Genetic control of carbohydrate uptake and utilization in *Corynebacterium glutamicum*

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Content

Abbreviations

Amp^R	Ampicillin resistance
ATCC	American Type Culture Collection
ATPase	adenosine triphosphatase
ChIP	Chromatin immunoprecipitation
DTT	dithiothreitol
e.g.	for example
et al.	*et alii*
i.e.	that is
IPTG	Isopropyl-thio-β-D-galactopyranosid
Kan^R	Kanamycin resistance
MES	2-(N-morpholino)-ethanesulfonic acid
MOPS	3-(N-morpholino)-propanesulfonic acid
ORF	open reading frame
RBS	ribosome binding site
rpm	revolutions per minute
v/v	volume per volume
WT	wild type
ε	molar extinction coefficient

Abbreviations not included in this section are according to international standards, as for example listed in the author guidelines of the *FEBS Journal.*

1 Summary

Corynebacterium glutamicum can utilize a variety of carbohydrates and organic acids. In contrast to other bacteria, *C. glutamicum* typically does not show diauxic growth on mixed carbon sources, but co-utilizes the present carbon sources. Uptake of the preferred carbon source glucose via the phospho*enol*pyruvate-dependent phosphotransferase system (PTS) is reduced during growth on substrate mixtures as compared to growth on glucose as sole carbon source. To cope with fast changing situations, especially with respect to nutrient availability, microorganisms have evolved a variety of different strategies to allow optimal growth.

In this work the control of carbohyrate uptake and utilization in *C. glutamicum* was studied to extend the knowledge of complex gene regulation in this industrial important organism. Thereby, the DeoR-type transcriptional regulator SugR was identified as repressor of *ptsG*, *ptsS* and *ptsF* expression encoding the PTS permeases specific for glucose, sucrose and fructose uptake, regions. A partly conserved 8 bp SugR binding-motif was found in the corresponding promoter fragments. On gluconeogenic carbon sources, e.g. pyruvate or acetate, SugR represses *ptsG* transcription. During growth on PTS sugars, the identified effector, fructose-6-phosphate, prevents SugR from binding, thus *ptsG* is derepressed. Repression of SugR is maximal when fructose-6-phosphate concentrations are low, representing a mechanism which allows *C. glutamicum* to adapt glucose uptake to carbon source availability.

Meanwhile, several transcriptional regulators of *pts* genes are known, among these are the two functionally equivalent regulators GntR1 and GntR2. GntR-type transcriptional regulators are involved in negative control of gluconate utilization genes in many bacteria and it was shown that gluconate itself interferes with their binding. A new and surprising aspect of this work was that GntR1 and GntR2 activate expression of *ptsG* and *ptsS*, too. This is the first example of *pts* gene control by gluconate. It is obvious that these transcriptional regulators are important players in a complex regulatory network, controlling uptake and metabolism of carbon sources in order to allow the most favourable combination of available substrates.

In addition, the results indicate that regulation of the central carbon metabolism in *C. glutamicum* comprises control of the glyoxylate and TCA cycle genes by the global regulators RamA and RamB on the one side and control of glycolysis and pentose phosphate pathway genes by SugR on the other side. The SugR regulon to date comprises at least 17 genes in 14 transcription units. The genes encoding e.g. 6-phosphofructokinase (*pfkA*), fructose-1,6-bisphosphate aldolase (*fba*), enolase (*eno*), pyruvate kinase (*pyk*), NAD-dependent L-lactate dehydrogenase (*ldhA*) and transketolase (*tkt*) were identified as direct SugR targets and enzymatic activity measeurements revealed that SugR-mediated repression affects the activities of PfkA, LdhA, Pyk and Fba *in vivo*, whereas maximum SugR control is on LdhA activity.

As SugR controls *pts* gene expression and the expression of genes involved in further consumption of these substrates in *C. glutamicum*, the gene encoding the initial step in sucrose metabolism, the sucrose-6-phosphate hydrolase (ScrB), was biochemically characterized and SugR-dependent control was analyzed. The results showed that SugR does not regulate *scrB*. Thus, control of *ptsS* and *scrB* with respect to the carbon source sucrose is clearly different.

In *C. glutamicum* L-lactate is produced from pyruvate by LdhA. The L-lactate utilization operon cg3226-*lldD*, encoding the quinone-dependent L-lactate dehydrogenase, is essential for growth on L-lactate as sole carbon source. Control of L-lactate metabolism by the FadR-type regulator LldR on the one hand ensures that the L-lactate utilization operon, is expressed only when L-lactate is present. Whereas, SugR control of *ldhA* on the other hand ensures that *ldhA* expression is maximal when i.e. supply of carbohydrate growth substrates entering glycolysis is sufficient and therewith fructose-6-phosphate concentrations are high. Under oxygen deprivation conditions, overexpression of *sugR* reduced L-lactate formation by about 25% and *sugR* deletion improved L-lactate formation three fold.

SugR negatively controls expression of *pts* genes and controls the glycolytic flux straight forward to pyruvate, which serves as precursor for L-lysine and L-lactate production in *C. glutamicum*, and also controls the flux through the pentose phosphate pathway leading to a better NADPH supply. Thus, this regulator is therefore a good candidate for improving L-lysine production with *C. glutamicum*. A *sugR* deletion in the strain DM1729 led indeed to an inceased lysine yield of about 20%, 50% and 70% on the PTS sugars glucose, fructose and sucrose, respectively.

2 Introduction

In the mid-1950s, *Corynebacterium glutamicum* (synonym *Micrococcus glutamicus*) was isolated by the group of Kinoshita due to its ability to excrete L-glutamate under biotin-limiting conditions (Kinoshita *et al.*, 1957). The primarily 1896 from Lehmann and Neumann defined genus *Corynebacterium* (Lehmann and Neumann, 1896) belongs to the class of *Actinobacteria*, which summarises eubacteria with a high GC content (51-68 mol%) (Liebl, 2005). Within this genus the Gram-positive *C. glutamicum* has become a model organism for the *Corynebacterineae*, a suborder of the *Actinomycetales*, which also comprises extremely pathogen species like *Mycobacterium tuberculosis, M. leprae* and *C. diphteriae*, but also non-pathogenic organisms like *C. efficiens* (Stackebrandt *et al.*, 1997). *C. glutamicum* is close related to these pathogen species but is itself classified as “safe”. Besides the already mentioned high GC content, *C. glutamicum* is characterized by biotin-auxotrophy, immobility and a rod-shaped and irregular morphology (“coryneform”) and the typical V-type arrangement of cell pairs due to “snapping division” (Liebl, 2005). Nowadays, this predominantly aerobic soil bacterium is widely used for the industrial production of more than two million tons of amino acids per year, mainly L-glutamate and L-lysine (Hermann, 2003; Leuchtenberger *et al.*, 2005). Monosodium glutamate was discovered in Japan as a new taste (“umami”) and enhances the flavor of food. L-lysine as an essential amino acid thus plays an important role as feed additive. A general view of this non-pathogenic bacterium can be found in the recent monographs (Burkovski, 2008; Eggeling and Bott, 2005).

To cope with different environmental situations which are often life-threatening, microorganisms have evolved a diversity of regulatory and adapted strategies. Carbon sources as example have to be converted to metabolites and precursors that enable the cells to build up energy for growth and synthesis of new cell material. Thus, the adaption to the availability of carbon and energy sources is in many cases connected with substrate-specific induction or repression of catabolic genes. The regulatory mechanisms behind these strategies play an important role in understanding the central carbon metabolism of microorganisms and in this case for the metabolic engineering in order to improve product formation with *C. glutamicum* as biotechnological important model organism (for detailed information see (Burkovski, 2008)).

2.1 Carbohydrate uptake by *C. glutamicum*

When two carbon sources are supplied simultaneously, one (e.g. glucose) is often preferred over the other (e.g. maltose, lactose). This observation of so-called diauxic growth was first

made in the early 1940s by Jacques Monod (Monod, 1942). A specific hierarchy exists for the utilization of carbon sources in each organism, usually with glucose at the top of it. It was also found that the preferred sugars such as glucose, fructose or sucrose repress the synthesis of enzymes required for the uptake of less favourable substrates as long as they are available in sufficient amounts. This phenomenon therefore was named carbon catabolite repression (CCR) (Contesse *et al.*, 1969). The regulatory mechanisms underlying CCR take place either on protein-DNA level (i.e. repression or activation due to protein binding to its target DNA) or on protein-protein level (i.e. protein phosphorylation) or due to protein-metabolite interactions (i.e. effector binding) which are in turn modulated via protein modifications (Wagner, 2000). *C. glutamicum* is able to grow on various carbon and energy sources, such as sugars, sugar alcohols and organic acids (Dominguez *et al.*, 1998; Eikmanns, 2005; Gerstmeir *et al.*, 2003; Kiefer *et al.*, 2002; Krämer *et al.*, 1990; Moon *et al.*, 2005; Wendisch, 2003; Yokota and Lindley, 2005). Among the substrates metabolized are glucose, sucrose, fructose, L-lactate, acetate and gluconate which all can also serve as substrates for amino acid production (Dominguez *et al.*, 1998; Kelle *et al.*, 2005; Kiefer *et al.*, 2002; Kimura, 2005). In contrast to *Escherichia coli* and *Bacillus subtilis*, which show distinct catabolite repression, *C. glutamicum* usually co-utilizes the carbon sources present in the mixtures without showing diauxic growth. Glucose is the preferred carbon source, but has been shown to be co-metabolized with e.g. acetate (Wendisch *et al.*, 2000), L-lactate (Stansen *et al.*, 2005), propionate (Claes *et al.*, 2002), pyruvate, serine (Netzer *et al.*, 2004b), protocatechuate, vanillate (Merkens *et al.*, 2005) and fructose (Dominguez *et al.*, 1997). The only known exceptions of carbon source co-utilization are the sequential consumption of glucose before glutamate (Kronemeyer *et al.*, 1995) and glucose before ethanol (Arndt *et al.*, 2007).

2.1.1 Glucose, fructose and sucrose uptake is mediated by the phospho*enol*pyuvate-dependent phosphotransferase system (PTS) in *C. glutamicum*

A sugar transport system which is widespread among various bacteria and which frequently plays a major role in CCR is the phospho*enol*pyuvate-dependent phosphotransferase system (PTS) (Deutscher *et al.*, 2006; Parche *et al.*, 2001; Postma *et al.*, 1993; Stülke and Hillen, 2000). Mori and Shiio (Mori and Shiio, 1987) first mentioned the presence of a PTS in *C. glutamicum*. The enzymes involved in sugar uptake via the PTS are well characterized (Kotrba *et al.*, 2001; Moon *et al.*, 2005; Parche *et al.*, 1999; Parche *et al.*, 2001), but detailed information on regulatory mechanisms with regard to this pathway are scare. In *C. glutamicum* uptake of glucose, fructose and sucrose is mediated by the PTS with the

substrate-specific enzyme II permeases EII^{Glc} (*ptsG,* cg1537), EII^{Fru} (*ptsF,* cg2120), and EII^{Suc} (*ptsS,* cg2925) (Figure 2.1), respectively (Dominguez and Lindley, 1996; Kiefer *et al.*, 2002; Kiefer *et al.*, 2004; Moon *et al.*, 2005; Mori and Shiio, 1987). The PTS is characterized by the uptake and concomitant phosphorylation of carbon sources resulting in intracellular sugar phosphates. The phosphoryl group transfer from phospho*enol*pyruvate (PEP) proceeds via the general PTS components EI (*ptsI,* cg2117) and HPr (*ptsH,* cg2121) to the sugar-specific permeases, which phosphorylate and transport their cognate substrates into the cell (Kotrba *et al.*, 2001; Lengeler *et al.*, 1994; Parche *et al.*, 2001). Thereby, glucose is converted to glucose-6-phosphate (Pons *et al.*, 1996) and fructose to fructose-1-phosphate (Dominguez *et al.*, 1998; Pons *et al.*, 1996; Sugimoto and Shiio, 1989). It is believed that sucrose uptake by EII^{Suc} leads to sucrose-6-phosphate, which subsequently is hydrolysed to glucose-6-phosphate and fructose (Kalinowski *et al.*, 2003; Shiio *et al.*, 1990). Unlike many sucrose-utilizing bacteria, *C. glutamicum* cannot phosphorylate fructose intracellularly due to the absence of fructokinase activity (Shiio *et al.*, 1990). Utilization of intracellular free fructose by *C. glutamicum* involves temporary efflux out of the cell. In a *ptsF* mutant, fructose accumulates in the medium as long as sucrose is present (Moon *et al.*, 2005). After complete consumption of the sucrose, fructose is slowly taken up by the glucose-PTS as a *ptsF*/*ptsG* double deletion mutant was not able to utilize the external fructose built during growth on sucrose minimal medium (Moon *et al.*, 2005). Subsequent re-uptake and phosphorylation of the fructose by the fructose-PTS yield fructose-1-phosphate and phosphorylation by the 1-phosphofructokinase (*fruK*, cg2119) yield the glycolytic intermediate fructose-1,6-bisphosphate (Figure 2.1) (Dominguez and Lindley, 1996; Dominguez *et al.*, 1998). The role of the sucrose- and fructose-specific PTS for sucrose metabolism has been studied to some detail (Moon *et al.*, 2005), while sucrose-6-phosphate hydrolase has not been characterized biochemically.

Sugar uptake via the PTS in *C. glutamicum* appears to be regulated, although *C. glutamicum* shows no diauxie, as glucose and fructose uptake during growth on glucose-acetate mixtures (Wendisch *et al.*, 2000) and glucose-fructose mixtures (Dominguez *et al.*, 1997) is reduced in comparison to growth on glucose or fructose alone. As mentioned above, the uptake of PTS sugars is strictly connected with the conversion of PEP to pyruvate, and therefore, the sugar uptake via the PTS in contrast to carbohydrate uptake via ABC transporters plays a special role for the organism. In the absence of the pyruvate kinase (*pyk*), which converts PEP to pyruvate in glycolysis (see Figure 2.1 and section 2.2), the uptake of sugars via the PTS and therewith the formation of pyruvate, is essential for growth of *C. glutamicum* (Gubler *et al.*, 1994; Netzer *et al.*, 2004a).

2.1.2 Uptake of non-PTS carbohydrates like D-ribose or gluconate

C. glutamicum can utilize the pentose sugar D-ribose and the sugar acid gluconate as sole carbon and engery sources. The transport and utilization of the pentose D-ribose is well understood in *E. coli* and *B. subtilis* (Lopilato *et al.*, 1984; Woodson and Devine, 1994). The ribose uptake system, belonging to the group of ATP binding cassette (ABC) transporters, is consisting of a soluble (in *C. glutamicum* membrane-attached) receptor protein, RbsB, an integral membrane component, RbsC, and an ATPase subunit, RbsA. In both organisms the genes coding for the ribose transporter form an operon together with the gene *rbsK*, encoding the ribokinase which phosphorylates ribose to ribose-5-phosphate, and *rbsD*, another component of the ABC transporter whose function is still unknown (Lopilato *et al.*, 1984; Woodson and Devine, 1994). The putative operon *rbsACBD* from *C. glutamicum* likely encodes the subunits of an ABC transport system and its binding protein for uptake of D-ribose as suggested based on comparison of global gene expression of wild type cultures grown either on glucose or ribose (Figure 2.1) (Wendisch, 2003). Moreover, *rbsK* encoding ribokinase, which phosphorylates ribose to the intermediate of the pentose phosphate pathway ribose-5-phosphate, showed a significantly increased RNA level during growth on ribose, too (Wendisch, 2003). In addition, it is known that growth on ribose requires pyruvate kinase activity, since a *C. glutamicum* Δ*pyk* mutant is not able to grow on ribose as sole carbon and energy source (Netzer *et al.*, 2004a). In *E. coli* and *B. subtilis,* expression of the *rbs* operon is controlled by a regulatory protein encoded by *rbsR* (Mauzy and Hermodson, 1992). However, such a transcriptional regulator has not been characterized in *C. glutamicum* to date.

In order to be metabolized, gluconate is first transported into the bacterial cytoplasm via a specific gluconate permease (GntP). Subsequently, gluconate kinase (GntK) phosphorylates intracellular gluconate to 6-phosphogluconate. In *C. glutamicum*, 6-phosphogluconate is further metabolized in the pentose phosphate pathway, as the alternative Entner-Doudoroff pathway is absent in this organism. The use of gluconate as an additional carbon source besides glucose was previously shown to have a positive effect on L-lysine production (Bianchi *et al.*, 2001; Lee *et al.*, 1998). Knowledge about transcriptional regulation of genes involved in gluconate metabolism and pentose phosphate pathway is scarce although in recent studies several transcriptional regulators involved in the regulation of central metabolic pathways in *C. glutamicum* were identified and characterized (see below).

GntR-type transcriptional regulators are involved in negative control of gluconate utilization genes in many bacteria. In the case of GntR of *B. subtilis* and *E. coli*, it was shown that gluconate itself interferes with the binding of these regulators to their target promoters

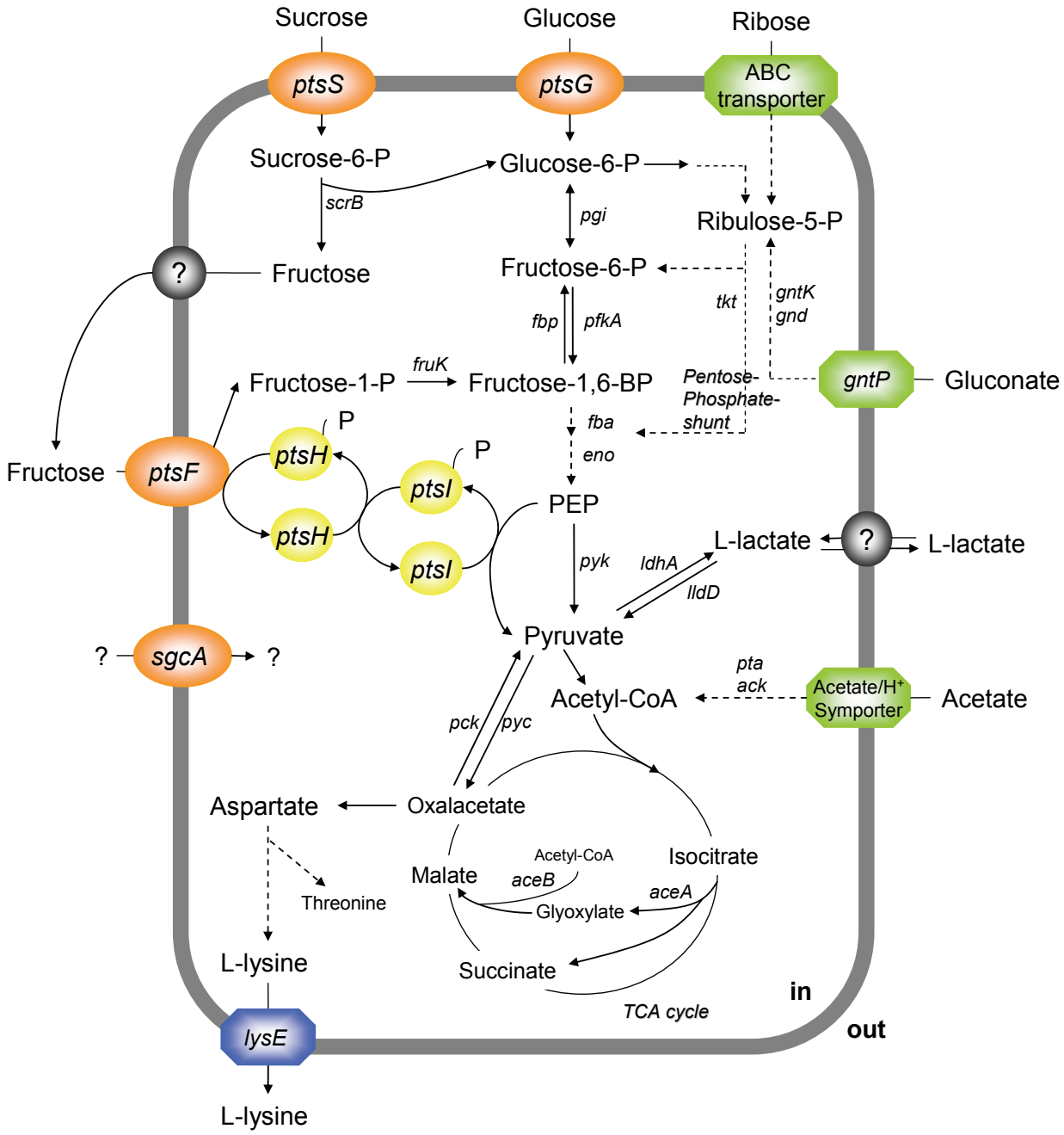

Figure 2.1. Carbohydrate uptake and utilization in *C. glutamicum*. The gene names of transport proteins and enzymes are given. *ptsG*, *ptsF*, *ptsS*, *sgcA*: EII PTS permeases specific for glucose, fructose, sucrose and a still unknown substrate, respectively, *ptsH* and *ptsI*: HPr and EI, the general PTS components, respectively, *scrB*: putative sucrose-6-phosphate hydrolase, *fruK*: 1-phosphofructokinase, *pgi*: glucose-6-phosphate isomerase, *fbp*: fructose-1,6-bisphosphatase, *pfkA*: 6-phosphofructokinase, *fba*: fructose-1,6-bisphosphate aldolase, *eno*: enolase, *tkt*: transketolase, *pyk*: pyruvate kinase, *ldhA*: NAD-dependent L-lactate dehydrogenase, *lldD*: quinone-dependent L-lactate dehydrogenase, *pyc*: pyruvate carboxylase, *pck*: PEP carboxykinase, *aceA*: isocitrate lyase, *aceB*: malate synthase, *pta*: phosphotransacetylase, *ack*: acetate kinase, *gntP*: gluconate permease, *gntK*: gluconokinase, *gnd*: 6-phosphogluconate dehydrogenase, ?: unidentified transporters, *lysE*: L-lysine exporter.

(Fujita and Fujita, 1987; Peekhaus and Conway, 1998). In several *Bacillus* species the genes encoding GntR, GntP, GntK, as well as the gene for a putative 6-phosphogluconate dehydrogenase (*gntZ*) are clustered in one operon. Expression of these genes is derepressed in the presence of gluconate and is also subject to carbon catabolite repression by the catabolite control protein A (CcpA) and the general PTS component HPr (Reizer *et al.*, 1996). In *E. coli,* the *gnt* genes are also repressed by GntR and they are activated by CRP (cAMP receptor protein) in complex with cAMP (Peekhaus and Conway, 1998). Thus, in *E. coli* as well as in *B. subtilis*, expression of the *gnt* genes is controlled by gluconate availability and the presence of a catabolite repressive carbohydrate like glucose. A transcriptional regulator has not been identified at the beginning of the thesis.

2.1.3 Uptake of the organic acids acetate and L-lactate by *C. glutamicum*

Growth of *C. glutamicum* on acetate and on glucose-acetate mixture has been studied in detail by Wendisch *et al.* (2000). It was shown that the consumption rate on glucose-acetate mixture of each of the two carbon sources is reduced to 50% of the rate observed for growth on acetate or glucose alone. Thus, this result indicated that either uptake or the following pathways are regulated directly or indirectly by the available carbon source (Wendisch *et al.*, 2000). Acetate uptake in *C. glutamicum* is mediated by a highly specific secondary carrier according to an acetate/proton symport mechanism (Ebbighausen *et al.*, 1991). This carrier also accepts propionate as substrate for transport but the gene(s) encoding the transport system has not been identified yet (Ebbighausen *et al.*, 1991). After uptake, acetate is activated to acetyl phosphate in an ATP-dependent reaction which is catalyzed by acetate kinase (AK; *ack*). Subsequently, acetyl phosphate is converted to acetyl-CoA by the phosphotransacetylase (PTA; *pta*). Acetyl-CoA can then be further metabolized via the TCA cycle or the glyoxylate cycle and is used in biosynthetic pathways e.g. for fatty acid biosynthesis (Wendisch *et al.*, 2000). Growth on acetate requires the operation of the glyoxylate cycle with its key enzymes isocitrate lyase (ICL encoded by *aceA*) and malate synthase (MS encoded by *aceB*) to fulfill the anaplerotic demand. The acetate activation enzymes AK and PTA as well as the glyoxylate pathway enzymes ICL and MS have been studied in detail and have been shown to be essential for growth of *C. glutamicum* on acetate minimal medium (Reinscheid *et al.*, 1994a, b; Reinscheid *et al.*, 1999; Shiio *et al.*, 1969; Wendisch *et al.*, 1997). The AK and PTA genes form an operon and two promoters were identified for the *pta-ack* operon (Reinscheid *et al.*, 1999). The *aceA* and *aceB* genes are clustered on the *C. glutamicum* chromosome and are transcribed in divergent directions from the transcriptional start site 112 bp upstream of the *aceA* gene and 466 and 468 bp upstream of the *aceB* gene, respectively (Reinscheid *et al.*, 1994a, b). The specific activities of AK,

PTA, ICL and MS and the mRNA levels of the respectives genes are significantly increased in the presence of acetate (Gerstmeir *et al.*, 2003; Muffler *et al.*, 2002; Reinscheid *et al.*, 1999; Wendisch *et al.*, 1997) due to coordinate control by the transcriptional regulators RamA, RamB and GlxR (see below).

Lactate is a major product of anaerobic metabolism, but it also serves as a carbon and energy source for anaerobic and aerobic microorganisms. Lactate can be fermented to acetate, propionate or butyrate by e.g. sulphate reducing bacteria, propionibacteria or *Eubacterium hallii* (Duncan *et al.*, 2004). Aerobic growth with L-lactate as sole carbon and energy source has been studied in *E. coli* to some detail. *C. glutamicum* can grow aerobically on L-lactate as sole carbon and energy sources (Stansen *et al.*, 2005) and is able to form L-lactate by the soluble NAD-dependent L-lactate dehydrogenase (EC 1.1.1.27) encoded by *ldhA* (Figure 2.1) (Bott and Niebisch, 2003; Inui *et al.*, 2004) under oxygen deprivation conditions (Inui *et al.*, 2004) and as a by-product during L-glutamate and L-lysine production (Kiefer *et al.*, 2002; Kiefer *et al.*, 2004; Stansen *et al.*, 2005). For L-lactate utilization, *C. glutamicum* requires the quinone-dependent L-lactate dehydrogenase LldD (EC 1.1.2.3), which is a peripheral membrane protein catalyzing the oxidation of L-lactate to pyruvate as central metabolite (Figure 2.1) (Bott and Niebisch, 2003; Schluesener *et al.*, 2005; Stansen *et al.*, 2005). The *C. glutamicum* L-lactate utilization operon comprises besides *lldD* a gene for a putative permease (cg3226) and its expression is maximal in the presence of L-lactate (Stansen *et al.*, 2005). Disruption of the cg3226-*lldD* operon resulted in the inability to grow on L-lactate as a sole carbon source unless the mutant was complemented with a plasmid carrying *lldD*, indicating that cg3226 is not essential for growth on L-lactate (Stansen *et al.*, 2005). Thus, additional carriers for uptake of L-lactate besides Cg3226 are likely to exist in *C. glutamicum*. The transcriptome analysis of L-lactate-grown *C. glutamicum* cells revealed higher mRNA levels of cg3112 and cg3399, and both encode putative permeases, which are thus possible candidates (Stansen *et al.*, 2005). *C. glutamicum* re-utilizes L-lactate formed during glutamate production in the presence of glucose (Stansen *et al.*, 2005) and co-utilizes L-lactate with glucose when grown on glucose/L-lactate mixtures as mentioned above. During co-utilization of glucose and L-lactate, the specific activity of quinone-dependent L-lactate dehydrogenase LldD was almost as high as on L-lactate alone, while it was about seven fold lower on glucose as sole carbon source (Stansen *et al.*, 2005). The apparent absence of glucose repression and the about 17 fold increased mRNA levels of cg3226-*lldD* during growth on L-lactate as compared to growth on pyruvate as sole carbon and energy source determined by transcriptome analyses (Stansen *et al.*, 2005) suggest that the cg3226-*lldD* operon is subject to L-lactate specific regulation. However, a putative regulatory gene is not encoded in the *C. glutamicum* lactate utilization operon cg3226-*lldD* and a transcriptional regulator had not been identified at the beginning of the thesis.

2.2 Central carbon metabolism pathways in *C. glutamicum*

Glycolysis and the pentose phosphate pathway

After sugar uptake and phosphorylation, further metabolism of the sugar phosphates occurs via both classical central metabolic pathways: the Embden-Meyerhof-Parnas pathway of glycolysis and the pentose phosphate pathway. The genes encoding all enzymes of glycolysis, gluconeogenesis, pentose phosphate pathway, TCA cycle and the glyoxylate cycle are encoded in the genome of *C. glutamicum* ATCC13032 and various were already known before sequencing the genome (Yokota and Lindley, 2005; Bott und Eggeling, 2005).

The glycolysis is the most important pathway of fueling reactions and the major control point is the pyruvate kinase (Figure 2.1). The importance of this enzyme in control of energy metabolism in *C. glutamicum* is also demonstrated by its regulation by adenine nucleotides, AMP which activates Pyk and ATP which inhibits the activity (Gubler *et al.*, 1994; Jetten *et al.*, 1994). Pyk is important for high-level lysine production with *C. glutamicum* (Gubler *et al.*, 1994). Fructose-1,6-bisphosphatase (encoded by *fbp*) is essential for gluconeogenesis as a Δ*fbp* mutant was unable to grow on the carbon sources acetate, citrate, glutamate and lactate (Rittmann *et al.*, 2003). Fbp catalyzes the conversion of fructose-1,6-bisphosphate to fructose-6-phosphate. The enzymatic activity is dependent on the divalent cations Mg^{2+} and Mn^{2+} and was inhibited by the monovalent cation Li^{+} (Rittmann *et al.*, 2003). *C. glutamicum* possesses single genes for 6-phosphofructokinase, Fbp and pyruvate kinase but two genes for the glyceraldehyde-3-phosphate dehydrogenase with presumably different physiological functions, GapA for glycolysis (NAD-dependent) and GapB for gluconeogenesis (NADP-dependent) (Hayashi *et al.*, 2002; Ikeda and Nakagawa, 2003). At that time, no essential control mechanism and no transcriptional regulator of glycolytic genes has been identified to explain the variations seen in gene expression on different substrates.

The pentose phosphate pathway serves as the main pathway for generation of NADPH required in many biosynthetic pathways (Figure 2.1). In all *Corynebacterineae* of which the genome sequence is available, e.g. *C. glutamicum*, *C. diphteriae*, *C. ammoniagenes*, *M. tuberculosis* and *M. leprae*, the five genes *tkt*, *tal*, *zwf*, *opcA,* and *pgl* encoding transketolase, transaldolase, glucose-6-phosphate dehydrogenase, and 6-phosphogluconolactonase, are clustered in this order on the chromosome and very likely form an operon (Yokota and Lindley, 2005). The pentose phosphate pathway involves all together seven enzymes of which the first three, glucose-6-phosphate dehydrogenase, 6-phosphogluconolactonase and 6-phosphogluconate dehydrogenase (*gnd*), constitute an oxidative route in which glucose-6-phosphate is converted into ribulose-5-phosphate with the formation of two moles NADPH as anabolic reducing power. The enzymes glucose-6-

phosphate dehydrogenase and the 6-phosphogluconate dehydrogenase are both inhibited by NADPH and the latter also by ATP, fructose-1,6-bisphosphate and glyceraldehyde-3-phosphate. Since glucose-6-phosphate dehydrogenase is the key enzyme of this pathway, it has been concluded that the flux through the pentose phosphate pathway is determined by the [$NADP^+$]/[NADPH] as well as by the specific activity of this enzyme (Moritz *et al.*, 2000). In contrast to the oxidative part of the pentose phosphate pathway described above, the non-oxidative part is completely reversible. This part serves as return of excessive pentoses into the glycolytic degradation path.

The TCA cycle and the glyoxylate shunt

One of the main and central pathways in *C. glutamicum* is the tricarboxylic acid (TCA) cycle which is a amphibolic pathway. The catabolic function of the TCA cycle is responsible for the complete oxidation of acetyl-CoA derived from different substrates, thereby generating CO_2, reducing equivalents, e.g. NADH for respiration (Bott and Niebisch, 2003), and ATP by substrate-level phosphorylation. The provision of precursors for amino acid production, e.g. the C_4-intermediate oxaloacetate for L-lysine biosynthesis (Figure 2.1) and 2-oxoglutarate for L-glutamate formation is the anabolic function of the cycle (Cronan and LaPorte, 1996; Guest and Russell, 1992). Therefore, anaplerosis is required by PEP carboxylase or pyruvate carboxylase or the glyoxylate cycle. During growth on carbon sources entering the central metabolism at the state of acetyl-CoA, e.g. acetate or ethanol, the glyoxylate cycle with its key enzymes isocitrate lyase and malate synthase provides oxaloacetate and thus fullfils the anaplerotic function (Kornberg, 1966) which is necessary during growth and especially under amino acid production conditions as mentioned above. Isocitrate lyase catalyses the cleavage of isocitrate to succinate and glyoxylate, whereas malate synthase condenses glyoxylate with acetyl-CoA forming malate (Figure 2.1). Both enzymes have been purified and biochemically characterized and their activity is controlled by allosteric regulation by a variety of intermediates of the central metabolism in *C. glutamicum*, e.g. ATP inhibits malate synthase and PEP or succinate inhibit isocitrate lyase (Reinscheid *et al.*, 1994a, b). Moreover, NADPH is formed by the isocitrate dehydrogenase reaction in the anabolic function of the TCA cycle. A comprehensive overview of the genes and enzymes of this pathway in *C. glutamicum* is available in the monograph on this organism (Eggeling and Bott, 2005; Eikmanns, 2005). The link between glycolysis and the TCA cycle represents the PEP-pyruvate-oxaloacetate node, which is therefore highly relevant for the correct distribution of the carbon flux.

2.3 Transcriptional regulators of carbon metabolism in *C. glutamicum*

From the 3002 protein-coding genes predicted in the genome of *C. glutamicum* ATCC13032 (Kalinowski *et al.*, 2003), the minimal repertoire of proteins that presumably act as transcriptional regulators was recently defined to 158 genes (Brinkrolf *et al.*, 2007). The large number of transcriptional regulators indicated that obviously many regulatory mechanisms have to be present in *C. glutamicum*. The prediction of regulators is often not very difficult, but a prediction which genes are influenced by a specific regulator in most cases is impossible. At the beginning of this PhD thesis, only four transcriptional regulators (Table 2.1) were identified to be involved in the regulation of carbon metabolism: AcnR, a repressor of the aconitase gene (Krug *et al.*, 2005) and RamA, RamB and GlxR, regulators of the acetate and ethanol metabolism (Cramer *et al.*, 2006; Gerstmeir *et al.*, 2004; Kim *et al.*, 2004).

