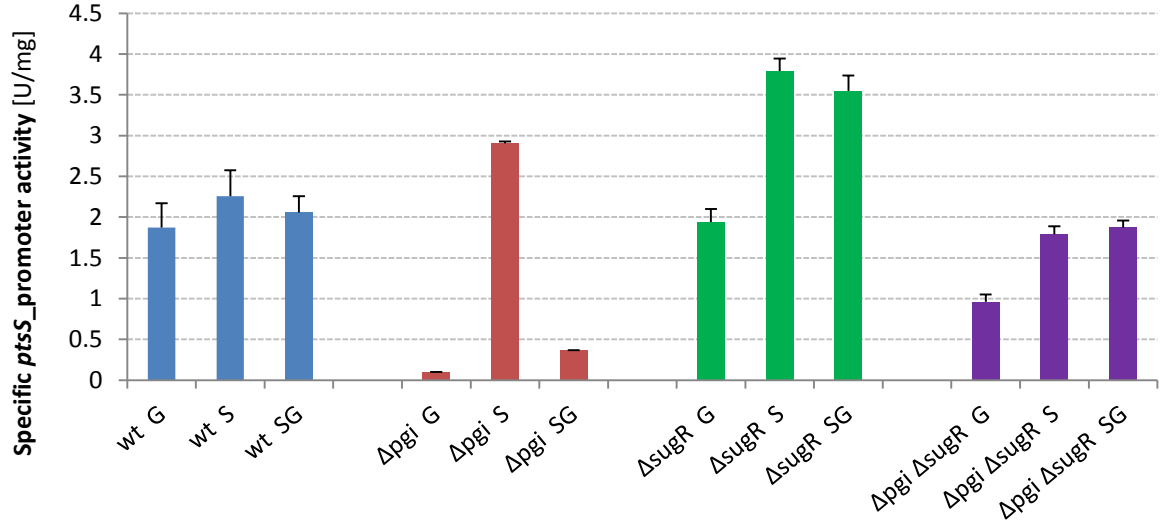


Figure 39: CAT-assay for the *ptsS*-promoter activity in *C. glutamicum*, *C. glutamicum Δpgi*, *C. glutamicum ΔsugR* and *C. glutamicum Δpgi ΔsugR* cells carrying the vector (pET-PR*ptsS*) and cultivated in MM with sucrose (S; 2 % [w/v]) or sugrose plus glucose (SG; 1 % [w/v] each) as substrates. For the assay each of the strains was transformed with the pET2-PR-*ptsS* plasmid, carrying *cat* under a *ptsS*-promoter region.

The strains *C. glutamicum, C. glutamicum* Δ*pgi, C. glutamicum* Δ*sugR* and *C. glutamicum* Δ*pgi* Δ*sugR* were transformed with the vector pET2-PR-*ptsS*, carrying the *cat* gene under the control of the *ptsS*-promoter region. The growth of the strains remained unchanged by the presence of the vector. Cells were harvested during the exponential growth phase and CAT-assays were performed in order to determine the *ptsS*-promoter activity.

Indeed, the *C. glutamicum* Δ*pgi* (pET2-PR-*ptsS*) cells cultivated on sucrose plus glucose had strongly diminished *ptsS*-promoter activity compared to the cultivated only with sucrose cells. This was not observed in the wild-type or *C. glutamicum* Δ*pgi* Δ*sugR* (pET2-PR-*ptsS*) strains (Fig. 39). These results were correlating with the observed previously *ptsS*-mRNA levels and gave a strong indication that in the Δ*pgi* mutant the presence of glucose activates the binding of SugR to the *ptsS*-promoter region thereby inhibiting *ptsS*-expression.

EMSA-analysis of SugR regulation – The transcriptional repressor SugR regulates all PTS genes as well as several genes of the central metabolism in *C. glutamicum* [58-60]. However, there is some discrepancy about the regulation of the SugR-binding to DNA. Fructose-6-P has been described as inhibitor of SugR-binding to the *ptsG*-promoter region [58]. On the other hand, later works revealed fructose-1-phosphate but not fructose-6-P as highly efficient, negative effector of SugR-binding to the *ptsI*- and *gapA*-promoters [60, 115]. Hence, the actual effector of SugR was not clear and it was even possible that the binding of SugR to different promoter regions is regulated by different metabolites.

To understand how the addition of glucose activates the SugR-dependent repression of *ptsS* in *C. glutamicum* Δ*pgi in vitro* DNA-binding studies were conducted. A C-terminally HIS-tagged SugR has been heterologously produced in *E. coli* cells and purified. Then the SugR-binding to the *ptsS*-promoter region in the presence of different effector candidates has been analyzed via electrophoretic mobility shift assays (EMSA). Most of the EMSA works were performed together with Vera Kerstens as part of her Bachelor Thesis[116].

From the studied substrates (glucose-1-P; glucose-6-P; fructose-1-P; fructose-1,6-BP; fructose-6-P; sucrose-6-P) only fructose-1-P and to a lower extent fructose-6-P reduced the binding of SugR to the *ptsS*-promoter region under the tested conditions (Fig. 40). The inhibiting effects of fructose-6-P required at least 10-fold higher concentrations than fructose-1-P. The binding of SugR to the *ptsG* and *pyk*-promoters was also inhibited by those two metabolites[116].

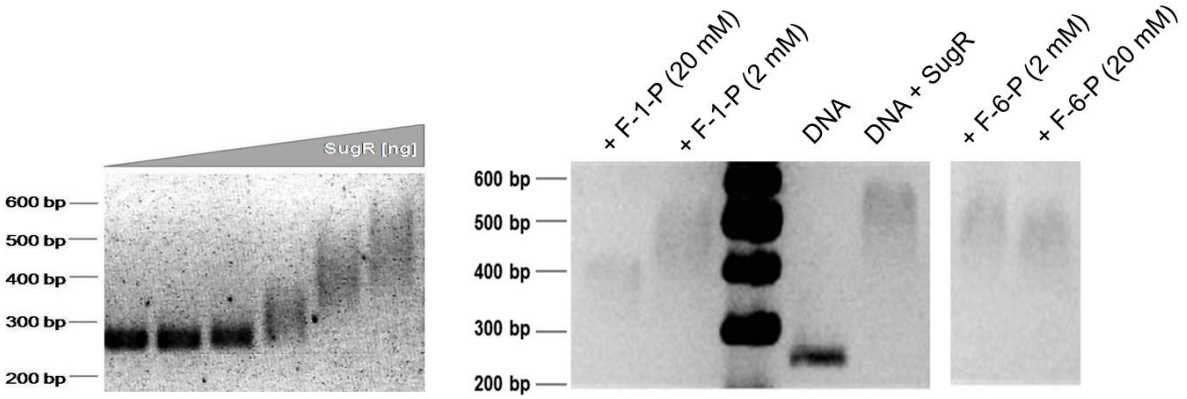


Figure 40: EMSA analysis of the SugR-binding to the *ptsS*-promoter region: Binding of increasing amounts of SugR to *ptsS*-promoter DNA (A); SugR-binding to ptsS-promoter DNA in presence of 2 or 20 mM fructose-1-P or fructose-6-P (B). As a negative control no SugR was added and as a positive control no inhibitor was added.

These results suggested fructose-1-P as the main inhibitor of the SugR-binding to the *ptsS*-promoter. As in the process of utilization sucrose leads to the formation of fructose-1-P it now seemed possible that the rapid sucrose uptake inhibition leads in an inducer exclusion-like manner to the reduction of fructose-1-P formation and thus activation of the SugR binding to its DNA targets. Additionally, in a Pgi-deficient background the formation of fructose-6-P is expected to be reduced, as well.

3.3.2. Response to non-PTS substrates:

***ptsS*-expression after maltose or glucose-6-P addition** – It was shown hitherto that maltose is able to replace glucose as a negative effector in the process of rapid sucrose uptake inhibition in *C. glutamicum Δpgi*. However, despite that *C. glutamicum* Δ*pgi* grew similarly poor with glucose or maltose as sole carbon sources, the addition of maltose to sucrose cultivated *C. glutamicum* Δ*pgi* cells caused lower growth reduction compared to the glucose addition. To understand the reason for that difference I analyzed the second part of the effects observed in *C. glutamicum Δpgi* after glucose addition: the transcriptional regulation by the repressor SugR.

In contrast to glucose, the addition of maltose did not cause reduction of the *ptsS*-mRNA amounts in sucrose cultivated *C. glutamicum* Δ*pgi* cells (Fig. 41). As expected the *ptsS*-promoter activities during cultivation on sucrose or sucrose plus maltose were comparable,

as well (Fig. 42). Similarly as maltose, the addition of glucose-6-P to sucrose cultivated *C. glutamicum* Δ*pgi* (pEKEx2*-*uhpT*) cells also did not lead to *ptsS*-repression (Fig. 41, B).

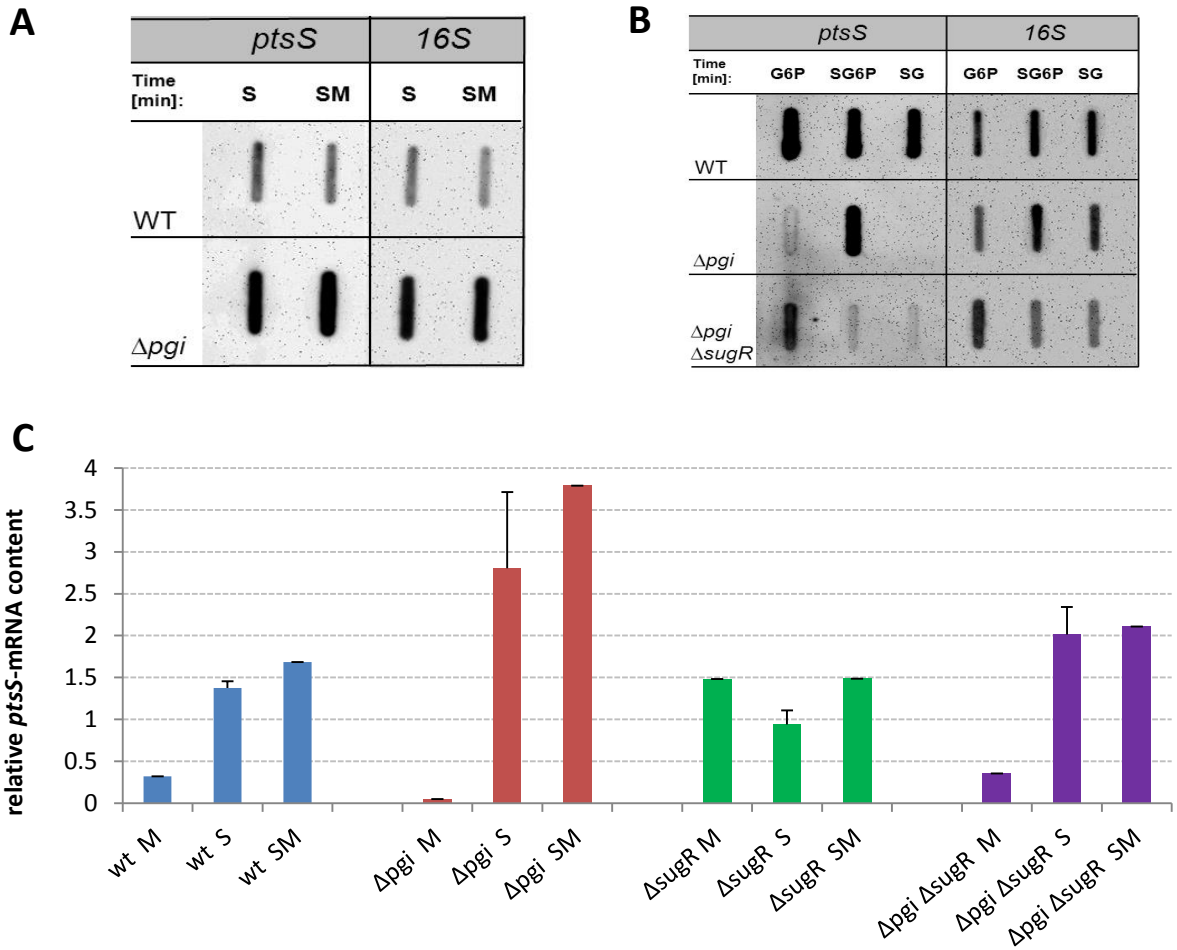


Figure 41: Effects of maltose on the *ptsS*-mRNA in *C. glutamicum* strains: Slot blot analysis of sucrose or sucrose plus maltose cultivated *C. glutamicum* Δ*pgi* cells (A) or glucose-6-P, sucrose plus glucose-6-P cultivated *C. glutamicum* Δ*pgi* (pEKEx2*-*uhpT*) cells (B). Real Time-PCR analysis of the *ptsS*-transcript in *C. glutamicum, C. glutamicum Δpgi, C. glutamicum ΔsugR* and *C. glutamicum Δpgi ΔsugR* cells cultivated in MM with glucose (G; 2 % [w/v]), sucrose (S; 2 % [w/v]), sugrose plus glucose (SG; 1 % [w/v] each), maltose (M; 2 % [w/v]) or sucrose plus maltose (SM; 1 % [w/v] each) as a substrate(C). The values represent the relative content compared to a cDNA-pool of all used samples. As a control the amounts of 16S were measured, resulting in comparable amounts.

These findings suggested that despite causing the same rapid sucrose uptake inhibition in *C. glutamicum* Δ*pgi* as glucose, the addition of non-PTS substrates like maltose or glucose-6-P did not lead to the later SugR-dependent transcriptional regulation of *ptsS*. As the formation of fructose-1-P should be reduced in both cases it seemed that either SugR is activated by a different mechanism or additional regulators are involved.

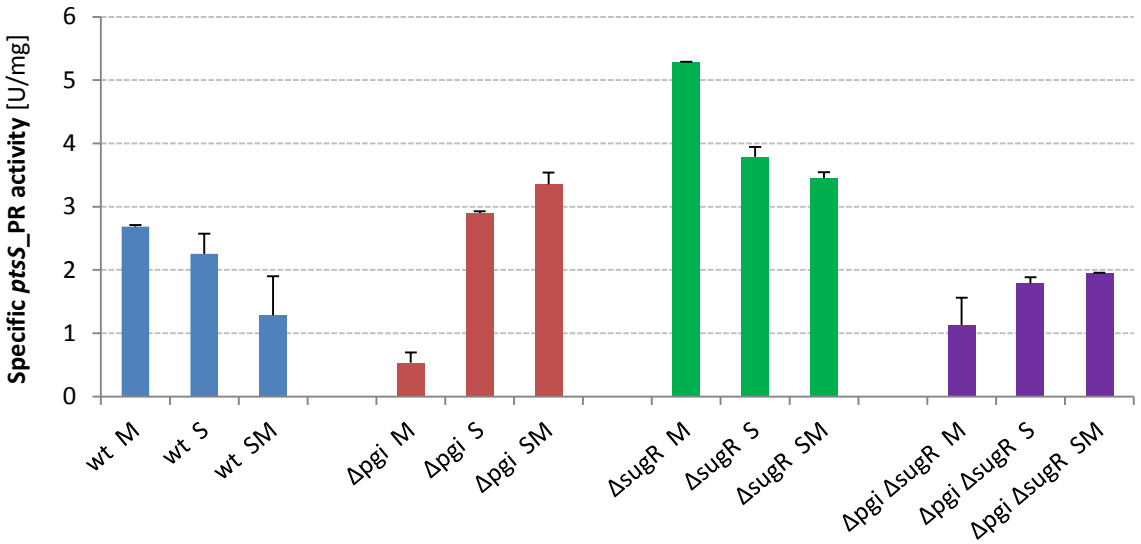


Figure 42: CAT-assay for the *ptsS*-promoter activity in *C. glutamicum, C. glutamicum Δpgi, C. glutamicum ΔsugR* and *C. glutamicum Δpgi ΔsugR* cells carrying the vector (pET-PR*ptsS*) and cultivated in MM with glucose (G; 2 % [w/v]), sucrose (S; 2 % [w/v]), sugrose plus glucose (SG; 1 % [w/v] each), maltose (M; 2 % [w/v]) or sucrose plus maltose (SM; 1 % [w/v] each) as a substrate.

Involvement of SugR in the maltose uptake regulation – *C. glutamicum* Δ*sugR* grows normally with all PTS substrates, like *e.g.* sucrose, but has significantly reduced growth rate (0.26 ± 0.02 h^{-1}) during cultivation with maltose as a sole carbon source [84]. This observation is surprising as SugR in not known to bind to the promoter regions of any of the genes encoding proteins of the maltose uptake system or essential for maltose utilization.

Complementation experiments with the strain *C. glutamicum* Δ*sugR* (pEKEx2-*sugR*) showed that the growth and maltose uptake reduction observed in the *C. glutamicum* Δ*sugR* mutant was caused specifically by the absence of SugR (Fig. 43, C & D).