Table 2.1. Transcriptional regulators affecting fundamental pathways of carbon metabolism described thus far in *C. glutamicum*.

Gene ID	Gene Name	Family	Function	Reference
cg1738	*acnR*	TetR	Repressor of aconitase gene of the TCA cycle	(Krug *et al.*, 2005)
cg2831	*ramA*	LuxR	Regulators of acetate and ethanol metabolism (RamA: activator; RamB and GlxR: repressors)	(Cramer *et al.*, 2006)
cg0444	*ramB*	HTH_3		(Gerstmeir *et al.*, 2004)
cg0350	*glxR*	Crp		(Kim *et al.*, 2004)

AcnR, a transcriptional regulator of the TetR-family, was identified as repressor of the aconitase gene *acn* (Krug *et al.*, 2005). The *acnR* deletion mutant showed a five fold higher *acn* mRNA level and a five fold higher specific aconitase activity. The transcriptome analysis also revealed that *acn* likely is the only target of the repressor AcnR. A putative 16 bp binding motif (CAGNAnnncGTACTG) was identified in the *acn* promoter region which leads to the assumption that AcnR binds as a dimer to this not perfectly conserved inverted repeat. To date, the effector of AcnR has not been identified (Krug *et al.*, 2005).

To grow on acetate as sole carbon and energy source, *C. glutamicum* requires besides the genes for acetate activation, PTA and AK, also the genes of the glyoxylate shunt, ICL and MS, as anaplerotic pathway as mentioned above. All four genes have in common that a

highly conserved 13 bp motif (AA/GAACTTTGCAAA) can be found in the vicinity of their transcriptional start sites (Gerstmeir *et al.*, 2004). RamB, regulator of acetate metabolism B, was identified by DNA affinity chromatography and features a helix-turn-helix motif in the N-terminus. RamB was shown to act as repressor of the *pta-ack* operon and the *aceA/aceB* genes in the presence of glucose (Gerstmeir *et al.*, 2004). In the presence of acetate the expression of the target genes is derepressed. The highly conserved 13 bp motif was also found in the promoter regions of further genes of the central carbon metabolism, such as aconitase, fumarase, EII^{Glc} and EII^{Fru} of the PTS and the alcohol dehydrogenase. Thus, RamB seems to belong to a larger regulatory network in *C. glutamicum*. Whereas RamB represses transcription of the *pta-ack* operon and the *aceA/aceB* genes in the absence of glucose, RamA activates their transcription in the presence of acetate. The regulator of acetate metabolism A was identified as a protein binding to single or tandem stretches $A/C/TG_{4-6}T/C$ or $AC_{4-5}A/G/T$ present in the *pta-ack* operon promoter region as well as the *aceA/aceB* gene promoter regions in *C. glutamicum* (Cramer *et al.*, 2006). RamA was shown to be essential for growth acetate as sole carbon and energy source and was shown to be present in acetate- as well as (in lower concentrations) in glucose-grown cells. The effector of RamA is still unknown (Cramer *et al.*, 2006).

Besides RamA and RamB, a third regulatory protein was shown to be involved in transcriptional control of the glyoxylate cycle genes *aceA* and *aceB*, namely GlxR (Kim *et al.*, 2004). GlxR belongs to the cAMP receptor protein family of transcriptional regulators which possess a helix-turn-helix motif in the C-terminus and a cAMP binding domain in the N-terminus. By gel retardation experiments it was shown that GlxR binds its target promoters in the presence of cAMP. Intracellular cAMP levels were about four fold higher in glucose-grown cells than in acetate-grown cells. Thus, Kim *et al.* (2004) supposed that GlxR represses the isocitrate lyase and the malate synthase genes in the presence of glucose.

2.4 L-lysine biosynthesis in *C. glutamicum*

As an essential amino acid, L-lysine is not synthesized in animals, thus it must be ingested as lysine or lysine-containing proteins. Addition of lysine as a feed additive augments the nutritional value of animal feed, which typically contains limiting lysine concentrations. Nowadays, *C. glutamicum* is one of the biotechnologically most important bacterial species with an industrial production of about 0.85 million tons of L-lysine per year (Leuchtenberger *et al.*, 2005). While wild-type *C. glutamicum* cells are able to produce glutamate when exposed to certain triggers, e.g. biotin limitation or ethambutol addition (Stansen *et al.*, 2005), L-lysine can only be produced by classically obtained mutants or genetically modified strains.

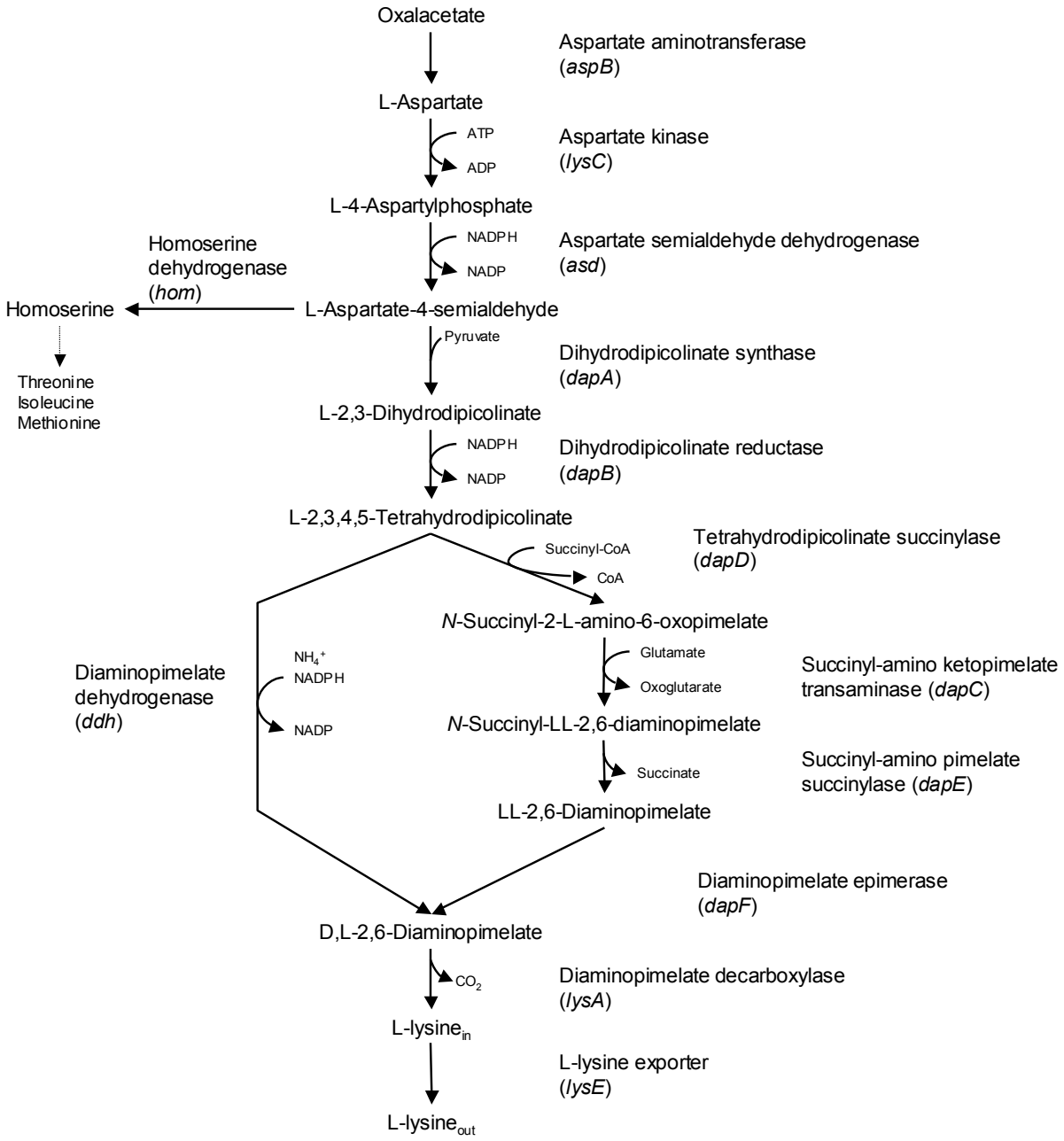

Figure 2.2. The L-lysine biosynthetic pathway in *C. glutamicum*.

During the last 30 years all genes involved in L-lysine biosynthesis have been identified (Cremer *et al.*, 1991; Hartmann *et al.*, 2003; Ishino *et al.*, 1987; Kalinowski *et al.*, 1990; Kalinowski *et al.*, 1991; Wehrmann *et al.*, 1994; Yeh *et al.*, 1988). In addition to genes of the lysine biosynthetic pathway a number of further molecular targets important for efficient

lysine production have been recognized with respect to pathways forming byproducts (e.g. other amino acids of the aspartate family), with respect to optimal carbon precursor supply or with respect to regeneration of the cofactor NADPH (Eggeling, 1994; Hermann, 2003; Pfefferle *et al.*, 2003; Sahm *et al.*, 2000). *C. glutamicum* possesses a split lysine biosynthesis pathway with a so-called succinylase branch and a diaminopimelate dehydrogenase branch (Figure 2.2). The key enzyme for lysine synthesis is the aspartokinase encoded by *lysC* (Schrumpf *et al.*, 1991). *C. glutamicum* only possesses a single aspartokinase enzyme which shows cumulative feedback inhibition by lysine and threonine. Overexpression of *lysC* and in particular of *lysC* alleles coding for aspartokinase variants not feedback-inhibited by lysine improved lysine production by *C. glutamicum* (Cremer *et al.*, 1991; Kalinowski *et al.*, 1991; Schrumpf *et al.*, 1992; Shiio and Miyajima, 1969; Thierbach *et al.*, 1990). Introduction of a *lysC* allele for a feedback-resistant aspartokinase into *C. glutamicum* WT already enables lysine production (Georgi *et al.*, 2005; Ohnishi *et al.*, 2002). Moreover, an improvement of lysine production was acchieved by a reduction of side product formation due to the introducing of *hom* alleles resulting in a restricted flux via homoserine dehydrogenase enzyme (Figure 2.2). As additional positive aspect, these *hom* mutants showed threonine concentrations too low to inhibit aspartokinase (Eikmanns *et al.*, 1991; Follettie *et al.*, 1988). One example for increasing the supply of oxaloacetate as carbon precursor for lysine biosynthesis is the overexpression of the pyruvate carboxylase gene *pyc* (Figure 2.1) (Peters-Wendisch *et al.*, 2001). The overexpression of *pyc* resulted in approximately 50% higher lysine accumulation in the culture supernatant indicating that the anaplerotic pyruvate carboxylase reaction is one major bottleneck for amino acid production in *C. glutamicum* (Peters-Wendisch *et al.*, 2001). Similarly, deletion of the PEP carboxykinase gene *pck* increased oxaloacetate availability due to reduced decarboxylation of oxaloacetate to PEP (Petersen *et al.*, 2000) and improved lysine production (Riedel *et al.*, 2001). Regeneration of NADPH, which is required in lysine biosynthesis, was achieved by deletion of *pgi* (Marx *et al.*, 2003), introduction of mutant *gnd* and *zwf* alleles (Ando *et al.*, 2002; Ohnishi *et al.*, 2005) or by overexpression of the *pntAB* genes coding for transhydrogenase from *E. coli* (Kabus *et al.*, 2007).

Glucose-, fructose- and sucrose-containing feedstocks are used for lysine production, but it was observed that lysine yields were lower on fructose or sucrose than on glucose (Kiefer *et al.*, 2002). In the WT, however, all three sugars are taken up via the PTS and sustain comparably high growth rates and biomass yields. While overproduction of malic enzyme, which generates NADPH by decarboxylation of malate, did not improve lysine production, increasing carbon flux entry into the pentose posphate pathway by overexpression of fructose-1,6-bisphosphatase gene led to improved lysine yields in *C. glutamicum* (Georgi *et al.*, 2005). The fact that NADPH regeneration limits lysine

production from sucrose in particular was evidenced on yield improvement by overexpression of the transhydrogenase genes *pntAB* was largest with sucrose (Kabus *et al.*, 2007).

Uptake of glucose, fructose and sucrose has not been a target of metabolic engineering approaches for lysine production by *C. glutamicum* until now. Incomplete utilization of high sucrose or fructose concentrations by lysine producing strains (Georgi *et al.*, 2005) and the observation that expression of *ptsG* increased after the switch from growth to lysine production, but was gradually decreased during the process by approximately 60% (Krömer *et al.*, 2004), suggested that engineering of PTS sugar uptake or engineering of genetic control of PTS gene expression might be a strategy to improve lysine production.

2.5 Aims of this work

As *C. glutamicum* lacks homologs of regulatory proteins involved in control of glucose uptake in *E. coli* (e.g. CRP and Mlc) and *B. subtilis* (e.g. CcpA, LicT and GlcT) and as PTS components are involved in regulation of sugar uptake in many bacteria, the genomic regions of the genes encoding PTS components were analysed for the presence of a gene coding for a potential transcriptional regulator of sugar uptake. One gene (cg2115, named *sugR* in the following) coding for a transcriptional regulator of the DeoR-family lies in the direct vicinity to the genes of the general PTS components HPr and EI in the genome of *C. glutamicum* and was therefore a good candidate for regulating the expression of PTS genes. The first aim of this work was to investigate the role of this regulator with respect to carbohydrate uptake and utilization in this organism. For this purpose, the identification of the direct target genes of SugR by biochemical and genome-wide approaches came to the fore. The intention was to provide insight into the regulon of this regulator in *C. glutamicum*. Another part of this PhD work was the try to make use of engineering control by SugR to improve lysine production in *C. glutamicum*. Glucose is directly phosphorylated to glucose-6-phosphate, an intermediate of glycolysis. Fructose is phosphorylated to fructose-1-phosphate by the PTS and subsequently converted to the glycolytic intermediate fructose-1,6-bisphosphate by FruK encoded in the *fruR-fruK-ptsF* operon in the *C. glutamicum* genome. Further conversion/hydrolyzation of sucrose-6-phosphate is unclear. Therefore, a putative sucrose-6-phosphate hydrolase in *C. glutamicum* was biochemically characterized. Taken together, the work on the transcriptional regulator SugR together with the results obtained in two collaborations with Julia Frunzke regarding gluconate metabolism and its regulation and with Tobias Georgi with respect to L-lactate metabolism will generate a more detailed knowledge about the genetic control of carbohydrate uptake and utilization in *C. glutamicum*.

3 Results

The regulation of sugar uptake and conversion in *C. glutamicum* was the major topic of this PhD. The results relating to this research have been summarized in three publications and three manuscripts which are already submitted or will be submitted in the near future.

In the first publication “The DeoR-type regulator SugR represses expression of *ptsG* in *Corynebacterium glutamicum*”, SugR was identified as the first transcriptional regulator of the glucose-specific EII PTS permease in *C. glutamicum*. Overexpression of *sugR* resulted in reduced *ptsG* mRNA levels, decreased glucose utilization and perturbed growth on media containing glucose. The deletion of *sugR* resulted in two to seven fold increased *ptsG*’-‘*cat* expression on gluconeogenic media but had no influence when the cells were grown on sugar-containing media. Purified SugR likely bound to a partly conserved 8 bp motif upstream of *ptsG, ptsS* and upstream of the *fruR-fruK-ptsF* operon. Fructose-6-phosphate interfered with SugR binding, which led to the assumption that SugR also regulates the other PTS permeases in *C. glutamicum* in a carbon source-dependent manner.

Regulation of *ptsG* was also shown in a collaboration with Julia Frunzke (Institute of Biotechnology 1, Research Center Jülich) which resulted in the following publication: “Co-ordinated regulation of gluconate catabolism and glucose uptake in *Corynebacterium glutamicum* by two functionally equivalent transcriptional regulators, GntR1 and GntR2”. In this study it was clearly shown that GntR1 and GntR2 strongly repress the genes encoding gluconate permease (*gntP*), gluconate kinase (*gntK*), and 6-phosphogluconate dehydrogenase (*gnd*) and weakly the pentose phosphate pathway genes organized in the *tkt-tal-zwf-opcA-devB* cluster. Gluconate itself interfered with GntR1 and GntR2 binding. In contrast, *ptsG* and *ptsS* are activated by GntR1 and GntR2. This is the first example of transcriptional regulation of *pts* genes with respect to gluconate availability. Thus, these transcriptional regulators are important players in a complex regulatory network that controls uptake and metabolism of carbon sources in *C. glutamicum* in order to allow the most favourable combination of the available substrates.

The previous work was continued in the following manuscript “The global repressor SugR controls expression of glycolysis and pentose phosphate pathway genes in *Corynebacterium glutamicum*”, where it was shown that SugR indeed acts as repressor of *ptsS* and *ptsF*. But perturbed growth on ribose minimal medium revealed that SugR has to be involved in the regulation of further genes of the central carbon metabolism in *C. glutamicum*. ChIP-to-chip analysis, gel retardation experiments and measurements of enzyme activities of genes directly bound by the regulator indicated the negative regulation of the NAD-dependent L-lactate dehydrogenase (*ldhA*), the 6-phosphofructokinase (*pfkA*)

and the fructose-1,6-bisphosphate adolase (*fba*) *in vivo* in *C. glutamicum*, whereas the maximum SugR control is on LdhA activity. Under oxygen-deprivation conditions the Δ*sugR* deletion mutant showed a three fold increased L-lactate formation whereas the *sugR* overexpressing strain formed approximately 25% less L-lactate. Thus, SugR turned out to be a new target for improvement of L-lactic acid production with *C. glutamicum*.

The "Regulation of L-Lactate utilization by the FadR-type regulator LldR of *Corynebacterium glutamicum*" (in collaboration with Tobias Georgi, Institute of Biotechnology 1, Research Center Jülich) describes the regulation of the quinone-dependent L-lactate dehydrogenase LldD (EC 1.1.2.3) which converts incorporated L-lactate to pyruvate in *C. glutamicum*. In the presence of L-lactate, the inducer binds to LldR preventing repression of the L-lactate utilization operon cg3226-*lldD* by LldR. Thus, transcription of the cg3226-*lldD* operon is controlled by L-lactate availability. In contrast to these observations, SugR control of LdhA on the one hand and SugR control by its inducer fructose-6-phosphate on the other hand ensures that L-lactate is only formed when the glycolytic flux is high, as described in the publication before.

Interestingly, "*sugR* deletion improves L-lysine production in *Corynebacterium glutamicum*". The deletion of the transcriptional regulator SugR in the lysine production strain DM1729 on the one hand led to an 20%, 50% and 70% increased L-lysine yield on glucose, fructose and sucrose containing media, but, however, this deletion led on the other hand to a 24 hours lag-phase combined with a reduced growth rate and sugar uptake rate afterwards. Since SugR negatively regulates *pts* genes, controls the glycolytic flux straight forward to pyruvate which serves as precursor for L-lysine production in *C. glutamicum* and also controls the flux through the pentose phosphate pathway leading to a better NADPH supply, SugR appears to be a good candidate to improve lysine formation on PTS sugars in *C. glutamicum* anyway.

The role of the sucrose- and fructose-specific PTS for sucrose metabolism has been studied to some detail, while sucrose-6-phosphate hydrolase has not been characterized biochemically. Due to the biotechnological importance of sucrose as carbon source of *C. glutamicum*, the cg2927 gene product was characterized in the last manuscript of this PhD thesis the "Functional and biochemical characterization of ScrB (Cg2927) as sucrose-6-phosphate hydrolase essential for sucrose utilization by *Corynebacterium glutamicum*" and it was shown that ScrB catalyzes sucrose-6-phosphate hydrolysis and is required for sucrose utilization.

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The DeoR-Type Regulator SugR Represses Expression of *ptsG* in *Corynebacterium glutamicum*▽

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Corynebacterium glutamicum **grows on a variety of carbohydrates and organic acids. Uptake of the preferred carbon source glucose via the phosphoenolpyruvate-dependent phosphotransferase system (PTS) is reduced during coutilization of glucose with acetate, sucrose, or fructose compared to growth on glucose as the sole carbon source. Here we show that the DeoR-type regulator SugR (NCgl1856) represses expression of *ptsG*, which encodes the glucose-specific PTS enzyme II. Overexpression of *sugR* resulted in reduced *ptsG* mRNA levels, decreased glucose utilization, and perturbed growth on media containing glucose. In mutants lacking *sugR*, expression of the *ptsG′-′cat* fusion was increased two- to sevenfold during growth on gluconeogenic carbon sources but remained similar during growth on glucose or other sugars. As shown by DNA microarray analysis, SugR also regulates expression of other genes, including *ptsS* and the putative NCgl1859-*fruK-ptsF* operon. Purified SugR bound to DNA regions upstream of *ptsG*, *ptsS*, and NCgl1859, and a 75-bp *ptsG* promoter fragment was sufficient for SugR binding. Fructose-6-phosphate interfered with binding of SugR to the *ptsG* promoter DNA. Thus, while during growth on gluconeogenic carbon sources SugR represses *ptsG*, *ptsG* expression is derepressed during growth on glucose or under other conditions characterized by high fructose-6-phosphate concentrations, representing one mechanism which allows *C. glutamicum* to adapt glucose uptake to carbon source availability.**

Corynebacterium glutamicum is a high-G+C content gram-positive soil bacterium which was isolated in 1957 as an L-glutamate-excreting bacterium (1, 32). It plays an important role in the large-scale biotechnological production of L-glutamate and L-lysine, with annual production of about 1.5 million tons/year and 0.85 million tons/year, respectively (21, 40). This species has become a model organism for the *Corynebacterianeae*, a suborder of the *Actinomycetales*, which also comprises *Nocardiaceae* and *Mycobacteriaceae*, and extensive knowledge about this nonpathogenic bacterium has accumulated since its discovery (summarized in reference 11).

C. glutamicum is able to grow on various carbon and energy sources, such as sugars, sugar alcohols, and organic acids (10, 12, 17, 27, 34, 42, 70, 73). In contrast to *Escherichia coli* and *Bacillus subtilis*, which show distinct catabolite repression, *C. glutamicum* usually coutilizes the carbon sources present in the mixtures without showing diauxic growth. Glucose is the preferred carbon source, but it has been shown to be cometabolized with, e.g., acetate (71), L-lactate (65), propionate (6), pyruvate and serine (46), protocatechuate (41), vanillate (41), and fructose (8). The only known exception is the sequential consumption of glucose before glutamate (35).

The central carbon metabolism of *C. glutamicum* has been characterized by enzymatic studies, carbon flux analysis, genetic analysis, and, since the complete genome sequence of ATCC 13032 is available (25), also by genome-wide studies (reviewed in references 12, 13, 59, and 73). For example, based on intensive studies on acetate metabolism and its regulation (see reference 17 and references therein), three transcriptional regulators of acetate metabolism, namely, RamA (7), RamB (16), and GlxR (29), as well as AcnR, the transcriptional repressor of the aconitase gene *acn* (36), have been identified. However, only a few studies investigated the sugar transport systems and their regulation on the molecular level (42, 48). Mori and Shiio (43) first mentioned the presence of a phosphoenolpyruvate-dependent sugar phosphotransferase system (PTS) in *C. glutamicum*. The PTS operates by phosphoryl group transfer from phosphoenolpyruvate via the general PTS components EI (encoded by *ptsI*) and HPr (encoded by *ptsH*) to the sugar-specific enzyme II permeases, which phosphorylate and transport their cognate substrates into the cell (33, 39, 48). *C. glutamicum* possesses the specific EII permeases EII^{Glc}, EII^{Fru}, and EII^{Suc} (encoded by *ptsG*, *ptsF*, and *ptsS*, respectively) for uptake of glucose, fructose, and sucrose, respectively (9, 27, 28, 42).

The glucose uptake in *C. glutamicum* appears to be regulated, as, e.g., during growth on glucose-acetate mixtures glucose uptake is reduced about twofold in comparison to growth on glucose alone (71). Since *C. glutamicum* lacks homologs of regulatory proteins involved in control of glucose uptake in *E. coli* (e.g., cyclic AMP [cAMP] receptor protein [CRP] and Mlc) and *B. subtilis* (e.g., CcpA, LicT, and GlcT) and since PTS components are involved in regulation of sugar uptake in many bacteria, we screened the genomic regions of the genes encoding PTS components for the presence of genes coding for potential transcriptional regulators of glucose uptake. Regarding the localization of the PTS genes in the genome of *C. glutamicum*, it was striking that the genes coding for the general PTS proteins (*ptsI* and *ptsH*) and for the fructose-specific EII (*ptsF*) lie next to two genes coding for transcriptional regulators of the DeoR family: NCgl1856 (renamed SugR in

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference
E. coli strains		
DH5α	F$^-$ *thi-1 endA1 hsdR17*(r^- m^-) *supE44* Δ*lacU169* (ϕ80*lacZ*ΔM15) *recA1 gyrA96 relA1*	Bethesda Research Laboratories (20)
BL21(DE3)	*ompT hsdS*$_B$(r_B^- m_B^-) *gal dcm*(DE3)	66
C. glutamicum strains		
ATCC 13032	Wild-type strain auxotrophic for biotin	1
Δ*sugR* mutant	In-frame deletion of the *sugR* gene	This study
Δ1859 mutant	In-frame deletion of the NCgl1859 gene	This study
Δ*sugR* Δ1859 mutant	In-frame deletion of the *sugR* and NCgl1859 genes	This study
Plasmids		
pGEM-T	Amp^r; PCR cloning vector (P_{T7}, P_{SP6})	Promega, WI, USA
pGEM-T-Δ*sugR*	Amp^r; pGEM-T with deletion fragment of 906 bp of *sugR*	This study
pGEM-T-Δ1859	Amp^r, pGEM-T with deletion fragment of 987 bp of NCgl1859	This study
pGEM-T-SugR^{His}	Amp^r; pGEM-T with a 786-bp fragment of the *sugR* gene	This study
pk19*mobsacB*	Kan^r; *E. coli*/*C. glutamicum* shuttle vector for construction of insertion and deletion mutants in *C. glutamicum* (pK18 *oriV*$_{Ec}$, *sacB*, *lacZ*α)	60
pk19*mobsacB*-Δ*sugR*	Kan^r; pk19*mobsacB* with the deletion construct of the *sugR* gene	This study
pk19*mobsacB*-Δ*1859*	Kan^r; pk19*mobsacB* with the deletion construct of the NCgl1859 gene	This study
pVWEx1	Kan^r; P_{tac} *lacI*^{q}	51
pVWEx1-*sugR*	Kan^r; pVWEx1 with an 807-bp fragment of the *sugR* gene and an artificial ribosome binding site	This study
pET16b	Amp^r; overproduction of proteins with a N-terminal decahistidine tag in *E. coli* (pBR322 *oriV*$_{Ec}$, *PT7*, *lacI*)	Novagen
pET16b-sugR^{His}	Amp^r; pET16b with a 786-bp fragment of the *sugR* gene	This study
pET2	Kan^r; promoter-probe vector	69
pET2-*ptsG*	Kan^r; pET2 with the full-length promoter fragment of 707 bp of the *ptsG* promoter	This study
pET2-*ptsG*/*F4*	Kan^r; pET2 with promoter fragment 4 of 188 bp of the *ptsG* promoter	This study

this study) and NCgl1859. Based on the assumption that one or both of these regulators is involved in the regulation of glucose uptake, we examined the role of the transcriptional regulators SugR and NCgl1859 in control of *ptsG* expression.

(Part of the work described here belongs to the planned dissertation of Verena Engels at the faculty of Mathematics and Natural Sciences of the Heinrich-Heine-Universität Düsseldorf.)

MATERIALS AND METHODS

Bacterial strains, plasmids, oligonucleotides, and culture conditions. The strains and plasmids used are listed in Table 1. For growth experiments, in the first preculture, 5 ml Luria-Bertani (LB) medium (58) was inoculated from a fresh LB agar plate and incubated at 30°C and 170 rpm. After washing the cells in medium without a carbon source, the second preculture and the main culture were inoculated to an optical density at 600 nm (OD_{600}) of 0.5 to 1.0 in 60 ml LB medium or in 60 ml CgXII minimal medium (26), which contained 0.03 g/liter protecatechuic acid. As carbon and energy sources, 100 mM glucose, 100 mM fructose, 50 mM glucose plus 50 mM fructose, 50 mM sucrose, 300 mM K-acetate, 100 mM Na_3-citrate plus 100 mM $MgCl_2$, 200 mM Na-pyruvate, 50 mM maltose, 120 mM ribose and 50 mM glucose plus 150 mM K-acetate were used. Precultures and main cultures were incubated at 30°C and 120 rpm on a rotary shaker in 500-ml baffled shake flasks. When appropriate, the medium was supplemented with kanamycin (50 μg/ml) or ampicillin (50 μg/ml). Growth of *C. glutamicum* was followed by measuring the OD_{600}. The biomass concentration was calculated from the OD_{600} values using an experimentally determined correlation factor of 0.25 g (dry weight) (0.25 gDW) for an OD_{600} of 1 (71). For all cloning purposes, *Escherichia coli* DH5α was used as the host, and for overproduction of SugR, *E. coli* BL21(DE3) (66) was used.

Recombinant DNA work. The oligonucleotides listed in Table 2 were obtained from Operon (Cologne, Germany) or MWG (Ebersberg, Germany). Standard methods such as PCR, restriction, and ligation were carried out as described previously (58). Plasmids from *E. coli* were isolated with the QIAprep spin miniprep kit (QIAGEN, Hilden, Germany). *E. coli* was transformed by the RbCl method (19), and *C. glutamicum* was transformed by electroporation (68) at 25 μF, 600 Ω, and 2.5 kV/cm (Bio-Rad Gene Pulser Xcell; Bio-Rad Laboratories, Hercules, Canada). All cloned DNA fragments were shown to be correct by sequencing (Agowa GmbH, Berlin, Germany).

Construction of mutants carrying in-frame deletions of *sugR* or NCgl1859. The in-frame *sugR* deletion mutants of *C. glutamicum* were constructed via a two-step homologous recombination procedure as described previously (47). The *sugR* up- and downstream regions were amplified using primer pairs *sugR*-A/*sugR*-B and *sugR*-C/*sugR*-D. The product of the subsequent crossover PCR was subcloned into the pGEM-T vector (Promega, WI) and was cloned as an EcoRI-BamHI fragment into pK19*mobsacB* vector (60). Transfer of the resulting plasmid, pK19*mobsacB*-Δ*sugR*, into *C. glutamicum* by electroporation and screening for the correct mutants was performed as described previously (47). Of eight clones tested by PCR (primer pair *sugR*-k-for/*sugR*-k-rev), two showed the wild-type (WT) situation and six had the desired in-frame deletion of the *sugR* gene. Similarly, three independent NCgl1859 deletion mutants were constructed in the WT background as well as in the Δ*sugR* deletion mutant by using the primers 1859-A to -D.

Homologous overexpression of *sugR*. For homologous overexpression of *sugR*, *sugR* was amplified from genomic DNA of *C. glutamicum* ATCC 13032 (referred to below as *C. glutamicum* WT) by using primers *sugR*-for and *sugR*-rev and was cloned into the expression vector pVWEx1 (51). The constructed vector, pVWEx1-*sugR*, allows the IPTG (isopropyl-β-D-thiogalactopyranoside)-inducible expression of *sugR* in *C. glutamicum*.

Construction of transcriptional fusions. The *ptsG* promoter region (−399 to +309 [see Fig. 4A]) including the first 16 codons of *ptsG* as well as promoter subfragment 4 (−125 to +62 [see Fig. 4A]) was amplified with primer pairs ptsG-1/ptsG-2 and F4-for/F4-rev, respectively (Table 2), and cloned into the corynebacterial promoter-probe vector pET2 (69). As the longer fragment contained *ptsG* coding sequence, a peptide of 46 amino acids containing the first 16 amino acids of PtsG may be formed. However, no fusion protein between chloramphenicol acetyltransferase and PtsG is formed. The resulting vectors, pET2-*ptsG* and pET2-*ptsG*/*F4*, were transferred into *C. glutamicum* WT, the Δ*sugR* mutant, and the Δ1859 deletion mutant by electroporation. The full-length promoter as well as subfragment 4 were tested for promoter activity by measuring chloramphenicol acetyltransferase activity (45).