Further, the reduced maltose utilization ability of the SugR-deficient strain correlated well with changes in the maltose uptake rates. As shown on Figure 43, the wild-type had an uptake rate of 14.3 ± 1.4 nmol/(min*mg DW) but the lack of *sugR* led to almost 50% lower maltose uptake ability in *C. glutamicum* Δ*sugR* and *C. glutamicum* Δ*pgi* Δ*sugR* (7.7 ± 1.2 and 7.5 ± 1.7 nmol/(min*mg DW), respectively). During cultivation on maltose the maltose uptake rate of the *Δpgi* mutant was reduced to comparable levels of 6.6 ± 1.1 nmol/(min*mg DW). However, the negative effects on the maltose uptake by the absence of Pgi or SugR were additive and thus probably independent processes as the maltose uptake rate of the maltose incubated *C. glutamicum Δpgi ΔsugR* cells was reduced even further to 2.8 nmol/(min*mg DW) (Fig. 43, B). Nonetheless, in all cases a significant maltose uptake rate

was still present in the cell, which ensures the function of maltose as an effector for sucrose uptake inhibition in *C. glutamicum* Δ*pgi*.

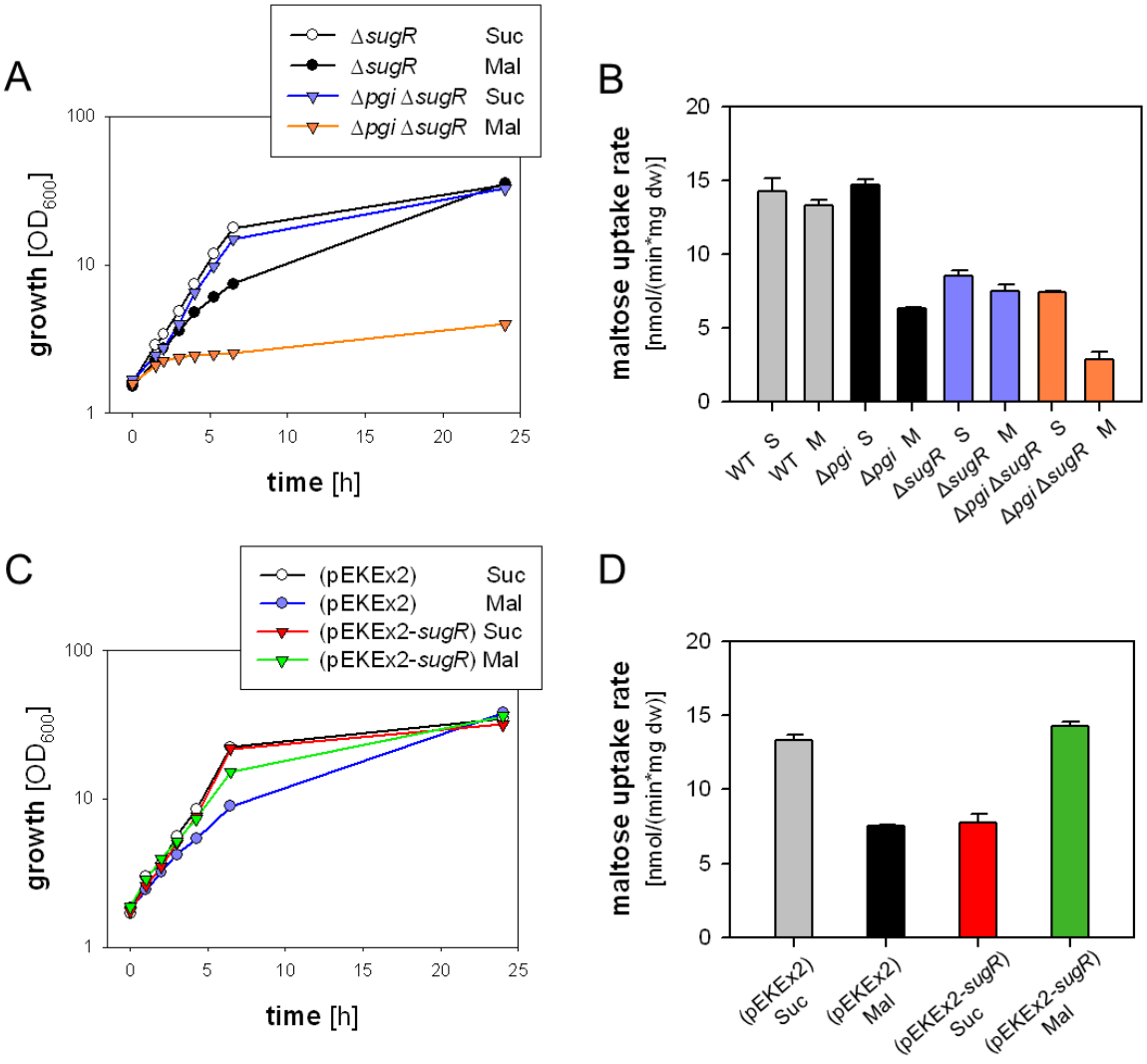


Figure 43: Absence of SugR inhibits the maltose utilization in *C. glutamicum*: Growth (A) and maltose uptake rate (B) of the Δ*sugR and Δpgi ΔsugR* mutants on sucrose or maltose; Growth (C) and maltose uptake (D) complementation in the strain *C. glutamicum* Δ*sugR* (pEKEx2-*sugR*) during growth on maltose or sucrose.

The involvement of SugR in the regulation of the maltose uptake in *C. glutamicum* in a so far unknown manner made the investigation of the question why SugR is not activated by maltose addition in *C. glutamicum* Δ*pgi* even more complicated. Furthermore, despite being described as transcriptional repressor, the absence of SugR led to decrease of the maltose uptake ability of the cell, thus suggesting an activating function. Hence, these results indicated that SugR is involved in a complex regulatory network involving so far unknown targets and/or interconnections with other regulators.

3.4. New targets: analysis of suppressor mutants

The results so far demonstrated an EII^{glc}-dependent sugar-P stress response in *C. glutamicum* Δ*pgi*, leading to a rapid inhibition of the sucrose uptake. Though, the mechanism of signal transduction from EII^{glc} to the actual uptake inhibition was unclear and did not seem to be similar to previously described mechanisms. It was also unclear why despite causing comparable rapid sucrose uptake inhibition in the Δ*pgi* mutant, maltose and glucose-6-P were not causing SugR-dependent *ptsS*-repression or why maltose utilization is affected by the absence of SugR. In order to identify unknown interaction sites of the already known players or to find potential new players involved in those two regulatory mechanisms, I generated and analysed suppressor mutants which have lost the observed regulatory phenomena.

3.4.1. C. glutamicum ΔscrB suppressor mutants:

Generation of *C. glutamicum* Δ*scrB* suppressor mutants – *C. glutamicum* Δ*scrB* cells were selected for improved growth on minimal medium with sucrose plus glucose. After each day of incubation the cells were transferred into fresh medium and the selective pressure was increased by adding 0.05%; 0.1 %; 0.5% and finally 1% [wt/vol] sucrose to a constant concentration of 1% [wt/vol] glucose. The experiment was performed with five cultures (flasks) simultaneously. At the end all isolates had improved growth rates (0.42 ± 0.01 h^{-1}) during growth on sucrose plus glucose (Fig. 44; A). In contrast to the parental strain *C. glutamicum* Δ*scrB,* the suppressor mutants had similar glucose uptake rates when cultivated on glucose or sucrose plus glucose and no rapid sucrose uptake inhibition was observed after sucrose addition (Fig. 44; B&C). Unfortunately, the measurement of sucrose uptake rates of the suppressor mutants showed that the reason for the lack of negative effects by the presence of sucrose was the lost sucrose uptake ability in all isolates (Fig. 44; D).

Sequencing analysis of the *scrB-ptsS* region identified a sequence with 98% homology to the *C. glutamicum* transposase genes *tnp1a*, *tanp1b* and *tnp1c* integrated in the *ptsS*-promoter

region, 73 bp before the transcriptional start of *ptsS.* It seemed that the transposase had selectively interrupted the *ptsS* transcription in all suppressor mutant isolates.

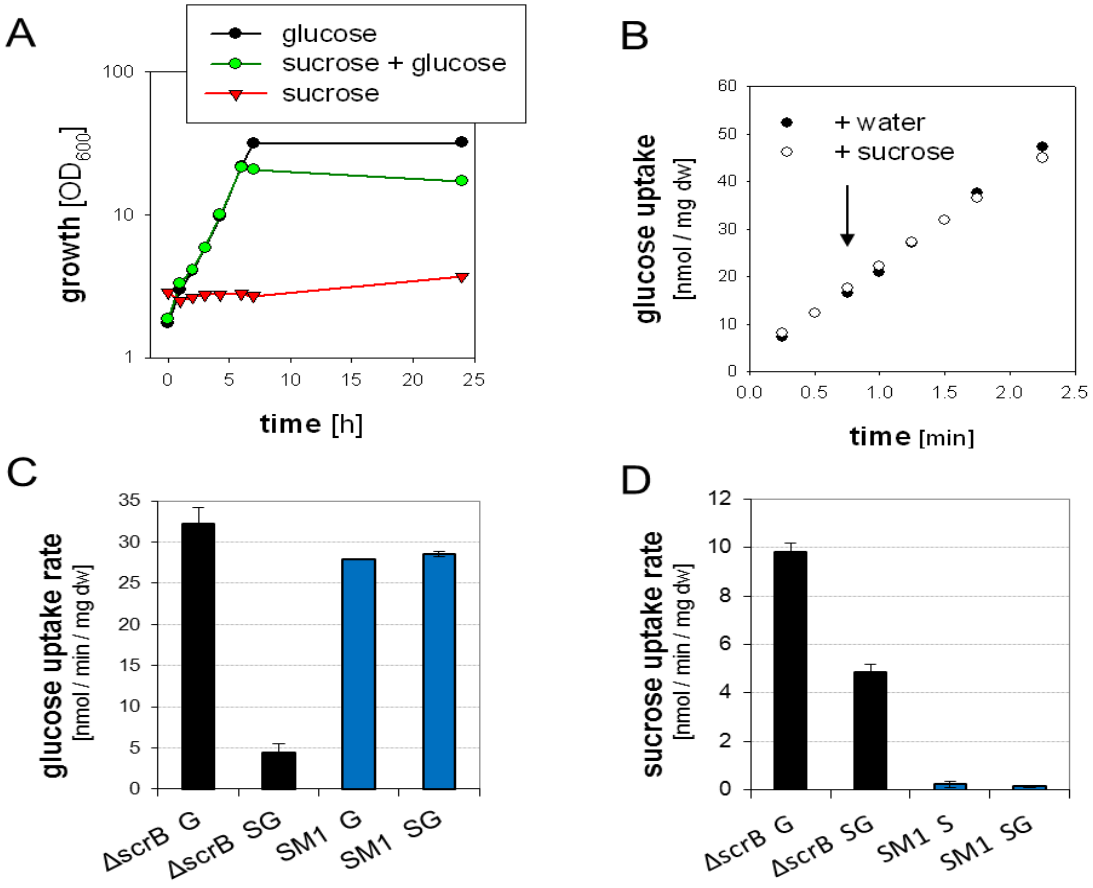


Figure 44: Analysis of *C. glutamicum* Δ*scrB* suppressor mutant: Shown are results for the *C. glutamicum* Δ*scrB* suppressor mutant SM1, which is representative for all other isolates; Growth of SM1 with 1% [wt/vol] glucose, 1% [wt/vol] sucrose or glucose plus sucrose (1% each) (A); Rapid glucose uptake inhibition after sucrose addition in SM1 (B). Comparison between the glucose (C) and sucrose (D) uptake rates of SM1 and the initial strain *C. glutamicum* Δ*scrB* during cultivation on glucose or glucose plus sucrose.

3.4.2. C. glutamicum Δpgi suppressor mutants:

Generation of *C. glutamicum* Δ*pgi* suppressor mutants – The screening criterion for the generation of *C. glutamicum Δpgi* suppressor mutants was again improvement of the growth with sucrose plus glucose. However, in contrast to the sucrose utilization in *C. glutamicum ΔscrB,* the utilization of glucose in the Pgi-deficient strain was not fully impaired. Hence, in order to avoid the loss of the glucose uptake ability which is clearly beneficial in those conditions, a strategy was chosen in which the cells were repeatedly switched between

cultivation on sucrose plus glucose and cultivation on glucose as a sole substrate. In that way only cells which retained their glucose uptake ability were further enriched.

Indeed, the strategy was successful and none of the mutants obtained after 7 days of cultivation had lost its glucose uptake ability. Three main cultures (flasks) were used and from each of them 4 clones were isolated at the end of the experiment, confirmed by *pgi*-specific PCR and analyzed. As shown on Figure 45 for one of the isolates designated as *C. glutamicum* Δ*pgi* SM-K6, the ability of the suppressor mutants to grow with glucose as well as with sucrose plus glucose was significantly improved compared to the parental strain *C. glutamicum Δpgi*. In fact, *C. glutamicum Δpgi* SM-K6 showed no significant difference in the growth rates during cultivation with sucrose as a sole carbon source or sucrose plus glucose (Fig. 45, B). However, it was noticeable that all isolates had a short (2-3 h) "lag phase" at the beginning of growth, during which the growth rate was slightly reduced and this phase was present even during cultivation on sucrose as a sole carbon source.

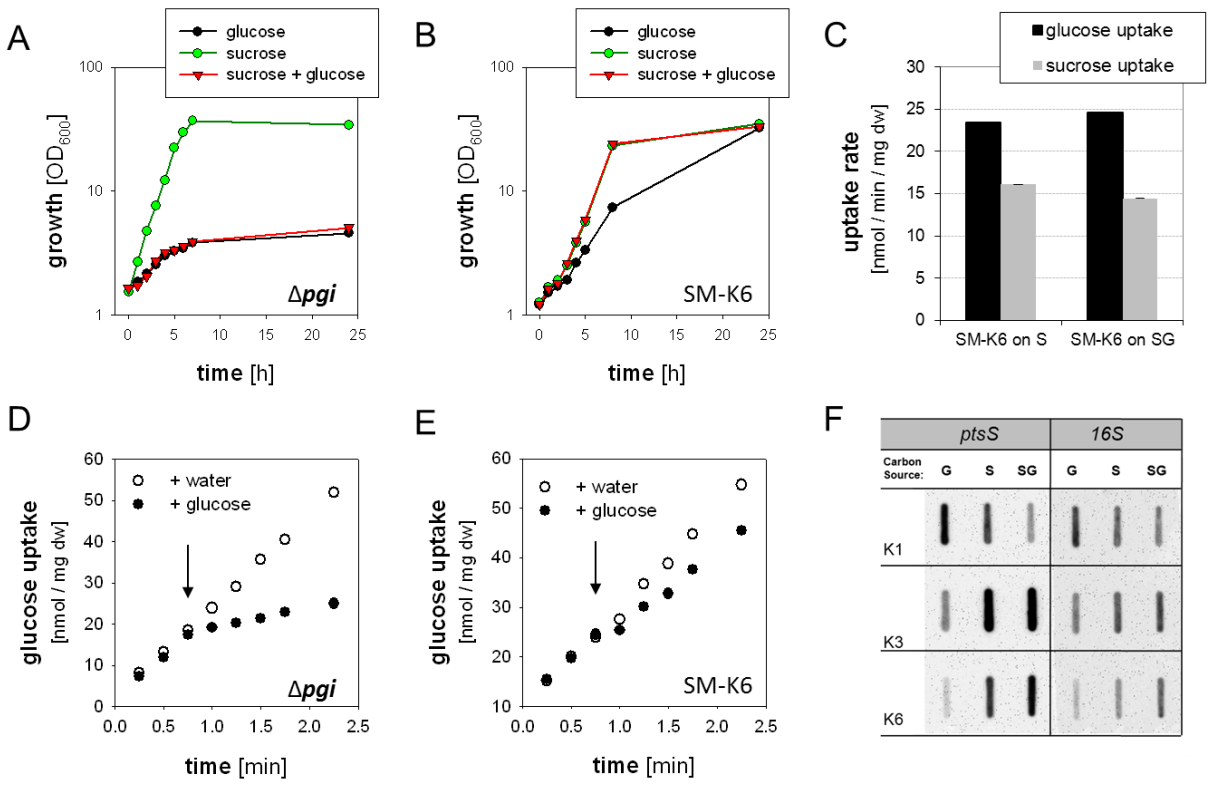


Figure 45: Analysis of *C. glutamicum* Δ*pgi* suppressor mutant: Shown are results for the suppressor mutant *C. glutamicum* Δ*pgi* SM-K6, which was representative for all other isolates; Growth of *C. glutamicum* Δ*pgi* (A) and SM-K6 (B) with glucose, sucrose or glucose plus sucrose (1% [wt/vol] each); Sucrose uptake inhibition after glucose addition in *C. glutamicum* Δ*pgi* (D) and SM-K6 (E). Comparison between the glucose and sucrose uptake rates of SM-K6 during cultivation on sucrose or sucrose plus glucose (C). Slot blot analysis of the ptsS-mRNA amounts in SM-K6 and two different suppressor mutant isolates during cultivation on glucose, sucrose or sucrose plus glucose (1% [wt/vol] each) (F).

The sucrose uptake of the sucrose plus glucose cultivated suppressor mutants was significantly improved, as well. As shown for SM-K6, the addition of glucose caused a much weaker rapid sucrose uptake reduction compared to the inhibition observed in the parental strain (Fig. 45, D&E). The reduced sensitivity towards glucose was not caused by lack of glucose uptake as all isolates had improved glucose uptake rates (*e. g.* 23.7 ± 2.1 nmol/(min*mg DW) for SM-K6) compared to the low glucose uptake rate (5.3 ± 0.9 nmol/(min*mg DW)) of the initial strain *C. glutamicum Δpgi* (Fig. 45; C). Last but not least, the glucose-triggered *ptsS*-repression in the tested isolates was also alleviated (Fig. 45, F). Apparently, the suppressor mutants had managed to overcome at least partially the sugar-P stress response triggered by the addition of glucose in *C. glutamicum* Δ*pgi*.

Gene specific sequencing analysis revealed that none of the *C. glutamicum* Δ*pgi* suppressor mutants had mutations in the coding sequences or promoter regions of *sugR, ptsG, ptsS*, *ptsI, zwf* and *gnd*. As no other players and respectively target genes were known or expected to lead to the observed phenotype improvement it seemed that indeed a novel player was involved in the regulatory cascade. Whole genome sequencing was required in order to get further information.

Whole genome sequencing analysis – The genomes of the most improved suppressor mutant *C. glutamicum* Δ*pgi* SM-K6 and the parental strain *C. glutamicum* Δ*pgi* were sequenced via 50bp paired-end sequencing. The obtained sets of over 31 million reads for each strain had high average phred quality scores and provided a reliable coverage of the *C. glutamicum* genome of 242 fold / bp (SupFig. 9).

The initial standard alignment of the reads with the *C. glutamicum* genome with the programme BWA[117] provided a high number (4792) of potential SM-K6 specific variations – base insertions/deletions (InDels) or single nucleotide polymorphisms (SNPs) – between the sequencsed as a reference genome of *C. glutamicum* Δ*pgi* and the genome of the SM6 suppressor mutant. However, a more restrictive and optimized for the analysis of smaller genomes alignment with the Bowtie algorithm[118], using only reads with phred quality score above 30 in at least 80% of the bases per read, limited the number of suppressor mutations to just two candidates (table 5):

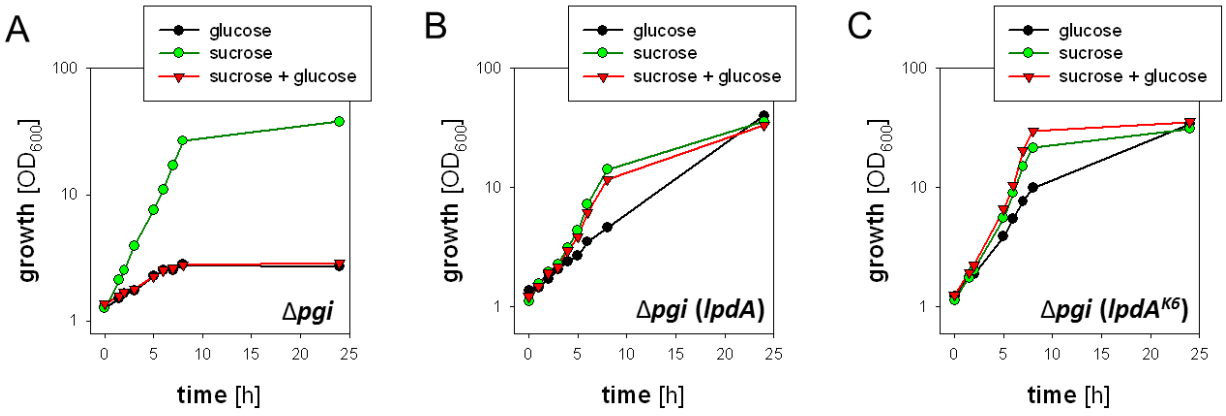
Table 5: Sequencing analysis of selected *C. glutamicum* Δ*pgi* suppressor mutants

Strain	Affected gene	Amino acid substitution	Codon change
Δ*pgi* SM-K6	cg2004	-	GAG → GAA
Δ*pgi* SM-K6	*lpdA*	A223T	GCA → ACA
Δ*pgi* SM-K3	*lpdA*	E226G	GAA → GGA
Δ*pgi* SM-K4	*lpdA*	P216S	CCT → TCT

- *Suppressor mutation one*: a guanine to adenine mutation on position 949 of the coding sequence of the hypothetical protein cg2004 resulted in a silent mutation and thus was not further investigated.

- *Suppressor mutation two*: a guanine to adenine mutation on position 1667 of the coding sequence of the gene *cg0790*, annotated as *lpdA,* led to an Aln^{223} → Thr exchange. Indeed, the identified mutation was verified via gene-specific Sanger sequencing. In fact, 3 out of 7 suppressor mutant isolates which were subsequently tested also had mutations in the *lpdA* gene. Moreover, the point mutations were on different places in the *lpdA* gene resulting accordingly in different amino acid exchanges: Gln^{226} → Gly and Pro^{216} → Ser (table 2). These results showed that *lpdA* was under high selective pressure and possibly plays an important role in the regulatory mechanism studied here. Furthermore, none of the mutations had resulted in a stop codon, which indicates that a simple loss of LpdA activity was not the reason for the observed positive effects.

Confirmation of the found suppressor mutant targets – To test if the identified mutation in *lpdA* is the reason for the weaker response to glucose addition in the SM-K6 suppressor mutant the gene $lpdA^{K6}$, encoding the mutated version $LpdA^{A223T}$, was expressed from a plasmid in the parental strain *C. glutamicum* Δ*pgi*. Indeed, *C. glutamicum* Δ*pgi* (pEKEx2-$lpdA^{K6}$) grew very similarly to SM-K6: the growth rate during cultivation on sucrose plus glucose (0.40 ± 0.02 h^{-1}) was significantly improved compared to the control strain *C. glutamicum* Δ*pgi* (pEKEx2) (0.09 ± 0.01 h^{-1}); the growth rate on glucose (0.26 ± 0.01 h^{-1}) was also higher compared to the control strain (0.09 ± 0.01 h^{-1}) (Fig. 46). These results demonstrated that the found suppressor mutation was sufficient to cause the observed in SM-K6 phenotype improvements and confirmed that LpdA is involved in the studied here regulatory processes.



Glc	Suc	Suc + Glc
0.09	0.40	0.09

Glc	Suc	Suc + Glc
0.14	0.26	0.25

Glc	Suc	Suc + Glc
0.26	0.38	0.40

Figure 46: Effects of *lpdA* and *lpdA*^{K6} overexpression in *C. glutamicum* Δ*pgi*: Growth of *C. glutamicum* Δ*pgi* (pEKEx2) (A), *C. glutamicum* Δ*pgi* (pEKEx2-*lpdA*) (B) and *C. glutamicum* Δ*pgi* (pEKEx2-*lpdA*^{K6}) (C) in minimal medium with 2% [wt/vol] glucose, 2% [wt/vol] sucrose or glucose plus sucrose (1% [wt/vol] each). All cultivations were performed in presence of 0.1 mM IPTG. The table below each graph represents the corresponding mean growth rates observed after the first 3-4h of cultivation.

Further, to elucidate if the mutation in LpdA had a significant function for the observed positive effects, the wild-type *lpdA* gene was expressed in *C. glutamicum* Δ*pgi,* as well. Interestingly, the overexpression of *lpdA* also improved the growth of *C. glutamicum* Δ*pgi* (pEKEx2-*lpdA*) on sucrose plus glucose (0.25 ± 0.02 h^{-1}) or glucose as a sole carbon source (0.14 ± 0.01 h^{-1}). However, the improvement was significantly weaker compared to the *C. glutamicum* Δ*pgi* (pEKEx2-*lpdA*^{K6}) results. The strain *C. glutamicum* Δ*pgi* (pEKEx2-*lpdA*) achieved similar growth rates during cultivation on sucrose (0.40 ± 0.03 h^{-1}) as the control strain *C. glutamicum* Δ*pgi* (pEKEx2) (0.42 ± 0.02 h^{-1}) or *C. glutamicum* Δ*pgi* (pEKEx2-*lpdA*^{K6}) (0.38 ± 0.02 h^{-1}), however, during the initial 3-4 h of incubation it had the characteristic for all suppressor mutants slower initial growth rate of 0.24 ± 0.02 h^{-1} which was not observed in the control strain (Fig. 46). This "lag phase" was significantly shorter and weaker in the suppressor mutants or in the *C. glutamicum* Δ*pgi* (pEKEx2-*lpdA*^{K6}) strain.

In conclusion, overexpression of *lpdA* led to improved growth of *C. glutamicum* Δ*pgi* with glucose or sucrose plus glucose suggesting that LpdA is involved in the regulatory mechanism of response to sugar-P stress. However, the effects were not as strong as by the overexpression of the suppressor mutated *lpdA*^{K6} which indicated a change in the functionality between the two variants.

Deletion mutant of *lpdA* – To analyze the possible function of *lpdA* in *C. glutamicum* and subsequently in the sugar-P stress response mechanism in *C. glutamicum* Δ*pgi* an LpdA-deficient mutant was created. The growth of *C. glutamicum* Δ*lpdA* with sucrose plus glucose or acetate as carbon sources was not affected compared to the wild-type, indicating that LpdA is not essential under those conditions (Fig. 47, A&B). However, in the later exponential growth phase during cultivation in TY complex medium the Δ*lpdA* mutant had slightly reduced growth rate compared to the wild-type. Again in the later exponential growth phase during cultivation in TY complex medium the growth of *C. glutamicum* Δ*pgi* (pEKEx2-*lpdA*^{K6}) seemed to be reduced compared to the *C. glutamicum* Δ*pgi* (pEKEx2-*lpdA*) strain (Fig. 47, C). Hence, LpdA did not seem essential for *C. glutamicum* but might be required in the late exponential growth phase for the utilization of specific substrates included in the TY complex medium.

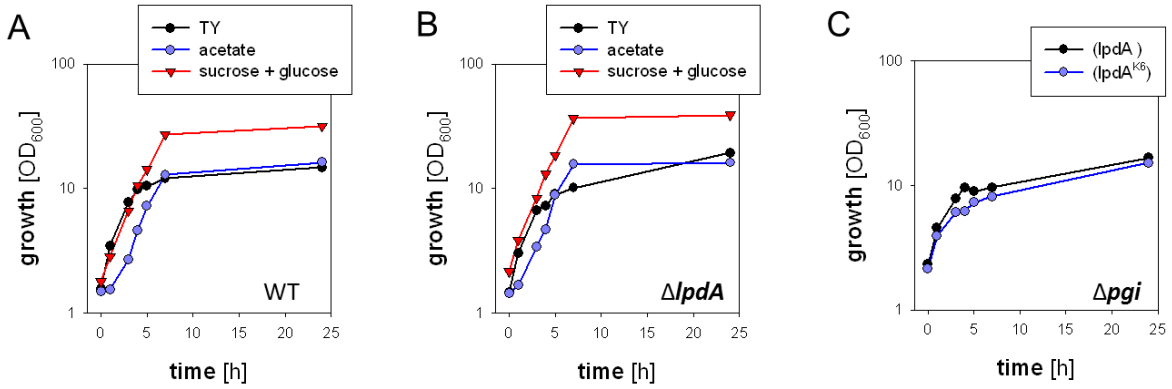


Figure 47: Growth of *C. glutamicum* Δ*lpdA*: Growth of wild-type *C. glutamicum* (A) and *C. glutamicum* Δ*lpdA* (B) in TY complex medium or minimal medium with acetate or glucose plus sucrose; Growth of *C. glutamicum* Δ*pgi* (pEKEx2-*lpdA*) or *C. glutamicum* Δ*pgi* (pEKEx2-*lpdA*^{K6}) in TY complex medium (C). All cultivations were performed in presence of 0.1 mM IPTG.

4. Discussion

C. glutamicum is able to co-utilize most carbon sources such as glucose, fructose and sucrose. Those three sugars are transported by the PTS, which plays a central role in the regulation of nutrient uptake and metabolism in bacteria. However, the regulatory functions of the PTS in *C. glutamicum* are unknown. This work showed that the glucose-specific PTS permease EII^{glc} is part of a novel sugar-P stress response in *C. glutamicum* which leads to instantaneous inhibition of the PTS phosphorylation cascade and thus regulation of the PTS activity of the cell. This rapid uptake inhibition was the first of a two-step regulatory mechanism responsible for the inhibitory effects of glucose addition on the growth of *C. glutamicum* Δ*pgi* with sucrose. In a second step, this fast response is further strengthened by a reduction of the *ptsS*-expression by the transcriptional repressor SugR. This regulatory cascade might be a general mechanism for the carbon uptake regulation in *C. glutamicum* as similar but inverted effects have been demonstrated for *C. glutamicum* Δ*scrB.* Further, a weaker but also rapid adjustment of the PTS-mediated uptake was shown to occur during co-utilisation of sucrose plus glucose or other substrate combinations like glucose plus fructose or glucose plus acetate in the wild-type, which showed that this organism adjusts the substrate uptake during co-utilization of carbon sources not only by transcriptional regulation but on the first place by a fast response directly on the level of protein activity.

4.1. A novel two-step sugar-P stress response mechanism

The accumulation of sugar phosphates as a consequence of disruption of metabolic pathways or uptake of non-metabolizable sugars such as the glucose analogue α-methyl glucoside (αMG) leads in many organisms to severe growth defects [68, 114, 119]. However, a clear regulatory mechanism responsible for that phenotype has been shown only for *E. coli* and *Salmonella*. As described in Introduction 1.5, those organisms react to a glucose-6-P stress with expression of the sRNA *sgrS* which with the aid of the chaperone Hfq causes degradation of *ptsG*-mRNA[72] and activates synthesis of the sugar-P phosphorylase SacP[73]. Additionally the sRNA sgrS and encodes a short peptide, SgrT, which is able to directly inhibit glucose uptake[75].

A sucrose-P stress response seemed to be present in *C. glutamicum* Δ*pgi*, as well. The growth and *ptsG*-expression of the mutant with glucose was drastically reduced compared to the wild-type[77]. What is more, the addition of glucose caused also inhibition of the otherwise normal growth of the *pgi* mutant on sucrose. However, since no Hfq or *sgrS* homologues are present in *C. glutamicum,* a sugar-P stress response mechanism different to that of *E. coli* could be expected.

Previous studies suggested that in *C. glutamicum* Δ*pgi* the presence of glucose activates the transcriptional repressor SugR, which reduces the *ptsS*-expression and as a consequence inhibits the sucrose uptake and eventually growth of the mutant with this substrate[83]. However, it was not clear how the addition of glucose would activate binding of SugR to the *ptsS*-promoter, as previous EMSA studies have shown no activating effects of glucose-6-P on SugR activity [58, 60]. An inactivation of SugR by sequestering to the membrane by EII^{glc}, as it is described for the Mlc regulator in *E. coli*[39], was excluded, as well, as no SugR-dependent *ptsS*-repression took place in *C. glutamicum Δpgi ΔptsG.*

Further concern about this initial model was the fact that the transcriptional regulation of *ptsS* offers no immediate remedy for the accumulation of sugar-P by the existing PTS transporters since protein half-lives normally exceed those of mRNAs and uptake would still be possible[120]. EII^{glc} in *E. coli* for example has a half-life of ~80 min so that *ptsG*-repression by *sgrS* required also the faster responses by SacP and SgrT for efficient sugar-P stress recovery[73]. Indeed, even though *ptsS* was derepressed in *C. glutamicum* Δ*pgi* Δ*sugR* the growth rate of the double mutant was still not recovered to wild-type levels and the sucrose uptake remained impaired[83]. This indicated that that the sucrose uptake inhibition might not be caused by *ptsS*-repression and additional regulatory processes are involved.