TABLE 2. Oligonucleotides used in this study

Oligonucleotide	Sequence (5′→3′)	Sequence details		
		Underlined nucleotide (position in NC003450)	Sequence in bold (restriction site)	Sequence in italic
sugR-A	GC**GAATTC**ACAAGGATTCATCTGGCATC	2037391	EcoRI	
sugR-B	*CCCATCCACTAAACTTAAACA*GCGCTCCTCTGCGTACAT	2037832		Linker
sugR-C	*TGTTTAAGTTTAGTGGATGGG*CGAGAACGCGATGTAGAAGTTGTG	2038559		Linker
sugR-D	GC**GGATCC**CAAATTGCCACCCAACAACACCC	2039001	BamHI	
sugR-k-for	GTTCGTCGCGGCAATGATTGACG	2037351		
sugR-k-rev	CTCACCACATCCACAAACCACGC	2039042		
1859-A	GC**TCTAGA**AACGCCGGTGCCCTTCAGTACA	2041268	XbaI	
1859-B	*CCCATCCACTAAACTTAAACA*TTCCGTTTGGCTGACCATGCTTAAC	2041745		Linker
1859-C	*TGTTTAAGTTTAGTGGATGGG*TTGCCCGACCACAACTTTCAGG	2042487		Linker
1859-D	GC**GAATTC**GACGCGCGTACCAGTCAACTGG	2042966	EcoRI	
1859-k-for	GGCGACGACTTCGCCTGCTTG	2041190		
1859-k-rev	TATCGACAGCCACGCGAACGTC	2043017		
sugR-for	GC**GGATCC***AAGGAGATATAGAT*ATGTACGCAGAGGAGCG	2037815	BamHI	Ribosome binding site
sugR-rev	GC**TCTAGA**GTAAGAATCATTCTGCAATCACAAC	2038601	XbaI	
SugRshift-for	GGAACC**CATATG**TACGCAGAGGAGCG	2037815	NdeI	
SugRshift-rev	GGG**GGATCC**AGAATCATTCTGCAATCACAAC	2038598	BamHI	
ptsG-for	TAGTTCCTGCACCTCTTAAGAGG	1422560		
ptsG-rev	CCAAGGTTTTCCAGAATATGTTGCG	1423266		
ptsG-1	GCGC**CTGCAG**TAGTTCCTGCACCTCTTAAGAGG	1422560	PstI	
ptsG-2	GCGC**GTCGAC**CCAAGGTTTTCCAGAATATGTTGC	1423266	SalI	
F2-rev	CCGAAGCGCCCGCCAAAAGC	1422734		
F3-for	GTGAGGCTTGGTTTTCGTACG	1422684		
F3-rev	GAATATGTCCGACCTGCATTTTTTG	1422890		
F4-for	GCATAATCTGACAGTGTGTCCGTTTTC	1422834		
F4-rev	GGCTCCCCCGCAATAGATTTGTGG	1423019		
F5-for	CCGCTTAATTGGAAAAAAGTGTGATCC	1422968		
F5-rev	CGCCGTTACGAATTGGGCC	1423148		
F6-for	GGTAGGCGCTGAGGAATC	1423095		
NCgl2027-for	AGCTTCAGAGGAGCCTGAAGCGG	2224999		
NCgl2027-rev	CGGAGTGGGTTGATTCTGGGATG	2225188		
NCgl1955-for	GTTCGCTACGTCCGAGTGATCACC	2146341		
NCgl1955-rev	GTCCCCCACGATTGTTTAAAAGTC	2145944		
mepA-for	GCACATCCGCTACCGAACCATC	2649369		
mepA-rev	CTAGTCGACGCTTCATGTTGCGG	2649635		
ptsG_30*	[*IRD800*]GTTGCGATGTCGTCGTCAGTTTG	1423247		Labeled with *IRD800*
ptsG_60*	[*IRD800*]GTCTGGTCCACCAAGGTTTTC	1423276		Labeled with *IRD800*
ptsG_90*	[*IRD800*]GAGTCGCACAGTGAGTCATCG	1423307		Labeled with *IRD800*
ptsG-Primex-rev	GCAGGGCCCACAGGATTGG	1423607		
*ptsS*_for	CTGCAGGTGATGGACAGGTTTCATTCG	2812022		
*ptsS*_rev	CGCCGCCAATGTCGCGCAGG	2811759		
*ptsF*_for	CCCAGAAGAAAACACTGGAGG	2043357		
*ptsF*_rev	CGCCGAAATCGACATCCAG	2043559		
NCgl1858/1859_for	CCGGTGCCCTTCAGTACAG	2041272		
NCgl1858/1859_rev	GGTGCCAGTAAAGAAGCAATTGCATG	2041777		
A6	GACCTGCATTTTTTGAAAATGAAAACGG	1422880		
B1	CCTTTTTGGTTTGTCTGTATTCAGC	1422901		
B6	GTCGGACATATTCAAAAGTATTACCTTTTTGG	1422878		
SF_1	GGTTAAGCTTTTGGCGGGCGC	1422709		
SF_2	GGCGAAAATACATGGGCCTACAACG	1422733		
SF_3	CGTGAAACTGGGGGATGGGG	1422767		
SF_4	CGGGGACGTGATGCGGTGG	1422802		
SF_5	GGTGCGGGCATAATCTGACAGTG	1422827		
SR_1	GTGGTGGATCACACTTTTTTCC	1422999		
SR_2	GCGGTAAGTCACCCATCATG	1422971		
SR_3	GCCGGAAACCCACCCAAAAC	1422947		
SR_4	GCTGAATACAGACAAACCAAAAAG	1422925		
SR_5	GTAATACTTTTGAATATGTCCGACC	1422901		

Overproduction and purification of SugR. For overproduction and purification of SugR carrying an N-terminal decahistidine tag, the *sugR* coding region was amplified using oligonucleotides that introduce an NdeI restriction site, including the start codon (SugRshift-for) and a BamHI restriction site after the stop codon (SugRshift-rev) for cloning into pET16b. For overproduction of SugR, the plasmid was transferred into *E. coli* BL21(DE3), and the resulting strain, *E. coli* BL21(DE3)(pET16b-SugR^{His}), was grown in 2 liters LB medium or 1 liter Overnight Express Instant TB Medium (Merck Biosciences Ltd, Germany) with 50 μg/ml ampicillin and 10 ml of 100% glycerol according to the manufacturer's recommendations. For cell extract preparation, washed (in 20 ml TNI5 buffer [20 mM Tris-HCl {pH 7.9}, 300 mM NaCl, 5% {vol/vol} glycerol, 5 mM imidazole) and thawed cells (about 5 to 6 g [wet weight]) from a 0.5- to 2-liter culture volume were resuspended in 10 ml of TNI5 buffer containing 1 mM diisopropylfluorophosphate and 1 mM phenylmethylsulfonyl fluoride. The cell suspension was passed five times through a French pressure cell (SLM Amino; Spectronic Instruments, Rochester, NY) at 150.8 MPa. After centrifugation (20 min, 5,292 × *g*, 4°C) and ultracentrifugation (1 to 1.5 h, 150,000 × *g*, 4°C), the supernatant was used to purify SugR by nickel affinity chromatography (Ni-nitrilotriacetic acid; Novagen, San Diego, CA). After washing with TNI175 buffer (which contained 175 mM imidazole), the SugR^{His} protein was eluted with TNI400 buffer (which contained 400 mM imidazole). Fractions with the highest protein content were pooled, and the elution buffer was exchanged against BS buffer (100 mM Tris-HCl, 20% [vol/vol] glycerol, 100 mM KCl, 20 mM $MgCl_2$, 1 mM EDTA, pH 7.5) using PD10 columns.

Gel shift assays. Gel shift assays with SugR^{His} were preformed as described previously (72). Briefly, various concentrations of purified SugR^{His} were mixed with full-length promoter DNA of *ptsG*; with promoter DNAs of *ptsS*, *ptsF*, and NCgl1858/1859; or with promoter subfragments of *ptsG* in 50 mM Tris-HCl, 10% (vol/vol) glycerol, 50 mM KCl, 10 mM $MgCl_2$, 0.5 mM EDTA, pH 7.5 (total volume of 20 μl). Different nontarget promoter fragments were added as a

negative control. The promoter DNA fragments were obtained by PCR with the primers listed in Table 2, using the following combinations: ptsG-for/ptsG-rev, ptsG-for/F2-rev, F3-for/F3-rev, F4-for/F4-rev, F5-for/F5-rev, F6-for/ptsG-rev, F3-for/SR_1, F3-for/SR_2, F3-for/SR_3, F3-for/SR_4, F3-for/SR_5, SF_1/F4-rev, SF_2/F4-rev, SF_3/F4-rev, SF_4/F4-rev, SF_5/F4-rev, SR_5/SF_5 (BS), F3-for/A6, B1/F4-rev, B6/F4-rev, *ptsF*-for/*ptsF*-rev, *ptsS*-for/*ptsS*-rev, and NCgl1858/1859-for/NCgl1858/1859-rev. After incubation for 30 min at room temperature, the samples were separated on 10 or 15% native polyacrylamide gels at a constant voltage of 170 V, stained, and photographed as described previously (72). To test for possible effectors, the protein was incubated with glucose-6-phosphate, fructose-6-phosphate, fructose-1,6-bisphosphate, phosphoenolpyruvate, dihydroxyacetone phosphate, pyruvate, L-lactate, D-lactate, acetyl coenzyme A (acetyl-CoA), and 3-phosphoglycerate (20 mM each) in the binding buffer for 15 min before addition of DNA fragment 4 and incubation for another 30 min.

Preparation of total RNA and primer extension analysis. Exponentially growing cells were harvested, and RNA was isolated using the RNeasy system (QIAGEN, Hilden, Germany) with on-column DNase I treatment as described previously (37). Purified RNA samples were analyzed for quantity and quality by UV spectrophotometry and stored at −20°C until use. Nonradioactive primer extension analysis with 10 to 13 μg of total RNA was performed using IRD800-labeled oligonucleotides (MWG Biotech, Ebersberg, Germany) as described previously (14), except with 200 U/μl SuperScript II (Invitrogen, Karlsruhe, Germany). The template for primer extension analysis for *ptsG* was designed by PCR amplification with the primers ptsG-for and ptsG-Primex-rev. Sequencing reactions and primer extension products were analyzed using denaturing 4.6% (wt/vol) Long Ranger (Biozym, Hamburg, Germany) sequencing gels in a Long Read IR DNA sequencer (Licor Inc., Lincoln, NE). The transcriptional start site of *ptsG* was determined using three different oligonucleotides (ptsG_30*, ptsG_60*, and ptsG_90*).

DNA microarray analysis. Generation of *C. glutamicum* whole-genome DNA microarrays (70), synthesis of fluorescently labeled cDNA from total RNA, microarray hybridization, washing, and gene expression analysis were performed as described previously (23, 36, 37, 54). Genes that exhibited mRNA levels that were significantly changed ($P \leq 0.05$ in Student's t test) by at least a factor of 2.0 were determined in three DNA microarray experiments performed with RNA isolated from three independent cultures.

Chloramphenicol acetyltransferase assay. For determination of chloramphenicol acetyltransferase activity, the cell pellet from a 50-ml culture volume was washed in 40 ml 0.08 M Tris-HCl (pH 7.0) buffer, centrifuged for 5 min at 5,422 × *g* and 4°C, resuspended in 1 ml of the same buffer, and disrupted by sonication (9 min, cycle 0.5, amplitude of 55%, on ice) (sonication processor UP200s; Hielscher Ultrasonics GmbH, Stuttgart, Germany). After centrifugation (1 to 1.5 h, 13,000 × *g*, 4°C), the supernatant was used for measuring the chloramphenicol acetyltransferase activity by the method of Shaw (63). Briefly, the assay mixture (1 ml) contained 90 mM Tris-HCl (pH 7.8), 0.09 mM acetyl-CoA, 0.36 mg/ml 5,5′-dithiobis-2-nitrobenzoic acid, 0.25 mM chloramphenicol, and crude extract. The formation of free 5-thio-2-nitrobenzoate was measured photometrically at 412 nm and 37°C. Protein concentrations were determined with the Bradford assay kit (Bio-Rad Laboratories, Hercules, CA) using bovine serum albumin as standard.

Determination of glucose and acetate concentrations. Samples (1 ml) of the cultures were centrifuged for 5 min at 16,060 × *g*, and aliquots of the supernatant were used directly for the determination. D-Glucose and acetate were quantified enzymatically with a D-glucose/D-fructose kit and an acetic acid kit, respectively (R-Biopharm, Darmstadt, Germany), as described by the manufacturer, by comparison of the sample probes with external standards.

RESULTS

Construction and growth properties of *C. glutamicum sugR* and NCgl1859 deletion mutants. The genome of *C. glutamicum* type strain ATCC 13032 (25) contains four genes (NCgl0105, NCgl0110, *sugR*/NCgl1856, and NCgl1859) encoding putative DeoR-type transcriptional regulators (5). The genes for two of these regulators, *sugR* and NCgl1859, are located in the vicinity of the genes for the general PTS components *ptsI* (NCgl1858) and *ptsH* (NCgl1862) as well as the gene of the fructose-specific enzyme II permease *ptsF* (NCgl1861) and might therefore be involved in the regulation of sugar uptake via the PTS and/or the initial consumption of the sugar-phosphates. For functional analysis, in-frame deletion mutants of *sugR* and NCgl1859 and a double deletion mutant were constructed by two-step homologous recombination. The resulting mutants, i.e., the Δ*sugR*, Δ1859 and Δ*sugR* Δ1859 mutants, which lack the *sugR* and/or the NCgl1859 coding region except for the 6 5′-terminal and the 12 3′-terminal codons, were verified by PCR analysis (see Materials and Methods).

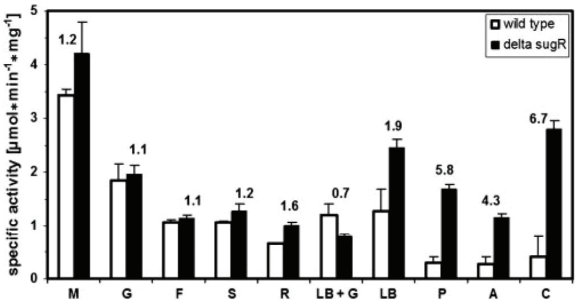

FIG. 1. Expression of a *ptsG′-′cat* fusion in *C. glutamicum* WT(pET2-*ptsG*) and *C. glutamicum* Δ*sugR*(pET2-*ptsG*). Expression of the *ptsG′-′cat* reporter gene fusion was determined by measuring the chloramphenicol acetyltransferase activity after cultivation on 50 mM maltose (M), 100 mM glucose (G), 100 mM fructose (F), 50 mM sucrose (S), 120 mM ribose (R), 200 mM Na-pyruvate (P), 300 mM K-acetate (A), 100 mM Na_3-citrate plus 100 mM $MgCl_2$ (C), LB medium (LB), and LB medium plus glucose (LB + G). The values represent averages and standard deviations from three independent experiments. The numbers above the bars indicate the ratio of *ptsG* expressions between the deletion mutant Δ*sugR* and the WT.

The growth behaviors of the Δ*sugR*, Δ1859 and Δ*sugR*Δ1859 mutants on different carbon and energy sources were compared to that of the WT. Only marginal differences between the Δ*sugR* and Δ*1859* deletion mutants, the Δ*sugR* Δ*1859* double deletion mutant, and the WT with respect to the growth rate and the biomass yield were observed on CgXII minimal medium with 100 mM glucose, 100 mM fructose, 50 mM sucrose, 50 mM glucose plus 50 mM fructose, 300 mM K-acetate, 100 mM Na_3-citrate plus 100 mM $MgCl_2$, 200 mM Na-pyruvate, 50 mM maltose, or 120 mM ribose (data not shown). Thus, neither *sugR* nor NCgl1859 is essential for growth on these carbon sources.

Expression of a *ptsG′-′cat* fusion in *C. glutamicum* WT and the Δ*sugR* and Δ1859 mutants. To test whether SugR and/or NCgl1859 exerts control on expression of the gene for the glucose-specific EII, *ptsG*, reporter gene fusion analyses were performed. A fusion between the *ptsG* promoter region and the promoterless chloramphenicol acetyltransferase gene (*cat*) was constructed using the promoter-probe vector pET2 (69). The resulting plasmid, pET2-*ptsG*, was transferred into *C. glutamicum* WT and the Δ*sugR* and Δ1859 mutants.

Expression of the *ptsG′-′cat* fusion in *C. glutamicum* WT was higher on glucose minimal medium (1.8 ± 0.3 U/mg of protein) than on LB medium (1.3 ± 0.4 U/mg of protein) or on LB medium plus glucose (1.2 ± 0.2 U/mg of protein) (Fig. 1). In *C. glutamicum* Δ1859(pET2-*ptsG*), expression of the *ptsG′-′cat* was comparable to that observed in *C. glutamicum* WT(pET2-*ptsG*) during growth on LB medium plus glucose (1.1 ± 0.1 U/mg of protein), while it was about 1.5-fold higher on LB

medium (1.9 ± 0.1 U/mg of protein). In *C. glutamicum* Δ*sugR* (pET2-*ptsG*), the *ptsG′-′cat* fusion showed an approximately twofold-increased expression on LB medium in comparison to the WT (2.5 ± 0.2 U/mg of protein) (Fig. 1). This difference in the expression of the *ptsG′-′cat* fusion was observed only on LB medium and not on either LB medium plus glucose (0.8 ± 0.1 U/mg of protein) or glucose minimal medium (2.0 ± 0.2 U/mg of protein) (Fig. 1). These results indicated a role of SugR in regulation of *ptsG* expression.

As *ptsG* expression was increased in the Δ*sugR* deletion mutant in comparison to the WT on LB medium but was comparable in both strains when glucose was present in the medium, we tested several carbon sources concerning their effects on expression of a *ptsG′-′cat* transcription fusion in the *C. glutamicum* WT and the Δ*sugR* deletion mutant (Fig. 1). Expression of the *ptsG′-′cat* transcription fusion in *C. glutamicum* WT depended on the carbon source. Expression of *ptsG′-′cat* was highest on maltose (3.4 ± 0.1 U/mg of protein), i.e., about 2-fold higher than on glucose (1.8 ± 0.3 U/mg of protein), 3.2-fold higher than on fructose (1.1 ± 0.05 U/mg of protein) or sucrose (1.1 ± 0.02 U/mg of protein), and more than 5-fold higher than on ribose (0.65 ± 0.01 U/mg of protein). On the gluconeogenic carbon sources pyruvate (0.3 ± 0.1 U/mg of protein), acetate (0.3 ± 0.1 U/mg of protein), and citrate (0.4 ± 0.4 U/mg of protein), the expression of *ptsG* was only 8 to 12% of the expression observed on maltose.

Some of these effects are due to the presence of SugR, but the absence of SugR had little effect on maltose (1.2-fold higher than in *C. glutamicum* WT), glucose (1.1-fold), fructose (1.1-fold), sucrose (1.2-fold), ribose (1.6-fold), and LB medium plus glucose (0.8-fold) (Fig. 1). In contrast, expression of the *ptsG′-′cat* transcription fusion was derepressed in the Δ*sugR* deletion mutant on LB medium and on media containing gluconeogenic carbon sources, namely, pyruvate (5.8-fold; 1.7 ± 0.1 U/mg of protein), acetate (4.3-fold; 1.2 ± 0.1 U/mg of protein), and citrate (6.7-fold; 2.8 ± 0.2 U/mg of protein).

These results show that SugR represses *ptsG* expression when *C. glutamicum* grows on media containing gluconeogenic carbon sources but lacking sugars.

Effect of SugR on growth and glucose utilization. To determine whether control of *ptsG* expression by SugR affects glucose utilization in vivo, we tested whether growth of *C. glutamicum* on LB medium with or without 100 mM glucose and on CgXII minimal medium either with 100 mM glucose or with 50 mM glucose plus 150 mM acetate is altered by overexpression of *sugR*. Therefore, we constructed *C. glutamicum* strains carrying either a *sugR* expression plasmid or, as a control, the expression vector alone. When the cells were grown on LB medium alone, the growth rate and final optical density of *C. glutamicum* WT(pVWEx1) (μ = 0.38 ± 0.02 h^{-1}; 1.9 ± 0.5 gDW $liter^{-1}$) were similar to those of the *sugR*-overexpressing strain *C. glutamicum* WT(pVWEx1-*sugR*) (μ = 0.35 ± 0.01 h^{-1}; 1.7 ± 0.1 gDW $liter^{-1}$) (Fig. 2A). But when glucose was present in the medium, the *sugR* overexpressing strain grew significantly slower (μ = 0.45 ± 0.05 h^{-1}) than the control strain WT(pVWEx1) (μ = 0.61 ± 0.03 h^{-1}). Moreover, the *sugR* overexpressing strain formed less than 1/3 of the biomass formed by the control strain (2.0 ± 0.1 compared to 7.5 ± 0.6 gDW l^{-1}) and utilized less than 15 mM of the 100 mM added glucose, while the control strain consumed the added glucose almost completely (Fig. 2A). In a similar experiment, it was shown that overexpression of *sugR* perturbed the growth of *C. glutamicum* on glucose minimal medium, as the biomass formed (4.6 ± 0.8 gDW $liter^{-1}$), the growth rate (μ = 0.17 ± 0.04 h^{-1}), and the glucose uptake rate (29 nmol mg^{-1} min^{-1}) were reduced compared to those of the control strain (7.3 ± 0.4 gDW $liter^{-1}$, μ = 0.40 ± 0.02 h^{-1}, and 93 nmol mg^{-1} min^{-1}). Growth inhibition during the first 3 h, i.e., prior to IPTG induction, might indicate that *sugR* expression is not completely repressed in plasmid pVWEx1-*sugR*.

On minimal medium containing 50 mM glucose and 150 mM acetate, WT as well as WT(pVWEx1) simultaneously utilized glucose (uptake rates of 43 and 43 nmol mg^{-1} min^{-1}) with acetate (132 and 130 nmol mg^{-1} min^{-1}) (Fig. 2B). On the glucose-acetate mixture, WT Δ*sugR* grew with a growth rate similar to that of WT (0.35 and 0.33 h^{-1}) but showed two phases with respect to carbon source utilization (Fig. 2B). Within the first 9 h of cultivation, glucose was utilized by WT Δ*sugR* with an uptake rate higher than that by WT (59 compared to 43 nmol mg^{-1} min^{-1}), while acetate uptake was reduced (81 compared to 130 nmol mg^{-1} min^{-1}). After glucose was exhausted, growth slowed and only acetate was utilized (34 nmol mg^{-1} min^{-1}). Thus, in the absence of SugR, glucose and acetate are coutilized, but the proportion between glucose and acetate is shifted towards glucose (glucose/acetate ratio of 42:58 compared to 25:75). On the glucose- and acetate-containing medium, overexpression of *sugR* resulted in slow growth (0.09 compared to 0.33 h^{-1}) (Fig. 2B). First, glucose and acetate were coutilized (13 and 68 nmol mg^{-1} min^{-1}, respectively) until acetate was exhausted, and then only glucose was utilized (36 nmol mg^{-1} min^{-1}). Overexpression of *sugR* thus perturbed growth on the glucose-acetate mixture and shifted the proportion of glucose and acetate coutilization towards acetate (16:84 compared to 25:75).

Taken together, the results show that overexpression of *sugR* has a negative influence on utilization of glucose as a carbon source for growth of *C. glutamicum*.

Identification of the transcriptional start site of *ptsG*. In order to determine whether overexpression of *sugR* reduces *ptsG* transcript levels and to determine the transcriptional start site of *ptsG*, primer extension analyses were performed. In these experiments, RNAs from *C. glutamicum* WT grown on LB medium and on glucose minimal medium, as well as RNAs from *C. glutamicum* WT(pVWEx1) and *C. glutamicum* WT(pVWEx1-*sugR*) grown on glucose or acetate minimal medium, were used.

A single primer extension product was detected using three independent primers (ptsG_30*, ptsG_60*, and ptsG_90* [Table 2]) when *C. glutamicum* WT was grown on glucose minimal medium or on LB medium (Fig. 3A and data not shown). The transcriptional start site identified by the primer extension experiments is located 258 bp upstream of the ATG start codon of *ptsG*. Analysis of the promoter region (Fig. 3B) revealed a sequence (5′-TATCAT-3′) similar to the consensus −10 sequence motif, 5′-tgngnTA(c/t)aaTgg-3′ (Uppercase, conserved; lowercase, less conserved), of *C. glutamicum* promoters (50) but no obvious −35 region as is typical for *C. glutamicum* promoters.

In contrast to the empty vector control strain *C. glutamicum* WT(pVWEx1), nearly no *ptsG* primer extension product could

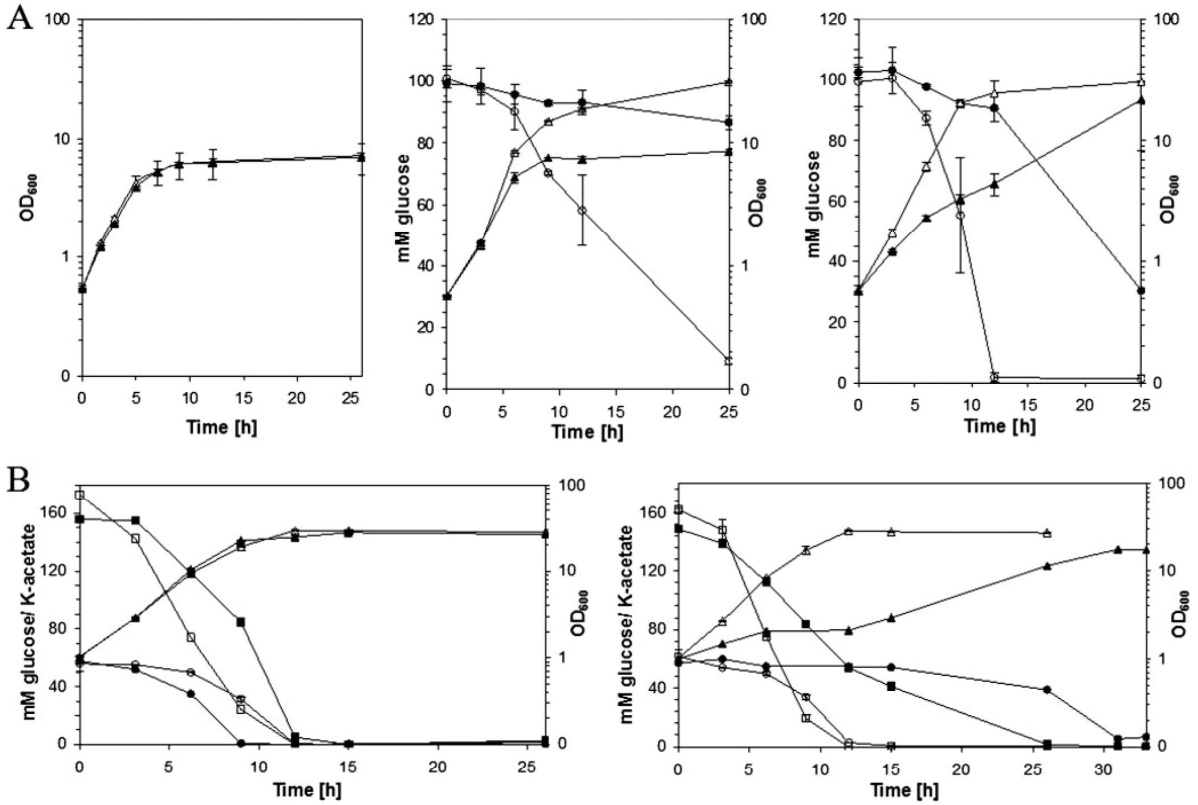

FIG. 2. Role of SugR in growth of *C. glutamicum* on glucose-containing media. (A) Growth of *C. glutamicum* WT(pVWEx1) (open symbols) and WT(pVWEx1-*sugR*) (filled symbols) on LB medium (left panel), LB medium with 100 mM glucose (middle panel), and CgXII minimal medium containing 100 mM glucose (right panel). The cultures were induced 3 hours after inoculation by addition of 1 mM IPTG. The ODs (triangles) and the glucose concentrations (circles) are indicated. (B) Growth of *C. glutamicum* WT (open symbols, left panel) and *C. glutamicum* Δ*sugR* (filled symbols, left panel) as well as of *C. glutamicum* WT(pVWEx1) (open symbols, right panel) and WT(pVWEx1-*sugR*) (filled symbols, right panel) on CgXII minimal medium containing 50 mM glucose and 150 mM K-acetate. The ODs (triangles), the glucose concentrations (circles), and the K-acetate concentrations (squares) are indicated. The cultures of *C. glutamicum* WT(pVWEx1) and WT(pVWEx1-*sugR*) were induced 3 hours after inoculation by addition of 1 mM IPTG. Values are averages and standard deviations from at least two independent cultivations are indicated.

be detected in the *sugR*-overexpressing strain *C. glutamicum* WT(pVWEx1-*sugR*) on glucose minimal medium. Thus, SugR negatively regulates *ptsG* expression in a direct or indirect manner. Only a very small amount of *ptsG* primer extension product was observed in the control strain when the cells were grown on acetate minimal medium, and no primer extension product could be detected in the *sugR*-overexpressing strain on acetate minimal medium (Fig. 3A). Taken together, the primer extension data support the assumption that expression of *ptsG* is dependent on the amount of SugR, which may function as a repressor of *ptsG* transcription.

Binding of purified SugR^{His} to the *ptsG* promoter region. To test whether the influence of SugR on *ptsG* expression is direct, we assayed the binding of purified SugR to the *ptsG* promoter in vitro. For that purpose, in several independent experiments the SugR protein containing an amino-terminal decahistidine tag (SugR^{His}) was overproduced in *E. coli* BL21(DE3), and 0.33 to 0.44 mg of SugR^{His} was purified to apparent homogeneity by affinity chromatography as described in Materials and Methods.

For the gel shift assays, the DNA fragments (10 to 102 nM) were mixed with various concentrations of the SugR^{His} protein (0 to 10 μM) and then separated on 10 or 15% native polyacrylamide gels. As shown in Fig. 4B, SugR^{His} bound the full-length *ptsG* promoter in vitro. A complete shift was observed at a 65-fold molar excess, whereas the negative control, a 190-bp promoter fragment of NCgl2027, was not shifted by SugR^{His}.

Binding of SugR to various subfragments of the *ptsG* promoter region was tested in order to confine the SugR binding site. Therefore, the full-length promoter fragment 1 (Fig. 4A) was first divided into five fragments (named fragments 2 to 6 [Fig. 4A]), overlapping by approximately 50 bp, and tested for SugR binding. SugR bound to fragments 3 and 4 (data not shown). Gel shift assays with 10 additional subfragments of the *ptsG* promoter covering different parts of fragments 3 and 4 (SF1 to -5 and SR1 to -5) suggested that SugR binds to a

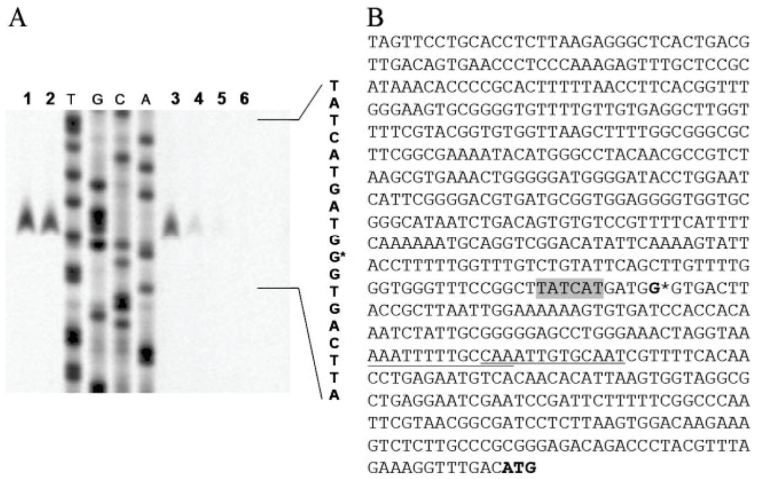

FIG. 3. Expression analysis and determination of the transcriptional start site of the *C. glutamicum ptsG* gene. (A) For primer extension analysis, 13 μg of total RNA isolated from *C. glutamicum* WT grown on LB medium (lane 1) and on CgXII minimal medium containing 100 mM glucose (lane 2), as well as 10 μg of total RNA from *C. glutamicum* WT(pVWEx1) grown on glucose (lane 3) and on CgXII minimal medium containing 300 mM acetate (lane 4) and from *C. glutamicum* WT(pVWEx1-*sugR*) grown on glucose (lane 5) and acetate (lane 6) were used. The transcriptional start site is indicated by an asterisk. The corresponding sequencing reactions (lanes T, G, C, and A) were performed using the same IRD800-labeled oligonucleotides that were used in the primer extension reactions and PCR products which covered the region of the corresponding transcriptional start site as template DNA. (B) Sequence of the intergenic region between NCgl1304 and *ptsG*. The start codon of *ptsG* is indicated in bold, the transcriptional start site is shown in bold and marked with an asterisk, the putative −10 region is highlighted in gray, and the putative RamB binding motifs are underlined.

sequence between positions −132 and −58 relative to the transcriptional start site of *ptsG* (data not shown). The 75-bp fragment BS covering the DNA region from position −132 to −58 was sufficient for SugR binding (Fig. 4A and C). In binding assays with fragment BS, SugR also bound to fragment B6 but did not bind to fragments B1 and A6 (Fig. 4A and C). Thus, the sequence from bp −81 to −58, which is present in fragments BS and B6 but absent from fragments B1 and A6, likely contains the SugR binding site.

To characterize the relevance of the SugR binding site in vivo, a fusion of the *ptsG* promoter fragment 4, which contains the transcriptional start site and the SugR binding site, to the promoterless chloramphenicol acetyltransferase gene was constructed, and its expression was tested in *C. glutamicum* WT and the Δ*sugR* mutant. Expression of the *ptsG/F4′-′cat* transcriptional fusion was derepressed in the absence of SugR during growth on LB medium (2.3 ± 0.7 U/mg of protein in WT compared to 5.0 ± 0.5 U/mg of protein in the Δ*sugR* mutant) and on acetate minimal medium (0.4 ± 0.5 U/mg of protein in WT compared to 4.9 ± 1.5 U/mg of protein in the Δ*sugR* mutant), but expression was comparable during growth on LB medium plus glucose regardless of the presence or absence of SugR (2.0 ± 0.1 U/mg of protein in WT compared to 1.6 ± 0.04 U/mg of protein in the Δ*sugR* mutant). Although under the tested conditions expression of the *ptsG/F4′-′cat* transcriptional fusion was generally higher in both *C. glutamicum* WT and the Δ*sugR* mutant compared to the *ptsG′-′cat* reporter gene fusion (Fig. 1), expression of both fusions depended on SugR in a comparable manner. Thus, *ptsG* promoter fragment 4 contains the sequences required for initiation and SugR-dependent control of *ptsG* transcription.

Fructose-6-phosphate prevents binding of SugR to the *ptsG* promoter. The carbon source-dependent effects of the absence of SugR on *ptsG* expression suggested that the binding of SugR might be affected by an inducer molecule. To test this hypothesis and to identify such an effector molecule, we assayed binding of $SugR^{His}$ protein to the *ptsG* promoter in the presence of several intermediates of the central metabolism (Fig. 4D). The possible effectors (20 mM each; see Materials and Methods) were incubated with the protein in the binding buffer for 15 min before addition of *ptsG* promoter fragment 4 (40 nM), and after incubation for another 30 min, free DNA and protein-DNA complexes were separated by 15% native polyacrylamide gel electrophoresis.

Neither glucose-6-phosphate nor phosphoenolpyruvate had an effect on the binding affinity of SugR to the *ptsG* promoter (Fig. 4D). Similarly, addition of dihydroxyacetone phosphate, fructose-1,6-bisphosphate, pyruvate, L-lactate, D-lactate, acetyl-CoA, or 3-phosphoglycerate did not affect formation of a SugR/*ptsG* promoter complex (data not shown). The inducer of SugR could be identified as fructose-6-phosphate, as at a 70-fold molar excess of protein to DNA the binding of SugR to *ptsG* promoter fragment 4 was inhibited almost completely at a concentration of 20 mM fructose-6-phosphate (Fig. 4D).