The comparison of the temporal development of the sucrose uptake inhibition and *ptsS*-repression in *C. glutamicum Δpgi* after glucose addition performed here showed that the two regulatory phenomena take place in reverse order compared to the initial model: First, glucose causes initial, rapid inhibition of sucrose uptake, which is a purely biochemical regulatory process as demonstrated by treatment of the cells with translational or transcriptional inhibitors. Then, in a second step, a transcriptional inhibition of the *ptsS*-expression by SugR is activated in order to strengthen the initial effect of sucrose uptake inhibition and to reduce the unnecessary production of EII^{suc}. This new temporal succession of the sucrose uptake inhibition and following *ptsS*-repression revealed also a possible

explanation for the activation of SugR. The EMSA analysis of purified SugR demonstrated that fructose-1-P and to a lower extent fructose-6-P are negative effectors of the SugR-binding to the promoter of *ptsS.* Hence, the rapid sucrose uptake inhibition seems to activate the SugR-dependent repression of *ptsS* in *C. glutamicum* Δ*pgi* by an inducer exclusion mechanism:

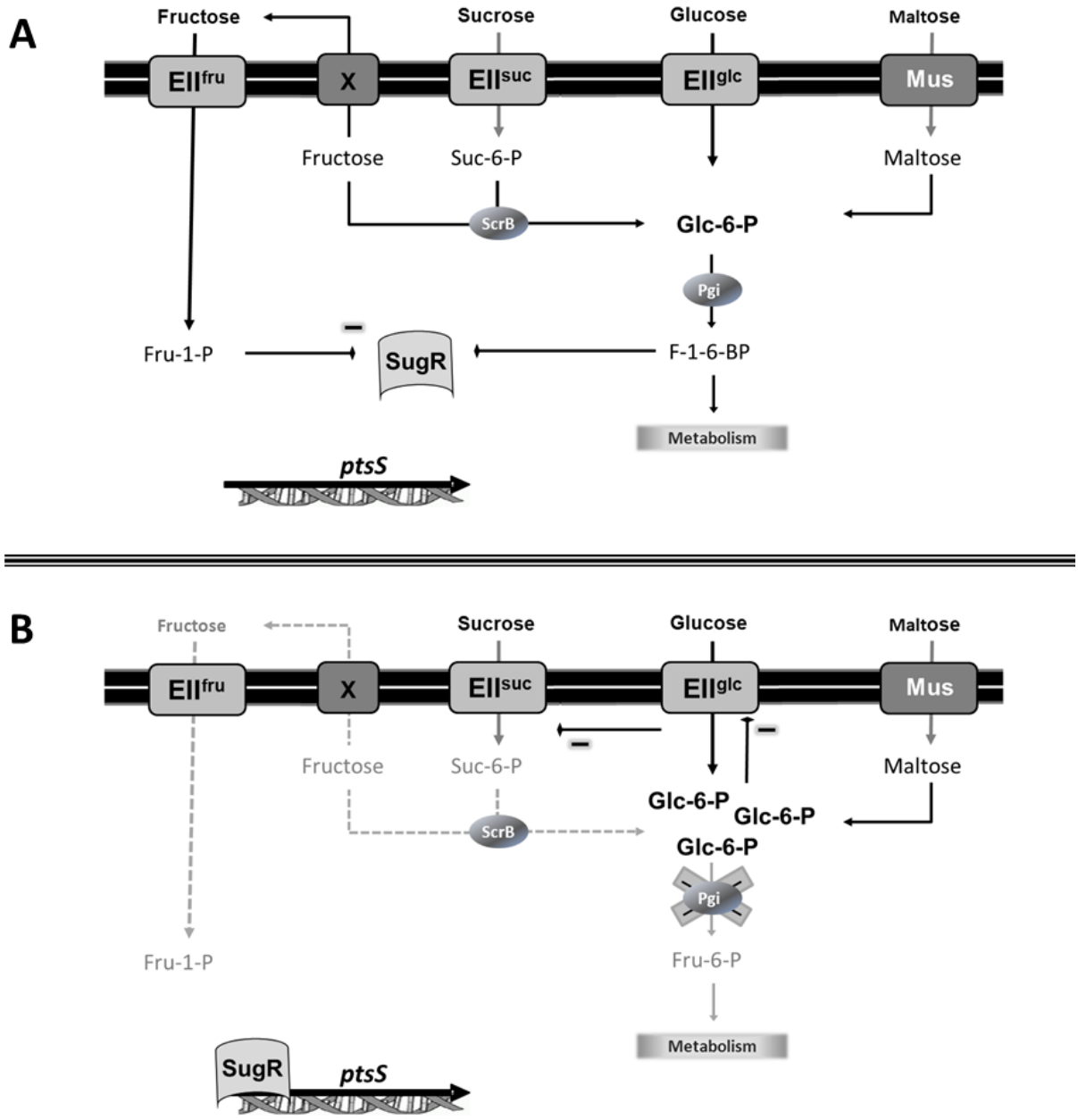


Figure 48: Model for the *ptsS* regulation by the transcriptional regulator SugR in *C. glutamicum* wild-type (A) and *C. glutamicum* Δ*pgi* (B) during minimal medium cultivation with sucrose plus glucose. In conditions of sugar-P stress SugR represses *ptsS* as a consequence of an inducer exclusion mechanism.

During growth on sucrose plus glucose both fructose-1-P and fructose-6-P are formed in the wild-type. They inhibit SugR-binding at the *ptsS* promoter and thereby stimulate expression of the sucrose-specific PTS permease when its substrates is available (Fig. 48, A). In *C. glutamicum* Δ*pgi*, however, the addition of glucose increases the formation of glucose-6-P, as demonstrated here by enzymatic measurements of the metabolite concentrations, which leads to sugar-P stress. This causes a rapid inhibition of sucrose uptake so that the intracellular fructose-1-P formation and consequently concentration drops and the SugR–binding to the *ptsS*-promoter is no longer inhibited (Fig. 48, B).

This two-step model emphasizes the rapid uptake regulation mechanism as a primary, initiating process in the sugar-P stress response. However, the transcriptional regulation is also important and mediates the long-term response to the presence of glucose. The growth of *C. glutamicum* Δ*pgi* with sucrose plus glucose was slow at the beginning and almost completely inhibited after 4-5 h of incubation. In contrast, despite the severely decreased growth rate, the *C. glutamicum* Δ*pgi* Δ*sugR* mutant continued to grow until complete consumption of the available substrates. Furthermore, it was also demonstrated here that the EII^{suc} / EII^{glc} ratio determines the strength of the rapid sucrose uptake inhibition. This shows that not only the rapid uptake inhibition determines the expression of the PTS genes via *e.g.* SugR but *vice versa* - the expression of the PTS genes (and thus amounts of EII in the cell) affects the extent of rapid uptake inhibition, as well.

The combination of initial reduction of the uptake and later transcriptional repression of the respective transporter gene was similar to the sugar-P stress response of *E. coli*. However, as expected due to the absence of Hfq and *sgrS* homologues, the mechanism in *C. glutamicum* is different. Sugar uptake in *C. glutamicum* Δ*pgi* is inhibited by a rapid, translation- and transcription-independent mechanism, whereas inhibition of the EII^{glc} activity by SgrT in *E. coli* requires expression of *sgrS* and translation of the SgrT peptide. In that aspect, as the mechanism of *sgrS* induction is not yet clarified[121], it might be interesting to study how fast the glucose uptake reduction in case of sugar-P stress takes place in *E. coli*. Another sign for mechanistic differences between the sugar-P stress responses of those two organisms was also the fact that the addition of pyruvate was able to improve the growth of *C. glutamicum* Δ*pgi* with glucose or glucose plus sucrose, whereas the addition of pyruvate during sugar-P stress in *E.coli* leads to even stronger growth inhibition and cell lysis[114]. Further, for *Streptococcus lactis* it has been shown that the accumulation of 2-deoxy-D-glucose-6-P

causes also a fast inhibition of the mannose-specific EII, which is followed by a repression of the gene encoding the maltose- specific EII [122]. Though, in this case uptake inhibition also starts later (after ~3 min) compared to the rapid uptake inhibition in *C. glutamicum* and this indicates that it might also function according to a different mechanism.

Surprisingly, whereas the addition of maltose caused comparable rapid sucrose uptake reduction in *C. glutamicum* Δ*pgi* as the addition of glucose, the following *ptsS*-repression takes place only in the case of glucose addition (see 3.3.2.). Indeed, the growth behaviour of the maltose plus sucrose cultivated Δ*pgi* cells was similar to the growth of *C. glutamicum Δpgi ΔsugR* on sucrose plus glucose, where the transcriptional regulation of *ptsS* was absent, as well. However, the molecular mechanism how the SugR-dependent repression of *ptsS* is triggered by the presence of glucose but not by the presence of maltose is unclear. Since in both cases the sucrose uptake is prevented and this should lead to inducer exclusion, an additional regulatory mechanism might play a role. Indeed, there is evidence for the involvement of an additional transcriptional regulatory processes: (i) Despite the absence of SugR, *ptsG* is still repressed in *C. glutamicum* Δ*pgi* Δ*sugR* during cultivation on glucose[83]; (ii) The absence of SugR led to reduction of the maltose uptake ability of *C. glutamicum*, even though none of the genes for the maltose uptake system or the enzymes specific for maltose utilization is a target of SugR. Moreover, SugR is a transcriptional repressor so that its absence should not have inhibiting but rather activating functions on the maltose utilization unless it is a part of a more complex regulatory network involving additional regulators; (iii) Former studies have shown that the presence of maltose increases the *ptsG*-expression in *C. glutamicum* on a not yet clarified but SugR-independent manner[58]. Hence, it is plausible to suggest that an additional mechanism which activates the expression of *ptsS* and/or avoids repression by SugR is responsible for the lack of *ptsS*-repression after maltose addition. Potential candidates could be for example the transcriptional activators GntR1 and GntR2 which bind to the *ptsS*-promoter region[53]. However, the mechanism of GntR1 and GntR2 activation is not clarified yet so that further studies are needed to understand why the potential *ptsS* activation would not take place in the sucrose plus glucose cultivated *pgi* mutant.

Similarly to maltose, the addition of glucose-6-P in *C. glutamicum Δpgi* (pEKEx2*-*uhpT*) did not cause *ptsS*-repression despite inhibiting the sucrose uptake. This result raised another

interesting question: whereas a maltose-specific metabolite or protein could be potentially acting as effector of SugR or the additional transcriptional regulator in order to induce different *ptsS*-regulation than in the case of glucose addition, it was not clear how the cell is able to discriminate between the addition of glucose or glucose-6-P, which both lead directly after their uptake to accumulation of glucose-6-P and to similar rapid sucrose uptake inhibition. There are three potential aspects which might be different after the addition of glucose or non-PTS substrates like glucose-6-P or maltose.

First, the phosphorylation state of EII^{glc}: For *E. coli* for example it is shown that glucose regulates the expression of the *lac* operon not only by inducer exclusion but also by affecting the cAMP-dependent activation of the operon by Crp (see Introduction 1.4). Thus, it is not excluded that the presence of glucose changes not only the activity of SugR in *C. glutamicum* Δ*pgi* by the described inducer exclusion mechanism but also affects additional regulators. This could be achieved in a cAMP-dependent manner by the direct or indirect involvement of GlxR or by another regulator like *e.g.* GntR1 and GntR2 via a mechanism similar to what has been shown for the regulation of Mlc of *E. coli*. However, this scenario would mean that inactive EII^{glc} (as in the case of gluose-6-P addition) will caouse activation of the *ptsS*-expression, which is not physiologically plausible.

Second, the activity of the PTS could reduce the PEP/pyruvate ratio in the *pgi* mutant due to PEP consumption by the PTS which is not sufficiently compensated by the utilization of the transported sugar. Thus, the differences of regulation on transcriptional level could be determined by a different PEP/pyruvate ratio in the case of addition to a PTS or a non-PTS substrate. However, as it would be discussed below, there is no evidence that the PEP/pyruvate ratio in those cases is different or even if it is different it could be similarly changed both in the case of glucose and in the case of glucose-6-P addition.

Finally, the different regulation of *ptsS* after glucose and maltose or glucose-6-P addition might be due to the responsiveness of a transcriptional regulator to the ATP or phosphate (P_i) balance of the cell. The conversion of maltose to glucose-6-P is connected with significant ATP and P_i consumption. Also the UhpT transporter mediates the exchange of P_i for glucose-6-P. In contrast, the uptake of glucose by the PTS is not expected to affect specifically the P_i levels in the cell. Indeed, a connection between the sugar-P stress response in *E. coli* and the regulation of genes involved in the phosphate metabolism has been demonstrated [123]. In this case the induction of the Pho regulon aided the recovery of a *sgrS*

mutant from glucose-phosphate stress. Hence, a better understanding of the transcriptional regulatory network in *C. glutamicum* would be required to clarify that question. Furthermore, it might be useful to analyse some of the generated but not yet sequenced *C. glutamicum Δpgi* suppressor mutants in order to potentially identify the unknown player involved in the *ptsS* regulation during sugar-P stress discussed here.

4.2. The sugar-P stress response: Mechanism of rapid uptake inhibition

The present work showed that the sucrose uptake in *C. glutamicum* Δ*pgi* is inhibited in a rapid, biochemical manner by the addition of glucose. An unspecific inhibition of EII^{suc} by extracellular glucose was excluded as the addition of glucose had no effect on the uptake of sucrose in *C. glutamicum* Δ*pgi* Δ*ptsG*. Hence, the accumulation of glucose-6-P and / or the EII^{glc} activity was necessary for induction of the stress response causing the rapid uptake inhibition. In order to uncouple those two processes and elucidate their specific role for this regulatory phenomenon I used non-PTS substrates, which also lead to the intracellular formation of glucose-6-P. The addition of maltose, which is transported by a primary active ABC transporter, or glucose-6-P, transported by a heterologously expressed secondary active MFS transporter, both triggered a rapid sucrose uptake inhibition in *C. glutamicum* Δ*pgi* similar to the one observed after glucose addition. This result suggested that the accumulation of glucose-6-P is the actual signal for the regulatory response. However, no sucrose uptake and growth inhibition was observed in the double mutant *C. glutamicum* Δ*pgi* Δ*ptsG* despite that maltose and glucose-6-P were still imported in the cells with unchanged rates. This result demonstrated two important aspects:

First, despite acting as a signal, the accumulation of glucose-6-P itself is not *per se* toxic for the cell. The growth inhibition is coupled to the inhibited uptake of available substrates but not to the glucose-6-P accumulation as such. When the uptake inhibition was abolished, growth was proceeding normally, despite the accumulation of sugar-P. Glucose-6-P accumulation did not cause growth inhibition of the *pgi* mutant with substrates like ribose or pyruvate, as well, indicating again that the uptake reduction and not the sugar-P stress is the reason for the growth inhibition during cultivation with sucrose plus glucose. An even more striking example for that was also the fact that both *C. glutamicum* WT (pEKEx2*-*uhpT*) and *C. glutamicum* Δ*pgi* (pEKEx2*-*uhpT*) had similar growth rates with glucose-6-P as a carbon

source. What is more, those growth rates were significantly higher than the growth rates of the *pgi* mutant with glucose or sucrose plus glucose, confirming that the accumulation of glucose-6-P allows better growth, but not if the uptake of the substrate is inhibited by a regulatory mechanism. In many organisms the severe growth reduction coincides with the accumulation of sugar phosphates which was therefore described as sugar-P stress[80, 81]. Though, a direct connection between the accumulation of *e.g.* glucose-6-P and a following sugar-P stress response mechanism has not been demonstrated and it is not clear why an intracellular sugar-P accumulation should be harmful for the cell. In fact, a recent study also suggested that glucose-6-P is not toxic *per se* and the absence of glycolytic intermediates rather than the sugar-P accumulation itself triggers the *sgrS*-mediated sugar-P stress response in *E. coli* and *Salmonella* [114].

Second, the unaffected growth and rapid sucrose uptake inhibition in *C. glutamicum* Δ*pgi* Δ*ptsG* after addition of maltose or glucose-6-P (in *C. glutamicum* Δ*pgi* Δ*ptsG* (pEKEx2*-*uhpT*), respectively) showed that the glucose-specific PTS permease EII^{glc} is required for the perception of sucrose-P stress and following induction of the rapid sucrose uptake inhibition independently whether it mediates the primary generation of sugar-P accumulation or not. EII^{glc} is involved in the regulation of carbohydrate utilization in numerous bacteria and archea[24, 37], however, regulatory functions of EII have not been described for *C. glutamicum* so far.

4.2.1. The target of inhibition:

EII^{glc} could mediate a biochemical inhibition of the PTS-mediated sucrose uptake by two main principles: (A) a specific inhibition of the EII^{suc} or by (B) inhibition of the PEP-EI-HPr phosphorylation cascade which energizes the uptake process. A direct way to discriminate between these two possibilities was the measurement of the HPr~P / HPr ratio in the cell under normal and stress-inducing conditions. The HPr~P / HPr ratio in *C. glutamicum* during exponential growth with glucose, sucrose or a combination of both was evenly balanced and both the phosphorylated and unphosphorylated forms were present in comparable amounts. When the substrates were exhausted at the stationary phase or during growth on non-PTS substrates, the PTS was not active and the HPr~P / HPr ratio was strongly shifted towards HPr~P. Both results are in good agreement with previous studies in Gram-positive organisms and the generally accepted model for the phosphorylation state of the PTS

components [28, 95, 124]. Similar results were observed also for *C. glutamicum* Δ*pgi* during cultivation on sucrose as a sole carbon source. However, the presence of a glucose-6-P stress source like *e.g.* glucose or maltose caused severe depletion of the HPr~P amount in *C. glutamicum* Δ*pgi*. Consistent with the observed rapid sucrose uptake inhibition, the depletion of HPr~P was also a rapid process taking place within 1 min after glucose addition. Further, as the inactive state of the PTS corresponded to a high HPr~P / HPr ratio, the drastic decrease of the HPr~P / HPr ratio after addition of glucose or maltose is not a consequence of the rapid sucrose uptake inhibition but rather the cause for it. Therefore, a specific, direct inhibition of the EII^{suc} permease seems unlikely.