Comparison of the expression profiles of *C. glutamicum* WT and the Δ*sugR* mutant by use of DNA microarrays. To investigate the effect of the *sugR* deletion on global gene expression and to identify further putative target genes of SugR, the transcriptomes of *C. glutamicum* WT and the Δ*sugR* mutant were compared using whole-genome DNA microarrays (70). RNA was isolated from cells grown on LB medium in the exponential growth phase. Table 3 shows those genes whose

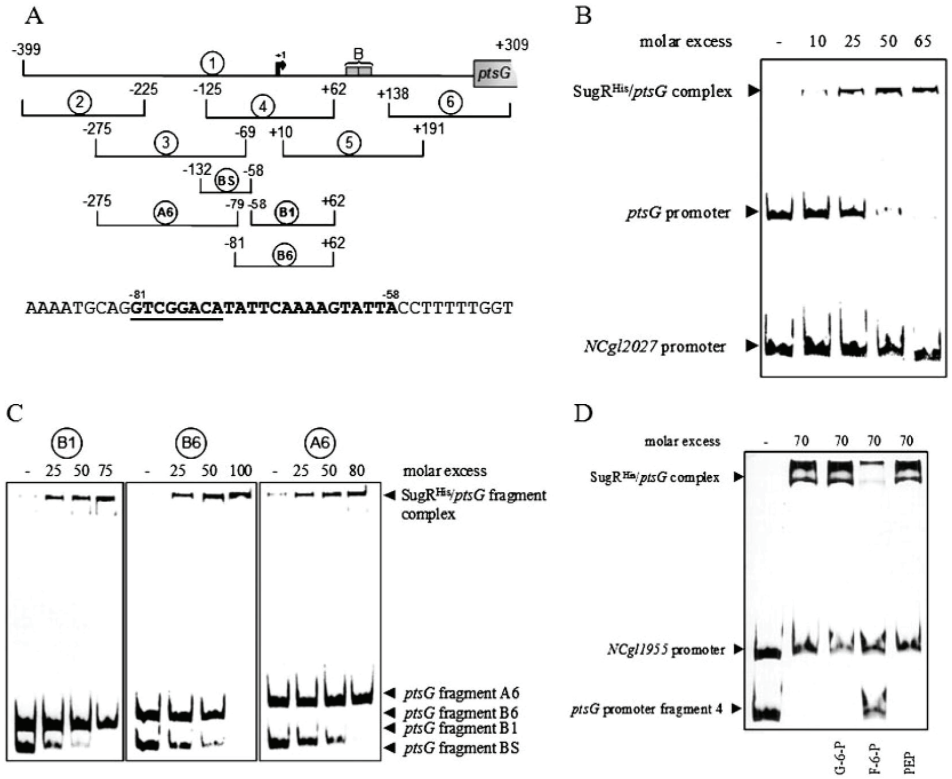

FIG. 4. Binding of SugR^{His} to the *ptsG* promoter. (A) DNA fragments (circled 1 to 6, A6, B1, B6, and BS) used to analyze SugR binding to the *ptsG* promoter by gel shift assays. The numbers indicate the ends of the fragments relative to the *ptsG* transcription start site (+1). Oligonucleotides used for amplification of the 10 fragments via PCR are listed in Table 2. The boxes labeled B indicate the regions where potential RamB binding sites are located. The sequence at the bottom shows the region between position −58 and −81 relative to the transcriptional start site of *ptsG* (bold) plus 10 bases upstream and downstream. The 8-bp motif contained within the putative binding site of SugR is underlined. (B) Gel showing binding of purified SugR^{His} (10- to 65-fold molar excess) to fragment 1 (10 nM, 707-bp fragment). A 190-bp NCgl2027 promoter fragment (40 nM) served as a negative control. (C) Gel showing binding of purified SugR^{His} (25- to 100-fold molar excess) to fragments A6, B1, B6, and BS (38, 64, 53 and 102 nM; 197-, 119-, 142-, and 75-bp fragments). (D) Gel showing binding of purified SugR^{His} (70-fold molar excess) to fragment 4 (40 nM, 186-bp fragment). A 400-bp NCgl1955 promoter fragment (19 nM) served as a negative control. The possible effectors of SugR shown here, tested at a concentration of 20 mM, were glucose-6-phosphate (G-6-P), frucose-6-phosphate (F-6-P), and phosphoenolpyruvate (PEP).

mRNA level was significantly ($P < 0.05$) changed by a factor of two or more in three biological replicates. Of these 25 genes, 14 genes showed a higher mRNA level and 11 genes a lower mRNA level in the Δ*sugR* mutant.

The mRNA level of *ptsG* was about twofold increased in the Δ*sugR* mutant compared to the WT, verifying the previous results that *ptsG* is repressed by SugR. Furthermore, the PTS genes *ptsS* (sucrose-specific PTS IIABC component) and *ptsF* (fructose-specific PTS IIABC component) with the adjacent NCgl1857 (1-phosphofructokinase-like protein) (42) and NCgl1859 (transcriptional repressor DeoR family) showed higher mRNA levels in the Δ*sugR* mutant than in the WT. Besides genes of propionate metabolism (coding for 2-methylisocitrate synthase and 2-methylcitrate synthase) and genes of the tricarboxylic acid (TCA) cycle (coding for succinyl-CoA synthetase), a cluster of genes of unknown function, NCgl0959 to NCgl0965, showed higher mRNA levels in the Δ*sugR* mutant than in the WT. While it is clear that SugR regulates *ptsG* expression directly, the effect of SugR on expression of these genes might be either direct or indirect.

Binding of purified SugR^{His} to additional target promoters. To determine whether SugR regulates expression of *ptsS* and the putative NCgl1859-*fruK-ptsF* operon directly, binding of SugR to the corresponding promoter regions was tested. As shown in Fig. 5, *ptsS* was shifted completely by SugR at a 50-fold molar excess, whereas the negative control, a 400-bp promoter fragment of NCgl1955, was not bound by SugR. The DNA region upstream of NCgl1859-*fruK-ptsF* (506 bp), but not the region upstream of *ptsF* (203 bp), was bound by SugR at a 70-fold molar excess. Binding of SugR to the DNA regions upstream of *ptsS* and NCgl1859-*fruK-ptsF* in vitro (Fig. 5) and their increased RNA levels in the absence of SugR (Table 3) indicate that SugR is a global regulator repressing transcription of *ptsG*, *ptsS*, and NCgl1859-*fruK-ptsF*.

DISCUSSION

In this study, we have shown that the DeoR-type transcriptional regulator SugR (NCgl1856) regulates *ptsG* in a carbon

TABLE 3. Genes whose average mRNA ratio was altered ≥2-fold ($P \leq 0.05$) on LB medium in *C. glutamicum* WT compared with the Δ*sugR* mutant

NCgl no.[a]	Annotation[a]	Ratio (WT/Δ*sugR*)[b]
0468	LSU ribosomal protein L10P	0.49
0492	LSU ribosomal protein L22P	0.42
0627	2-Methylisocitrate synthase, *prpD2*	2.56
0630	2-Methylcitrate synthase, *prpC2*	2.61
0917	Hypothetical cytosolic protein	3.82
0959	Phosphinothricin *N*-acetyltransferase	0.31
0960	Allophanate hydrolase subunit 2	0.33
0962	Lactam utilization protein, LamB	0.16
0963	Transporter	0.08
0965	Transcriptional regulator, GntR family	0.49
1206	ABC-type sugar transport system, periplasmic component	0.48
1305	Glucose-specific PTS enzyme II, *ptsG*	0.43
1368	Acetyltransferase	6.59
1418	Hypothetical secreted protein	2.08
1857	1-Phosphofructokinase homolog	0.37
1859	Transcriptional regulator, DeoR family	0.21
1861	Fructose-specific PTS enzyme II, *ptsF*	0.17
2252	Hypothetical cytosolic protein	2.36
2439	Ferritin-like protein, *ftn*	2.01
2477	Succinyl-CoA synthetase beta chain, *sucC*	2.47
2552	Cysteinyl-tRNA synthetase	0.43
2553	Sucrose-specific PTS enzyme II, *ptsS*	0.06
2657	Phosphate acetyltransferase, *pta*	2.43
2787	Predicted flavoprotein involved in K^+ transport	2.98
2804	DNA binding protein	2.05

[a] Gene numbering, gene designations, and descriptions of gene products are given according to the National Center of Biotechnology (NCBI) for the genome of *C. glutamicum* ATCC 13032 (GI:58036263).

[b] The mRNA ratios represent average values obtained from three DNA microarray experiments performed with RNA isolated from three independent cultures in LB medium.

source-dependent manner. Thus, we identified a novel transcriptional regulator of the central carbon metabolism of *C. glutamicum*. The inducer of SugR could be identified as fructose-6-phosphate, since of the glycolytic intermediates tested only fructose-6-phosphate inhibited binding of SugR to the *ptsG* promoter (Fig. 4D).

While regulation of glucose uptake in *C. glutamicum* is only poorly understood, in *E. coli* CRP and Mlc are known to be the major regulators of *ptsHIcrr* (coding for enzyme I, HPr, and $EIIA^{Glc}$) and *ptsG* (coding for $EIICB^{Glc}$) in response to the availability of carbon sources (18, 53). Transcription of *ptsG* is stimulated by cAMP · CRP and is repressed by Mlc (18, 30, 52, 53, 57, 76). Mlc binds upstream of *ptsG* and represses its transcription in the absence of glucose. During growth on glucose, $EIICB^{Glc}$ occurs in its unphosphorylated form due to glucose uptake and binds to Mlc, which results in derepression of *ptsG* (4, 38). FIS, a nucleoid-associated protein, facilitates rapid adaptation of *E. coli* to different carbon and energy sources through the formation of nucleoprotein complexes either with cAMP · CRP in the presence of glucose or with Mlc in the absence of glucose (64). In addition, expression of *ptsG* is regulated posttranscriptionally via modulation of *ptsG* mRNA stability by RNase E-mediated mRNA degradation in response to the glycolytic flux (24, 31). Moreover, *ptsG* transcription appears to be inhibited by the response regulator ArcA, which is phosphorylated and activated by the histidine kinase ArcB under reducing conditions. Phosphorylated ArcA binds to the CRP binding site of *ptsG*, thus interfering with CRP activation of *ptsG* transcription (24). In *B. subtilis*, *ptsGHI* transcription terminates in the absence of glucose due to the inactivation of the RNA binding antitermination protein GlcT by phosphorylation by the phosphorylated EII^{Glc}. In the presence of the inducer glucose, phosphorylated EII^{Glc} transports and phosphorylates the incoming glucose. Under these conditions, the GlcT protein is not phosphorylated and binds to the ribonucleic antiterminator (RAT) sequence of the nascent *ptsGHI* operon RNA and prevents premature termination of transcription (61, 62, 67). Although not characterized functionally, it was found that the upstream region of *ptsG* from *Corynebacterium diphtheriae* contains a RAT-like sequence, and a GlcT-like protein (DIP1150) is encoded in its genome (49). However, the *C. glutamicum* genome does not code for a functional GlcT-like protein, as the putative 197-amino-acid protein encoded by NCgl2743, which shows the highest sequence similarity to amino acids 93 to 275 of *C. diphtheriae* DIP1150, lacks the 92 N-terminal amino acids of *C. diphtheriae* DIP1150, including the RNA binding domain (25). In addition, the region upstream of *C. glutamicum ptsG* is lacking a RAT-like sequence (49). As *C. glutamicum* lacks functional homologs of regulatory proteins such as Mlc, CRP, CcpA, or GlcT and as in neither *E. coli* nor *B. subtilis* was a DeoR-type regulator shown to be involved in the regulation of PTS genes, the mechanism of *ptsG* regulation in *C. glutamicum* is distinct from that in other bacteria.

SugR belongs to the transcriptional regulators of the DeoR family, which in most instances act as transcriptional repressors in sugar metabolism (44, 75). For example, DeoR of *B. subtilis* represses the transcription of the *dra-nupC-pdp* operon, which is important for utilization of deoxyribonucleosides and deoxyribose (75). Molecular genetic studies indicated that a palindromic sequence located between nucleotides −60 and −43 relative to the transcriptional start site of the *dra-nupC-pdp* operon as well as a direct repeat of the 3′ half of the palindrome located between the −35 and the −10 regions [5′-ATTGAA-(6)-TTCAAT-(16)-TTCAA-3′] were both required for repression of this operon by DeoR (74). DeoR of *E. coli*, on

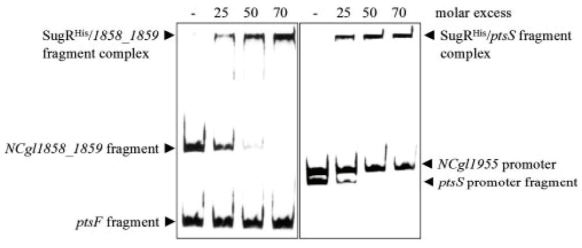

FIG. 5. Binding of $SugR^{His}$ to the promoter fragments of *ptsS* and the putative NCgl1859-*fruK-ptsF* operon. On the left side, a gel demonstrating binding of purified $SugR^{His}$ (25- to 70-fold molar excess) to the intergenic region between NCgl1858 and NCgl1859 (15 nM, 506 bp) is shown. The *ptsF* fragment (37 nM, 203 bp) was not bound by $SugR^{His}$. On the right side, a gel demonstrating binding of purified $SugR^{His}$ (25- to 70-fold molar excess) to the *ptsS* promoter fragment (29 nM, 264 bp) is shown. A 400-bp NCgl1955 promoter fragment (19 nM) served as a negative control.

the other hand, represses the initiation of transcription of the *deo* operon, which consists of four structural genes encoding ribonucleoside- and deoxyribonucleoside-catabolizing enzymes (44). The DeoR protein of *E. coli* binds to the putative 16-bp palindromic sequence 5′-TGTTAGAA · TTCTAACA-3′ in either of two of the three operator sites O_1, O_2, and O_E, forming a single or double DNA loop (44). By gel shift experiments, the 75-bp *ptsG* promoter fragment BS was shown to be sufficient for SugR binding, and additional experiments suggested that SugR binds to a DNA region from bp −81 to −58 (Fig. 4A and C). As SugR also bound to DNA regions upstream of *ptsS* and the putative NCgl1859-*fruK-ptsF* operon, we compared the sequences for similarities by using the MEME software (http://bioweb.pasteur.fr/seqanal/motif/meme/meme.html). A partly conserved 8-bp motif was found upstream of *ptsG* (5′-GTCGGACA-3′ within the DNA region from bp −81 to −58 [Fig. 4A]), of *ptsS* (5′-TGTACAAA-3′) and of NCgl1859-*fruK-ptsF* (5′-TGTGCAAC-3′). Thus, it is likely that the SugR binding sites contain this 8-bp motif.

Although the DeoR proteins of *E. coli* and *B. subtilis* share little sequence similarity and the DNA sequences to which they bind are dissimilar, their binding is inhibited by the same effector, i.e., deoxyribose-5-phosphate (44, 75). Effector molecules for DeoR-type regulators are generally phosphorylated intermediates of the relevant metabolic pathways (including, for example, besides deoxyribose-5-phosphate, fructose-1-phosphate for FruR of *Lactococcus lactis* [2]). However, nonphosphorylated inducers also have been described, e.g., opine for AccR from *Agrobacterium tumefaciens* (3), fucose for FucR from *Bacteroides thetaiotaomicron* (22), and likely *N*-acetylglucosamine or galactosamine for AgaR from *E. coli* (55). In *C. glutamicum*, the sugar phosphate fructose-6-phosphate was shown to inhibit binding of SugR to the *ptsG* promoter, thus linking regulation of glucose uptake to the first glycolytic intermediate after the branch point to the pentose phosphate pathway (Fig. 4D).

Intracellular fructose-6-phosphate concentrations of 13 mM, 1 mM, and 5 mM were determined in *C. glutamicum* DM1730 during growth on glucose, sucrose, and fructose, respectively (15), and these are in the same range as the fructose-6-phosphate concentrations affecting binding of purified $SugR^{His}$ to the *ptsG* promoter in vitro. During growth on fructose and sucrose, which enter glycolysis as fructose-6-phosphate and/or fructose-1,6-bisphosphate, slightly lower expression levels of the *ptsG′-′cat* fusion than on glucose were observed, but this difference was not dependent on the presence of SugR (Fig. 1). Similarly, the absence of SugR did not affect expression of the *ptsG′-′cat* fusion during growth on glucose, as under these conditions fructose-6-phosphate should prevent binding of SugR to the *ptsG* promoter. However, during growth on the gluconeogenic carbon sources pyruvate, acetate, and citrate, expression of the *ptsG′-′cat* fusion, which was lower than that during growth on sugars, increased four- to sevenfold in the absence of SugR (Fig. 1). Although intracellular fructose-6-phosphate concentrations have not been reported for *C. glutamicum* grown on gluconeogenic carbon sources, they are expected to be much lower than those during growth on sugars. Thus, the observed expression levels of the *ptsG′-′cat* fusion in *C. glutamicum* WT and the Δ*sugR* mutant during growth on sugars or gluconeogenic carbon sources can be explained by fructose-6-phosphate-dependent regulation by SugR.

The transcriptome comparison of *C. glutamicum* WT and the Δ*sugR* mutant during growth on LB medium (Table 3) and binding of purified SugR to DNA regions upstream of *ptsS* and the putative operon NCgl1859-*fruK-ptsF* indicate a role for SugR as a global regulator of glucose, fructose, and sucrose uptake. The transcriptome comparison also revealed direct or indirect regulation of the putative operon NCgl0959-NCgl0963 and NCgl0965, which code for proteins of unknown function and showed increased expression in the absence of SugR. In contrast, the mRNA level of, e.g., the phosphotransacetylase gene *pta* was lower in the absence of SugR. The *pta-ack* operon (mRNA levels for *ack* could not be determined), which also encodes acetate kinase, is required for acetate activation in *C. glutamicum* (56). On a glucose-acetate mixture, glucose uptake was increased in the absence of SugR due to derepression of *ptsG*. It is conceivable that the decreased acetate uptake observed under these conditions is due to direct or indirect regulation of *pta-ack* expression by SugR. Commensurate with this view, overexpression of *sugR* reduced glucose uptake during growth on a glucose-acetate mixture, while acetate uptake was increased.

Regulation of *ptsG* in *C. glutamicum* does not depend solely on SugR but appears to be more complex. Expression of the *ptsG′-′cat* fusion during growth on the sugars maltose, glucose, fructose, and sucrose was similar in the absence and presence of SugR but was twofold and fourfold higher on maltose than on glucose and fructose or sucrose, respectively (Fig. 1). Gerstmeir et al. (16) suggested a putative binding site for the global regulator RamB in the *ptsG* promoter region. In *C. glutamicum*, repression of genes of acetate metabolism by RamB was observed only during growth on glucose, i.e., in the absence of acetate (16). As the putative binding sites of RamB are located downstream of the transcriptional start site of *ptsG* (Fig. 3), RamB might function as a negative regulator of *ptsG*. It is noteworthy that expression of the transcriptional fusion containing *ptsG* promoter fragment 4, which lacks the putative RamB binding sites, was about twofold higher than expression of the full-length *ptsG* promoter fusion.

In summary, we have identified the DeoR-type regulator SugR as a transcriptional regulator of *ptsG*, *ptsS*, and NCgl1859-*fruK-ptsF* expression in *C. glutamicum*. Fructose-6-phosphate interferes with SugR binding to the promoter of *ptsG*, and thus *ptsG* is derepressed during growth on sugars or under conditions characterized by high fructose-6-phosphate concentrations. During growth on gluconeogenic carbon sources such as acetate, pyruvate, or citrate, however, *ptsG* is repressed by SugR. While SugR is the first transcriptional regulator of *ptsG* described to date for *C. glutamicum*, carbon source-dependent differences of *ptsG* expression in the absence of SugR indicate the involvement of additional regulatory systems allowing *C. glutamicum* to fine-tune *ptsG* expression according to the availability of carbon and energy sources.

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Co-ordinated regulation of gluconate catabolism and glucose uptake in *Corynebacterium glutamicum* by two functionally equivalent transcriptional regulators, GntR1 and GntR2

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Summary

Corynebacterium glutamicum **is a Gram-positive soil bacterium that prefers the simultaneous catabolism of different carbon sources rather than their sequential utilization. This type of metabolism requires an adaptation of the utilization rates to the overall metabolic capacity. Here we show how two functionally redundant GntR-type transcriptional regulators, designated GntR1 and GntR2, co-ordinately regulate gluconate catabolism and glucose uptake. GntR1 and GntR2 strongly repress the genes encoding gluconate permease (*gntP*), gluconate kinase (*gntK*), and 6-phosphogluconate dehydrogenase (*gnd*) and weakly the pentose phosphate pathway genes organized in the *tkt-tal-zwf-opcA-devB* cluster. In contrast, *ptsG* encoding the EII^{Glc} permease of the glucose phosphotransferase system (PTS) is activated by GntR1 and GntR2. Gluconate and glucono-δ-lactone interfere with binding of GntR1 and GntR2 to their target promoters, leading to a derepression of the genes involved in gluconate catabolism and reduced *ptsG* expression. To our knowledge, this is the first example for gluconate-dependent transcriptional control of PTS genes. A mutant lacking both *gntR1* and *gntR2* shows a 60% lower glucose uptake rate and growth rate than the wild type when cultivated on glucose as sole carbon source. This growth defect can be complemented by plasmid-encoded GntR1 or GntR2.**

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Introduction

Corynebacterium glutamicum is a predominantly aerobic, biotin-auxotrophic Gram-positive soil bacterium that was isolated in Japan owing to its ability to excrete L-glutamate under biotin-limiting growth conditions (Kinoshita *et al.*, 1957). It is used today for the industrial production of more than two million tons of amino acids per year, mainly L-glutamate and L-lysine. Additionally, this species has become a model organism of the *Corynebacterineae*, a suborder of the *Actinomycetales* which also comprises the genus *Mycobacterium*. An overview on the current knowledge on *C. glutamicum* can be found in a recent monograph (Eggeling and Bott, 2005).

Corynebacterium glutamicum is able to grow on a variety of sugars, sugar alcohols and organic acids (e.g. acetate, lactate or citrate) as carbon and energy sources. The use of gluconate as an additional carbon source besides glucose was previously shown to have a positive effect on L-lysine production (Lee *et al.*, 1998; Bianchi *et al.*, 2001). In order to be metabolized, gluconate is first transported into the bacterial cytoplasm via a specific gluconate permease (GntP). Subsequently, it is phosphorylated to 6-phosphogluconate by gluconate kinase (GntK). In *C. glutamicum*, 6-phosphogluconate is further metabolized in the pentose phosphate pathway, as the alternative Entner–Doudoroff pathway is absent in this organism. Although in recent studies several transcriptional regulators involved in the regulation of central metabolic pathways in *C. glutamicum* were identified and characterized, knowledge about transcriptional regulation of genes involved in gluconate metabolism and pentose phosphate pathway is scarce (Gerstmeir *et al.*, 2004; Kim *et al.*, 2004; Krug *et al.*, 2005; Cramer *et al.*, 2006; Engels and Wendisch, 2007; Wennerhold *et al.*, 2005; Bott, 2007).

In many bacteria genes involved in gluconate utilization are subject to negative control by GntR-like transcriptional regulators. In the case of GntR of *Bacillus subtilis* and *Escherichia coli*, it was shown that gluconate itself interferes with the binding of these regulators to their target promoters (Fujita and Fujita, 1987; Peekhaus and Conway, 1998). In several *Bacillus* species the genes encoding GntR, GntP, GntK, as well as a putative

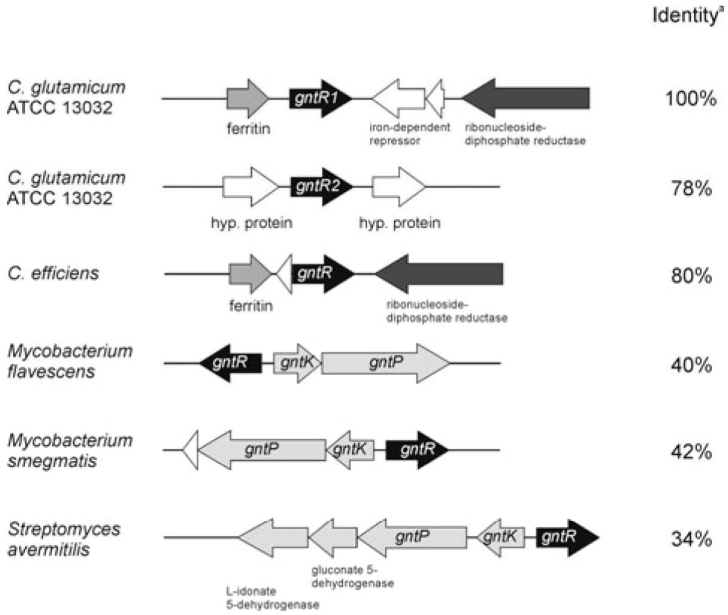

Fig. 1. Genomic organization of GntR-type regulators with high sequence identity to GntR1. Genes for GntR-type regulators with high sequence identity to GntR1 from *C. glutamicum* are shown in black. In several *Mycobacterium* species and *Streptomyces avermitilis* genes encoding gluconate kinase (*gntK*) and gluconate permease (*gntP*) are located divergently to *gntR*. Data were taken from the bioinformatics software ERGO (Integrated Genomics). [a] Identity of the amino acid sequence to GntR1 (encoded by *cg2783*) of *C. glutamicum*.

6-phosphogluconate dehydrogenase (*gntZ*) are clustered in one operon. Expression of these genes is derepressed in the presence of gluconate and also subject to carbon catabolite repression by the catabolite control protein CcpA and the phosphocarrier protein HPr (Reizer *et al.*, 1996). In *E. coli* the *gnt* genes are also repressed by the gluconate repressor GntR and activated by CRP (cAMP receptor protein) in complex with cAMP (Peekhaus and Conway, 1998). These data demonstrate that expression of the *gnt* genes is controlled in dependency of gluconate availability and the presence of a catabolite repressive carbohydrate-like glucose.

Recently, it was reported that the genes encoding gluconate permease and gluconate kinase (*gntP* and *gntK*) in *C. glutamicum* are also subject to carbon catabolite repression, presumably via the cAMP-dependent regulator GlxR which binds to the promoter regions of *gntP* and *gntK* (Letek *et al.*, 2006). *C. glutamicum* GlxR contains a cAMP-binding motif and shows 27% sequence identity with the CRP protein of *E. coli*. GlxR was first identified as a repressor of *aceA* and *aceB* encoding the key enzymes of the glyoxylate cycle, isocitrate lyase and malate synthase respectively (Kim *et al.*, 2004). Letek *et al.* (2006) reported that expression of *gntP* and *gntK* are not induced (or derepressed) by gluconate.

In this study, we have identified two paralogous GntR-type regulators in *C. glutamicum*, designated GntR1 and GntR2, which repress the expression of genes involved in gluconate metabolism (e.g. *gntK*, *gntP* and *gnd*) in the absence of gluconate. Surprisingly, these regulators function at the same time as activators of *ptsG* and *ptsS* encoding the permeases EII^{Glc} and EII^{Suc} of the PEP-dependent phosphotransferase system (PTS) for glucose and sucrose uptake in *C. glutamicum* (Lengeler *et al.*, 1994; Kotrba *et al.*, 2001; Parche *et al.*, 2001; Moon *et al.*, 2005). To our knowledge, this is the first example for a gluconate-dependent transcriptional control of PTS genes.

Results

Identification of putative gluconate-dependent transcriptional regulators in C. glutamicum

In *C. glutamicum* genes involved in gluconate metabolism (*gntP*, *gntK*, *gnd*) are not clustered in an operon, like in *E. coli* or *B. subtilis*, but are scattered on the genome of this organism. In their close vicinity, no genes for transcriptional regulators belonging to the GntR family, which might act as gluconate-dependent regulators of these genes, could be detected. The genome of *C. glutamicum* ATCC 13032 contains 11 genes which encode GntR-type transcriptional regulators (Brune *et al.*, 2005); two of them (*cg1935* and *cg2783*) show 78% sequence identity on the level of amino acid sequence and may have arisen by gene duplication. Interestingly, orthologs of *cg1935* and *cg2783* could also be found in *Mycobacterium flavescens* (Mflv_0501) and *Mycobacterium smegmatis* (MSMEG_0454) where they are located divergently to *gntK* and *gntP* (Fig. 1). This finding indicated a possible function of *cg1935* and *cg2783* in the regulation of gluconate metabolism in

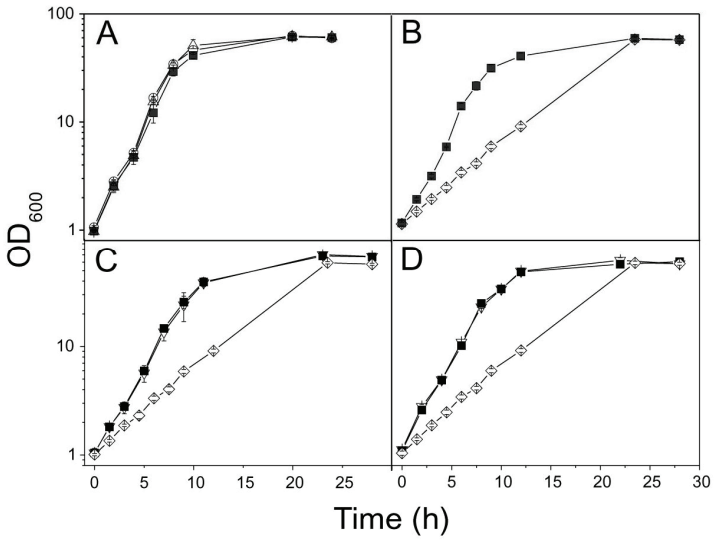

Fig. 2. Growth of *C. glutamicum* wild type and different deletion mutants in CGXII minimal medium with 4% (w/v) glucose. In experiments C and D, the medium contained in addition 25 μg ml^{-1} kanaymycin.
A. wild type (■), Δ*gntR1* (Δ) and Δ*gntR2* (○).
B. wild type (■) and Δ*gntR1*Δ*gntR2* (◇).
C. wild type/pAN6 (■), Δ*gntR1*Δ*gntR2*/pAN6 (◇) and Δ*gntR1*Δ*gntR2*/pAN6-*gntR2* (∇).
D. wild type/pAN6 (■), Δ*gntR1*Δ*gntR2*/pAN6 (◇) and Δ*gntR1*Δ*gntR2*/pAN6-*gntR1* (∇).

C. glutamicum. In *Corynebacterium efficiens*, an orthologous gene (CE2422) was located in a similar genomic context as *cg2783* in *C. glutamicum* (Fig. 1). Because of their proposed function in gluconate catabolism, the *C. glutamicum* genes were designated as *gntR1* (*cg2783*) and *gntR2* (*cg1935*). The sequence identity of GntR1 and GntR2 to GntR of *B. subtilis* and *E. coli*, which are known to control the expression of genes involved in gluconate metabolism, is below 30%.

GntR1 and GntR2 of *C. glutamicum* consist of an N-terminal GntR-type helix–turn–helix motif (PFAM: PF00392) responsible for DNA-binding and a C-terminal putative ligand-binding domain (PFAM: PF07729) typical for many GntR-type regulators. GntR-type regulators constitute to a large family of transcriptional regulators which typically share a highly conserved N-terminal DNA-binding motif, whereas the C-terminal parts show large divergence. Therefore, GntR members were classified into four subfamilies designated as FadR, HutC, MocR and YtrA (Rigali *et al.*, 2002). Because of the presence of an FCD domain (FCD stands for FadR C-terminal domain) in GntR1 and GntR2 of *C. glutamicum*, these regulators most probably belong to the FadR family, which also includes GntR of *B. subtilis*. The coding region of *gntR2* (*cg1935*) lies within the prophage region CGP3 of the *C. glutamicum* genome (Kalinowski, 2005) which spans more than 180 kb covering approximately 200 coding regions for proteins most of which lack any significant similarities to known bacterial genes. In *C. glutamicum* strain R (Yukawa *et al.*, 2007) and *C. efficiens* (Nishio *et al.*, 2003), only orthologs of *gntR1* are present and located in the same genomic environment as *gntR1* of *C. glutamicum*.

The genes gntR1 *and* gntR2 *are functionally redundant*

In order to explore the regulatory function of GntR1 and GntR2 in *C. glutamicum* ATCC 13032, in-frame deletion mutants of the genes *cg2783* (Δ*gntR1*) and *cg1935* (Δ*gntR2*) as well as a double deletion mutant (Δ*gntR1*Δ*gntR2*) were constructed. Subsequently, growth of the different mutant strains was compared with that of the wild type using CGXII minimal medium containing either 4% (w/v) glucose or 2% (w/v) gluconate as carbon and energy source. When cultivated in minimal medium with 2% (w/v) gluconate, all four strains showed the same growth rate ($0.46 \pm 0.02\ h^{-1}$) and the same final cell density ($OD_{600} = 25 \pm 1.5$). In minimal medium with 4% (w/v) glucose, the mutant strains Δ*gntR1* and Δ*gntR2* displayed the same growth behaviour ($\mu = 0.41 \pm 0.02\ h^{-1}$, final $OD_{600} = 60 \pm 1.2$) as the wild type (Fig. 2A). In contrast, the double mutant Δ*gntR1*Δ*gntR2* showed a strongly reduced growth rate of only $0.16 \pm 0.01\ h^{-1}$, but reached the same final cell density as the other strains after 24 h (Fig. 2B). As shown in Fig. 2C and D, the growth defect of mutant Δ*gntR1*Δ*gntR2* on glucose could be reversed by transformation with a plasmid carrying either the *gntR1* or the *gntR2* gene under control of the non-induced *tac* promoter. This result confirms that the simultaneous absence of GntR1 and GntR2 is responsible for the reduced growth rate in glucose minimal medium and indicates that GntR1 and GntR2 can replace each other. Complementation of the growth defect of strain Δ*gntR1*Δ*gntR2* on glucose was only possible when *gntR1* or *gntR2* were expressed at low levels owing to a basal activity of the *tac* promoter. Strong overexpression of

either *gntR1* or *gntR2* in strain Δ*gntR1*Δ*gntR2* by addition of 1 mM isopropylthiogalactoside (IPTG) to the medium resulted in a growth defect in glucose and gluconate minimal medium, but not in acetate minimal medium (data not shown). Thus, high cellular levels of either GntR1 or GntR2 are inhibitory if glucose or gluconate are used as carbon source.

*Transcriptome analyses of the Δ*gntR1*, Δ*gntR2 *and Δ*gntR1ΔgntR2 *mutant strains*

The growth experiments described above revealed that the single deletion mutants Δ*gntR1* and Δ*gntR2* grow like wild type under all tested conditions, whereas the Δ*gntR1*Δ*gntR2* deletion mutant shows a strongly reduced growth rate when cultivated on glucose, but not on gluconate. In order to elucidate the molecular basis of this phenotype, expression profiles of the different deletion mutants were compared with that of the *C. glutamicum* wild type using DNA microarray analysis. For this purpose, strains were cultivated in CGXII minimal medium with either 100 mM glucose or 100 mM gluconate. Additionally, expression profiles of wild type and the mutant Δ*gntR1*Δ*gntR2* were also compared after cultivation in CGXII minimal medium with 50 mM glucose and 50 mM gluconate. For each comparison, a set of two to three experiments starting from independent cultures was performed. RNA was isolated from cells harvested in the early exponential phase (OD_{600} 4–6) and always the expression levels of wild type and a deletion mutant were compared. No remarkable differences were observed between the expression levels of the single mutants Δ*gntR1* and Δ*gntR2* and the wild type, both for glucose- and gluconate-grown cells. A similar result was obtained in the comparison of wild type and the double mutant Δ*gntR1*Δ*gntR2* when the strains were cultivated either on gluconate alone or on glucose plus gluconate. In contrast, a variety of significant differences in gene expression was detected between wild type and strain Δ*gntR1*Δ*gntR2* when cells were cultivated with glucose as sole carbon source (Table 1).

Figure 3 shows a hierarchical cluster of all genes which showed a ≥ fourfold altered mRNA level in the Δ*gntR1*Δ*gntR2* mutant cultivated on glucose. Under the chosen criteria, 26 genes showed a decreased and 19 genes an increased mRNA level in the Δ*gntR1*Δ*gntR2* mutant. Interestingly, one of the genes with the most significantly decreased mRNA level (factor 25) is *ptsG*, encoding the permease EII^{Glc} of the phospho*enol*pyruvate-dependent sugar PTS responsible for glucose uptake in *C. glutamicum* (Lee *et al.*, 1994; Moon *et al.*, 2005). Additionally, also the mRNA level of the *ptsS* gene encoding the EII^{Suc} permease involved in sucrose uptake was lower by a factor of four in the double mutant. On the other hand, the genes involved in gluconate uptake and metabolism showed a strongly increased mRNA level in the Δ*gntR1*Δ*gntR2* mutant (*gntP* 25-fold, *gntK* 2700-fold, *gnd* 12-fold). Besides the mRNA level of 6-phosphogluconate dehydrogenase (*gnd*), also the mRNA levels of other pentose phosphate pathway genes (*tkt-tal-zwf-opcA-devB*) showed a 1.6-fold to threefold increased mRNA level. Although the mRNA ratios of these genes did not exceed a factor of four, they were also included in the hierarchical cluster analysis shown in Fig. 3. The microarray data indicate an important function of GntR1 and GntR2 in gluconate metabolism and sugar uptake in *C. glutamicum*. Additionally, they support the assumption that GntR1 and GntR2 are able to complement each other, because no significant gene expression differences were detected between the single deletion mutants Δ*gntR1* and Δ*gntR2* and the wild type.

Influence of GntR1 and GntR2 on the activity of gluconate kinase, 6-phosphogluconate dehydrogenase and glucose 6-phosphate dehydrogenase

The microarray data indicated that GntR1 and GntR2 act as repressors of the genes required for gluconate catabolism, i.e. *gntP*, *gntK*, *gnd* and other pentose phosphate pathway genes. To test whether the differences observed at the mRNA level are also present at the protein level, we determined the specific activities of gluconate kinase, 6-phosphogluconate dehydrogenase and glucose 6-phosphate dehydrogenase in cell-free extracts of wild type and the deletion mutant Δ*gntR1*Δ*gntR2*. For this purpose, the strains were cultivated in CGXII minimal medium with either 4% glucose or 2% gluconate or 1% of glucose and gluconate or 2% acetate and harvested in the early exponential phase (OD_{600} 4–6). As shown in Table 2, the activities of all three enzymes were significantly increased in the Δ*gntR1*Δ*gntR2* mutant when the cells were grown with glucose or acetate as carbon source. As expected from the transcriptome analysis, gluconate kinase showed the strongest increase, as its activity was below the detection limit (0.01 U mg^{-1}) in wild type cells cultivated on glucose or acetate. The activities of 6-phosphogluconate dehydrogenase and glucose 6-phosphate dehydrogenase were increased ~10-fold and approximately threefold, respectively, in the Δ*gntR1*Δ*gntR2* mutant grown on glucose, which is in very good agreement with the increase in the mRNA levels. When extracts of cells grown on gluconate or glucose plus gluconate were tested, the enzyme activities were also increased in strain Δ*gntR1*Δ*gntR2*, but to a much lower extent (≤ twofold). These data support the assumption that GntR1 and GntR2 act as gluconate-responsive repressors of genes involved in gluconate catabolism and the pentose phosphate pathway.

Table 1. Genome-wide comparison of mRNA levels in *C. glutamicum* wild type with the mutant strains Δ*gntR1*, Δ*gntR1* or Δ*gntR1*Δ*gntR2* using DNA microarrays.

		mRNA ratio						
		Δ*gntR1*Δ*gntR2*/wild type			Δ*gntR1*/wild type		Δ*gntR2*/wild type	
Gene	Annotation	Glu	Glu + Gnt	Gnt	Glu	Gnt	Glu	Gnt
cg1935	Transcriptional regulator of GntR family, *gntR2*	< 0.01	0.05	< 0.01	2.09	1.00	0.05	< 0.01
cg2783	Transcriptional regulator of GntR family, *gntR1*	< 0.01	0.03	< 0.01	< 0.01	0.02	1.00	1.23
cg2940	Dipeptide transport ATP-binding protein, *dppF*	0.01	0.73	1.17	1.05	1.11	0.99	0.93
cg0211	Myo-inositol 2-dehydrogenase	0.02	1.12	2.66	1.18	1.32	1.13	0.93
cg2691	Hypothetical protein	0.02	0.92	0.77	0.99	1.02	0.90	0.84
cg0751	Hypothetical protein	0.03	1.08	3.23	1.10	1.09	0.87	0.85
cg0082	Chloride channel protein	0.03	1.36	1.69	1.04	1.06	1.10	0.95
cg1493	D-alanine–D-alanine ligase	0.03	1.28	0.90	1.25	1.19	1.00	1.16
cg1369	F_1F_0 ATP synthase ε subunit, *atpC*	0.03	1.05	5.36	1.44	1.14	0.84	0.97
cg1537	PTS system, glucose-specific IIABC component, *ptsG*	0.04	0.57	1.11	1.31	1.37	0.81	1.13
cg0993	Transcriptional regulator of ArsR family	0.05	0.74	7.14	0.93	0.98	0.93	0.95
cg2725	Transposase	0.08	0.69	0.45	0.37	0.65	1.34	2.83
cg0658	Hypothetical membrane spanning protein	0.08	0.89	0.91	1.13	1.06	0.99	0.90
cg1488	3-isopropylmalate dehydratase small subunit, *leuD*	0.10	1.23	1.09	1.27	1.15	0.98	0.98
cg1451	D-3-phosphoglycerate dehydrogenase, *serA*	0.12	1.10	0.86	1.00	1.09	0.67	1.14
cg2125	Uracil permease, *uraA*	0.14	1.11	1.22	0.86	1.10	1.03	0.98
cg0564	LSU ribosomal protein L1P	0.19	0.75	0.97	0.80	1.43	0.86	0.85
cg0770	ABC-type siderophore transport system, permease component	0.19	1.03	0.71	0.71	0.72	0.81	0.70
cg0687	O-sialoglycoprotein endopeptidase, *gcp*	0.19	1.24	0.67	0.80	1.13	0.88	1.03
cg1002	Hypothetical protein	0.19	0.91	0.99	0.81	0.86	0.93	1.22
cg0286	Transporter	0.21	0.67	1.20	0.90	1.16	0.99	1.10
cg1487	3-isopropylmalate dehydratase large subunit, *leuC*	0.21	1.76	1.31	1.05	1.24	1.34	0.92
cg2399	Glucose kinase, *glk*	0.22	0.93	0.82	0.66	1.09	0.91	0.81
cg2925	PTS system, sucrose-specific IIABC component, *ptsS*	0.26	1.05	1.37	1.17	1.47	1.14	1.28
cg2178	N utilization substance protein A	0.26	1.03	0.68	1.09	1.26	0.93	0.85
cg3398	Superfamily II DNA and RNA helicase	0.27	1.14	1.10	1.13	1.19	1.04	1.10
cg1778	Glucose 6-phosphate dehydrogenase, *zwf*	1.60	1.16	0.89	0.97	1.02	1.14	0.87
cg1779	Glucose 6-phosphate dehydrogenase, *opcA*	1.77	1.03	0.88	0.89	1.09	1.15	0.74
cg1776	Transaldolase, *tal*	2.01	1.21	0.81	1.06	1.02	1.12	0.97
cg1780	6-phosphogluconolactonase, *devB*	2.51	1.28	0.89	1.14	1.02	0.91	1.08
cg1774	Transketolase, *tkt*	2.87	1.13	0.83	1.07	1.09	1.03	1.12
cg2836	Succinyl-CoA synthetase α chain, *sucD*	4.48	0.93	0.52	1.41	0.75	0.81	0.97
cg3399	Hypothetical protein	4.50	1.85	0.76	0.83	1.01	1.22	0.97
cg0143	Mannitol 2-dehydrogenase	4.52	1.19	2.11	1.27	0.98	1.13	1.76
cg0291	Protocatechuate 3,4-dioxygenase β chain	4.64	0.75	0.72	0.91	1.14	1.19	1.11
cg1454	Taurine-binding protein	5.03	0.97	0.89	0.83	0.90	1.01	1.13
cg1642	Cytoplasmic siderophore-interacting protein	5.03	1.15	1.12	n.d.	1.11	1.15	1.55
cg2616	Vanillate O-demethylase oxygenase subunit	5.87	1.21	0.79	0.61	1.24	1.04	1.36
cg0797	Methylisocitrate lyase, *prpB1*	5.91	1.01	0.90	1.51	0.86	0.85	0.89
cg0796	2-methylcitrate dehydratase, *prpD1*	6.34	1.08	0.78	0.93	0.73	0.89	0.81
cg0798	2-methylcitrate synthase, *prpC1*	6.69	1.14	0.68	1.24	0.88	1.09	1.29
cg0144	Transporter	8.11	1.03	1.87	1.01	0.98	1.17	1.29
cg1589	Hypothetical protein	9.33	1.06	1.21	0.98	1.08	1.15	1.11
cg1643	6-phosphogluconate dehydrogenase, *gnd*	12.40	1.12	1.76	1.36	1.28	0.97	1.19
cg3216	Gluconate permease, *gntP*	25.08	0.79	0.83	1.38	0.91	1.33	1.07
cg2810	Na^+/H^+-dicarboxylate symport protein	65.46	1.04	1.29	1.20	1.04	1.01	2.41
cg1255	HNH endonuclease family protein	129.02	1.01	1.60	1.33	0.94	1.01	1.44
cg2733	HNH endonuclease family protein	155.10	1.05	1.46	1.01	0.95	1.05	11.53
cg0385	Periplasmic β-glucosidase/β-xylosidase, *bglS*	935.79	1.36	0.01	n.d.	1.27	1.77	21.08
cg2732	Gluconate kinase, *gntK*	2716.50	1.05	1.06	1.11	1.10	1.19	4.69

The mRNA ratios shown represent mean values from two or three independent microarray experiments starting from independent cultures (see *Experimental procedures*). In total, 17 microarray experiments were performed for the three comparisons Δ*gntR1* versus wild type, Δ*gntR1*Δ*gntR2* versus wild type and Δ*gntR2* versus wild type. The strains were cultivated in CGXII minimal medium with either 100 mM glucose (Glu), or 100 mM gluconate (Gnt), or 50 mM glucose and 50 mM gluconate (Glu + Gnt) and mRNA was prepared from cells in the exponential growth phase. The table includes those genes which showed a ≥ fourfold changed mRNA level (increased or decreased) in at least two of the three experiments comparing the double mutant Δ*gntR1*Δ*gntR2* versus wild type on glucose minimal medium and which had a *P*-value of ≤ 0.05. The genes are ordered according to the mRNA ratio of this comparison. In addition, the gene cluster encoding enzymes of the pentose phosphate pathway have been included, although their mRNA ratio was changed less than fourfold.

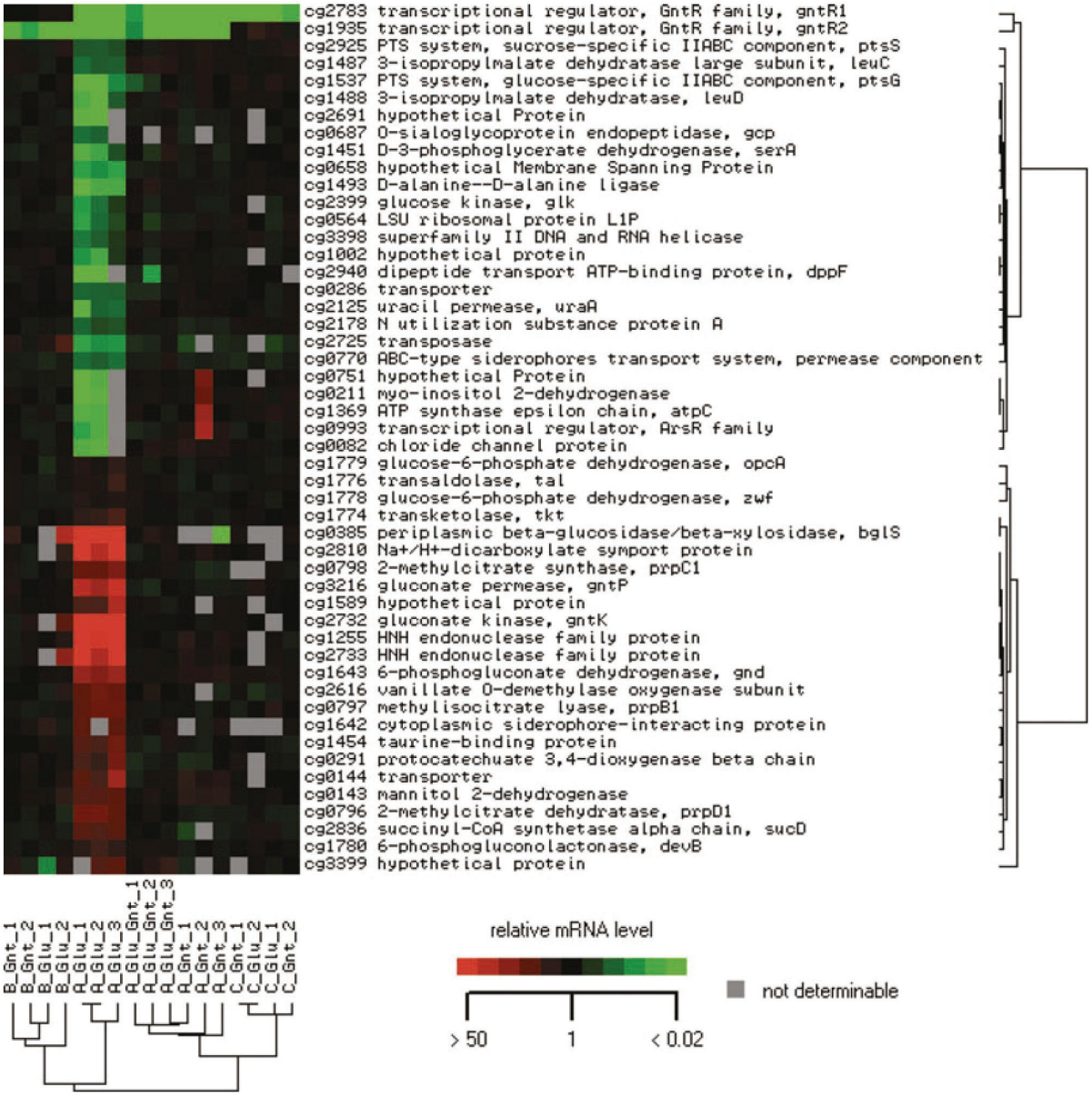

Fig. 3. Hierarchical cluster analysis of gene expression changes in three series of DNA microarray experiments. The expression profiles of three different deletion mutants were compared with *C. glutamicum* wild type in totally 17 microarray experiments: (A) Δ*gntR1*Δ*gntR2* versus wild type; (B) Δ*gntR2* versus wild type; (C) Δ*gntR1* versus wild type. The strains were cultivated in CGXII minimal medium with either 100 mM glucose (Glu), or 100 mM gluconate (Gnt), or 50 mM glucose and 50 mM gluconate (Glu_Gnt). The cluster includes those genes which showed a ≥ fourfold changed mRNA level (increased or decreased) in at least two of the experiments A_Glu and had a *P*-value of ≤ 0.05. The relative mRNA level represents the ratio of mutant/wild type.

The activity of all three enzymes measured in the derepressed background of a Δ*gntR1*Δ*gntR2* mutant was higher (~25–60%) in glucose-grown cells compared with gluconate-grown cells. This difference could be due to a regulatory effect on the transcriptional level elicited by the influence of GntR1 and GntR2 on glucose uptake (see below).

Activation of PTS-dependent sugar uptake via GntR1 and GntR2

In contrast to genes involved in gluconate metabolism and the pentose phosphate pathway, the genes *ptsG* and *ptsS* encoding the permeases EII^{Glc} and EII^{Suc} of the PTS system showed 25-fold or fourfold decreased mRNA levels in the

Table 2. Specific activity of gluconate kinase, 6-phosphogluconate dehydrogenase and glucose 6-phosphate dehydrogenase in *C. glutamicum* wild type and the Δ*gntR1*Δ*gntR2* mutant.

		Specific activity (U mg $^{-1}$)		
Strain	Carbon source	Gluconate kinase	6-Phosphogluconate DH	Glucose 6-phosphate DH
Wild type	Glucose	n.d.[a]	0.19 ± 0.02	0.15 ± 0.02
Δ*gntR1*Δ*gntR2*		2.37 ± 0.3	2.62 ± 0.18	0.49 ± 0.08
Wild type	Gluconate	0.92 ± 0.1	0.84 ± 0.01	0.35 ± 0.02
Δ*gntR1*Δ*gntR2*		1.50 ± 0.2	1.69 ± 0.03	0.40 ± 0.03
Wild type	Glucose + gluconate	0.60 ± 0.1	0.69 ± 0.03	0.11 ± 0.01
Δ*gntR1*Δ*gntR2*		1.20 ± 0.1	1.13 ± 0.10	0.16 ± 0.03
Wild type	Acetate	n.d.[a]	0.12 ± 0.01	0.03 ± 0.01
Δ*gntR1*Δ*gntR2*		1.27 ± 0.3	1.79 ± 0.08	0.16 ± 0.04

a. n.d., not detectable (below 0.01 U mg^{-1}).
The two strains were grown in CGXII minimal medium containing either 4% (w/v) glucose or 2% (w/v) gluconate or 1% glucose plus 1% gluconate or 2% acetate. Cells were harvested in the early exponential growth phase (OD_{600} ~5). Enzyme activities were determined in cell-free extracts. The values for the specific activities represent means ± standard deviations from at least three independent cultivations.

Δ*gntR1*Δ*gntR2* mutant, respectively. In order to investigate a potential activation of *ptsG* expression by GntR1 and GntR2, reporter gene fusion analyses were performed. The plasmid pET2-*ptsG* containing the *ptsG* promoter region in front of a promoterless chloramphenicol acetyltransferase gene (Engels and Wendisch, 2007) was transferred into *C. glutamicum* wild type and the Δ*gntR1*Δ*gntR2* mutant. Subsequently, the two strains were grown in CGXII minimal medium with either a single carbon source (100 mM glucose or 100 mM gluconate) or mixed carbon sources (50 mM glucose + 50 mM gluconate). When cultivated on glucose, expression of the *ptsG–cat* fusion was ninefold lower in the Δ*gntR1*Δ*gntR2* mutant in comparison to the wild type (Table 3); showing that the reduced *ptsG* mRNA level observed in the microarray experiments is caused by reduced transcription. When cultivated on gluconate or glucose plus gluconate, the CAT activity of the mutant was only 1.5- to 1.8-fold lower than the activity of the wild type. These results can be explained by the assumption that *ptsG* expression is strongly activated by GntR1 and GntR2 in the absence of gluconate.

Binding of purified GntR1 and GntR2 to the promoter regions of putative target genes

The microarray experiments reported above identified *gntP*, *gntK*, *ptsG*, *ptsS* and the gene cluster *tkt-tal-zwf-opcA-devB* as putative target genes of GntR1 and GntR2. In order to test for a direct interaction of GntR1 and GntR2 with the promoter regions of these genes, the binding of the purified proteins was tested *in vitro*. For this purpose, GntR1 and GntR2 were overproduced in *E. coli* BL21(DE3)/pLysS and purified to homogeneity by means of an amino-terminal decahistidine tag (see *Experimental procedures*). The histidine tag does not interfere with the functionality of the proteins, as His-tagged GntR1 and GntR2 were able to complement the growth defect of the Δ*gntR1*Δ*gntR2* mutant on glucose (data not shown). In gel shift assays, DNA fragments covering the corresponding promoter regions were incubated with increasing concentrations of purified GntR1 or GntR2 and subsequently separated on a 10% native polyacrylamide gel. As shown in Fig. 4, all six promoter regions were shifted by GntR1 as well as by GntR2. A complete shift was observed at a fivefold to 10-fold molar excess of protein. Interestingly, at a 10- to 20-fold molar excess of protein, the formation of multiple GntR/DNA complexes was observed with all tested promoter regions. This observation could indicate the presence of several GntR1/2-binding motifs within the target promoter regions and/or the oligomerization of the protein once it is bound to DNA. Different DNA fragments covering for example the promoter regions of *acn* (aconi-

Table 3. Specific chloramphenicol acetyltransferase (CAT) activities of *C. glutamicum* wild type and the mutant Δ*gntR1*Δ*gntR2*, both carrying the promoter-probe plasmid pET2-*ptsG*.

	Specific CAT activities (U mg $^{-1}$)	
Carbon source(s)	*C. glutamicum* wild type/pET2-*ptsG*	*C. glutamicum* Δ*gntR1*Δ*gntR2*/pET2-*ptsG*
Glucose	1.84 ± 0.30	0.21 ± 0.05
Gluconate	0.61 ± 0.13	0.41 ± 0.05
Glucose + gluconate	0.77 ± 0.03	0.43 ± 0.05

The cells were grown in CGXII minimal medium with either 100 mM glucose or 100 mM gluconate or with 50 mM of both carbon sources. Enzyme activities were determined in cell-free extracts. The values for the specific activities represent means ± standard deviations from three independent cultivations.

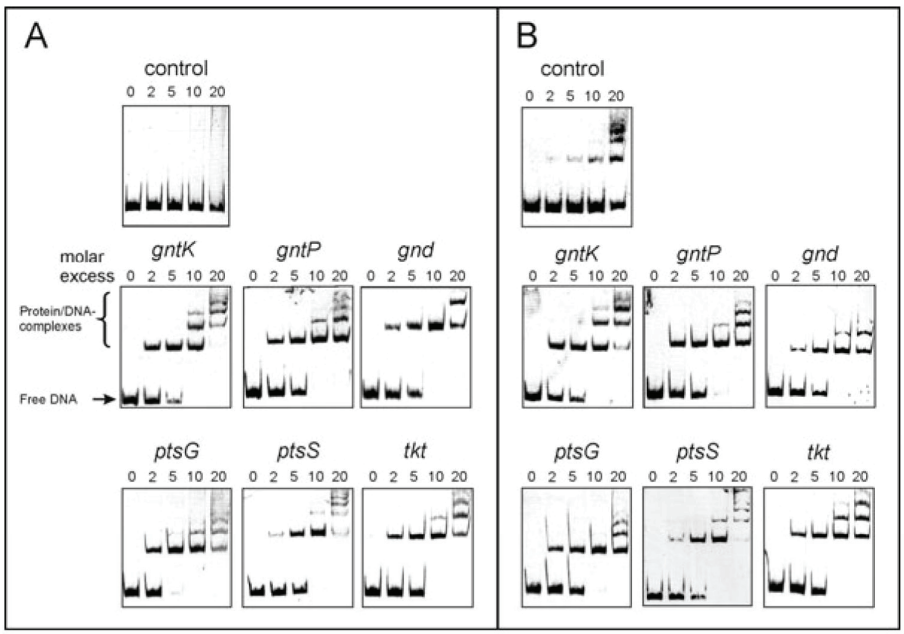

Fig. 4. Binding of GntR1 (A) and GntR2 (B) to the promoter regions of the predicted target genes. DNA fragments (550 bp, 14 nM) covering the promoter regions of the putative target genes *gntP*, *gntK*, *gnd*, *ptsG*, *ptsS* and *tkt* were incubated for 20 min at room temperature either without protein or with a twofold, fivefold, 10-, or 20-fold molar excess of either purified GntR1 (A) or GntR2 protein (B). A DNA fragment containing the *acn* (aconitase) promoter region was used as a negative control. The samples were separated by native PAGE (10%) and stained with SybrGreen I.

tase) or *sdhCAB* (succinate dehydrogenase) served as negative controls and were incubated with the same protein concentrations as the putative target genes. The GntR2 protein also bound to these control DNA fragments, but with much lower affinity compared with the promoter regions of the identified target genes (Fig. 4B), indicating that this binding is unspecific.

In subsequent experiments the exact location of the binding sites of GntR1 and GntR2 was determined for four of the target genes (see below). In the case of *gntK*, the binding site was found to extend from position –45 to –59 with respect to the transcriptional start site reported by Letek *et al.* (2006), which is located 17 bp upstream of the ATG start codon. As the position of the binding site is unusual for a regulator acting as a repressor, we determined the transcriptional start site of *gntK* by primer extension analysis. A single primer extension product was detected using two independent oligonucleotides (PE-gntK-1 and PE-gntK-2, Table S1) and total RNA isolated from *C. glutamicum* wild type cultivated on minimal medium with 100 mM gluconate as carbon source. The transcriptional start site identified by these experiments is located 65 bp upstream of the start codon of *gntK* (Fig. 5). The extended '–10' region derived from this start site (agagtTATGATag) shows a good agreement with the corresponding consensus sequence [tgngnTA(c/t)aaTgg] (Patek *et al.*, 2003). No evidence for the previously reported transcriptional start site 17 bp upstream of the start codon was obtained in the primer extension experiments.

In order to identify the binding sites of GntR1 and GntR2 in the promoter region of *gntK*, the originally used DNA

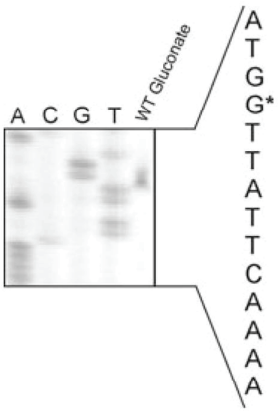

Fig. 5. Identification of the transcriptional start site of the *gntK* gene by primer extension analysis using the oligonucleotide PE-gntK-1 (Table S1). Ten micrograms of total RNA isolated from *C. glutamicum* wild type grown on CGXII minimal medium with 100 mM gluconate was used as template. The transcriptional start site is indicated by an asterisk. The Sanger sequencing reactions (lanes A, C, G and T) were generated with a PCR product covering the corresponding DNA region as template and oligonucleotide PE-gntK-1.

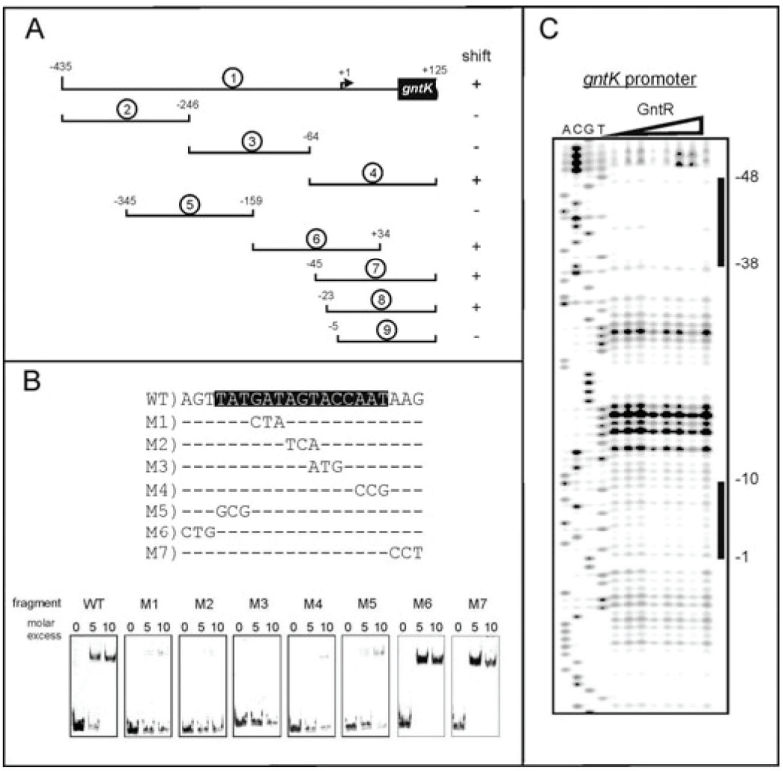

Fig. 6. Identification of the GntR1/2 binding site in the promoter region of *gntK*.
A. DNA fragments used to determine the location of the GntR1/2 binding site in the *gntK* promoter. The numbers indicate the position of the fragments relative to the transcription start site (+1) determined in this work (see Fig. 5). Oligonucleotides used for amplification by PCR are listed in Table S1. At the right, it is indicated whether the fragment, when tested in bandshift assays with purified GntR2, was shifted (+) or not (–).
B. Mutational analysis of the putative GntR1/2 binding site (shaded in black) within the *gntK* promoter region. Mutations introduced are listed below the wild type sequence. Oligonucleotides used for amplification of the corresponding fragments are listed in Table S1. The fragments were incubated with purified GntR1 and the samples were separated on a 10% non-denaturating polyacrylamide gel and stained with SybrGreen I.
C. DNase I footprinting analysis with GntR2 and the *gntK* promoter region. Two nM of IRD-800-labelled *gntK* template strand was incubated with increasing concentrations of GntR2 (0–2 μM). The first and the last lane were loaded with samples containing no protein. Regions protected from digestion by DNase I are indicated by the black bars. The DNA sequencing reactions were set up using the same IRD-800-labelled oligonucleotide as for generating labelled footprinting probes as well as suitable PCR template.

fragment was divided into several subfragments which were then also tested in gel shift assays with purified GntR1 and GntR2. As shown in Fig. 6A, GntR2 bound to fragments 4 and 6 which cover an overlapping region of approximately 100 bp. A further refinement using fragments 7–9 showed that an essential part of the GntR2 binding site is located between position –5 and –23 with respect to the transcription start site identified in this work. Further inspection of this region revealed a potential binding motif of GntR2 extending from position +4 to –11. Subsequently, the relevance of this motif was tested by mutational analysis. To this end, seven mutated DNA fragments were synthesized by PCR, each of which contained three nucleotide exchanges. All mutations within the postulated motif (fragments M1–M5) abolished binding of GntR2 (data not shown) and also of GntR1 (Fig. 6B) nearly completely, whereas the mutations outside the motif (fragments M6 and M7) had no effect on binding. These data confirm the relevance of the identified motif and show that GntR1 and GntR2 share the same binding site.

In an independent approach, the binding site of GntR1 and GntR2 within the *gntK* promoter was searched by DNase I footprinting. A protected region could be detected on the template strand extending from position –1 to –10 relative to the transcription start site, which completely overlaps with the binding motif previously identified by gel shift assays (Fig. 6C). This site was also confirmed by DNase I footprinting analysis with GntR1 and GntR2 and the non-template strand (data not shown). Interestingly, an additional protected region was present on the template strand between –38 and –48 (Fig. 6C). This indicates the existence of at least one

Promoter	Sequence	Location	Orientation
gntK	TATGATAGTACCAAT	-3	+
gntP	TTTGATCATACTAAT	+2	+
gnd	ATTGATCGTACTTGA	-11	-
ptsG	AAAAGTATTACCTTT	-60	+
Consensus	WWtgaTMNTACYWNt		

Fig. 7. Experimentally identified GntR1/2 binding sites in the promoter regions of *gntK*, *gntP*, *gnd* and *ptsG*. The location of the central nucleotide of the 15 bp binding sites is indicated with respect to the transcriptional start site for *gntK*, *gntP* and *ptsG*, but with respect to the start codon for *gnd*. The orientation of the binding sites is indicated by plus and minus signs. The relevance of each binding site was confirmed by mutational analysis using gel shift assays with purified GntR1 and GntR2. Nucleotides shaded in black are conserved in all binding sites, those shaded in grey are identical in three of four binding sites.

additional GntR1/2 binding site, whose sequence shows no obvious similarity to those of the other identified GntR1/2 binding sites. Repression of *gntK* by GntR1 and GntR2 might involve formation of a DNA loop between the two binding sites.

Analysis of the promoter regions of *gntP*, *gnd* and *ptsG* by gel shift analyses with subfragments of the promoter regions also led to the identification of distinct sites involved in GntR1/2 binding (Fig. 7). The relevance of these sites was again confirmed by mutation studies which showed that an exchange of 3 bp within these sites prevented binding (data not shown). The binding sites were centred at position +2 with respect to the recently reported transcriptional start site of *gntP* (Letek *et al.*, 2006) and at position −11 with respect to the start codon of *gnd*. In the case of *ptsG*, the binding site was centred at position −60 with respect to the transcriptional start site determined previously by primer extension experiments (Engels and Wendisch, 2007). These positions fit with a repressor function for *gntP* and *gnd* and an activator function for *ptsG* of GntR1/2. All GntR1/2 binding sites identified in this work are in reasonable agreement (1–2 mismatches) with a consensus operator site deduced for GntR-type regulators of the FadR subfamily (TNGTNNNACNA) (Rigali *et al.*, 2002).

Gluconate interferes with the binding of GntR1 and GntR2 to their target promoters

The transcriptome comparisons as well as the measurement of enzyme activities (gluconate kinase, 6-phosphogluconate dehydrogenase and glucose 6-phosphate dehydrogenase) indicated that the activity of GntR1 and GntR2 is dependent on the carbon source available. In order to identify putative effector molecules, the binding of GntR1 and GntR2 to the *gntK* promoter was assayed in the presence of glucose, gluconate, glucono-δ-lactone, 6-phosphogluconate, glucose 6-phosphate, fructose, sucrose, mannitol, sorbitol and glucuronate. For this purpose, purified GntR1 or GntR2 was incubated with the potential effector substances (50 mM) for 5–10 min before addition of a DNA fragment covering the *gntK* promoter and another 20 min of incubation. Subsequently, the samples were separated on a 10% native polyacrylamide gel. Of the 10 compounds tested only gluconate and, to a lower extent, glucono-δ-lactone inhibited binding of GntR1 and GntR2 to its target DNA (Fig. 8). In further studies it was shown that already a concentration of 1 mM gluconate led to a partial inhibition of binding. However, as even a concentration of 50 mM gluconate led only to a partial inhibition of binding, the possibility that a contaminating compound rather than gluconate or glucono-δ-

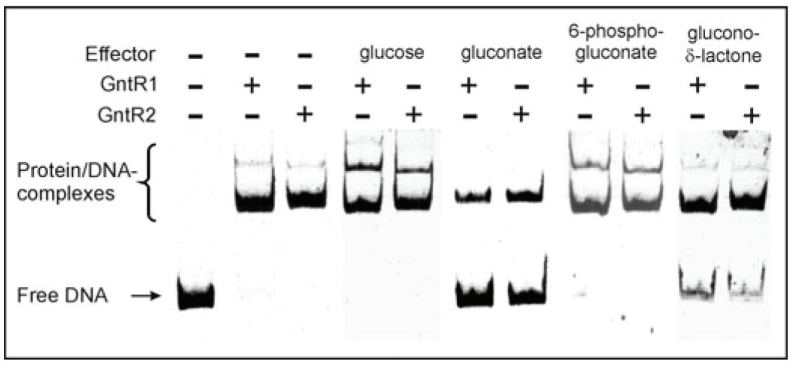

Fig. 8. Search for putative effector molecules of GntR1 and GntR2. Various carbohydrates were tested for their influence on GntR1/2 binding to a DNA fragment containing the promoter region of *gntK*. Approximately 0.28 pmol of the 550 bp *gntK* fragment was incubated with either 2.8 pmol purified GntR1 or GntR2 protein in the presence of the following carbohydrates (50 mM each): glucose, gluconate, 6-phosphogluconate, glucono-δ-lactone. Not shown are the experiments with glucuronic acid, glucose 6-phosphate, fructose, sucrose, mannitol and sorbitol, which had no influence on DNA binding.