Taken together, those results show that in response to the accumulation of glucose-6-P the glucose specific PTS permease EII^{glc} induces instantaneous inhibition of the common PTS phosphorylation cascade, which decreases the general PTS activity of the cell (see 4.2.3.). In agreement with that, the fructose uptake of *C. glutamicum* Δ*pgi* was also rapidly reduced by the addition of a substrate leading to glucose-6-P formation.

4.2.2. PEP limitation vs. glucose-6-P accumulation:

The PTS-mediated uptake of glucose or sucrose is connected with a constant consumption of PEP by the PEP-EI-HPr phosphorylation cascade. Normally this consumption is compensated by the following metabolisation of the transported sugar which even leads to a net gain of 1 mol PEP per 1 mol transported monosaccharide. However, the rate of PEP-regeneration in *C. glutamicum* Δ*pgi* might be insufficient as the utilization of glucose-6-P is restricted only to the potentially feedback inhibited PPP. This might lead to a decrease in the PEP/pyruvate ratio in the cell and consequent reduction of the PTS activity. Indeed, it has been suggested that upon activation of the PTS the adjustment of the PEP/pyruvate ratio to a new stable steady state level requires less than 1 sec, which is in agreement with the rapid uptake regulation observed here [125].

Strong evidence that a PEP-limitation is not the reason for the rapid sucrose uptake inhibition after glucose addition was given by the fact that addition of non-PTS substrates such as maltose or glucose-6-P also caused a rapid inhibition of the PTS activity. Those substrates lead to the intracellular formation of glucose-6-P but do not use PEP for uptake so that they would not increase the PEP consumption of the cell. However, there is a potential scenario when this would not be true.

If the accumulating glucose-6-P is dephosphorylated and the resulting glucose is exported from the cell and eventually reimported by EII^{glc} this would create a futile cycle leading to a loss of PEP even if the glucose-6-P accumulation is not initially generated by the PTS. Hence, if such a futile cycle is present, this would be a tempting explanation for the EII^{glc}–depending rapid depletion of HPr~P in case of glucose-6-P accumulation. Indeed, it has been shown that the glucose-6-P phosphorylase SacP is involved in the sugar-P stress response of *E. coli* [73]. No *sacP* homologue is present in the genome of *C. glutamicum* but the genes *cg1048* and *cg3199* encode theoretical proteins, which share high similarity to respectively YbiV and YidA - two of the five glucose dephosphorylating enzymes found in *E. coli* [126]. A fructose exporter has also been suggested [24], so that it is not excluded that the same or similar exporter is able to export glucose, as well. However, if a futile cycle was responsible for the rapid uptake reduction, the glucose-6-P dephosphorylation and expulsion should take place in less than 15 sec, as well. Furthermore, as the rapid sucrose uptake was not changed by the treatment of the cells with transcriptional or translational inhibitors, the hypothetical G6Pase should be already present in the cell. The SacP involvement in the sugar-P stress response in *E.coli* is activated by a combination of transcriptional and posttranscriptional steps and an increase in the SacP amounts was detected 20 min after addition of a glucose-6-P stress inducing substrate, so that a similar mechanism could not be expected.

Direct evidence that a PEP-consuming futile cycle is not the reason for the rapid uptake inhibition observed in *C. glutamicum Δpgi* after addition of maltose or glucose-6-P was also provided. If the addition of [^{14}C]maltose or [^{14}C]glucose-6-P leads to the described futile cycle then [^{14}C]glucose should be exported from the cell. As the exported [^{14}C]glucose would be rapidly reimported by the highly affine EII^{glc}, a significant accumulation of external [^{14}C]glucose would not necessarily be expected. Though, the addition of unlabelled excess glucose should mask the reimport of [^{14}C]glucose so that (i) the labelled glucose should start to accumulate outside the cell and (ii) the radioactivity inside the cell should decrease due to the replacement of the [^{14}C]glucose with unlabelled glucose. This was not the case as shown for example for the glucose-6-P uptake in *C. glutamicum* Δ*pgi* (pEKEx2*-*uhpT*) after addition of glucose (Fig. 14). Furthermore, if the accumulation of glucose-6-P leads to continuous expulsion of glucose it would be expected that *C. glutamicum* Δ*pgi* Δ*ptsG* (pEKEx2*-*uhpT*) would have a lower substrate yield during growth with sucrose plus glucose-6-P as the EII^{glc}-mediated reimport would rather not be taking place or would be at least very slowly, as the

unspecific glucose uptake by the inositol permeases is observed only at high extracellular glucose concentrations, which would not be expected in this case. Though, a reduction of the reached final OD_{600} or a biphasic growth was not observed. Also during cultivation with sucrose plus maltose *C. glutamicum* Δ*pgi* Δ*ptsG* and the wild-type reached similar final optical densities. Moreover, the presence of a futile cycle which burns energy in terms of PEP would increase the proportion of the total substrate consumed for maintenance, giving as a result less available substrate for biomass synthesis. Though, the *pgi* mutant grown on sucrose plus maltose reached similar final optical density as the wild-type cultivated with those substrates.

Taken together, these results give strong evidence that a futile cycle generating an uncompensated PEP-consumption is not the reason for the rapid sucrose uptake inhibition in *C. glutamicum* Δ*pgi* after addition of non-PTS substrates like maltose or glucose-6-P. Furthermore, the addition of ribose, which neither increases the amount of active EII permeases nor increases the generation of glucose-6-P but increases through its utilization the generation of PEP in the cell, did not alleviate the sucrose uptake inhibition by glucose. This result also indicates that sucrose uptake inhibition is uncoupled form the PEP-generation rate in the *pgi* mutant. Thus, it could be concluded that sugar-P accumulation and not PEP-limitation is the primary inducer of the rapid sucrose uptake inhibition in *C. glutamicum* Δ*pgi*. Furthermore, this conclusion is consistent with former studies which have shown that in glucose cultivated *E. coli* mutants lacking different glycolytic enzymes like phosphoglycerate kinase (Pgk), glyceraldehyde-3-P dehydrogenase (Gap) or enolase (Eno) (Fig. 1) the concentrations of intermediates above the missing enzyme was increased but the intermediates below that point, inclusively PEP, had unchanged concentrations when compared to the wild-type[117]. It has been also demonstrated that an allosteric regulation of the PEP carboxylase and pyruvate kinase turns off PEP consumption when the amount of fructose-1,6,-BP starts to drop as a consequence of a sugar exhaustion, in order to preserve a sufficient PEP-pool for the case when glucose becomes available again [127-129].

4.2.3. A novel regulatory function of EII^{glc}

The results presented here suggest that EII^{glc} has a regulatory function, which enables it to sense glucose-6-P accumulation and induce a biochemical inhibition of the PTS phosphorylation cascade (Fig. 49). Such a regulatory function has not been attributed to EII^{glc}

so far. Indeed, as glucose-6-P is the product of the PTS activity this process could be regarded as a product inhibition. Though, in contrast to the majority of enzymes, the PTS has a highly complex multi protein composition. For *E. coli* the different components of the PTS are even spatially divided – EII permeases seem equally distributed throughout the cell membrane whereas EI is positioned at the cell poles with HPr circulating between the two components through the cytosol [130, 131]. Thus, even if the accumulation of glucose-6-P leads to specific product inhibition of EII^{glc}, it is not clear how and why the signal is transmitted to the common part of the PTS phosphorylation cascade thereby blocking the activity of the entire PTS.

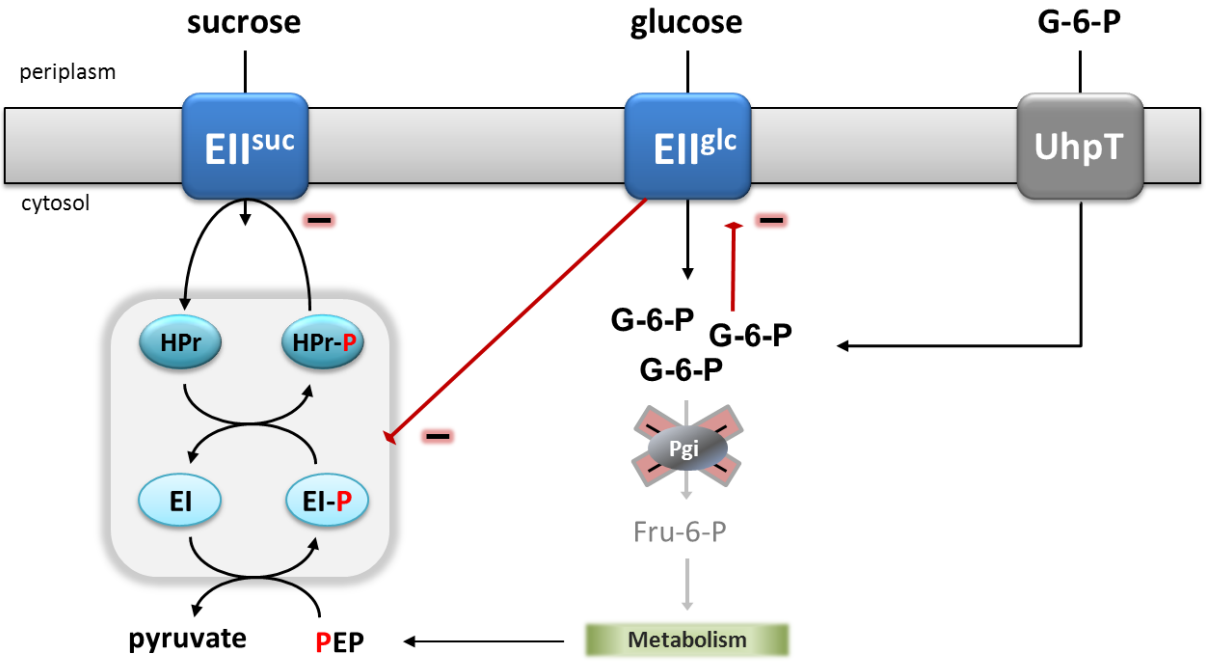


Figure 49: Model for the EII^{glc} dependent sucrose uptake inhibition by after addition of glucose or glucose-6-P in *C. glutamicum* Δ*pgi*

As PEP-limitation does not seem to be the reason for the rapid uptake inhibition in *C. glutamicum* Δ*pgi*, the EII^{glc}-dependent reduction of the HPr~P/HPr ratio should be caused by blockage of the PEP-EI and / or EI-HPr phosphotransfer reaction. A similar rapid (within 15 sec) inhibition of PTS activity of the cell by inhibition of the PTS phosphorylation cascade as a response to increasing amounts of metabolic intermediates has been described for low-GC Gram-positive bacteria like *e.g. B. subtillis* or *Lactococcus lactis* [24, 132]. In those organisms regulation is mediated by an HPr kinase, which phosphorylates HPr at a regulatory Ser46

residue, which reduces drastically the affinity of HPr-Ser-~P towards EI or EII [132]. Nevertheless, the HPr kinase of low-GC Gram-positive bacteria is activated by increased frucose-1,6-BP or ATP levels in the cell, which are not expected in *C. glutamicum* Δ*pgi* after addition of glucose. In fact, no HPr kinase activity could be found in *C. glutamicum*[24, 112]. Hence, for the mechanism of PTS activity inhibition in *C. glutamicum* Δ*pgi* it is rather unlikely that EII^{glc} blocks the phosphorylation cascade by activating a so far unknown HPr kinase. Though, it is possible that the presence of HPr kinase was not tested under the right conditions: the HPr kinase in *C. glutamicum* might be active only during *e.g.* sugar-P stress. Furthermore, it is not excluded that EII^{glc} is required for the activity of an HPr kinase-like protein or even that EII^{glc} modifies HPr directly. In that aspect, it might be interesting to investigate more precisely possible chemical modifications like *e.g.* Ser-phosphorylation or acetylation of HPr during the observed regulatory processes.

Alternatively, EII^{glc} could be affecting the PTS phosphorylation cascade indirectly by regulating the activity of another metabolic or regulatory protein. As described also in the introduction, there are various examples in literature showing EII-dependent regulation of glycolytic or regulatory proteins[133]. Interestingly, the additional player involved in the rapid sucrose uptake inhibition as a response to sugar-P stress which was identified from suppressor mutants was none of the potential targets of EII^{glc} known from other organisms (see 4.3) and the found target did not seem to affect directly PEP-EI or EI-HPr transfer. Therefore, the analysis of other suppressor mutant isolates, in which this target was not found, could be useful in order to understand which might be additional targets affecting the PEP-EI or EI-HPr phosphotransfer. Furthermore, as functional and tagged versions of all PTS components involved in the regulatory process were created, in future work pull down experiments or direct biochemical studies of phosphotransfer inhibition could be performed in order to search for possible signal transducers or direct regulatory interactions. Additionally, an *in vitro* PTS assay for the analysis of PEP-dependent ^{14}C glucose phosphorylation was used in this work (data not shown). Unfortunately, the use of cytosolic and membrane fractions instead of purified enzymes caused significant difficulties in the analysis of the obtained results. Hence, a significant improvement of the assay would be the use of defined amounts of purified HPr and EI instead of cytosolic fractions, which would then be used to test potential inhibitors.

4.3. Suppression of the sugar-P stress inhibition – involvement of LpdA

The growth inhibition of sucrose cultivated *C. glutamicum Δpgi* cells by glucose is caused by EII^{glc}-dependent blockage of the PTS phosphorylation cascade leading to rapid sucrose uptake inhibition followed by a SugR-dependent *ptsS*-repression. The detailed investigation of the mechanisms behind those two regulatory processes, however, indicated that additional players might be involved in both of them. Therefore, suppressor mutants of *C. glutamicum* Δ*pgi* which had improved growth on sucrose plus glucose but still retained their glucose uptake ability were generated and analyzed.

Surprisingly, none of the expected genes which were known to play a role in the regulatory mechanism was mutated in any of the obtained suppressor mutants. Such genes were for example *sugR* and *ptsG* which encode the two proteins required for induction of the respectively biochemical and transcriptional regulatory response. No mutations were found in *ptsS* and *ptsI*, which are at the receiving end of the response, either. In contrast, with a high frequency (3 out of 7 tested isolates) a mutation of the gene *lpdA* was found, indicating a high selective pressure at that particular point. The isolates carrying this mutation were also the most improved suppressor mutants in terms of growth, *ptsS*-derepression and sucrose uptake in presence of glucose. What is more, the *lpdA* mutations were independent from each other as they were single nucleotide exchanges on different positions in the gene. Expression of the mutant version $lpdA^{K6}$ in *C. glutamicum* Δ*pgi* confirmed that the mutation of the *lpdA* gene alone was sufficient for the observed suppression effects. Hence, this so far unknown player in the regulatory cascade seems to have a crucial role in the response of the *pgi* mutant to glucose-6-P stress.

The function of *lpdA* in *C. glutamicum* is unknown. In the genome of this organism there is also a second gene, *lpd*. Both *lpd* and *lpdA* encode proteins which have a high homology to the enzyme lipoamide dehydrogenase - a member of the flavoprotein disulfide reductases (FDRs). Sequence comparison indicated that the two genes might encode two different types of lipoamide dehydrogenases. The *lpd* gene has a higher similarity to the gene *lpd* of *E. coli*, encoding a flavoprotein disulfide reductase which is a component of the pyruvate dehydrogenase and α-ketoglutarate dehydrogenase complexes[134]. This enzyme is responsible for oxidation of the reduced intermediate forms in the two complexes and uses FAD and NADH as cofactors. Though, the protein encoded by *lpdA*, which was the target

found in the suppressor mutants, lacks one of the two highly conserved cysteine residues that comprise the catalytic redox-active disulfide involved in the dithiol-disulfide interchange with lipoyl substrates of the lipoamide dehydrogenase of *E. coli* [135]. Thus, it seems likely that the encoded by *lpd* protein is the component of the pyruvate dehydrogenase and α-ketoglutarate dehydrogenase complexes in *C. glutamicum* whereas LpdA has a different function. Indeed, it has been shown previously that the LpdA protein from *M. tuberculosis,* which has a high sequence homology to LpdA of *C. glutamicum* and also lacks one of the two catalytically important cysteine residues, has no catalytic ability to reduce disulfide-bonded substrates. Instead, it catalyses the NADH- and NADPH-dependent reduction of quinones as alternative electron acceptors[136]. Hence, it is reasonable to suggest that LpdA of *C. glutamicum* might possess similar catalytic activity. In that case it is interesting how the *lpdA* mutation leads to suppression of the inhibitory effects in *C. glutamicum Δpgi*.