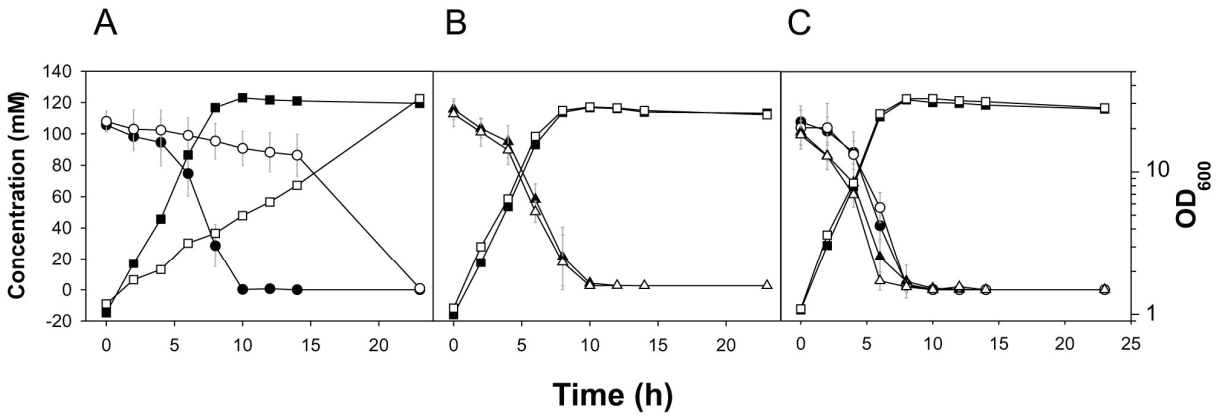

Fig. 9. Growth (squares) and carbon source consumption of *C. glutamicum* wild type (filled symbols) and the mutant Δ*gntR1*Δ*gntR2* (open symbols). The two strains were cultivated in CGXII minimal medium containing as carbon source either 100 mM glucose (A), or 100 mM gluconate (B), or 50 mM glucose + 50 mM gluconate (C). The values are means obtained from three independent cultivations. Glucose and gluconate concentrations are indicated by circles and triangles, respectively.

lactone itself is responsible for the effect cannot be completely excluded. Similar results as described above for the *gntK* promoter were also obtained with the promoter regions of *gntP*, *gnd*, *tkt*, *ptsG* and *ptsS* (data not shown).

Co-utilization of glucose and gluconate by C. glutamicum

It has previously been reported that *C. glutamicum*, like several other bacteria, is able to consume glucose and gluconate simultaneously (Lee *et al.*, 1998). The results described above have uncovered that the genes involved in gluconate catabolism, including the pentose phosphate pathway, and the *ptsG* gene encoding the permease EII^{Glc} of the glucose PTS are co-ordinately regulated by GntR1 and GntR2. We therefore investigated whether the deletion of both transcriptional regulators has an effect on the co-consumption of glucose and gluconate. *C. glutamicum* wild type and the Δ*gntR1*Δ*gntR2* mutant were cultivated in CGXII minimal medium containing either 100 mM glucose, or 100 mM gluconate, or 50 mM glucose plus 50 mM gluconate and growth as well as glucose and gluconate uptake rates were monitored (Fig. 9). As described before, the Δ*gntR1*Δ*gntR2* mutant showed a drastically reduced growth rate when cultivated in minimal medium with 100 mM glucose ($\mu = 0.15 \pm 0.01\ h^{-1}$) in comparison to the wild type ($\mu = 0.43 \pm 0.02\ h^{-1}$). As expected from this observation, the glucose uptake rate of the Δ*gntR1*Δ*gntR2* mutant (33 nmol mg^{-1} min^{-1}) was only one-third of that of the wild type (90 nmol mg^{-1} min^{-1}) (Table 4). In contrast, cultivation on gluconate as carbon source resulted in almost identical growth rates of both strains ($\mu = 0.46 \pm 0.02\ h^{-1}$) and nearly identical gluconate uptake rates (99 nmol mg^{-1} min^{-1}). The final cell density reached in gluconate medium (OD_{600} = 25.3 ± 0.5) was somewhat lower than the one reached in glucose medium ($OD_{600} = 30.1 \pm 1.1$), which might be caused by an increased loss of substrate carbon as CO_2 in the 6-phosphogluconate dehydrogenase reaction. In contrast to glucose, gluconate has to be metabolized completely via the oxidative pentose phospate pathway. Interestingly, when cells were cultivated with glucose plus gluconate, both *C. glutamicum* wild type and the Δ*gntR1*Δ*gntR2* mutant showed a significantly increased growth rate ($\mu = 0.52 \pm 0.02\ h^{-1}$). In this case, the final cell density ($OD_{600} = 27.5 \pm 0.3$) was in between that obtained for glucose and gluconate as single carbon sources. Determination of the uptake rates confirmed that both strains consumed glucose and gluconate simultaneously. In the wild type, comparable uptake rates

Table 4. Carbon consumption rates of *C. glutamicum* wild type and the Δ*gntR1*Δ*gntR2* mutant during growth in CGXII minimal medium with either 100 mM glucose or gluconate or with 50 mM of both carbon sources.

	Carbon source consumption rates (nmol min^{-1} mg^{-1})		
Strain	Glucose	Gluconate	Glucose + gluconate
Wild type	90 ± 8	98 ± 9	56 ± 8; 52 ± 4
Δ*gntR1*Δ*gntR2*	33 ± 6	99 ± 8	52 ± 7; 65 ± 3

The represent means ± standard deviations for at least three independent cultivations.

between 50 and 60 nmol mg^{-1} min^{-1} were determined (Table 4). Whereas the reduced glucose uptake in the wild type during cultivation in the presence of gluconate is presumably a consequence of the missing *ptsG* activation by GntR1 and GntR2, the reduced gluconate uptake in the presence of glucose might be caused by repression of *gntP* and *gntK* by the GlxR–cAMP complex, as suggested previously (Letek *et al.*, 2006). In the Δ*gntR1*Δ*gntR2* mutant glucose uptake was slightly decreased compared with the wild type (52 versus 56 nmol mg^{-1} min^{-1}), whereas gluconate uptake was slightly increased (65 versus 52 nmol mg^{-1} min^{-1}). These minor differences might be explained by the assumption that in the wild type, but not in the Δ*gntR1*Δ*gntR2* mutant, there is some weak residual activation of *ptsG* and repression of *gntP*, *gntK* and *gnd* by GntR1 and GntR2 even in the presence of gluconate. Such a behaviour fits with the observation that even high gluconate concentrations did not completely prevent binding of GntR1/2 to its target promoters (see above). The finding that the glucose uptake rate of the Δ*gntR1*Δ*gntR2* mutant during growth on glucose plus gluconate was 50% higher than during growth on glucose alone indicates that gluconate has not only a negative effect on glucose uptake via GntR1/2, but also a positive effect via another transcriptional regulator or another regulatory mechanism.

Discussion

In this study we have identified two functionally redundant GntR-type regulators in *C. glutamicum*, GntR1 and GntR2, which co-ordinately control gluconate catabolism and glucose uptake, presumably in dependency of the intracellular concentration of gluconate and glucono-δ-lactone. Whereas the negative control of genes involved in gluconate metabolism by GntR-type regulators has previously been demonstrated, e.g. in *E. coli* (Izu *et al.*, 1997; Porco *et al.*, 1997; Peekhaus and Conway, 1998) or *B. subtilis* (Miwa and Fujita, 1988; Fujita and Miwa, 1989; Reizer *et al.*, 1991), the simultaneous positive control by these regulators of the *ptsG* gene encoding the key protein for glucose uptake via the PTS is a novel and surprising aspect. If the activation of *ptsG* expression is abolished by deletion of *gntR1* and *gntR2*, the growth rate and the glucose uptake rate of the corresponding strain in glucose minimal medium is reduced by about 60%. The question arises why this type of opposite co-regulation of glucose and gluconate metabolism has been established in *C. glutamicum*. One reason might be the fact that this species, in contrast to, e.g. *E. coli* or *B. subtilis*, usually prefers the simultaneous consumption of different carbon sources rather than their sequential utilization. Examples are the co-utilization of glucose with acetate (Wendisch *et al.*, 2000), lactate (Stansen *et al.*, 2005), propionate (Claes *et al.*, 2002), fructose (Dominguez *et al.*, 1997) or citrate (von der Osten *et al.*, 1989). In the case of glucose-acetate co-metabolism it was shown that both the acetate consumption rate [270 nmol min^{-1} (mg protein)$^{-1}$] and the glucose consumption rate [72 nmol min^{-1} (mg protein)$^{-1}$] were twofold decreased compared with growth on acetate or glucose as sole carbon source, resulting in a comparable rate of total carbon uptake of about 1000 nmol C min^{-1} (mg protein)$^{-1}$ under all three growth conditions (Wendisch *et al.*, 2000). The carbon uptake rates [nmol C min^{-1} (mg protein)$^{-1}$, based on the assumption that protein constitutes 50% of the cell dry weight] determined in this work for the wild type were in the same order of magnitude (Table 4): 1080 for growth on glucose, 1180 for growth on gluconate and 1290 for growth on glucose (670) plus gluconate (620). These two examples show that *C. glutamicum* is able to adjust the uptake rates for different carbon sources in such a way that they match its metabolic capacities. The co-metabolism of glucose and gluconate is advantageous for *C. glutamicum* as its growth rate (0.52 h^{-1}) is increased by 20% compared with growth on glucose alone (0.43 h^{-1}) and by 13% compared with growth on gluconate alone (0.46 h^{-1}). Thus, activation of *ptsG* expression by GntR1 and GntR2 can be interpreted as one of the mechanisms that allow *C. glutamicum* the simultaneous consumption of carbon sources and thereby a maximization of its growth rate and a selective advantage in the competition with other microorganisms. Gluconate is likely to be a frequent substrate in nature, as (i) many bacteria, such as pseudomonads, acetic acid bacteria or enterobacteria (Neijssel *et al.*, 1989; Anthony, 2004), possess membrane-bound glucose dehydrogenases that catalyse the extracytoplasmic oxidation of glucose to gluconic acid and (ii) a high number of bacteria possess gluconate permeases and are able to utilize gluconate either via the Entner–Doudoroff pathway or via the pentose phosphate pathway.

Besides its negative influence on *ptsG* expression mediated by GntR1 and GntR2, gluconate appears to have also a positive effect on *ptsG* expression: in the *ptsG–cat* fusion assays, expression of *ptsG* in the Δ*gntR1*Δ*gntR2* mutant was twofold higher on gluconate or glucose plus gluconate than on glucose alone (Table 3). Similarly, the glucose consumption rate of the double mutant was ~60% higher during growth on glucose and gluconate than during growth on glucose alone (Table 4). These differences might be caused by the SugR protein, which was recently identified as a repressor of *ptsG* and other PTS genes during growth on gluconeogenic carbon sources (Engels and Wendisch, 2007). The activity of SugR is controlled by fructose 6-phosphate, which was shown to abolish binding of SugR to the *ptsG* promoter region *in vitro*. When gluconate is catabolized via the pentose phosphate pathway, it enters glycolysis at the level of fructose 6-phosphate and glyceraldehyde

3-phosphate. Therefore, it seems possible that the intracellular fructose 6-phosphate concentration is increased in the presence of gluconate and repression of *ptsG* by SugR is diminished. Analysis of *ptsG* expression in a Δ*gntR1*Δ*gntR2*Δ*sugR* triple mutant and measurement of the intracellular fructose 6-phosphate concentration might allow confirming or disproving this explanation.

The genomes of the closely related organisms *C. glutamicum* strain R (Yukawa *et al.*, 2007) and *C. efficiens* contain just one *gntR* orthologous gene. Thus, the presence of *gntR2*, which most likely resulted from of a gene duplication event of *gntR1*, seems to be a characteristic of the *C. glutamicum* type strain ATCC 13032. As all results obtained in this work show that GntR1 and GntR2 can fully replace each other, the question arises why both *gntR* genes are retained in the chromosome. A convincing answer to this question is not yet available. The possibility exists that differences in the expression of the two genes or not yet uncovered individual functions of the regulators allow the cell a better adaptation to certain growth conditions.

In this work 10 direct target genes of GntR1 and GntR2 have been identified. Those involved in gluconate transport and metabolism (*gntP*, *gntK*, *gnd*, *tkt-tal-zwf-opcA-devB*) are repressed by GntR1 and GntR2, whereas *ptsG* and *ptsS* encoding the permeases EII^{Glc} and EII^{Suc} of the PTS system are activated. Activation of gene expression by GntR-type regulators has also been demonstrated for other members of this family, e.g. MatR, an activator of genes involved in malonate metabolism of *Rhizobium leguminosarum* (Rigali *et al.*, 2002). Binding of GntR1 and GntR2 to all of its target promoters was inhibited by gluconate and glucono-δ-lactone (Fig. 8), which fits with their function in gluconate metabolism. The same metabolites were previously shown to interfere with binding of the *B. subtilis* GntR protein to its target promoters (Miwa and Fujita, 1988). Binding of *E. coli* GntR to the *gntT* promoter was likewise inhibited by gluconate, but at higher concentrations also by 6-phosphogluconate (Peekhaus and Conway, 1998). One millimolar and 20 mM gluconate were sufficient to completely inhibit binding of *E. coli* GntR and *B. subtilis* GntR to target promoters, respectively. In the case of *C. glutamicum* GntR1 and GntR2, only a partial inhibition of DNA binding was achieved with 50 mM gluconate, indicating a lower affinity for gluconate. Although the possibility exists that a contaminant present in the source of gluconate or glucono-δ-lactone could be responsible for inhibition of binding, this seems not very likely.

Besides being induced by gluconate, genes involved in the catabolism of this sugar acid are often subject to catabolite repression, e.g. in *E. coli* or *B. subtilis* (Reizer *et al.*, 1996; Tong *et al.*, 1996; Peekhaus and Conway, 1998; Titgemeyer and Hillen, 2002; Warner and Lolkema, 2003). Recently, it was reported that *gntK* and *gntP* of *C. glutamicum* are also subject to catabolite repression, mediated by the transcriptional regulator GlxR in complex with cAMP (Letek *et al.*, 2006). Kim *et al.* (2004) reported that in *C. glutamicum* the cAMP concentration is 10-fold higher during growth on glucose than during growth on acetate, indicating that GlxR is active in the presence of glucose. Our finding that the gluconate consumption rate of *C. glutamicum* wild type is about twofold lower during growth on glucose plus gluconate compared with growth on gluconate alone (Table 4) could be due to catabolite repression of *gntP* and *gntK* by the GlxR–cAMP complex. A prerequisite for this explanation is that cells cultivated in the presence of glucose plus gluconate have a higher cAMP level than cells grown on gluconate alone.

In a previous study on gluconate metabolism in *C. glutamicum* it was reported that *gntP* and *gntK* are not induced by gluconate (Letek *et al.*, 2006). Our results clearly show that *gntP* and *gntK* together with pentose phosphate pathway genes are induced by gluconate via GntR1 and GntR2. Simultaneously these regulators control glucose uptake by activation of *ptsG* expression in the absence of gluconate. In conclusion, these transcriptional regulators are important players in a complex regulatory network that controls uptake and metabolism of carbon sources in *C. glutamicum* in order to allow the most favourable combination of the available substrates.

Experimental procedures

Bacterial strains, media and growth conditions

All strains and plasmids used in this work are listed in Table 5. The *C. glutamicum* type strain ATCC 13032 (Kinoshita *et al.*, 1957) was used as wild type. Strain Δ*gntR1* and strain Δ*gntR2* are derivatives containing an in-frame deletion of the genes *gntR1* (*cg2783*) and *gntR2* (*cg1935*), respectively. In strain Δ*gntR1*Δ*gntR2* both genes were deleted. For growth experiments, 5 ml of brain–heart infusion medium (Difco Laboratories, Detroit, USA) was inoculated with colonies from a fresh Luria–Bertani (LB) agar plate (Sambrook *et al.*, 1989) and incubated for 6 h at 30°C and 170 r.p.m. After washing with 5 ml of 0.9% (w/v) NaCl, the cells of this first preculture were used to inoculate a 500 ml shake flask containing 50 ml of CGXII minimal medium (Keilhauer *et al.*, 1993) with either glucose, or gluconate, or glucose plus gluconate in the indicated concentrations as carbon source(s). Additionally, the medium was supplemented with 30 mg l^{-1} 3,4-dihydroxybenzoate as iron chelator. This second preculture was cultivated overnight at 30°C and then used to inoculate the main culture to an optical density at OD_{600} of ~1. The trace element solution was always added after autoclaving. For all cloning purposes, *E. coli* DH5α (Invitrogen, Karlsruhe, Germany) was used as host, for overproduction of the proteins Cg2783 (= GntR1) and Cg1935 (= GntR2) *E. coli* BL21(DE3)/pLysS. The *E. coli* strains were cultivated aerobically in LB medium at 37°C (strain DH5α) or at 30°C [strain

Table 5. Bacterial strains and plasmids used in this study.

Strains or plasmids	Relevant characteristics	Source or reference
Strains		
C. glutamicum		
ATCC 13032	Biotin-auxotrophic wild type	Kinoshita *et al.* (1957)
Δ*gntR1*	In-frame deletion of the *gntR1* (*cg2783*) gene	This work
Δ*gntR2*	In-frame deletion of the *gntR2* (*cg1935*) gene	This work
Δ*gntR1*Δ*gntR2*	In-frame deletion of the genes *gntR1* and *gntR2*	This work
E. coli		
DH5α	*supE44* Δ*lacU169* (ϕ80*lacZ*ΔM15) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Invitrogen
BL21(DE3)/pLysS	F^-*ompT hsdS*$_B$($r_B^-m_B^-$) *gal dcm* (DE3); contains plasmid pLysS (Cam^R)	Studier and Moffatt (1986)
Plasmids		
pK19*mobsacB*	Kan^R; vector for allelic exchange in *C. glutamicum*; (pK18 *oriV*$_{E.c.}$, *sacB*, *lacZ*α)	Schäfer *et al.* (1994)
pK19*mobsacB*-Δ*cg1935*	Kan^R; pK19*mobsacB* derivative containing a crossover PCR product covering the up- and downstream regions of *cg1935* (*gntR2*)	This work
pK19*mobsacB*-Δ*cg2783*	Kan^R; pK19*mobsacB* derivative containing a crossover PCR product covering the up- and downstream regions of *cg2783* (*gntR1*)	This work
pAN6	Kan^R; *C. glutamicum*/*E. coli* shuttle vector for regulated gene expression; derivative of pEKEx2 (P_{tac}, *lacI*^{q}, pBL1 *oriV*$_{C.g.}$, pUC18 *oriV*$_{E.c.}$); for details see *Experimental procedures*	This work
pAN6-*gntR1*	Kan^R; pAN6 derivative containing the *gntR1* gene (*cg2783*) under control of the *tac* promoter	This work
pAN6-*gntR2*	Kan^R; pAN6 derivative containing the *gntR2* gene (*cg1935*) under control of the *tac* promoter	This work
pEKEx2	Kan^R; *C. glutamicum*/*E. coli* shuttle vector for regulated gene expression (P_{tac}, *lacI*^{Q}, pBL1 *oriV*$_{C.g.}$, pUC18 *oriV*$_{E.c.}$)	Eikmanns *et al.* (1991)
pEKEx2-*gntR1*-His	Kan^R; pEKEx2 derivative encoding GntR1 with an aminoterminal decahistidine tag	This work
pEKEx2-*gntR2*-His	Kan^R; pEKEx2 derivative encoding GntR2 with an aminoterminal decahistidine tag	This work
pET16b	Amp^R; vector for overexpression of genes in *E. coli*, adding a C-terminal hexahistidine affinity tag to the synthesized protein (pBR322 *oriV*$_{E.c.}$, P_{T7}, *lacI*)	Novagen
pET16b-*gntR1*	Kan^R; pET16b derivative for overproduction of GntR1 with an N-terminal decahistidine tag.	This work
pET16b-*gntR2*	Kan^R; pET16b derivative for over-production of GntR2 with an N-terminal decahistidine tag.	This work
pET2-*ptsG*	KanR; *C. glutamicum* promoter-probe plasmid with a 707 bp fragment covering the *C. glutamicum ptsG* promoter	Engels and Wendisch (2007)

BL21(DE3)/pLysS]. When appropriate, the media contained chloramphenicol [34 μg ml^{-1} for cultivation of *E. coli* BL21 (DE3)/pLysS], ampicillin (100 μg ml^{-1} for *E. coli*), or kanamycin (25 μg ml^{-1} for *C. glutamicum*, 50 μg ml^{-1} for *E. coli*).

Recombinant DNA work

The enzymes for recombinant DNA work were obtained from Roche Diagnostics (Mannheim, Germany) or New England Biolabs (Frankfurt, Germany). The oligonucleotides used in this study are listed in Table S1 and were obtained from Operon (Cologne, Germany), except for the IRD800-labelled oligonucleotides, which were purchased from MWG Biotech (Ebersberg, Germany). Routine methods like PCR, restriction or ligation were carried out according to standard protocols (Sambrook *et al.*, 1989). Chromosomal DNA from *C. glutamicum* was prepared as described (Eikmanns *et al.*, 1994). Plasmids from *E. coli* were isolated with the QIAprep spin miniprep Kit (Qiagen, Hilden, Germany). *E. coli* was transformed by the RbCl method (Hanahan, 1985), *C. glutamicum* by electroporation (van der Rest *et al.*, 1999). DNA sequencing was performed with a Genetic Analyzer 3100-Avant (Applied Biosystems, Darmstadt, Germany). Sequencing reactions were carried out with the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Darmstadt, Germany).

In-frame deletion mutants of *C. glutamicum* were constructed via a two-step homologous recombination procedure as described previously (Niebisch and Bott, 2001). The primers used for this purpose are listed in Table S1. The chromosomal deletions were confirmed by PCR with oligonucleotides annealing outside the deleted regions.

In order to complement the Δ*gntR1*Δ*gntR2* mutant, the *gntR1* (*cg2783*) and *gntR2* (*cg1935*) coding regions were amplified using oligonucleotides (2783NdeN, 2783Ex1, 1935NdeN and1935Ex1) introducing an NdeI restriction site that included the start codon and an NheI restriction site behind the stop codon. The resulting PCR products were cloned into the expression vector pAN6, resulting in plasmids pAN6-*gntR1* and pAN6-*gntR2*. These plasmids and as a control pAN6 were used to transform *C. glutamicum* wild type and the Δ*gntR1*Δ*gntR2* strain. The vector pAN6 is a derivative of pEKEx2 (Eikmanns *et al.*, 1991) that contains a 56 bp insertion between the PstI and EcoRI restriction sites. This insertion harbours a ribosome binding site (GGAGATA) in an optimal distance to a unique NdeI cloning site. Downstream of the NdeI site, there is a unique NheI cloning site which is followed by a *Strep*Tag-II-coding sequence and a stop codon before the EcoRI site. For the construction of pAN6, the original NdeI restriction site of pEKEx2 was first removed by Klenow fill-in and religation and subsequently a DNA fragment of the sequence 5′-GACCTGCAGAAGGAGATATACATATG

ACCTGAGCTAGCTGGTCCCACCCACAGTTCGAGAAGTA AGAATTCGTC-3′ was cut with PstI and EcoRI and ligated with the modified pEKEx2 vector cut with the same enzymes.

For overproduction and purification of GntR1 and GntR2 with an N-terminal decahistidine tag, the corresponding coding regions were amplified using oligonucleotides that introduce an NdeI restriction site including the start codon and an XhoI restriction site after the stop codon. The purified PCR products were cloned into the expression vector pET16b (Novagen, Darmstadt, Germany), resulting in plasmids pET16b-*gntR1* and pET16b-*gntR2*. The GntR proteins encoded by these plasmids contain 21 additional amino acids (MGHHHHHHHHHHSSGHIEGRH) at the amino terminus. The PCR-derived portion of the constructed plasmids were analysed by DNA sequence analysis and found to contain no spurious mutations. For overproduction of the GntR proteins, the plasmids were transferred into *E. coli* BL21 (DE3)/pLysS.

Global gene expression analysis

Preparation of RNA and synthesis of fluorescently labelled cDNA were carried out as described (Möker *et al*., 2004). Custom-made DNA microarrays for *C. glutamicum* ATCC 13032 printed with 70mer oligonucleotides were obtained from Operon (Cologne, Germany) and are based on the genome sequence entry NC_006958 (Kalinowski *et al*., 2003). Hybridization and stringent washing of the microarrays were performed according to the instructions of the supplier. Hybridization was carried out for 16–18 h at 42°C using a MAUI hybridization system (BioMicro Systems, Salt Lake City, USA). After washing the microarrays were dried by centrifugation (5 min, 1600 *g*) and fluorescence was determined at 532 nm (Cy3-dUTP) and 635 nm (Cy5-dUTP) with 10 μm resolution using an Axon GenePix 6000 laser scanner (Axon Instruments, Sunnyvale, USA). Quantitative image analysis was carried out using GenePix image analysis software and results were saved as GPR-file (GenePix Pro 6.0, Axon Instruments). For data normalization, GPR-files were processed using the BioConductor/R-packages limma and marray (http://www.bioconductor.org). Processed and normalized data as well as experimental details (MIAME, Brazma *et al*. 2001) were stored in the in-house microarray database for further analysis (Polen and Wendisch, 2004).

Using the DNA microarray technology, the genome-wide mRNA concentrations of *C. glutamicum* wild type were compared with those of the mutant strains Δ*gntR1*Δ*gntR2* (A), Δ*gntR2* (B), and Δ*gntR1* (C). The strains were cultivated in CGXII minimal medium with either 100 mM glucose, or 100 mM gluconate, or 50 mM glucose plus 50 mM gluconate (only for comparison A). RNA used for the synthesis of labelled cDNA was prepared from cells in the exponential growth phase. For each of the seven comparisons, two or three independent DNA microarray experiments were performed, each starting from an independent culture. To filter for differentially expressed genes and reliable signal detection in each of the seven comparisons, the following quality filter was applied: (i) flags ≤ 0 (GenePix Pro 6.0), (ii) signal/noise ≥ 3 for Cy5 (F635Median/B635Median, GenePix Pro 6.0) or Cy3 (F532Median/B532Median, GenePix Pro 6.0), (iii) ≥ fourfold change in the comparison Δ*gntR1*Δ*gntR2* mutant versus wild type in glucose minimal medium, and (iv) significant change ($P < 0.05$) in Student's *t*-test (Excel, Microsoft).

Primer extension analysis

For non-radioactive primer extension analysis of the *gntK* gene total RNA was isolated from exponentially growing *C. glutamicum* wild type cultivated in CGXII minimal medium with 100 mM gluconate as carbon source. Primer extension analysis with 10–13 μg of total RNA was performed using IRD800-labelled oligonucleotides (PE-gntK-1 and PE-gntK-2, Table S1) (MWG Biotech, Ebersberg, Germany) as described previously (Engels *et al*., 2004). The template for the DNA sequence analysis used to localize the 3′ end of the primer extension product was amplified in a standard PCR reaction using the unlabelled oligonucleotides gntK-seq-for and gntK-seq-rev (Table S1). The oligonucleotides PE-gntK-1 or PE-gntK-2 served as primers for the sequencing reactions.

Measurement of enzyme activities

For the measurement of enzyme activities, cells of *C. glutamicum* wild type and the double deletion mutant Δ*gntR1*Δ*gntR2* were cultivated in CGXII minimal medium with either 4% (w/v) glucose or 2% (w/v) gluconate up to the exponential growth phase (OD_{600} ~5). Then cells of 20 ml culture were harvested with ~25 g of crushed ice (precooled to −20°C) by centrifugation at 4000 *g* for 5 min. The cell pellet was resuspended in 900 μl of Tris/HCl (50 mM, pH 7.5) and the cells were mechanically disrupted by 3 × 20 s bead beating with 1 g of zirconia-silica beads (diameter 0.1 mm; Roth, Karlsruhe, Germany) using a Silamat S5 (Vivadent, Ellwangen, Germany). After centrifugation (5 min, 18 320 *g*, 4°C), the supernatant was used immediately for the enzyme assay.

For the determination of glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase activity, the assay mixtures (1 ml total volume) contained 50 mM Tris/HCl pH 7.5, 10 mM $MgCl_2$, 1 mM $NADP^+$, 200 mM potassium glutamate and 3–20 μl cell-free extract (1–5 mg protein ml^{-1}). The reaction was initiated by the addition of 4 mM glucose 6-phosphate or 1 mM 6-phosphogluconate, and the increase in absorption at 340 nm was monitored at 30°C using a Jasco V560 spectrophotometer (Jasco, Gross-Umstadt, Germany).

Gluconate kinase activity was determined in a coupled assay with 6-phosphogluconate dehydrogenase. The assay mixture (1 ml total volume) contained 50 mM Tris/HCl pH 8.0, 0.25 mM $NADP^+$, 1 mM ATP, 1.2 U 6-phosphogluconate dehydrogenase, and 5–50 μl cell-free extract (1–5 mg protein ml^{-1}). After preincubation for 5 min at 30°C, the reaction was started by the addition of 50 μl of a 200 mM gluconic acid solution (pH 6.8) and the increase in absorption at 340 nm was measured at 30°C.

Chloramphenicol acetyltransferase assay

For analysing the expression of the *ptsG* gene, *C. glutamicum* wild type and the double mutant Δ*gntR1*Δ*gntR2* were transformed with plasmid pET2-*ptsG* (Engels and Wendisch, 2007), which is based on the corynebacterial promoter-probe vector pET2 (Vasicova *et al*., 1998) and contains the *ptsG* promoter region (−399 to +309) in front of a promoter-less *cat* (chloramphenicol acetyltransferase) gene. The promoter activity was tested by measuring chloramphenicol acetyl-

transferase activity in cell extracts. For this purpose, 5 ml of LB medium was inoculated with colonies from a fresh LB agar plate and incubated for 6 h at 30°C and 170 r.p.m. After washing the cells in CGXII medium without carbon source, the second preculture and subsequently the main culture (both 60 ml of CGXII minimal medium with 25 μg ml^{-1} kanamycin) were inoculated to an OD_{600} of 0.5. As carbon and energy source either 100 mM glucose, or 100 mM gluconate, or 50 mM glucose plus 50 mM gluconate was used. Precultures and main cultures were incubated at 30°C and 120 r.p.m. on a rotary shaker in 500 ml baffled shake flasks. The preparation of the crude extract and the measurement of its chloramphenicol acetyltransferase activity were performed as described by Engels and Wendisch (2007).

Overproduction and purification of GntR1 and GntR2

The *C. glutamicum* proteins GntR1 and GntR2 containing 21 additional amino acids at the N-terminus (MGHHHHHHHH HHSSGHIEGRH) were overproduced in *E. coli* BL21(DE3)/pLysS using the expression plasmids pET16b-*gntR1* and pET16b-*gntR2*, respectively. Expression was induced at an A_{600} of 0.3 with 1 mM isopropyl β-D-thiogalactoside. Four hours after induction, cells were harvested by centrifugation and stored at –20°C. For cell extract preparation, thawed cells were washed once and resuspended in 10 ml of TNGI5 buffer (20 mM Tris/HCl pH 7.9, 300 mM NaCl, 5% (v/v) glycerol, and 5 mM imidazol). After the addition of 1 mM diisopropylfluorophosphate and 1 mM phenylmethylsulfonyl fluoride, the cell suspension was passed three times through a French pressure cell (SLM Aminco, Spectronic Instruments, Rochester, NY, USA) at 207 MPa. Intact cells and cell debris were removed by centrifugation (15 min, 5000 *g*, 4°C), and the cell-free extract was subjected to ultracentrifugation (1 h, 150 000 *g*, 4°C). GntR1 or GntR2 present in the supernatant of the ultracentrifugation step was purified by nickel chelate affinity chromatography using nickel-activated nitrilotriacetic acid-agarose (Novagen, Darmstadt, Germany). After washing the column with TNGI50 buffer (which contains 50 mM imidazol), specifically bound protein was eluted with TNGI100 buffer (which contains 100 mM imidazol). Fractions containing GntR1 or GntR2 were pooled, and the elution buffer was exchanged against TG buffer (30 mM Tris/HCl pH 7.5, 10% (v/v) glycerol).

Gel shift assays

For testing the binding of GntR1 and GntR2 to putative target promoters, purified protein was mixed with DNA fragments (100–700 bp, final concentration 8–20 nM) in a total volume of 20 μl. The binding buffer contained 20 mM Tris/HCl pH 7.5, 50 mM KCl, 10 mM $MgCl_2$, 5% (v/v) glycerol, and 0.5 mM EDTA. Approximately 13 nM promoter fragments of putative non-target genes of GntR1/2 (*acn*, *sucCD* and *sdh*) were used as negative controls. The reaction mixtures were incubated at room temperature for 20 min and then loaded onto a 10% native polyacrylamide gel. Electrophoresis was performed at room temperature and 170 V using 1× TBE (89 mM Tris base, 89 mM boric acid, 2 mM EDTA) as electrophoresis buffer. The gels were subsequently stained with SybrGreen I according to the instructions of the supplier (Sigma-Aldrich, Taufkirchen, Germany) and photographed. All PCR products used in the gel shift assays were purified with the PCR purification kit (Qiagen, Hilden, Germany) and eluted in EB buffer (10 mM Tris/HCl pH 8.5).

DNase I footprinting

Labelled DNA fragments were obtained by amplification with 5′-IRD800-labelled oligonucleotides (MWG Biotech, Ebersberg, Germany). The *gntK* promoter region was amplified using the oligonucleotides gntK-2-for-M* and gntK-prom-rev-M (labelled template strand). Binding reactions, DNase I digestion and DNA precipitation were performed as described previously (Engels *et al.*, 2004). A sample of 1.4 μl was then loaded onto a denaturating 4.6% (w/v) Long Ranger (Biozym, Hamburg, Germany) sequencing gel (separation length 61 cm) and separated in a Long Read IR DNA sequencer (Licor, Bad Homburg, Germany). The DNA sequencing reactions were set up using one of the IRD-800-labelled oligonucleotides and a suitable unlabelled PCR product of the promoter region as template.