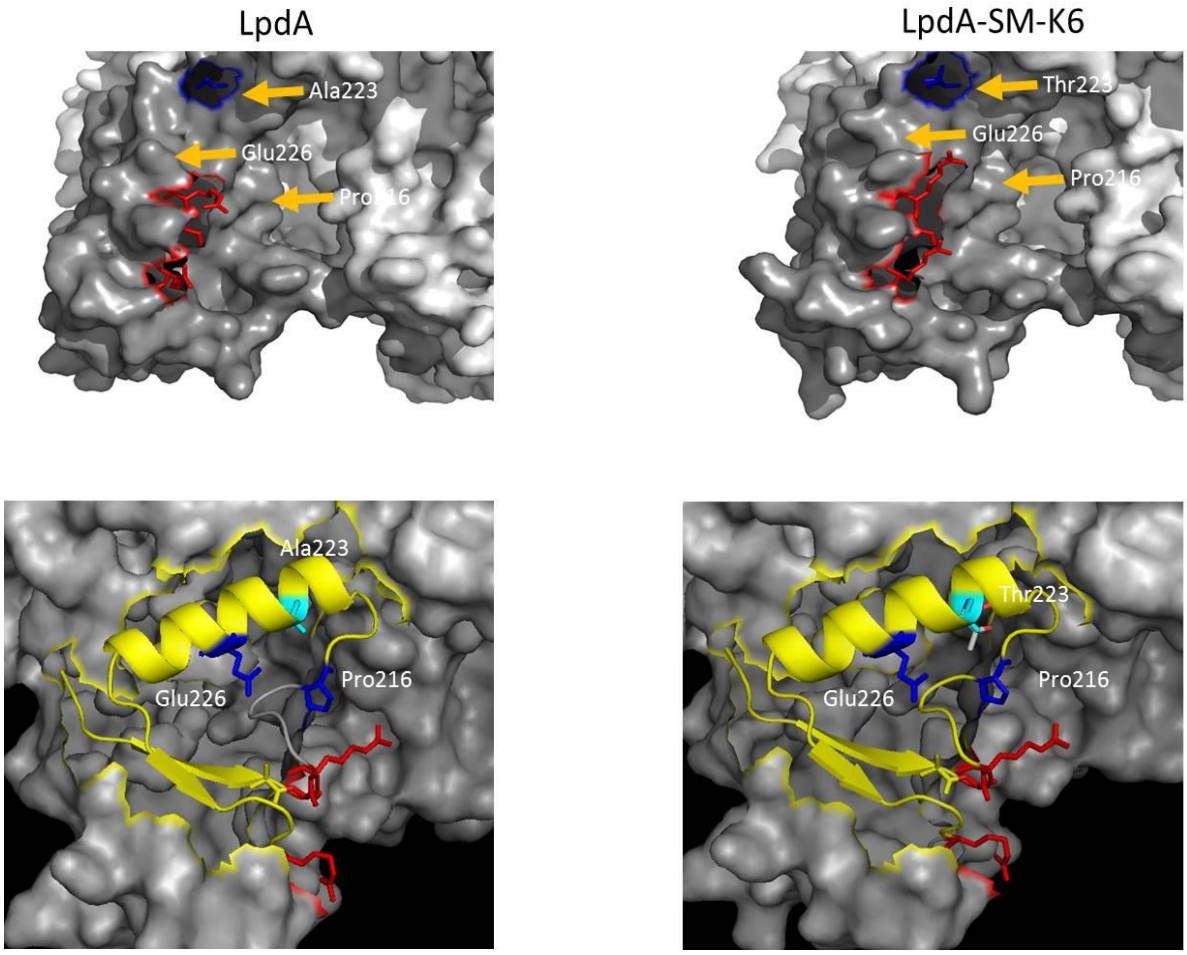


Figure 50: Structure models of the pyridine nucleotide binding pocket of LpdA and the suppressor mutant version LpdA^{A223T} based on the structure of LpdA of M. tuberculosis [136] (PDB entry 1DXI). The cyan colored Ala223 residue in the wild-type (left) is exchanged with a polar Thr group in the SM-K6 *pgi* suppressor mutant (right). Shwon in blue are the residues found in the other tree *pgi* suppressor mutants. Colored in red are the two residues suggested to be interacting with NAD(P)H for the LpdA from *M. tuberculosis*.

The feedback inhibition of the PPP enzymes glucose-6-P dehydrogenase and 6-phosphogluconate dehydrogenase by their product NADPH has been suggested as a reason for the inhibited glucose utilization in *C. glutamicum Δpgi* [137]. Therefore, the possible NAD(P)H-dependent quinone reductase activity of LpdA might be involved in the sugar-P stress response mechanism described here by enableing the transfer of NADPH towards the electron transport chain and thus reducing the feedback inhibition of the PPP, which is directly connected with the glucose-6-P accumulation in the *pgi* mutant. The growth deficit in *E. coli Δpgi* during cultivation with glucose has been also attributed to inbalances in the NADPH homeostasis and indeed a decrease of the NADPH levels, achieved in that case by proper affection of the transhydrogenase reactions mediated by UdhA or PntA, has led to significant growth improvement [69, 70].

Notably, none of the *lpdA* mutations found in the *C. glutamicum Δpgi* suppressor mutants was in the promoter region of the gene or was leading to a stop codon. Hence, an increased or decreased expression of the *lpdA* gene is an unlikely reason for the observed improvement in the phenotype of the mutants. In fact, all mutations were in the same narrow region between residues 210 and 225 which was shown in the crystal structure of LpdA of *M. tuberculosis* to form the pyridine nucleotide-binding site of the enzyme[136] (Fig. 50). Hence, it seems that the mutations might have changed the kinetic properties of the LpdA enzyme towards NADPH, NADH and/or its substrates. Indeed, all mutations were leading to changes in the polarity of the respective residues. For example, in the most improved variant, found in the SM-K6 mutant, the hydrophobic alanine 223 residue, which is in close proximity to the binding site for the phosphor-group of NADPH shown for the LpdA of *M. tuberculosis*, was exchanged for a polar and spatially bigger threonine residue, which is consistent with the hypothesis of changed affinity towards NADPH/NADH (Fig. 50).

If the kinetic properties of the mutant versions of LpdA were changed in terms of affinity towards NADPH this might have lowered the steady state level of the NADPH pool and consequently relieved the feedback inhibition of the PPP. Indeed, in an *E. coli* strain designed to produce increased NADPH amounts and unable to perform transhydrogenase conversion of NADPH to NADH, a single point mutation of the NuoF subunit of the NADH:ubiquinone oxidoreductase (respiratory complex I) led to the ability of the enzyme to use NADPH instead of NADH as energy source for the respiratory chain and this consequently improved the growth of the strain on glucose[138].

Interestingly, though, in previous experiments the heterologous overexpression of *E. coli*'s transhydrogenase encoding gene *udhA* in *C. glutamicum* Δ*pgi* improved only slightly the growth ot the strain during cultivation on glucose[77]. It is possible that in that case the decrease of the NADPH pool was not sufficient for a stronger growth improvement due to kinetic limitations of the heterologous UdhA, which is adjusted to a redox steady state of *E.coli* and not to the steady state in *C. glutamicum*. On the other hand, this could be the maximal improvement which is achieved by a decrease of NADPH and LpdA might have a stronger suppressive effect than the overexpression of *udhA* because it affects not only the redox but also the metabolic state of the cell. For example, *lpdA* is positioned in the genome of *C. glutamicum* directly in front of the *pyc* gene, encoding the anaplerotic enzyme pyruvate carboxylase, which often indicates functional connections. Also, the putative quinone reducing function of LpdA indicates similarity to *e.g.* the flavoprotein subunit of the succinate dehydrogenase (Sdh), the malate:quinone oxidoreductase (Mqo) or pyruvate:quonone oxidoreductase (Pqo). All these enzymes are connected to anaplerotic reaction of the TCA cycle. If the anaplerotic flux is changed by possible interaction with mutated LpdA this could affect the amount of α-ketoglutarate in the cell, which is an inhibitor of the EI dimerization and thus regulates the general PTS activity of the cell[139]. As the genes for the flavoprotein subunit of Sdh and Mqo are known and deletion mutants indicated that no redundant enzymes are present in this organism[140, 141], the LpdA does not seem to originally catalyse the reaction mediated by those two enzymes but a redundant function to Pqo is not excluded. Additionally, the mutation of residues near the reaction center might have extended the substrate specificity of LpdA thus enabling even more distant effets in terms of metabolic changes. However, it is unlikely that the suppressing ability of LpdA has emerged by a completely novel enzymatic activity of the protein since overexpression of wild-type LpdA was also able to improve growth of *C. glutamicum* Δ*pgi* in the presence of glucose, albeit more weakly than the mutant versions.

Taken together, LpdA was identified as a novel player affecting the glucose-6-P stress response described here for *C. glutamicum Δpgi*. The high sequence homology to previously charcterized lipoamide dehydrogenase of *M. tuberculosis,* which catalyse the NADPH- and NADH-dependent reduction of quinones, and the result that the putative co-factor binding site of LpdA was target of mutation suggest that LpdA might be affecting positively the growth of *C. glutamicum Δpgi* in the presence of glucose by a decrease of the feedback

inhibition of PPP enzymes by NADPH. However, a biochemical characterization of the LpdA enzyme would be needed in order to verify and understand more precisely its function and the reason for the observed suppressing effects during glucose-6-P stress. It might be also interesting to test if metabolic modifications leading to conditions of increased NAPDH demand in the cell, such as during oxidative stress or disproportionally high anabolism, might also have a positive effect on the glucose-6-P stress response of *C. glutamicum* Δ*pgi.*

4.4. Specificity of the novel mechanism of rapid PTS regulation

4.4.1. The sugar-P stress response in *C. glutamicum* Δ*scrB*

To test if the novel sugar-P stress response in *C. glutamicum Δpgi* described here might be a more general mechanism for the regulation of PTS activity in this organism I studied if other metabolic perturbations leading to sugar-P stress cause a similar reaction of the cell. The strain *C. glutamicum ΔscrB*, which lacks the only known pathway for the utilization of sucrose-6-P in this organism, grows normally on glucose but when the strain was cultivated with sucrose plus glucose a severe growth reduction had been reported[33]. Hence, this mutant seemed to represent another case of sugar-P stress response.

Indeed, similar effects as those observed in *C. glutamicum* Δ*pgi* after glucose addition were caused by the addition of sucrose in *C. glutamicum ΔscrB*: growth and glucose uptake were rapidly inhibited and the expression of *ptsG* was reduced. Furthermore, the rapid uptake inhibition in the *scrB* mutant also correlated with a rapid sucrose-6-P accumulation as well as reduced HPr~P levels in the cell after addition of sucrose. The studies of the glucose uptake inhibition by sucrose in *C. glutamicum ΔscrB* suggested that similarly as in the *pgi* mutant the PEP-production rate is not the limiting factor leading to glucose uptake inhibition after addition of sucrose. Hence, those results suggest that the sugar-P stress response observed in the *pgi* mutant might be a general sugar-P stress response mechanism in *C. glutamicum*. Additionally, in the *pgi* mutant the glucose-6-P accumulation and NADPH imbalance are tightly connected due to the feedback regulated initial enzymes of PPP so that potentially both factors could be regarded as inducers of the studied sugar-P stress response. Though, as in the *scrB* mutant an increased NADPH production after sucrose addition is not expected,

those results showed that the accumulation of sugar-P is sufficient as a trigger of the sugar-P stress response and does not require changes of the redox balance in the cell.

However, even though in terms of regulation of PTS activity and gene expression the responses to glucose addition in the *pgi* mutant and to sucrose addition in the *scrB* mutant are similar, it seems that in the case of sucrose-6-P accumulation additional regulatory processes are also activated. The overexpression of *ptsH* in *C. glutamicum* Δ*scrB* led to improvement of the glucose uptake rate reduction by sucrose and improved growth rate on sucrose plus glucose. No such positive effects were observed in the *pgi* mutant after increase of the HPr amounts in the cell. Further, the growth rate of the *scrB* mutant with maltose or ribose was significantly reduced when compared to the wild-type if sucrose was present in the medium, even though both maltose and ribose are non-PTS substrates and sucrose-6-P does not interfere directly with their metabolism. Hence, unlike the accumulation of glucose-6-P in the *pgi* mutant, the sucrose-6-P accumulation in *C. glutamicum ΔscrB* is toxic for the cell not only due to its function in the regulation of PTS activity. As the addition of sucrose caused reduction of the HPr~P amounts in the cell, the inhibitory effect on maltose utilization might still be connected with the regulation of PTS due to the recently identified *malP* repression and respectively low MalP activity in case of absence of HPr~P[75]. However, the reason for the negative effect on growth with ribose was not clear and it was also in contrast to the absence of such a negative effect on the growth on ribose plus glucose in the *pgi* mutant. The ribose utilization could not be caused by the low HPr~P amounts suggested for the reduction of maltose utilization as it was shown here that the absence of HPr reduces only the growth on maltose but not on ribose. Additionally, the *pgi* strain cultivated on sucrose, glucose and ribose together grew normally despite that the sucrose uptake was rapidly inhibited, *i. e*. despite depletion of HPr~P. The latter shows also that the lack of inhibition of the ribose utilization in the *pgi* mutant was not due to absence of glucose-6-P stress. Hence, the mechanistic explaination for those differences is not clear. They did not seem to be due the fact that the utilization of sucrose-6-P is abolished in the *scrB* mutant, whereas in the *pgi* mutant the glucose-6-P stress rather depends on the feedbach in inhibition of the first enzymes of PPP by their product NADPH, which in the case of ribose addition might be relieved by a better metabolic balance in the cell. Therefore, better understanding of the reasons for those differences would require a more precise analysis of the processes in *C. glutamicum* Δ*scrB* in future work.

4.4.2. PTS regulation in the wild-type

The well-studied mechanisms of CCR do not seem to apply for *C. glutamicum* which utilizes most carbon sources simultaneously. Though, the nutrients uptake during mixed substrate cultivation in this organism still seems regulated. During co-utilization of glucose-acetate[48], glucose-fructose[55] or sucrose-glucose[78] mixed substrates *C. glutamicum* reduces the uptake of each carbon source so that the total carbon consumption rate remains similar as during growth with each of the substrates alone. However, the regulatory mechanism behind those processes has not been clarified.

A rapid biochemical mechanism which reduces sucrose uptake after addition of glucose was described here for *C. glutamicum* Δ*pgi*. In this case the uptake regulation was a response to the increased flux towards glucose-6-P, which could be utilized only through the feedback inhibited PPP. Even in the presence of Pgi, however, flux studies in wild-type *C. glutamicum* have shown that during growth on glucose the predominant part (68%) of the formed glucose-6-P is utilized through the PPP [142]. Thus, it was interesting if the regulatory mechanism shown for the *pgi* mutant could be causing the sucrose and glucose uptake rate adjustment also in the wild-type, where the uptake reduction would be weaker because of the possibility of the cell to use also the glycolytic pathway for the utilization of glucose-6-P. Indeed, in the wild-type the sucrose uptake reduction after addition of glucose was also a rapid, biochemical process. The addition of glucose caused a similar, rapid reduction of the PTS-mediated fructose uptake, as well. Nonetheless, in contrast to the effects observed in the *pgi* mutant, the addition of the non-PTS substrate maltose instead of glucose did not cause a rapid reduction of the sucrose uptake in the wild-type, despite that it also leads to increased formation of glucose-6-P. Moreover, whereas the addition of maltose to sucrose-cultivated *C. glutamicum* Δ*pgi* cells caused significant reduction of the growth and total carbon consumption rate, in the wild-type the addition of maltose or glucose-6-P resulted in an increase of the growth rates. These results show that the described above sugar-P stress response mechanism is not responsible for the adjustment of the PTS mediated uptake during sucrose-glucose co-utilization in the wild-type.