Determination of glucose and gluconate

To determine the concentration of glucose or gluconate in culture supernatants, 1 ml sample of the culture was centrifuged for 2 min at 16 060 *g* and aliquots of the supernatant were used directly for the assay or stored at –20°C. D-glucose and D-gluconate were quantified enzymatically using a D-glucose/D-fructose or a D-gluconic acid/glucono-δ-lactone Kit, respectively (R-Biopharm, Darmstadt, Germany), as described by the manufacturer. Concentrations were calculated based on calibration curves with standards of glucose or gluconate. Uptake rates (nmol min^{-1} (mg dry weight)$^{-1}$) for glucose and gluconate (Table 4) were calculated according to the following equation:

$$\left(\frac{S}{M}\right)\times\mu\left[\left(\frac{\text{mmol}\times l^{-1}\times OD^{-1}}{\text{gDW}\times l^{-1}\times OD^{-1}}\right)\times h^{-1}\right]=\left[\frac{\text{mmol}}{\text{gDW}\times h}\right]$$

Where S is the slope of a plot of the substrate concentration in the medium versus the OD_{600} (mmol × l^{-1} × OD_{600}^{-1}), M the correlation between dry weight and OD (g dry weight × l^{-1} × OD^{-1}) and μ the growth rate (h^{-1}). An OD_{600} of 1 corresponds to 0.25 g dry weight l^{-1} (Kabus *et al.*, 2007).

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Supplementary material

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The Global Repressor SugR Controls Expression of Glycolysis and Pentose Phosphate Pathway Genes in *Corynebacterium glutamicum*

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The transcriptional regulator SugR from *Corynebacterium glutamicum* represses genes of the phosphoenolpyruvate-dependent phosphotransferase system (PTS). Growth experiments revealed that overexpression of *sugR* not only perturbed growth of *C. glutamicum* on the PTS-sugars glucose, fructose and sucrose, but also led to a significant growth inhibition on ribose, which is not taken up via the PTS. Therefore, ChIP-to-chip analysis and gel retardation experiments were performed to identify novel target genes of SugR. Gel retardation analysis confirmed that SugR bound to the promoter regions of genes of the glycolytic enzymes 6-phosphofructokinase (*pfkA*), fructose-1,6-bisphosphate aldolase (*fba*), enolase (*eno*), pyruvate kinase (*pyk*), and NAD-dependent L-lactate dehydrogenase (*ldhA*) and of transketolase (*tkt*), an enzyme of the pentose phosphate pathway. Enzyme activity measurements revealed that SugR-mediated repression affects the activities of PfkA, Fba, Pyk, and LdhA *in vivo*. As deletion of *sugR* led to eight fold increased LdhA activity under aerobic growth conditions, L-lactate production by *C. glutamicum* was determined under oxygen-deprivation conditions. Overexpression of *sugR* reduced L-lactate production by about 25% and *sugR* deletion increased L-lactate formation under oxygen-deprivation conditions three fold. Thus, SugR functions as a global repressor of genes of the PTS, pentose phosphate pathway, glycolysis and fermentative L-lactate dehydrogenase in *C. glutamicum*.

Corynebacterium glutamicum, which was isolated as L-glutamate excreting soil bacterium (1, 39), is a predominantly aerobic, biotin-auxotrophic Gram-positive bacterium widely used for the industrial production of more than two million tons of amino acids per year, mainly L-glutamate and L-lysine (31, 43). A general view of this non-pathogenic bacterium, which has become a model organism for the *Corynebacterineae*, a suborder of the *Actinomycetales* that also comprises the genus *Mycobacterium* (56), can be found in two recent monographs (10, 18).

C. glutamicum is able to grow on a variety of sugars, sugar alcohols and organic acids as sole carbon and energy sources. As in many other Gram-positive and Gram-negative bacteria, the phosphoenolpyruvate-dependent phosphotransferase system (PTS) is the major sugar uptake system (16, 37, 45, 49). The PTS-mediated glucose, fructose and sucrose uptake in *C. glutamicum* operates by phosphoryl group transfer from phosphoenolpyruvate via EI (encoded by *ptsI*) and HPr (*ptsH*) to the sugar-specific permeases EII^{Glc}, EII^{Fru} and EII^{Suc}, respectively (*ptsG, ptsF* and *ptsS*, respectively). Unlike many other bacteria, *C. glutamicum* usually co-utilizes the carbon sources present in mixtures without showing diauxic growth (65). Glucose as the preferred carbon source has been shown to be co-metabolized with acetate (67), L-lactate (57) or fructose (15). When glucose is co-utilized with another carbon source, e.g. acetate or fructose (17, 67), its uptake is reduced due to repression of *ptsG* by the recently identified transcriptional SugR (19). It was shown that SugR acts as repressor not only of *ptsG* expression in *C. glutamicum*, but also controls genes of the fructose- and sucrose-specific PTS permeases (*fruR-fruK-ptsF* and *ptsS*, respectively) (19) as well as the genes of the general components of the PTS (*ptsH* and *ptsI*) (21). In *Escherichia coli* and *Bacillus subtilis*, regulators like Crp (cyclic AMP receptor protein) and CcpA (catabolite control protein A) are not only regulating *pts* genes, but are global regulators of the carbon metabolism in these bacteria. CcpA is the master regulator of carbon catabolite regulation in *B. subtilis* (30, 55) and regulates more than 300 genes by either activation (e.g. the α-acetolactate synthase gene *alsS* and the acetate kinase gene *ackA*) or repression (e.g. the gluconate operon

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repressor gene *gntR*) (29, 44, 55). Activation and repression mediated by CcpA may utilize different conformational changes of the protein (55). Because *ccpA* mutants are unable to activate glycolysis or carbon overflow metabolism CcpA appears to control a superregulon of glucose catabolism in this organism (63). In *E. coli*, Crp, one of the global regulators known to regulate >50% of this bacterium's transcription units (26), is activated by cAMP, which is synthesized from ATP by adenylate cyclase (*cyaA*) (40). Chip-to-chip analysis and DNA microarrays showed that Crp binds to dozens of regions in the *E. coli* chromosome, e.g. *rbsD* (D-ribose high affinity transport system), *gnt* (gluconate transporter), *aceA* and *aceB* (isocitrate lyase, malate synthase), *gnd* (6-phosphogluconate dehydrogenase) or *pckA* (phosphoenolpyruvate carboxykinase) (25, 26).

In this study, it was determined that in addition to *pts* genes the DeoR-type transcriptional regulator SugR also regulates genes of the central carbon metabolism in *C. glutamicum*. Thus, SugR was shown to be a pleiotropic regulator with its regulon comprising genes of the PTS, pentose phosphate pathway, glycolysis and fermentative L-lactate dehydrogenase in *C. glutamicum*.

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions. The strains and plasmids used are listed in Table 1 and the oligonucleotides used are listed in Table 2. The *C. glutamicum* type strain ATCC 13032 (36) was used as wild type (WT). Growth experiments were performed using CgXII minimal medium as described previously (19). As carbon and energy sources 100 mM glucose, 100 mM fructose, 50 mM sucrose or 120 mM ribose were used. For all cloning purposes, *E. coli* DH5α was used as host and for overproduction of SugR *E. coli* BL21(DE3) (58) was used. The *E. coli* strains were cultivated aerobically in Luria-Bertani (LB) medium (51) at 37°C.

Recombinant DNA work. The enzymes for recombinant DNA work were obtained from Roche Diagnostics (Mannheim, Germany). The oligonucleotides were obtained from Operon (Cologne, Germany). Standard methods like PCR, restriction or ligation were carried out according to (51). Plasmids from *E. coli* were isolated with the QIAprep spin miniprep kit (Qiagen, Hilden, Germany). *E. coli* was transformed by the RbCl method (27), and *C. glutamicum* was transformed by electroporation (19, 64). DNA sequencing was performed by Agowa GmbH (Berlin, Germany).

Construction of *sugR-Strep*Tag strain. To generate a strain derived from *C. glutamicum* ATCC 13032, which synthesizes C-terminally *Strep*-tagged SugR from the genomic *sugR* locus, the plasmid pK19*mobsacB-sugR*^{Strep} was constructed. The base pairs 349-777 of *sugR* were amplified using primers *sugR* and *sugR-Strep* (Table 2) introducing the *Strep*Tag II sequence at the C-terminus of the protein (N-SAWSHPQFEK-C) (54). The PCR product was subcloned into the pGEM-T vector (Promega, WI, USA), and was cloned as *Eco*RI-/*Bam*HI-fragment into pK19*mobsacB* vector (52). *C. glutamicum* was transformed with the resulting plasmid pK19*mobsacB-sugR*^{Strep} by electroporation and site-specific integration of the plasmid into the *sugR* genomic locus was verfified by PCR using the primers *sugR* and M13 (Table 2). As expected, only *C. glutamicum* WT-*sugR*^{Strep} yielded in a PCR product of the expected size, while no signal was obtained with ATCC 13032 WT.

Overproduction and purification of SugR. The *C. glutamicum* SugR protein containing an N-terminal decahistidine tag was overproduced in *E. coli* BL21(DE3) using the expression plasmid pET16b-SugR^{His} and purified by Ni^{2+}-chelate affinity chromatography as described previously by Engels and Wendisch (19).

Gel shift assays. Gel shift assays with SugR^{His} were preformed as described previously (19). Briefly, purified SugR^{His} (in concentrations ranging from 0-3.3 µM) was mixed with various promoter fragments (Fig. 2, 186-967 bp, final concentrations 7-37 nM) in a total volume of 20 µl and contained 50 mM Tris-HCl, 10% (v/v) glycerol, 50 mM KCl, 10 mM $MgCl_2$, 0.5 mM EDTA, pH 7.5. Approximately 90 nM of a nontarget promoter fragment (cg2228) was added as a negative control. The primers used for amplification of the promoter fragments are listed in Table 2. All PCR products used in the gel shift assays were purified with the PCR purification Kit (Qiagen, Hilden, Germany) and eluted in 10 mM Tris-HCl, pH 8.5. After incubation for 30 min at room temperature, the samples were separated on a 10% native polyacrylamide gel at room temperature and 170 V constant using 1x TBE (89 mM Tris base, 89 mM boric acid, 2 mM EDTA, pH 8.3) as electrophoresis buffer. The gels were subsequently stained with SybrGreen I according to the instructions of the supplier (Sigma, Rödermark, Germany) and photographed.

ChIP-to-chip analysis. From a fresh LB agar plate with *C. glutamicum* WT as control strain and the *C. glutamicum* WT-*sugR*^{Strep} strain the first preculture, 5 ml LB medium, was inoculated. After washing the cells in culture medium without any carbon source, the second preculture and the main culture were inoculated to an optical density at 600 nm (OD_{600}) of 0.5 either in 500 ml CgXII minimal medium in eight separate flasks, which contained 0.03 g/l protecatechuic acid and 0.2 mg/l biotin, or in LB medium. As carbon and energy sources 100 mM glucose, 100 mM fructose or 50 mM sucrose were used. The main cultures were cultivated to mid-exponential phase (OD_{600} 4-6). 50 ml of these main cultures were harvested by centrifugation (10', 11325 x *g*, 4°C) and the cells were washed with 50 ml buffer A (100 mM Tris, 1 mM EDTA, pH 8.0). Subsequently, the cells were resuspended in 10 ml of buffer A that was supplemented with 1% (v/v) formaldehyde. After incubation for 20 min at room temperature, glycine was added to a final concentration of 125 mM and the cultures were incubated for another 5 min. Then the cells were harvested (10', 3500 x *g*, 4°C) and washed twice in buffer A. The cell pellet was stored at -20°C until use. After thawing, cells were resuspended in 10 ml buffer A with one pill Complete Mini Protease Inhibitor (Roche, Mannheim, Germany) and 100 µg RNase A and disrupted by six passages at 172 MPa through a French pressure cell (SLM Aminco® Spectronic Instruments, Rochester, NY). The chromosomal DNA of the lysate was shared by sonication (2 x 30s with a Branson sonifier W-250, Danbury, USA, using a pulse length of 40% and an intensity of 1) to give an average fragment size of 200-750 bp. Cell debris was removed by centrifugation (20', 5300 x *g*, 4°C) and ultracentrifugation (1 h, 150000 x *g*, 4°C). The cytosolic fraction was then used for

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	References
E. coli strains		
DH5α	F⁻*thi*-1 *endA*1 *hsdr*17(r^-, m^-) *supE*44 Δ*lacU*169 (ϕ80*lacZ*ΔM15) *recA*1 *gyrA*96 *relA*1	Bethesda Research Laboratories; 28
BL21(DE3)	*ompT hsdS*$_B$($r_B^-m_B^-$) *gal dcm* (DE3)	Novagen
C. glutamicum strains		
WT	ATCC13032; wild-type strain, auxotrophic for biotin	ATCC
Δ*sugR*	*in-frame* deletion of the *sugR* gene of WT	19
sugR^{Strep}	pk19*mobsacB*- *sugR*^{Strep} integration in WT	This work
Plasmids		
pGEM-T	Amp^R, PCR cloning vector (P_{T7}, P_{SP6})	Promega, WI, USA
pGEM-T-*sugR*^{Strep}	Amp^R, pGEM-T with 424 bp fragment of the 3'-end of *sugR* with an C-terminal *Strep*Tag II sequence	19
pk19*mobsacB*	Kan^R, mobilizable *E. coli* vector for the construction of insertion and deletion mutants in *C. glutamicum*, (*ori*$V_{E.c.}$, *sacB*, *lacZα*)	52
pk19*mobsacB*- *sugR*^{Strep}	Kan^R, pk19*mobsacB* with the 424 bp integration construct comprising the 3'-end of *sugR* with added codons for a C-terminal *Strep*Tag II	This work
pVWEx1	Kan^R, P_{tac}, *lacI*^{q}	50
pVWEx1-*sugR*	Kan^R, pVWEx1 with a 807 bp fragment of the *sugR* gene and a artificial RBS	19
pET16b	Amp^R, overproduction of proteins with a N-terminal decahistidine tag in *E. coli* (pBR322 *ori*$V_{E.c.}$, *PT7*, *lacI*)	Novagen
pET16b-SugR^{His}	Amp^R, pET16b with a 786 bp fragment of the gene *sugR* with a N-terminal decahistidine tag	19

immunoprecipitation and therefore spiked with 5 µg/ml avidin and incubated 5 min on ice. The SugR^{Strep}-DNA complexes in the supernatant were precipitated by loading the supernatant onto an equilibrated (two times washed with 10 ml buffer W (100 mM Tris, 1 mM EDTA, 100 mM NaCl, pH 8.0)) column with 4 ml 50% (w/v) *Strep*Tactin-Sepharose (IBA GmbH, Göttingen, Germany). After washing the column three times with 10 ml buffer W, the bound SugR^{Strep}/DNA complexes were eluted in eight 1-ml-fractions of buffer E (100 mM Tris, 1 mM EDTA, 100 mM NaCl, 2.5 mM desthiobiotin, pH 8.0). After adding 1% SDS to the elution fractions they were incubated overnight at 65°C and then treated for 3 h at 55°C with proteinase K (400 µg/ml). The DNA was purified by phenol-chloroform extraction, precipitated with ethanol, washed with 70% (v/v) ethanol, dried and resuspended in 100 µl EB buffer (10 mM Tris-HCl, pH 8.5; Qiagen, Hilden, Germany). Fluorescent labelling of genomic DNA was performed as described previously (20, 53). Hybridisation to the *C. glutamicum* whole-genome microarray and array scanning were performed as described previously (19).

Determination of glucose, fructose and ribose concentrations. D-glucose and D-fructose were quantified enzymatically with the D-glucose/D-fructose kit (R-Biopharm, Darmstadt, Germany) according to the manufacturers' instructions as described previously by (19). D-ribose was separated by HPLC with a MultoHigh 100 RP18-5 column (150 x 2 mm, 5µ; CS Chromatographie Service; Langerwehe, Germany) using injection volumes of 10 µl, a flow rate of 0.2 ml/min and an overall run time of 20 min. A Q 4000 TRAP™ linear ion trap mass spectrometer (Applied Biosystems, Foster City, CA, USA) equipped with an electron ion spray source (Turbo Ion Spray) operating in the negative mode was used as detector. The ion source parameters were as follows: ion spray voltage, -4000 V; source temperature, 350°C; nebulizer gas (GS1), auxiliary gas (GS2), curtain gas (CUR) and collision gas (CAD) at setting 30, 20, 10 and 5 (arbitrary units), respectively. Data were acquired and processed using Analyst™ software. Concentrations were determined by comparison of the sample probes with external standards.

Measurement of enzyme activities. For measurements of enzyme activities, *C. glutamicum* WT, WTΔ*sugR*, WT(pVWEx1) and WT(pVWEx1-*sugR*) were cultivated in LB medium to an OD_{600} of 2-3.5. The cells were harvested by centrifugation (10', 4°C, 3220 x *g*), washed twice in 100 mM Tris-HCl pH 7.3 plus 10%(v/v) glycerol and stored at -70°C until use. For preparation of cell-free extracts, the cell pellet was resuspended in 500 µl of the washing buffer and the cells were mechanically disrupted by 3 x 20 s beat beating with 0.5 g of zirconia-silica beads (diameter 0.1 mm; Roth, Karlsruhe, Germany) using a Silamat S5 (Vivadent, Ellwangen, Germany). After centrifugation (45', 4°C, 12100 x *g*) the supernatant was used immediately for the enzyme assay. Protein concentrations were determined with the Bradford assay kit (Bio-Rad Laboratories, Hercules, Canada) using bovine serum albumin as standard.

6-phosphofructokinase PfkA (EC 2.7.1.11): Determination of the specific activity of the 6-phosphofructokinase PfkA in crude extracts was performed as described previously (60). The two different enzyme tests

TABLE 2. Oligonucleotides used in this study

Name	Sequence (5' → 3')	nt underlined = nt in NC003450	sequence in **bold** (restriction site)	sequence in *italics*
sugR	CG**GAATTC**TAGCTCCAAGCAGTGGTCGATCG	2038164	**EcoRI**	-
sugR-Strep	CG**GGATCC***TTATTTTTCGAACTGCGGGTGGCTCCAAGCGCT*TTCT GCAATCACAACTTCTACATCG	2038591	**BamHI**	*Strep*TagII
M13-rev	CACACAGGAAACAGCTATGACCATG	-	-	-
P0441-for	GCTAAAAAAGTAGAAATTATCGACC	387199	-	-
P0441-rev	GCCGGCTCCGAGTACTAC	387730	-	-
P1110-for	GTGATGCCTGCGGTTGCTG	1033597	-	-
P1110-rev	CAAATGAGATCACCATGATGACGGC	1034029	-	-
P1111-for	CTAAAACTTAACAAGCGCAACCCCC	1034739	-	-
P1111-rev	CCGCGGGAGTCGAGAATTTC	1034998	-	-
P1142_1143-for	GCGGTGATTCGAGCCGCTAAATCAAG	1060191	-	-
P1142_1143-rev	GTGCTGGCTGAGACTAGCTGGC	1059882	-	-
P1408_1409-for	GCGACGACGGTGGCAGTGCTGACC	1314735	-	-
P1408_1409-rev	CCGGGGCAGTCGCCGCCTGAC	1315095	-	-
F4(*ptsG*)-for	GCATAATCTGACAGTGTGTCCGTTTTC	1422834	-	-
F4(*ptsG*)-rev	GGCTCCCCCGCAATAGATTTGTGG	1423019	-	-
P1773_1774-for	GATCGTGTCCAAGGGTTCTCCTCC	1663548	-	-
P1773_1774-rev	GCGTACAGTGAGCGCCTGAAGTTC	1664450	-	-
P2115-for	GTCGCTTTTCAGGTTCCCGC	2037511	-	-
P2115-rev	CTCAACTGCCGTTAATGAGGCAATC	2037865	-	-
P2121-for	TCGGACCTTGACCCGATGTCTGGTC	2045575	-	-
P2121-rev	CGTGCGTGCAGGCCAACGGAG	2045811	-	-
P2157-for	GGCTTTAGGAGCCTAGTGGC	2073290	-	-
P2157-rev	CCAGCAATCACTGCGATAGTGATTAG	2072856	-	-
P2291-for	GTTTCAACCATAGGCCTGACCTGGC	2207298	-	-
P2291-rev	CTAGCCACCGCTGGGCCTAG	2207043	-	-
P3068-for	CCCCGATAGTGTATGTGCTGAC	2955520	-	-
P3068-rev	CTTAGCACGATCGAGCATCTCGTTA	2955223	-	-
P3169_3170-for	GGTAAGACCGCAGCGTAGCTTTTGG	3054803	-	-
P3169_3170-rev	CCTTATTCTTGGTCGGCGCCTCG	3053838	-	-
P3219-for	GTTGCCAGGCGAGTGGTGAGC	3113669	-	-
P3219-rev	CATCTCCTGCGCCAATGAGGACAATC	3113343	-	-
P3366_3367-for	GCTGTGAACGACCCAAAACTCAAACTTAG	3242913	-	-
P3366_3367-rev	CGGTCGAGTTCTATGCGTTCTGC	3242640	-	-
cg2228_for	GTTCGCTACGTCCGAGTGATCACC	2146341	-	-
cg2228_rev_short	CTCAGGCATGATGATGTCAGGC	2146264	-	-
P1145_for	CGTTTCGCAGTACGGAGCG	1063987	-	-
P1145_rev	CCTTCACTTCACCCATGGTGTC	1063566	-	-
P2837_for	CGCAGCAATGTGATAACAGAGG	2727080	-	-
P2837_rev	GGCACACCATGGGTTTCAAAG	2726526	-	-
P3048-for	GATTCCTGCTGGACCTGCAGCG	2938454	-	-
P3048-rev	CGAAGCTGCGGTTGACCGTGG	2937840	-	-

described in this publication were named test A (coupling to pryuvate kinase/lactate dehydrognase) and test B (coupling to aldolase, triosephosphate isomerase and glycerophosphate dehydrogenase) in this study. Both assay mixtures (500 µl total volume) contained 100 mM Tris-HCl pH 7.5, 0.2 mM NADH, 10 mM $MgCl_2$, 1 mM ATP, 20-75 µl crude extract. In addition, assay A contaied 2.75 U/ml NAD-dependent L-lactate dehydrogenase, 2.2 U/ml pyruvate kinase and assay B contained 0.4 U/ml aldolase, 3.06 U/ml α-glycerophosphate dehydrogenase, 0.033 U/ml triosephosphate isomerase. The reaction was started by the addition of 4 mM fructose-6-phosphate and the increase of absorption at 340 nm (ε_{340nm}(NADH) = 6.3 mM^{-1} cm^{-1}) was monitored at 30°C for 5-30 min using Shimadzu UV-1202 spectrophotometer (Shimadzu, Duisburg, Germany).

Fructose-1,6-bisphosphat aldolase Fba (EC 4.1.2.13): Determination of the specific activity of the fructose-1,6-bisphosphate aldolase Fba in crude extracts was performed as described in (6). The assay mixture (500 µl total volume) contained 100 mM Tris-HCl pH 7.4, 0.13 mM NADH, 1.67 U/ml α-glycerophosphate dehydrogenase, 0.018 U/ml triosephosphate isomerase and 20-50 µl crude extract. The reaction was started by the addition of 2 mM fructose-1,6-bisphosphate and the increase of absorption at 340 nm was monitored at 30°C for 5-10 min using Shimadzu UV-1202 spectrophotometer (Shimadzu, Duisburg, Germany).

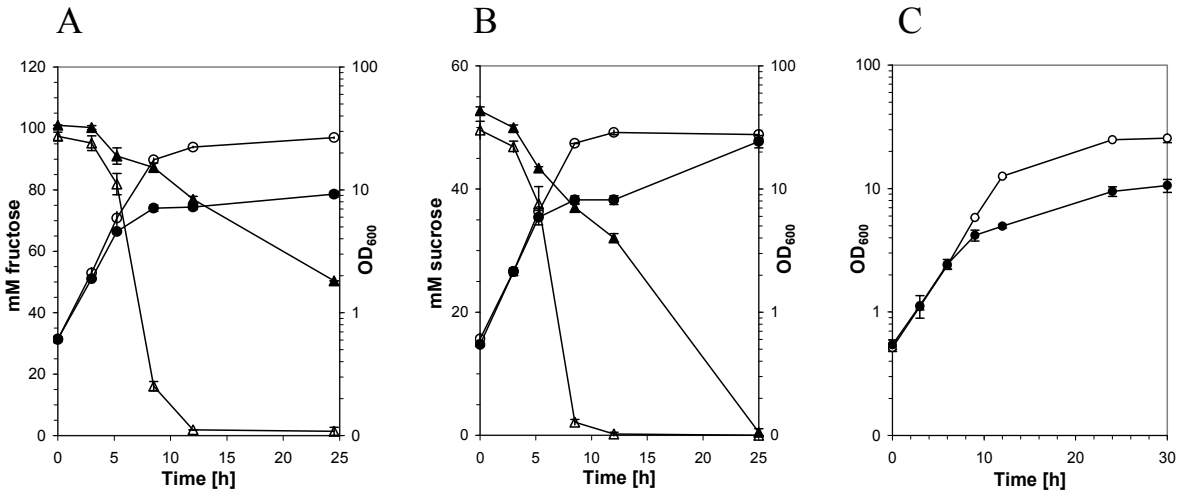

FIG. 1. Role of SugR for growth of *C. glutamicum* on minimal medium with fructose, sucrose or ribose. Growth of *C. glutamicum* WT(pVWEx1) (open symbols) and WT(pVWEx1-*sugR*) (filled symbols) on CgXII minimal medium containing 100 mM fructose (A) or 50 mM sucrose (B) or 120 mM ribose (C). The cultures were induced three hours after inoculation by addition of 1 mM IPTG. The optical densities (circles) and the fructose and sucrose concentrations (triangles) are indicated.

NAD-dependent L-lactate dehydrogenase LdhA (EC 1.1.1.27): Determination of the specific activity of the NAD-dependent L-lactate dehydrogenase LdhA in crude extracts was performed as described (9). The assay mixture (500 µl total volume) contained: 20 mM MOPS pH 7.0, 0.2 mM NADH and 1-20 µl crude extract. The reaction was started by the addition of 30 mM pyruvate and the increase of absorption at 340 nm was monitored at 30°C for 5 min using Shimadzu UV-1202 spectrophotometer (Shimadzu, Duisburg, Germany).

Pyruvate kinase Pyk (EC 2.7.1.40): Determination of the specific activity of the pyruvate kinase Pyk in crude extracts was performed as described (35). The assay mixture (500 µl total volume) contained: 200 mM Tris-HCl pH 7.0, 5 mM NADH, 200 mM $MgCl_2$, 20 mM ATP, 110 U/ml NAD-dependent L-lactate dehydrogenase and 1-20 µl crude extract. The reaction was started by the addition of 240 mM phosphoenolpyruvate and the increase of absorption at 340 nm was monitored at 30°C for 5-30 min using Shimadzu UV-1202 spectrophotometer (Shimadzu, Duisburg, Germany).

Fumerase Fum (EC 4.2.1.2): Determination of the specific activity of the fumerase Fum in crude extracts was performed as described (22). The assay mixture (500 µl total volume) contained: 100 mM sodium phosphate buffer pH 7.3 and 0.5-2 µl crude extract. The reaction was started by the addition of 50 mM L-malate and the increase of absorption at 240 nm (ε_{240nm}(fumarate) = 2.44 mM^{-1} cm^{-1}) was monitored at 26°C for 5 min using Shimadzu UV-1202 spectrophotometer (Shimadzu, Duisburg, Germany).

L-lactic acid production. For L-lactic acid production, the *C. glutamicum* strains WT, Δ*sugR*, WT(pVWEx1) and WT(pVWEx1-*sugR*) were cultivated aerobically at 30°C for about 16 h in 100 ml LB complex medium with 4%(w/v) glucose as carbon source. When appropriate, media were supplemented with 50 µg/ml kanamycin. The precultures were harvested by centrifugation (5', 11325 x *g*, 4°C), cells were washed in CgXII medium (pH 7.2) without any carbon source, the washed cells were resuspended in 1 ml of the same medium and used to inoculate the oxygen-deprivation culture with 80 ml CgXII minimal medium, pH 7.2, with 200 mM glucose, 0.03 g/l protecatechuic acid and 0.2 mg/l biotin, 30 mM potassium nitrate as electron acceptor, 1 µg/ml resazurin as oxygen indicator and when appropriate 50 µg/ml kanamycin and 1 mM IPTG. The medium was flushed for 24 h with nitrogen prior to inoculation. The cell suspension was subsequently incubated at 30°C in a lidded 100 ml medium bottle and gently shaking for three hours.

RESULTS

Effect of *sugR* overexpression on growth on fructose or sucrose. SugR binds to promoters of PTS genes and was previously shown to affect utilization of the PTS substrate glucose *in vivo* (19). To test whether SugR also affects utilization of the PTS-substrates sucrose and fructose *in vivo*, growth of *C. glutamicum* strain overexpressing *sugR* was compared to the control strain on minimal medium with 100 mM fructose or 50 mM sucrose (Fig. 1A and B). The strain overexpressing *sugR* grew significantly slower and formed less biomass than the control strain on 100 mM fructose. Moreover, the strain overexpressing *sugR* utilized the added fructose with an approximately two fold decreased uptake rate in comparison to the control strain (Fig. 1A). Overexpression of *sugR* also perturbed growth of *C. glutamicum* on sucrose minimal medium and the sucrose uptake rate was reduced about 13% as compared to the control strain (Fig. 1B). Taken together, overexpression of *sugR* had a negative influence on utilization of all known PTS-substrates (glucose, fructose and sucrose) in *C. glutamicum*.

Effect of *sugR* overexpression on growth on ribose. To test whether control of substrate utilization by SugR is limited to PTS substrates, the empty vector

TABLE 3. Genes enriched in SugR^{Strep}-DNA complexes identified by ChIP-to-chip analysis[a].

Gene ID	Gene name, Gene product or deducted function	enrichment factor ChIP-to-chip			
		glucose	fructose	sucrose	LB
presence and absence of sugars					
cg0641	*fabG2,* probable short chain dehydrogenase, secreted	**4,27**	**3,41**	**3,25**	**13,62**
cg0682	predicted ATPase or kinase	**2,77**	**2,50**	**2,65**	**8,91**
cg1110	conserved hypothetical protein	**5,07**	**6,51**	**7,26**	**8,58**
cg1111	*eno,* Enolase	**4,27**	**4,72**	**4,60**	**10,64**
cg1408	hypothetical membrane protein	**2,01**	**2,14**	1,06	**6,35**
cg2157^{P}	*terC,* membrane protein TerC	**2,76**	**2,60**	**2,52**	**7,75**
cg3068^{P}	*fba,* fructose-1,6-bisphosphate aldolase	**4,12**	**3,76**	**3,46**	**28,24**
cg3219	*ldhA,* L-lactate dehydrogenase	**2,81**	**3,93**	**2,35**	**4,28**
absence of sugars					
cg0441^{P}	*lpd,* diaminolipoamide dehydrogenase	1,38	1,23	0,95	**3,97**
cg0448	hypothetical protein	1,12	1,10	1,07	**2,07**
cg1174^{P}	*tkt,* transketolase	1,93	1,36	1,18	**7,28**
cg1283	*aroE2,* putative Shikimate / Quinate 5-dehydrogenase	1,17	1,23	0,98	**2,39**
cg1493	conserved hypothetical protein	1,13	0,99	1,05	**3,58**
cg1537^{P}	*ptsG,* glucose-specific IIABC PTS component	1,24	1,13	0,98	**3,79**
cg1696	permease of the major facilitator superfamily	1,34	1,29	1,07	**4,80**
cg2115^{P}	*sugR* , transcriptional regulator DeoR family	1,30	1,45	1,45	**4,39**
cg2121	*ptsH,* phosphocarrier protein HPr	1,91	1,87	1,47	**9,64**
cg2262^{P}	*ftsY,* signal recognition particle GTPase	1,29	1,10	1,07	**2,55**
cg2291	*pyk,* pyruvate kinase	1,32	1,26	1,12	**2,41**
cg2794	conserved hypothetical protein	1,33	1,26	1,45	**3,49**
cg2831^{P}/cg2833^{P}	*ramA,* transcriptional regulator LuxR-family / *cysK,* Cysteine synthase	1,22	1,28	1,51	**2,47**
cg2908	hypothetical trehalose-binding protein	1,46	1,29	0,98	**2,85**
cg2909	*otsB,* trehalose-phosphatase	1,56	1,48	1,39	**4,48**
cg3366	*sgcA,* putative phosphotransferase enzyme II, A component	1,12	1,19	1,74	**2,33**
others					
cg0974	conserved hypothetical protein	1,43	**3,15**	0,80	
cg1074	conserved hypothetical protein	**2,30**	1,83	0,25	**2,71**
cg1142	Na^+/proline, Na^+/panthothenate symporter	**2,03**			1,06

[a] Genes or genomic regions listed showed enrichment factors of two or more in three independent ChIP-to-chip analyses during growth on LB (*P* value ≤0.05). In addition, those genes which were enriched in three additional ChIP-to-chip experiments during growth on CgXII minimal medium containing 100 mM glucose, 100 mM fructose or 50 mM sucrose, respectively, are listed. [p] Enrichment factors based on intergenic regions, of which few were present on the DNA microarray, are marked.

control strain and the strain overexpressing *sugR* were grown on minimal medium containing 120 mM ribose, which is taken up into the cell via an ABC transport system, as sole carbon and energy source (Fig. 1C). *C. glutamicum* and the WT(pVWEx1-*sugR*) grew slower on ribose minimal medium than the control strain WT(pVWEx1), formed less biomass and only consumed 70% of the added ribose, while the empty vector control strain utilized ribose completely (Fig. 1 and data not shown). Whereas the negative effect of *sugR* overexpression on the PTS substrates glucose, fructose and sucrose can be explained by SugR control of its known targets, the PTS genes, the negative effect on the non-PTS substrate ribose suggested that SugR controls expression of additional target genes, e.g. genes coding for ribose uptake proteins or enzymes of the central carbon metabolism.

Identification of possible SugR targets using ChIP-to-chip analysis. In order to identify further direct target genes of SugR on the genome-wide scale, a modified method of chromatin immunoprecipitation combined with DNA microarray analysis (ChIP-to-chip; (34)) was applied. *C. glutamicum* WT-*sugR*^{Strep}, which produces an affinity-tagged SugR protein instead of native SugR, was constructed in order to facilitate purification of SugR^{Strep}-DNA complexes formed *in vivo*. A vector containing the 3' end of the *sugR* gene extended by codons for addition of a C-terminal *Strep*Tag was inserted into the *sugR* locus, thus allowing *sugR*^{Strep} expression from the native *sugR* promoter. Protein-DNA complexes formed *in vivo* in *C. glutamicum* WT-*sugR*^{Strep} and in the control *C. glutamicum* WT were crosslinked by treating intact cells with formaldehyde. After cell disruption and DNA sharing by sonification SugR^{Strep}-DNA complexes were enriched by *Strep*Tactin-Sepharose chromatography. After reversal of the crosslinks, the co-precipitated DNA was purified, fluorescently labelled and hybridized to *C. glutamicum* DNA microarrays.