The growth rates of the wild-type and *C. glutamicum* (pEKEx2*-*uhpT*) during cultivation on sucrose-maltose or sucrose-glucose-6-P mixed substrates were even higher than the growth rates during growth of the wild-type on sucrose plus glucose. As those substrates all require identical metabolic pathways for utilization, this result demonstrates that the metabolic

capacity of the wild-type is not the limiting factor for a higher sugar utilization rate during growth on sucrose plus glucose. Consequently, it seems that the PTS capacity of the cell is the limiting factor leading to a similar total carbon consumption rate during growth on sucrose plus glucose and on either of the sugars as a sole carbon source. Furthermore, the overexpression of *ptsG* or *ptsS* did not increase the total carbon consumption rate of PTS substrates like glucose or sucrose in the wild-type. This indicates that specifically the common PTS phosphorylation cascade might be the limiting factor. In conclusion, these results suggest that the rapid adjustment of the PTS-mediated uptake in the wild-type during co-utilization of sucrose plus glucose or glucose plus fructose is caused by a competition of the EII permeases for a limited rate of the common PEP-EI-HPr phosphorylation cascade, *i.e*. limited amount of generated HPr~P. Indeed, the addition of the non-PTS substrate maltose had no effect on the sucrose uptake and *vice versa* - the addition of glucose reduced the sucrose or fructose uptake of the cell but not the uptake of maltose. The idea of competition of the EII permeases for limited amount of HPr~P is supported also by the result that changes in the EII^{glc} / EII^{suc} ratio in the cell resulted in different proportions between the sucrose and glucose uptake rate reductions but in all cases total carbon uptake rates remained similar.

The activity of the PTS phosphorylation cascade is tightly coupled to the PEP/pyruvate ratio in the cell. In *E. coli* changes in the PEP/pyruvate ratio correlate with changes in the phosphorylation state of $EIIA^{glc}$ [28, 143]. Further, overexpression of the *ppS* gene, encoding for a PEP synthase, stimulated the glucose consumption rate in *E. coli* [143]. Also in *C. glutamicum* the addition of maltose led to increase of both total carbon and glucose consumption rates[54], which is consistent with the idea of a PEP/pyruvate limitation of the PTS activity. The adjustment of a higher PEP/pyruvate ratio after addition of a non-PTS carbon source might not be a rapid process, though. The addition of maltose or ribose was shown here to have no positive effect on the sucrose or respectively glucose uptake rate in the wild-type for the initial 2 min after addition. This was also in agreement with the previous observations that the increase in glucose consumption after PEP synthase induction is not a rapid process, as well[143]. Further, consistent with the idea that the rate of PTS phosphotransfer is limited by the PEP/pyruvate ratio, the increase in the amounts of either EI or HPr in the cell did not lead to an increase of the PTS activity in the wild-type, indicating that their amount might not be the bottleneck determining the limited phosphotransfer rate. However, it should be

considered that increased amounts of both common PTS components might be required, in order to increase the rate of the PTS phosphorylation cascade and thus PTS activity of the cell.

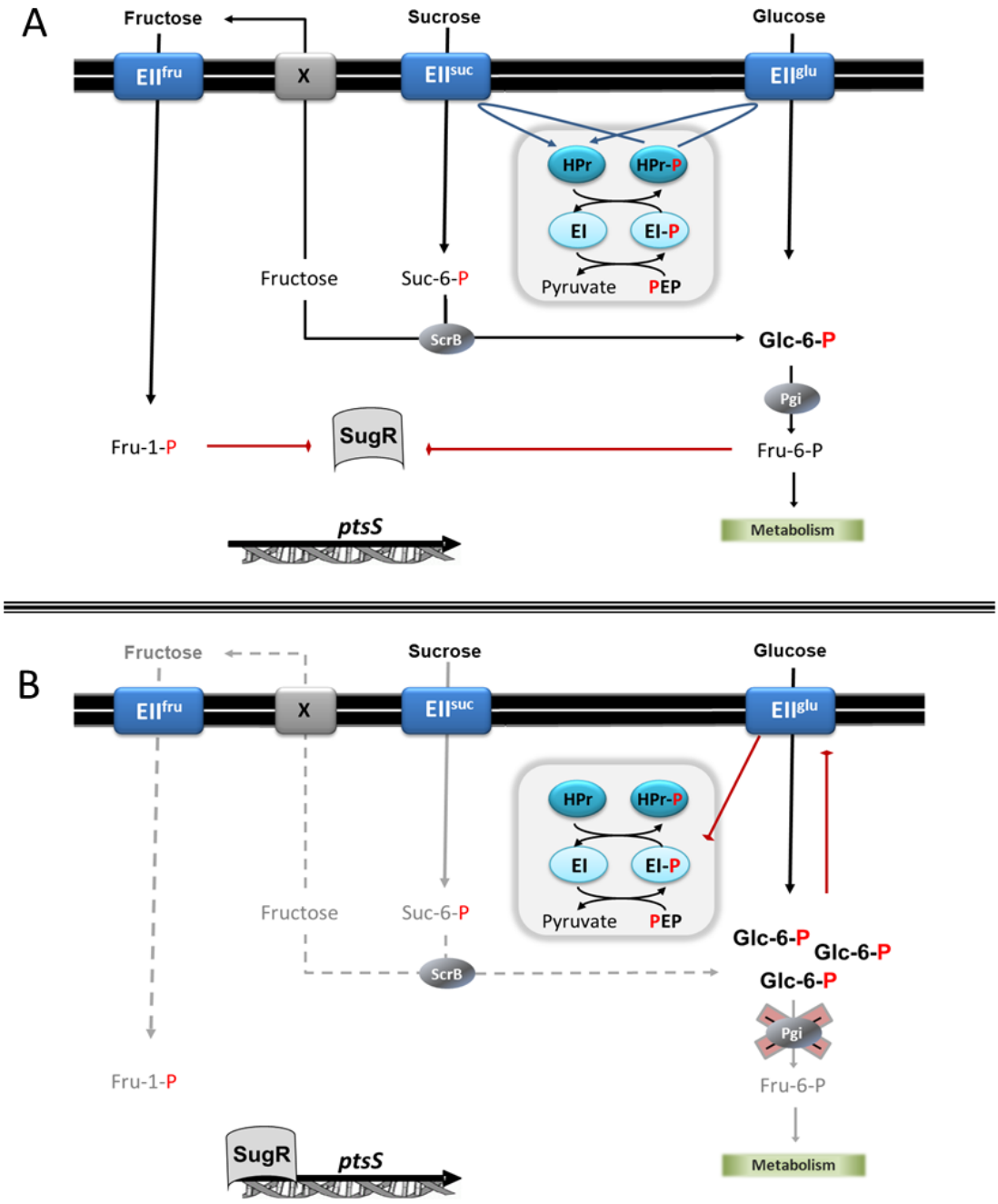


Figure 51: Models for the regulation of PTS activity in *C. glutamicum* during co-utilization of sucrose and glucose. (A): wild-type *C. glutamicum*: the total carbon uptake rate remains similar but the specific sucrose and glucose uptake rates are adjusted due to competition between EII^{glc} and EII^{suc} for a limited amount of HPr~P. High amounts of glycolytic intermediates like fructose-1-P and fructose-6-P are generated, which prevents repression of the PTS genes by SugR. **(B):** in *C. glutamicum Δpgi* the perturbation of metabolism leads to glucose-6-P accumulation sensed by EII^{glc}, which induces a sugar-P stress response reducing the PEP-EI-HPr phosphorylation cascade and thus general PTS activity. As a consequence of the low sugar uptake the formation of fructose-1-P and fructose-6-P is diminished and SugR gets activated.

Taken together, the adjustment of PTS activity in *C. glutamicum* during co-utilization of sucrose plus glucose seems to originate from direct competition of the EII^{suc} and EII^{glc} permeases for a limited amount of HPr~P (Fig. 51, A). The limitation of the phosphotransfer rate is likely to be determined simply by the PEP/pyruvate ratio in the cell, as discussed also for the co-utilization of PTS substrates in other organisms like *e.g. E. coli*[144] or *S. lactis*[122]. The expression of *ptsG* and *ptsS* proceeds normally as glycolytic intermediates acting as negative effectors of the SugR repressor are produced in the cell. However, in *C. glutamicum Δpgi* the increased concentrations of glucose-6-P activate an EII^{glc}-dependent regulatory mechanism which causes additional inhibition of the PTS phosphorylation cascade. EII^{glc} and EII^{suc} in this case start to compete for a much lower level of HPr~P and thus the general substrate uptake activity and growth rate of the cell drops. As a consequence the concentration of the upper glycolytic intermediates decreases and SugR binds to its targets, leading to inhibition of the *ptsS*-expression (Fig. 51, B).

Reduction of the glucose uptake rate during co-utilization of glucose with acetate in *C. glutamicum* could not be explained with direct competition of EII permeases for HPr~P as acetate is transported by the secondary active trasnporter MctC. The presence of acetate has been suggested to decrease the glucose uptake by a SugR-dependent repression of *ptsG*[54]. However, here it was shown that the reduction of the glucose uptake after acetate addition is a rapid, biochemical response, which occurs within 15 sec. This result indicates that the transcriptional regulation of *ptsG* suggested previously is not the only and also not the primary reason for the reduced glucose uptake rate during cultivation of *C. glutamicum* on glucose plus acetate. Moreover, it was also demonstrated, that the addition of acetate causes a rapid reduction of the sucrose uptake in *C. glutamicum*, as well. Hence, the initial biochemical regulation of glucose and sucrose uptake could be the reason for the *ptsG*-repression by SugR due to a reduction of the concentration of glycolytic intermediates, similarly as described above for the mechanism of inducer exclusion in the *pgi* mutant. Carbon flux studies in *C. glutamicum* cells grown on glucose plus acetate have also shown a significantly increased flux from fructose-6-P to glucose-6-P compared to growth on glucose as a sole carbon source[48]. Thus, it was interesting if the sugar-P stress response mechanism described for *C. glutamicum Δpgi* was involved in the glucose uptake rate adjustment during growth on glucose-acetate and sucrose-acetate mixed substrates. More detailed studies

showed that this is not the case. Sucrose uptake reduction by acetate addition was still taking place to the same extent in *C. glutamicum Δpgi*, despite that the conversion of fructose-6-P to glucose-6-P by Pgi in this strain is abolished. Furthermore, the sucrose uptake inhibition by acetate was not abolished by the absence of EII^{glc}, which is required for the regulatory mechanism described here. Therefore, these results show that the regulatory mechanism demonstrated for the control of the PTS activity in the *pgi* mutant was not involved in the regulation of the glucose or sucrose uptake in *C. glutamicum* after addition of acetate.

In the absence of acetate kinase (Ack) or phosphotransacetylase (Pta), which catalyse the first two steps of acetate utilisation to acetyl-CoA, no glucose uptake reduction by acetate was observed, indicating that downstream products of the acetate metabolism are required in order to influence the activity of the PTS. It was also excluded that an unspecific acidification of the cytosol is the reason for the acetate-dependent glucose uptake regulation. Further, the fact that acetate caused a similar reduction of both the glucose and sucrose uptake but had no effect on the uptake of the non-PTS substrate maltose indicated that the presence of acetate might be affecting specifically the common PTS phosphorylation cascade. This is also consistent with the fact that the total carbon consumption during growth of the wild-type on glucose-acetate mixed substrate remained similar as during cultivation on glucose alone[48].

In contrast to other non-PTS substrates like maltose and ribose, which did not affect the PTS activity and even increased the total carbon consumption rate in *C. glutamicum*, acetate is a gluconeogenetic substrate, which enters the central metabolism at the end of glycolysis. Thus, acetate competes with the pyruvate flux coming from glycolysis for their further utilization in the TCA cycle. Indeed, flux studies performed by Wendisch *et al.* have shown that during growth of *C. glutamicum* on glucose plus acetate the proportion of glucose-derived carbon oxidized to acetyl-CoA is greatly diminished compared to that during growth on glucose as a sole carbon source[48]. Accordingly, acetyl-CoA was shown to be derived in the acetate plus glucose cultivated wild-type almost exclusively from acetate and the flux from pyruvate to oxaloacetate or to biosynthetic products did not seem to compensate for the low flux from pyruvate towards acetyl-CoA [48]. Hence, the limited flux of pyruvate towards the TCA cycle in presence of acetate could be resulting in a product inhibition of EI by pyruvate and following reduction of the PEP to pyruvate conversion rate. Thereby a lower

PEP to pyruvate conversion rate would lead to the rapid biochemical reduction of the glucose and sucrose uptake. Indeed, a rapid inhibition of PTS activity was demonstrated here also after addition of pyruvate, indicating directly that a product inhibition of EI is able to cause the effects observed after acetate addition. Further, the absence of a pyruvate dehydrogenase, which catalyses the reaction of pyruvate to acetyl-CoA creates a comparable situation to the case when the pyruvate coming from glycolysis competes with acetyl-CoA derived from the addition of acetate. Indeed, during growth on the glycolytic, non-PTS substrate glucuronate the conversion flux of PEP to pyruvate was significantly reduced in an *E. coli* mutant lacking pyruvate dehydrogenase when compared to the wild-type [145].

On the other hand, for *E. coli* and *Salmonella typhimurium* a direct reversible phosphoryl group transfer between the acetate kinase and the catalytic histidine of EI has been shown[146A]. Therefore, it is possible that during co-utilization of glucose and acetate EI~P is used not only for the uptake of glucose by the PTS but also for the conversion of acetate to acetyl-P. In that sense, the uptake and utilization of acetate might still be acting analogously as the activation of a second PTS permease but in the case of acetate addition the two substrates might be competing for EI~P. The lack of glucose uptake reduction in the *pta* mutant was not contorversial to this hypothesis as Ack would be inhibited by the accumulation of its product acetyl phosphate and thus would no longer be competitor for EI~P [147B]. Nonetheless, further studies would be necessary to understand more precisely that phenomenon.

Taken together, it was shown that *C. glutamicum* controls its PTS activity during co-utilization of carbon sources not only by long-term transcriptional regulation but also by immediate response directly on the level of protein activity. Moreover, since this rapid uptake adjustment occurs as initial response it might be affecting transcriptional regulation, as discussed for the case of SugR. Further, the rapid uptake responses seem to rely on specific mechanisms in dependence of the different metabolic changes as the novel EII^{glc}-dependent glucose-6-P stress response mechanism described here for *C. glutamicum* Δ*pgi* is not responsible for the rapid adjustments of the PTS activity in the wild-type. In all cases, however, the common PEP-EI-HPr phosphorylation cascade seems to be the target for PTS regulation. Hence, despite not showing the CCR behaviour and regulatory components known from Gram-negative or low-GC Gram-positive bacteria this organism still has a

sophisticated regulatory network to control its substrate uptake and PTS activity. Considering the biotechnological importance of the efficient substrate uptake as well as the fact that the regulation of and by the PTS in *C. glutamicum* seems different compared to well-studied Gram-negative and low-GC Gram-positive bacteria, further investigation of this regulatory network would be interesting and could reveal additional, novel mechanisms for regulated co-utilization of mixed substrates.

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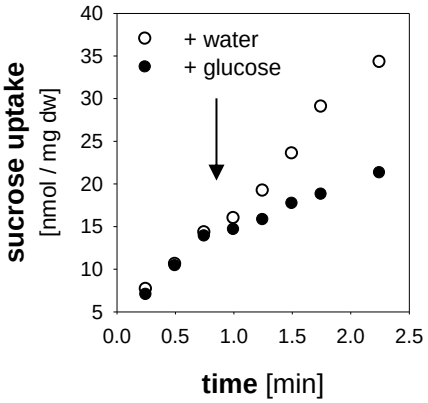
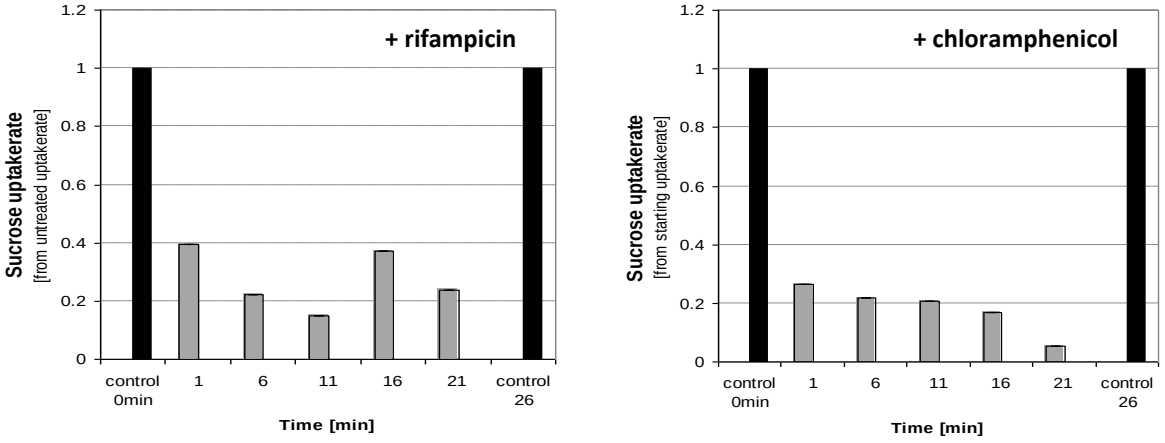
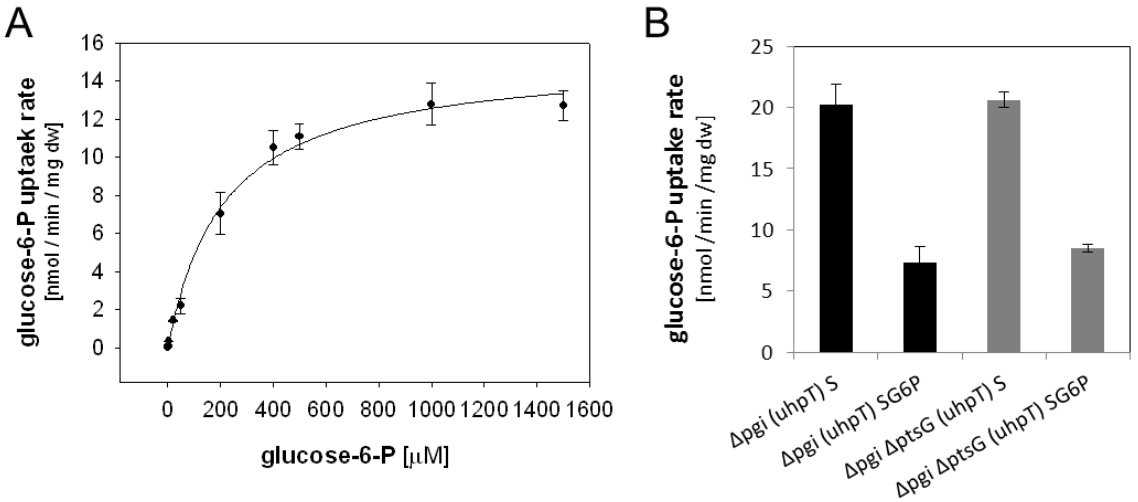
SUPPLEMENTARY DATA:



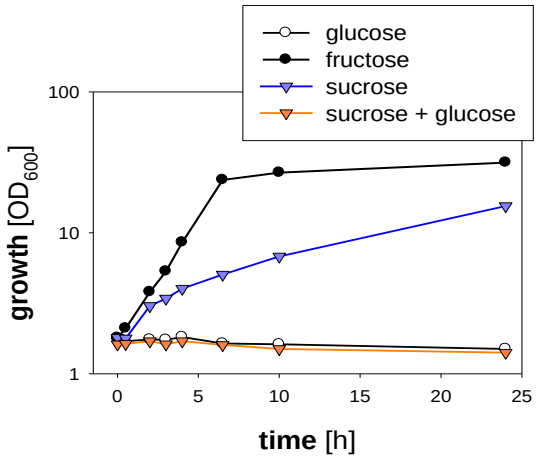
Supplementary Figure 1: Rapid sucrose uptake inhibition in *C. glutamicum* Δ*pgi* Δ*sugR*



Supplementary Figure 2: Sucrose uptake rates after addition of glucose (20 mM) in sucrose cultivated *C. glutamicum Δpgi* cells, after treatment for 10 min with 30µg/ml rifampicin or 75µg/ml chloramphenicol.



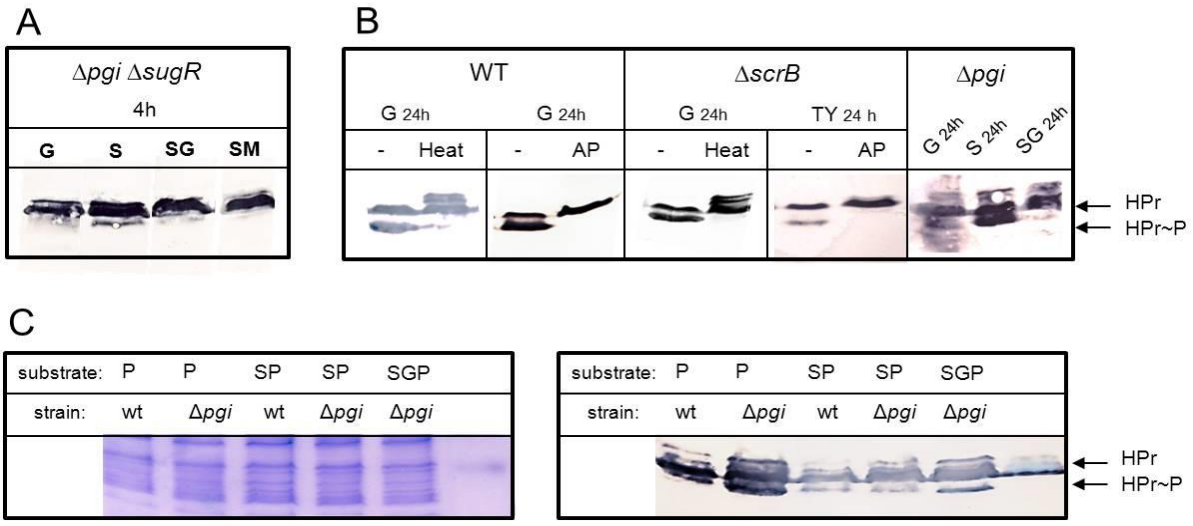
Supplementary Figure 3: Glucose-6-P uptake characterization of *C. glutamicum* Δ*pgi* (pEKEx2*-*uhpT*) and maximal glucose-6-P uptake rates of *C. glutamicum* Δ*pgi* (pEKEx2*-*uhpT*) and *C. glutamicum* Δ*pgi* Δ*ptsG* (pEKEx2*-*uhpT*) cells cultivated on sucrose (1% [w/v]) or sucrose plus glucose-6-P (1%[w/v] each). The cells were incubated in presence of 0.1 mM IPTG.



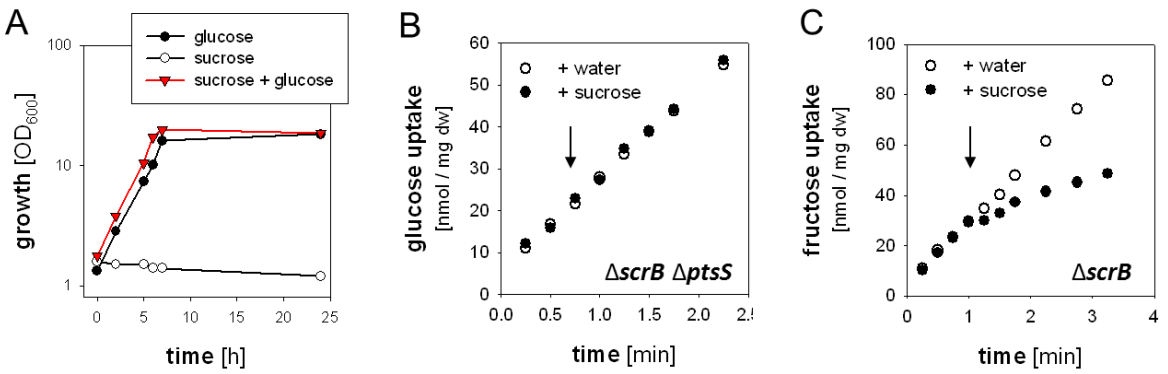
Supplementary Figure 4: Growth of *C. glutamicum* Δ*pfkA*: cells cultivated in MM with glucose (2% [w/v]), fructose (2% [w/v]), sucrose (2% [w/v]) and sucrose plus glucose (1 + 1 % [w/v]) as a substrate;

C. glutamicum strain	IPTG [mM]	Growth rate on sucrose [1/h]	Sucrose uptake [nmol/min/mg dw]
wt (pEkEx2)	0.2	0.42 ± 0.02	18.9 ± 1.2
wt (pEkEx2-*ptsH*)	0	0.42 ± 0.02	
wt (pEkEx2-*ptsH*)	0.2	0.42 ± 0.02	19.2 ± 1.7
Δ*pgi* (pEkEx2)	0.2	0.42 ± 0.02	17.8 ± 1.3
Δ*pgi* (pEkEx2-*ptsH*)	0	0.42 ± 0.02	17.8 ± 2.2
Δ*pgi* (pEkEx2-*ptsH*)	0.01	0.41 ± 0.01	17.0 ± 1.5
Δ*pgi* (pEkEx2-*ptsH*)	0.2	0.42 ± 0.02	16.6 ± 2.2

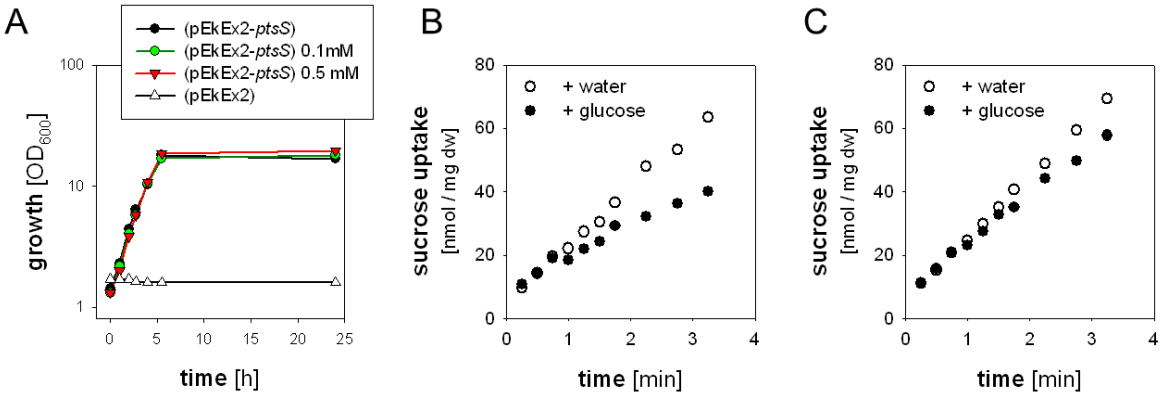
Supplementary Figure 5: Growth rates during cultivation on sucrose as a sole carbon source and sucrose uptake rates of *C. glutamicum* (pEKEx2-*ptsH*_FLAG) and *C. glutamicum* Δ*pgi* (pEKEx2-*ptsH*_FLAG) induced with different IPTG concentrations.



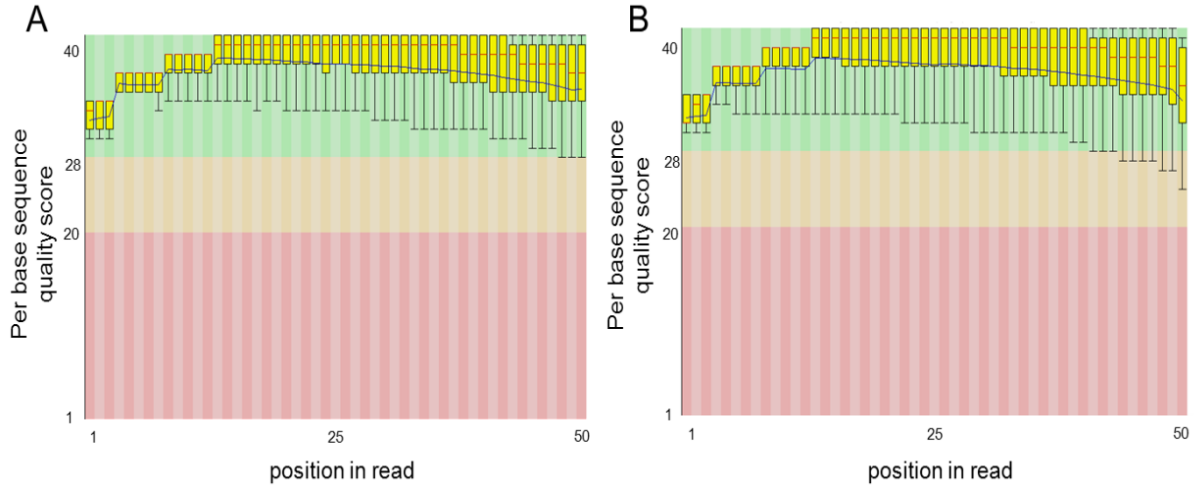
Supplementary Figure 6: Analysis of the HPR phosphorylation state of (A): *C. glutamicum* Δ*pgi* Δ*sugR* cells cultivated in MM with glucose (G; 2% [w/v]), sucrose (S, 2% [w/v]), sucrose plus maltose (SM; 1 + 1% [w/v]) and sucrose plus glucose (SG; 1 + 1 % [w/v]) as a substrate; **B:** wild-type, Δ*pgi* and Δ*scrB* cells harvested after 24 h of cultivation on glucose, sucrose, glucose plus sucrose or TY complex medium. C: wild-type and Δ*pgi* cells cultivated with pyruvate, sucrose plus pyruvate or sucrose plus glucose plus pyruvate 1% w/v] each. The samples were analysed after heat or alkaline phosphatese (AP) treatment, as well.



Supplementary Figure 7: Inhibitory effects of sucrose in *C. glutamicum* Δ*scrB*: growth on glucose (G; 2% [w/v]), sucrose (S, 2% [w/v]) and sucrose plus glucose (SG; 1 + 1 % [w/v]) (A) and glucose uptake after sucrose addition (B) of *C. glutamicum* Δ*scrB* Δ*ptsS*; Fructose uptake inhibition after sucrose addition in Δ*scrB*.



Supplementary Figure 8: Effects by *ptsS* overexpression in *C. glutamicum* Δ*ptsS*: growth sucrose (S, 2% [w/v]) (A) and glucose uptake after sucrose addition (B) of *C. glutamicum* Δ*scrB* Δ*ptsS*; Fructose uptake inhibition after sucrose addition in Δ*scrB*.



Supplementary Figure 9: Per base sequence quality of the raw sequencing data from the whole genome re-sequencing of the original *C. glutamicum* Δ*pgi* strain (A) and the *C. glutamicum* Δ*pgi* SM6 suppressor mutant (B). The quality analysis was performed with FastQC.

Affirmation:

Hereby, I declare to have prepared the present dissertation autonomously without illegitimated assistance. No other supplemental material or references have been used than those, which are annotated. This dissertation has not been submitted to any other faculty or university for examination.

Köln, April 24, 2015 Dimitar P. Petrov

Scientific activities

Publications:

Kuhlmann N / Petrov D P, Henrich A W, Lindner S N, Wendisch V F, and Seibold G M., Transcription of *malP* is target of PTS-dependent regulation in *Corynebacterium glutamicum***,** submitted to *Microbiology*

Lindner SN / Petrov DP, Hagmann CT, Henrich A, Krämer R, Eikmanns BJ, Wendisch VF, Seibold GM. (2013), Phosphotransferase system-mediated glucose uptake is repressed in phosphoglucoisomerase-deficient *Corynebacterium glutamicum* strains, *Appl Environ Microbiol.* 79(8):2588-95.

Conference participation:

2013: Annual conference of the association for general and applied microbiology (VAAM) in Bremen. Oral presentation "Novel response on central metabolism perturbations in *Corynebacterium glutamicum*: Instantaneous stop of phosphotransferase system-mediated sugar uptake"

2014: Annual conference of the association for general and applied microbiology (VAAM) in Dresden. Poster presentation "Phosphosugar stress causes EII^{Glc}–dependent inhibition of the PTS phosphorylation cascade in *Corynebacterium glutamicum*"

2014: International conference „50 years PTS"in Münster. Poster presentation "PTS controls maltose metabolism in *Corynebacterium glutamicum*: Identification of *malP*-transcription as target of PTS-dependent regulation"

2015: Annual conference of the association for general and applied microbiology (VAAM) in Marburg. Poster presentation "Sucrose causes rapid glucose uptake inhibition and *ptsG*-repression in *Corynebacterium glutamicum* Δ*scrB*"

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Erklärung

Ich versichere, dass ich die von mir vorgelegte Dissertation selbständig angefertigt, die benutzten Quellen und Hilfsmittel vollständig angegeben und die Stellen der Arbeit − einschließlich Tabellen, Karten und Abbildungen −, die anderen Werken im Wortlaut oder dem Sinn nach entnommen sind, in jedem Einzelfall als Entlehnung kenntlich gemacht habe; dass diese Dissertation noch keiner anderen Fakultät oder Universität zur Prüfung vorgelegen hat; dass sie − abgesehen von unten angegebenen Teilpublikationen − noch nicht veröffentlicht worden ist sowie, dass ich eine solche Veröffentlichung vor Abschluss des Promotionsverfahrens nicht vornehmen werde. Die Bestimmungen der Promotionsordnung sind mir bekannt. Die von mir vorgelegte Dissertation ist von Prof. Dr. Reinhard Krämer betreut worden.

Teilpublikationen:

Kuhlmann N / Petrov D P, Henrich A W, Lindner S N, Wendisch V F, and Seibold G M., Transcription of *malP* is target of PTS-dependent regulation in *Corynebacterium glutamicum***, submitted to *Microbiology***

Lindner SN / Petrov DP, Hagmann CT, Henrich A, Krämer R, Eikmanns BJ, Wendisch VF, Seibold GM. (2013), Phosphotransferase system-mediated glucose uptake is repressed in phosphoglucoisomerase-deficient *Corynebacterium glutamicum* strains, ***Appl Environ Microbiol.* 79(8):2588-95.**

Köln, den 24.04.2015 Unterschrift