Table 3 lists all genes showing average enrichment factors of two or more in three independent ChIP-to-chip analyses during growth on LB (*P* value, ≤0.05) and those genes or genomic regions which were

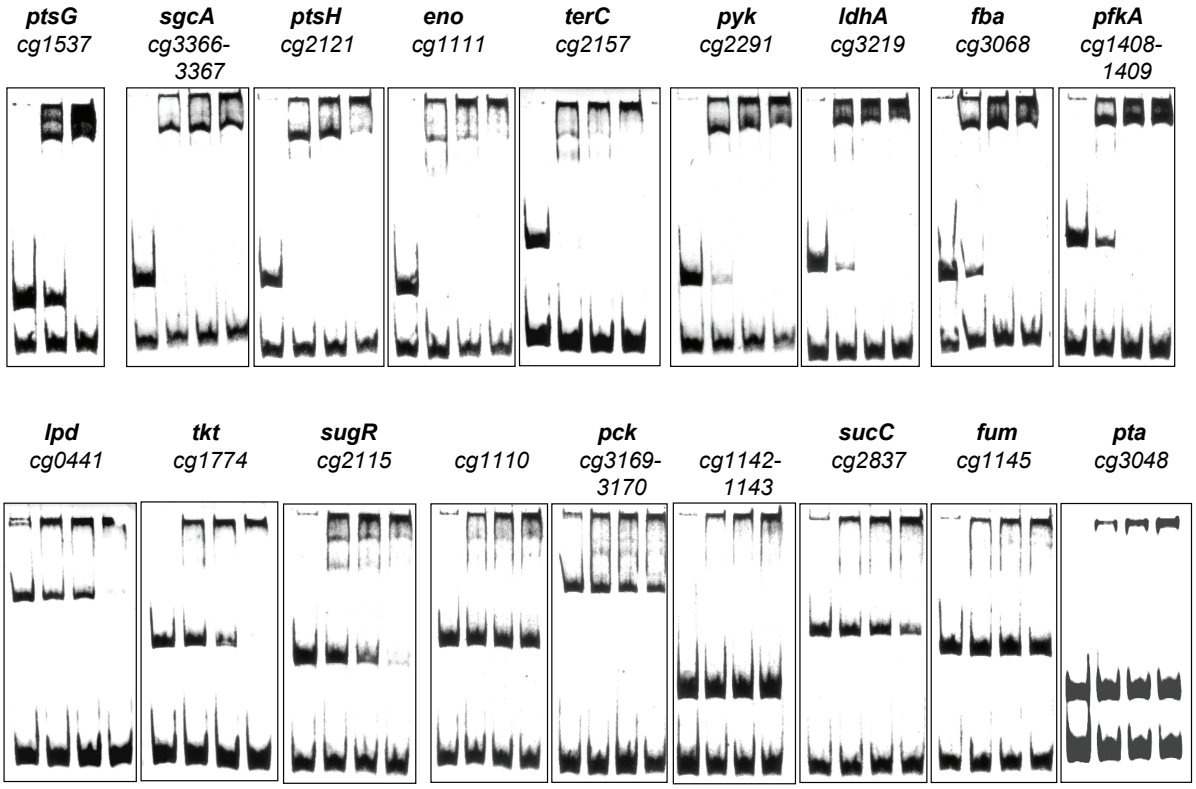

FIG. 2. Binding of $SugR^{His}$ to candidate target genes. DNA fragments (186-967 bp; final concentration 7-37 nM) covering the promoter regions of further putative target genes of SugR were incubated for 30 min at room temperature without $SugR^{His}$ protein (left lane) or with a 30, 60 or a 90 fold molar excess (lanes 2-4, respectively) of purified $SugR^{His}$ protein before separation by native polyacrylamide gel electrophoresis (10%) and staining with SybrGreen I. A 78 bp cg2228 promoter fragment (90 nM) served as negative control. Oligonucleotides used for amplification of these fragments via PCR are listed in Table 2.

enriched in three additional ChIP-to-chip experiments during growth on CgXII minimal medium containing either 100 mM glucose, 100 mM fructose or 50 mM sucrose. The genes could be subdivided into three groups: those bound by SugR in the presence and absence of sugars (group I), those bound by SugR only in the absence of sugars (group II) and other genes, which could not be classified in the first two categories (group III). Group I comprised eight genes including the genes for enolase (cg1111, *eno*), fructose-1,6-bisphosphate aldolase (cg3068, *fba*), fermentative NAD-dependent L-lactate dehydrogenase (cg3219, *ldhA*) which is crucial for anaerobic L-lactate production (32) and a hypothetical membrane protein (cg1408). Enrichment of cg1408, which lies divergent to the 6-phosphofructokinase (cg1409, *pfkA*) was found during growth on LB, on glucose and on fructose, but not on sucrose. Group II comprised 17 genes or genomic regions and included genes of the previously known SugR targets *ptsG* and *ptsH*. Possibly due to the absence of suitable probes on the DNA microarrays, the enrichment factors for *ptsI* and the operon *fruR-fruK-ptsF* were only 1.6 fold (data not shown) and enrichment of *ptsS* was not identified. As expected from previous work (19, 21), SugR bound to *ptsG* and *ptsH* only in the absence of sugars. Group II also included *sugR* itself and gene for the fourth EII permease in *C. glutamicum* possibly transporting a yet unknown substrate (cg3366, *sgcA*) as well as genes for dihydrolipoamide dehydrogenase (cg0441, *lpd*), pyruvate kinase (cg2291, *pyk*), trehalose phosphatase *otsB* (cg2909), and the genomic region between the genes for polyprenyltransferase and transketolase (cg1774, *tkt*). Group III includes four genes or genomic regions of unknown function: cg0974 was enriched only during growth on fructose and cg1142 only on glucose, while cg1074 was enriched on LB, glucose and fructose, but not on sucrose. In summary, the ChIP-to-chip analysis revealed new candidate SugR target genes including genes for enzymes of central carbon metabolism such as 6-phosphofructokinase, NAD-dependent L-lactate dehydrogenase or transketolase.

Gel retardation analysis of candidate SugR target genes and identification of a SugR consensus binding site. In order to test for direct interaction of SugR with the promoter regions of the possible new target genes *in vitro*, gel retardation analysis was performed with purified $SugR^{His}$ protein and DNA fragments covering the corresponding promoter regions. As shown in Figure 2, SugR bound a number of the promoter fragments tested with different affinities. SugR bound the promoter fragments of *sgcA*, *ldhA*, *eno*, *pyk*, *terC*, and *ptsH* with a higher affinity than the *ptsG* promoter fragment (Fig. 2). The promoter fragments of *fba* and *pfkA* were bound with affinities comparable to that of the *ptsG* promoter region. SugR bound its own promoter region as well as the promoter regions of *lpd* and *tkt*, but with a weaker affinity than the others. SugR binding to the promoter fragments of the phosphoenolpyruvate carboxykinase (*pck*), the succinyl-CoA-synthetase (*sucC*) and the fumarase (*fum*), and the phosphotransacetylase (*pta*) was very weak or even absent.

To identify a consensus SugR binding site, the promoter sequences of those genes which were bound by SugR as evidenced by ChIP-to-chip and gel retardation analysis were compared for sequence similarities using the free available MEME software ((4); http://bioweb.pasteur.fr/seqanal/motif/meme/meme.html). Two putative SugR binding motives were identified in each promoter fragment with the consensus sequence NN**TCRRACA**N**W**N**W**N shown at the bottom of Table 4. The binding sites have in common that they are located at or near the transcriptional start sites suggesting that SugR acts as repressor of those genes.

Specific activities of NAD-dependent L-lactate dehydrogenase, 6-phosphofructokinase, fructose-1,6-bisphosphate aldolase, pyruvate kinase and fumarase in *C. glutamicum* WT, Δ*sugR*, WT(pVWEx1) and WT(pVWEx1-*sugR*). The specific activities of NAD-dependent L-lactate dehydrogenase, 6-phosphofructokinase, pyruvate kinase and fructose-1,6-bisphosphate aldolase were determined in strains lacking *sugR* or overexpressing *sugR* because SugR was found to bind to the promoter regions of their genes. Fumarase activity was also determined although SugR binding was very weak (or absent). As shown in Figure 3, the activities of the tested enzymes were comparable in *C. glutamicum* WT and WT(pVWEx1). In *C. glutamicum* Δ*sugR*, the LdhA, Fba, PfkA and Pyk activities were increased 8.1 fold, 1.6 fold, 1.2 fold and 1.3 fold respectively, as compared to WT (Fig. 3A-D). In *C. glutamicum* WT(pVWEx1-*sugR*), the specific activities of LdhA, Fba and PfkA were 65%, 16% and 44%, respectively, of the specific activities measured in the empty vector control (Fig. 3A-C), while the specific activity of Pyk was not significantly changed due to overexpression of *sugR* (Fig. 3D). The specific activity of fumerase was comparable in the all strains tested (data not shown). Taken together, the results indicate that SugR acts as repressor of the genes for NAD-dependent L-lactate dehydrogenase, 6-phosphofructokinase, fructose-1,6-bisphosphate aldolase and pyruvate kinase and affects their activities *in vivo*.

Influence of deletion and overexpression of *sugR* on L-lactate formation by *C. glutamicum*. Formation of L-lactate, which is secreted into the medium during anaerobiosis or as a by-product during glutamate production (37, 57), requires *ldhA* (32), which was shown to be repressed by SugR (Fig. 2, Fig. 3A). To test the physiological relevance of *ldhA* control by SugR, L-lactate formation by *C. glutamicum* WT, Δ*sugR*, WT(pVWEx1) and WT(pVWEx1-*sugR*) was compared under oxygen-deprivation conditions. As shown in figure 4, the Δ*sugR* deletion mutant showed three fold increased L-lactate formation compared to the WT, whereas the strain overexpressing *sugR* formed approximately 30% less L-lactate than the control. Thus, SugR repression of *ldhA* is important for anaerobic L-lactate formation by *C. glutamicum*.

DISCUSSION

SugR has previously been shown to repress the PTS-genes for the glucose-, fructose and sucrose-specific enzymes II (19) and for the general components enzyme I and HPr (21, 61). The ChIP-to-chip and gel retardation analysis shown here revealed that SugR not only represses PTS-genes, but also a number of further genes, mainly encoding enzymes of central carbon metabolism. The SugR regulon therefore comprises at least 17 genes in 14 transcription units, e.g. the genes encoding 6-phosphofructokinase (*pfkA*), fructose-1,6-bisphosphate aldolase (*fba*), enolase (*eno*), pyruvate kinase (*pyk*), NAD-dependent L-lactate dehydrogenase (*ldhA*) and transketolase (*tkt*) (Table 3, Fig. 2). Thus, SugR coordinately controls expression of genes for uptake of carbohydrates via the PTS and for their further metabolism in the central pathways of glycolysis (*pfkA, fba, eno, pyk*) and the pentose phosphate pathway (*tkt*, which is part of the *tkt-tal-zwf-opcA-pgl* operon also encoding the pentose phosphate pathway enzymes transaldolase, glucose-6-phosphate dehydrogenase and 6-phosphogluconolactonase; (69)). In contrast, fewer targets have been identified to be regulated by the carbon regulators AcnR (represses aconitase gene *acn*; (41)), GntR1 and GntR2 (represses gluconate utilization genes *gntP, gntK* and *gnd*, activates *ptsG* and *ptsS*; (11)), GlxR (represses *gntP, gntK* and isocitrate lyase and malate synthase genes *aceA* and *aceB*; (38, 42)) and LldR (represses the L-lactate utilization operon cg3226-*lldD*; (23)). With respect to the number of regulated genes, the SugR regulon resembles the regulons of RamA und RamB (3, 65). RamB was shown to repress its own gene, *aceA, aceB*, the acetate activation operon *pta-ack* and the alcohol dehydrogenase gene *adhA* and to activate *aceE*, which encodes subunit E1 of pyruvate

TABLE 4. SugR binding sites in the DNA fragments verified by bandshift analysis[a].

gene name/gene ID	putative SugR binding site	strand	shift	position/location	
ptsG (cg1537)	GG**TCGGACA**T**A**T**T**C	+	++	-82 to -69	TS
	AAA**CGGACA**C**A**C**T**G	-		-101 to -114	
ptsS (cg2925)	GA**TCGGACA**C**AAA**T	+	+	-33 to -20	TS
	AT**TCAGACA**TGA**A**T	-		+30 to +17	
ptsH (cg2121)	GG**TCGGACA**T**T**G**T**T	+	++	-116 to -153	TL
	AG**T**G**AGACA**T**T**G**T**T	-		-113 to -126	
ptsI/fruR-fruK-ptsF (cg2117-cg2120)	AT**TCAAACA**T**T**TGG	+	+	-343 to -330	TL
	CA**TCAG**C**CA**C**A**G**T**A	+		-425 to -412	
pyk (cg2291)	GAA**CAGACA**A**T**G**T**T	-	+	-82 to -98	TL
	AAAA**AGAC**TA**A**A**T**T	-		-126 to -113	
ldhA (cg3219)	GG**TCAGAC**CA**A**G**T**G	-	+	-126 to -139	TL
	TT**TCGGACA**T**A**A**T**C	+		-121 to -108	
sgcA (cg3366)	GG**TCAAACA**T**AA**G	-	++	-38 to -51	TL
	GTA**CAAACA**T**A**AGT	-		+10 to -4	
eno (cg1111)	GA**TCAGACA**T**A**A**T**C	-	++	-126 to -175	TL
	TT**TCAG**C**CA**C**T**G**T**G	-		+10 to -4	
cg1408 (cg1408)	GG**TCGGACA**C**A**T**T**T	-		-172 to -185	
	GG**TCAGA**G**A**C**A**G**T**T	+	+	-89 to -79	TL
	GC**TCGGACA**A**T**G**T**C	+		-144 to -131	
sugR (cg2115)	AT**TCGGACA**T**A**T**T**T	+	(+)	-45 to -32	TL
	AG**T**G**AGACA**A**T**GGG	+		-65 to -52	
fba (cg3068)	AA**TCAGACA**T**T**G**T**G	+	+	-108 to -95	TL
	GA**TCAAACA**T**T**G**A**T	-		-115 to -128	
terC (cg2157)	AA**TCGGACA**TGC**T**C	-	++	-291 to -304	TL
	GT**TCG**C**ACA**G**TT**G	+		-334 to -331	
tkt (cg1174)	AC**T**CT**GACA**G**A**AGC	+	+	-733 to -719	TL
	TT**TCAAAC**CA**TTT**G	+		-627 to -613	
consensus	NN**TCRRACA**N**W**N**W**N				

[a]The SugR binding sites shown in this table were identified by a motif search using the MEME software ((4); http://bioweb.pasteur.fr/seqanal/motif/meme/meme.html) and the promoter fragments used in the gel shift analysis. The column labelled „shift“ indicates whether SugR showed a high [++], medium [+], or low [(+)] affinity to the corresponding DNA fragments. The positions of the binding sites relative to the transcriptional start site (TS) or the translational start site (TL) are given by the numbers in the „position/location“ column.

Dehydrogenase (2, 7, 24). The occurrence of RamB binding motifs suggests that *ptsG*, the TCA cycle genes *gltA* and *acn,* and the PEP carboxykinase and malic enzyme genes *pck* and *malE* are regulated by RamB (3). RamA activates *adhA, aceA, aceB, pta-ack* and *ramB* (2, 3, 12, 13) and, in addition, likely regulates the TCA cycle genes *sdhCAB* for succinate dehydrogenase and *acn*, as well as *pck* and *malE* as RamA bindings site motifs are found in the promoter regions of these genes (3). It is noteworthy that genes for enzymes of glycolysis and the pentose phosphate pathway were not found to be regulated by either RamA or RamB, while SugR regulates *pfkA, fba, eno, pyk, ldhA* and *tkt* (s. above). In a first approximation, regulation of the central carbon metabolism in *C. glutamicum* comprises control of the glyoxylate and

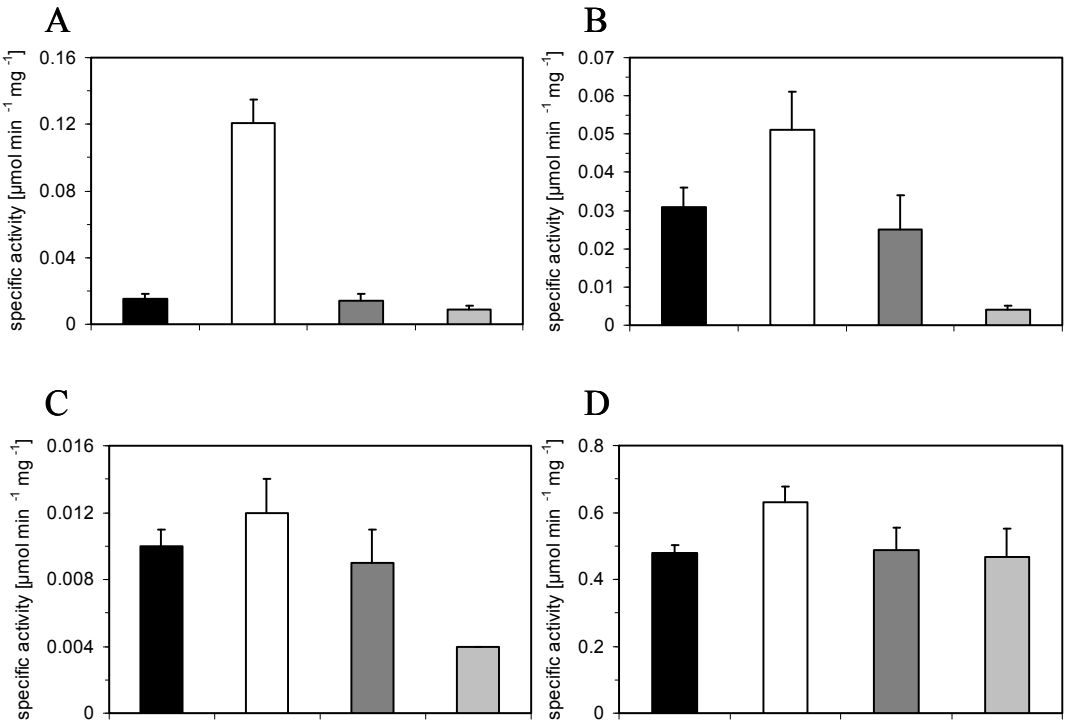

FIG 3. Specific activites of (A) NAD-dependent L-lactate dehydrogenase (LdhA), (B) fructose-1,6-bisphosphate aldolase (Fba), (C) 6-phosphofructokinase (PfkA), (D) pyruvate kinase (Pyk) in *C. glutamicum* WT (black bars), WTΔ*sugR* (white bars) and WT(pVWEx1) (dark grey bars) and WT(pVWEx1-*sugR*) (light grey bars). All data are mean values of at least four determinations of one to two independent cultivations on LB medium.

TCA cycle genes by the global regulators RamA and RamB on the one side and control of glycolysis and pentose phosphate pathway genes by SugR on the other side. However, expression of genes for sugar uptake via the PTS is controlled by several transcriptional regulators, e.g. *ptsG* by RamB, SugR, GntR1 and GntR2 (3, 11, 19) or *fruR-fruK-ptsF* by SugR and FruR (19, 21, 61, 62).

The regulons of the carbon catabolite regulators cAMP-CRP from *E. coli* and CcpA from *B. subtilis* comprise genes of glycolysis and the TCA cycle and are larger than the SugR regulon from *C. glutamicum*. In *E. coli*, at least 200 genes, which encode enzymes of many different pathways including glycolysis and the TCA cycle, are controlled by cAMP-CRP (25, 26). The carbon catabolite control protein CcpA of *B. subtilis* positively regulates genes for glycolytic enzymes and carbon overflow pathways and represses genes of the TCA cycle and for utilization of carbon sources other than glucose (14). In both *E. coli* and *B. subtilis*, carbon catabolite control is a dominant regulatory mechanism responsible for diauxic growth phenomena. In contrast, in *C. glutamicum*, which generally co-utilizes carbon sources present in substrate mixtures, the carbon regulators SugR, RamA and RamB primarily fine-tune central metabolic pathways for simultaneous substrate utilization.

A conserved sequence motif (NN**TCRRACA**N**W**N**W**N, Table 4) is found two times in the promoter regions of all identified SugR targets and coincides with those sequences upstream of *ptsG* (19) and in the intergenic region between *ptsI* and *fruR* (21) shown by mutational analysis to be essential for SugR binding. The locations and the relative orientations between the two sequence motifs within a given promoter region vary (Table 4). *In vitro* evidence suggests that a $TG(T)_{2\text{-}5}G$ sequence might in addition be involved in SugR binding in *C. glutamicum* R, a related strain providing high lactate yields (62). Typical representatives of the DeoR-type family proteins, to which SugR from *C. glutamicum* belongs, are DeoR from *E. coli*, which binds to a 16 bp palindromic sequence 5'-TGTTAGAA· TTCTAACA-3' in either two of the three operator sites O_1, O_2 and O_E forming a single or double DNA loop (46), and FruR from *Lactococcus lactis*, which potentially binds to four repeating non-palindromic sequences upstream of the fructose-PTS gene cluster (5). Currently, it is not known how different orientations and locations of the binding sequence motifs affect the action of the SugR from *C. glutamicum in vivo* and if SugR occurs in different multimeric forms as described for DeoR of *E. coli*. In this regard, its is interesting to note that ChIP-to-chip analysis revealed binding of SugR to some targets, mainly PTS-genes (e.g. *ptsG* or *ptsH,* but also *pyk* and

tkt), primarily in the absence of PTS-sugars, while binding to other targets (e.g. *eno, ldhA* or *fba*) was readily detected during growth in the absence or presence of sucrose, fructose or glucose (Table 3). The physiological relevance of these observations remains to be studied.

SugR control of PTS genes is physiologically relevant as utilization of glucose (19), fructose or sucrose (Fig. 1) is negatively affected by overexpression of *sugR*, while deletion of *sugR* resulted in increased glucose uptake during growth on glucose-acetate mixtures (19). In addition, the physiological significance of SugR control of non-PTS genes became obvious by the facts that *sugR* overexpression perturbed utilization of ribose (Fig. 1) and that deletion of *sugR* resulted in increased L-lactate formation under oxygen-deprivation conditions (Fig. 4). In *B. subtilis* the well characterized *rbs* operon (*rbsRKDACB*) is essential for utilization of ribose as sole carbon source (59, 68). Expression of the ribose transport operon from *B. subtilis* is negatively regulated by the transcriptional repressor RbsR encoded within this operon (59), it is also subject to catabolite repression and to control by Spo0A~P during the late exponential phase (48, 68).

In *C. glutamicum*, the putative operon *rbsACBD* encoding the subunits of an ABC transport system and an operon including the ribokinase gene *rbsK1* were suggested to be induced by ribose based on DNA microarray experiments and enzyme activity assays (66). The ribose transporter genes are clustered with the regulatory gene *rbsR* in the genome of *C. glutamicum* (36). The role of *rbsR* with regard to regulation of the ribose operon genes has not been investigated to date. The *C. glutamicum* genome contains a second gene encoding a ribokinase, *rbsK2* (36). Growth experiments with the *rbsK1* and *rbsK2* deletion mutants and a *rbsK1/rbsK2* double mutant indicated that at least one functional ribokinase is necessary for utilization of uridine or ribose as sole carbon source in *C. glutamicum* (8). Moreover, it was shown that the uridine utilization regulator UriR binds to the promoter region of *rbsK2* but regulation was suggested to be more complex and occurs in conjunction with another unknown regulatory protein (8). Although SugR affected growth on ribose, SugR was not found to be involved in the regulation of the ribose operons (Table 3). Growth of *C. glutamicum* on ribose requires pyruvate kinase activity, since a Δ*pyk* mutant was not able to grow on ribose as sole carbon and energy source (47). As SugR was found to bind upstream of *pyk*, growth perturbation due to overexpression of *sugR* can be explained by repression of *pyk* by SugR. In addition, as the pentose phosphate pathway enzyme transketolase is important for growth on ribose, SugR repression of the transketolase gene *tkt* likely augments growth perturbation on ribose by *sugR* overexpression.

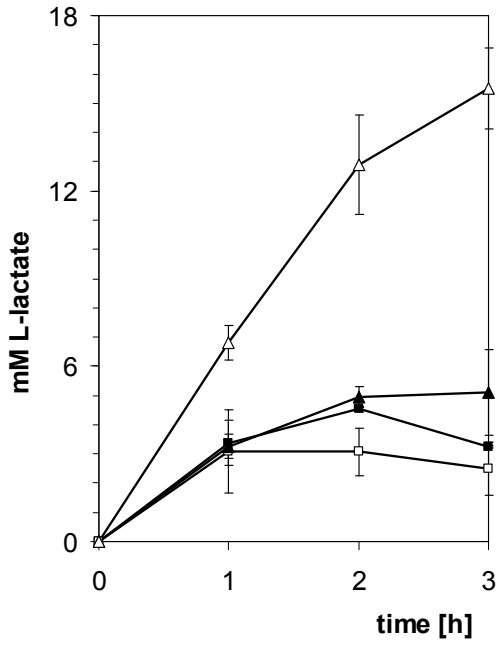

FIG. 4. Production of L-lactate by *C. glutamicum* under oxygen-deprivation conditions. Concentration of L-lactate produced by *C. glutamicum* WT (closed trianles), Δ*sugR* (open trianles), WT(pVWEx1) (closed squares) and WT(pVWEx1-*sugR*) (open squares) are indicated. The data represent averages of two independent cultivations.

L-lactate is both a metabolic product as well as a carbon substrate for growth. Growth of *C. glutamicum* on L-lactate requires quinone-dependent L-lactate dehydrogenase LldD (cg3227; EC 1.1.2.3) (57). The cg3226-*lldD* operon, which contains a gene for a putative lactate transport system besides *lldD*, is repressed by the FadR-type transcriptional regulator LldR in the absence of its effector L-lactate (23). *C. glutamicum* is able to secrete L-lactate into the medium, e.g. as by-product during glutamate and lysine production (37, 57) or under oxygen-deprivation conditions (32). L-lactate formation requires the NAD-dependent L-lactate dehydrogenase LdhA (32) and *ldhA* mRNA levels increased about 9 fold under oxygen deprivation conditions (33) as expected for fermentative enzymes. However, the regulatory mechanism for anaerobic induction or aerobic repression of *ldhA* is currently unknown. Here, it was shown that *ldhA* is a target of SugR and that SugR represses *ldhA* (Fig. 2, Table 3, Fig. 3A). In the absence of SugR, LdhA activities were about eight fold higher than in *C. glutamicum* WT (Fig. 3A), which was associated with a three fold increased L-lactate formation on glucose medium under oxygen-deprivation conditions (Fig. 4). As the glycolytic intermediates glucose-6-phosphate, fructose-6-phosphate, fructose-1,6-bisphosphate or fructose-1-phosphate interfere with SugR binding to its target promoters (19, 21), SugR control of *ldhA* ensures that *ldhA* expression is maximal under oxygen-deprivation conditions only if supply of carbohydrate growth substrates entering glycolysis is sufficient.

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Regulation of L-Lactate Utilization by the FadR-Type Regulator LldR of *Corynebacterium glutamicum*▽

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Corynebacterium glutamicum **can grow on L-lactate as a sole carbon and energy source. The NCgl2816-*lldD* operon encoding a putative transporter (NCgl2816) and a quinone-dependent L-lactate dehydrogenase (LldD) is required for L-lactate utilization. DNA affinity chromatography revealed that the FadR-type regulator LldR (encoded by NCgl2814) binds to the upstream region of NCgl2816-*lldD*. Overexpression of *lldR* resulted in strongly reduced NCgl2816-*lldD* mRNA levels and strongly reduced LldD activity, and as a consequence, a severe growth defect was observed in cells grown on L-lactate as the sole carbon and energy source, but not in cells grown on glucose, ribose, or acetate. Deletion of *lldR* had no effect on growth on these carbon sources but resulted in high NCgl2816-*lldD* mRNA levels and high LldD activity in the presence and absence of L-lactate. Purified His-tagged LldR bound to a 54-bp fragment of the NCgl2816-*lldD* promoter, which overlaps with the transcriptional start site determined by random amplification of cDNA ends-PCR and contains a putative operator motif typical of FadR-type regulators, which is $^{-1}$TNGTNNNACNA^{10}. Mutational analysis revealed that this motif with hyphenated dyad symmetry is essential for binding of LldD to the NCgl2816-*lldD* promoter. L-Lactate, but not D-lactate, interfered with binding of LldR^{His} to the NCgl2816-*lldD* promoter. Thus, during growth on media lacking L-lactate, LldR represses expression of NCgl2816-*lldD*. In the presence of L-lactate in the growth medium or under conditions leading to intracellular L-lactate accumulation, the L-lactate utilization operon is induced.**

Lactate is a major product of anaerobic metabolism, but it also serves as a carbon and energy source for anaerobic and aerobic microorganisms. Lactate can be fermented to acetate, propionate, or butyrate by, e.g., sulfate-reducing bacteria, propionibacteria, or *Eubacterium hallii* (11). When oxygen becomes available but glucose is limiting, *Lactobacillus plantarum* converts its fermentation product, lactate, to acetate (18). Aerobic growth with L-lactate as the sole carbon and energy source has been studied in *Escherichia coli* in some detail. L-Lactate is taken up into the *E. coli* cell either by the L-lactate permease LldP or by the glycolate permease GlcA (39). L-Lactate is oxidized to the central metabolite pyruvate by quinone-dependent L-lactate dehydrogenase (LldD; EC 1.1.2.3) (10). For growth on L-lactate, *E. coli* requires *lldD*, which forms an operon with *lldP* and the putative lactate regulator gene *lldR* (10). Transcription of *lldDRP* is repressed by ArcAB under anaerobic reducing conditions (24) and is maximal in the presence of L-lactate (10); however, regulation of *lldDRP* by the putative regulator LldR encoded in this operon has not been analyzed yet in detail.

Recently, we identified the L-lactate utilization operon in *Corynebacterium glutamicum*, a nonpathogenic gram-positive soil bacterium that is widely used for biotechnological production of amino acids such as L-glutamate and L-lysine. *C. glutamicum* can grow aerobically on a variety of sugars, sugar alcohols, and organic acids, including L-lactate, as sole carbon and energy sources (9, 17, 27, 31, 36, 59). *C. glutamicum* forms L-lactate with the soluble NAD^+-dependent L-lactate dehydrogenase (EC 1.1.1.27) encoded by *ldhA* (3, 22) under oxygen deprivation (22) and as a by-product during glutamate and lysine production (27, 28, 53). For L-lactate utilization, on the other hand, *C. glutamicum* requires the quinone-dependent L-lactate dehydrogenase LldD (EC 1.1.2.3) (53), which is a peripheral membrane protein (51) catalyzing oxidation of L-lactate to pyruvate (3, 53).

The *C. glutamicum* L-lactate utilization operon comprises the quinone-dependent L-lactate dehydrogenase gene *lldD* and a gene encoding a putative permease (NCgl2816), and its expression is maximal in the presence of L-lactate (53). *C. glutamicum* reutilizes L-lactate formed during glutamate production in the presence of glucose (53) and coutilizes L-lactate with glucose when it is grown on glucose–L-lactate mixtures. Coutilization of glucose with acetate (57), propionate (5), protocatechuate and vanillate (35), serine (37), and fructose (8) has also been observed, while *C. glutamicum* utilizes glucose before it utilizes glutamate and ethanol (2, 31). During coutilization of glucose and L-lactate, the specific activity of the quinone-dependent L-lactate dehydrogenase LldD was almost as high as it was with L-lactate alone, while it was about sevenfold lower with glucose as a sole carbon source (53). The apparent absence of glucose repression and the approximately 17-fold-higher levels of mRNA of NCgl2816-*lldD* during growth on L-lactate than during growth on pyruvate as a sole carbon and energy source as determined by transcriptome analyses (53) suggest that the NCgl2816-*lldD* operon is subject to L-lactate-specific regulation. However, a putative regulatory

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source and/or reference
E. coli strains		
DH5α	F$^-$ *thi-1 endA1 hsdr17*(r_K^- m_K^-) *supE44* Δ*lacU169* (Φ80*lacZ*Δ*M15*) *recA1 gyrA96 relA1*	Bethesda Research Laboratories (20)
BL21(DE3)	*ompT hsdS*$_B$(r_B^- m_B^-) *gal dcm* (DE3)	54
C. glutamicum strains		
WT	Wild-type strain ATCC 13032, auxotrophic for biotin	1
WTΔ*lldR*	In-frame deletion of *lldR* gene	This study
Plasmids		
pK19*mobsacB*	Kan^r, *E. coli*/*C. glutamicum* shuttle vector for construction of insertion and deletion mutants in *C. glutamicum* (pK18 *ori*$V_{E.c.}$ *sacB lacZ*α)	49
pK19*mobsacB*-Δ*lldR*	Kan^r, pK19*mobsacB* with deletion construct of the *lldR* gene	This study
pVWEx1	Kan^r, P_{tac} *lacI*^{q}	43
pVWEx1-*lldR*	Kan^r, pVWEx1 with a 761-bp fragment of the *lldR* gene and an artificial ribosome-binding site	This study
pET16b	Amp^r, overproduction of proteins with an N-terminal decahistidine tag in *E. coli* (pBR322 *ori*$V_{E.c.}$ P_{T7} *lacI*)	Novagen
pET16b-*lldR*^{His}	Amp^r, pET16b with a 707-bp fragment of the *lldR* gene	This study

gene is not present in the *C. glutamicum* NCgl2816-*lldD* lactate utilization operon. Here, we identified a previously unknown FadR-type regulator of the NCgl2816-*lldD* operon, which we designated LldR, and characterized its role in L-lactate-dependent regulation of NCgl2816-*lldD* expression.

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions. All strains and plasmids used in this work are listed in Table 1. *C. glutamicum* type strain ATCC 13032 (30) was used as the wild type (WT). For growth experiments, determination of the LldD activity, and DNA microarray experiments, the first preculture, in 70 ml Luria-Bertani (LB) medium (48), was inoculated from a fresh LB agar plate and incubated in a 500-ml baffled shake flask at 30°C and 120 rpm. For DNA microarray experiments, cells were washed in culture medium without any carbon source, and the second preculture and the main culture were inoculated to obtain optical densities at 600 nm of 0.1 and 0.5, respectively, in 60 ml CgXII minimal medium (26), which contained 0.03 g/liter protocatechuic acid and 0.2 mg/liter biotin. For growth experiments and determination of the LldD activity, the main culture was inoculated to obtain an optical density of 1. The following compounds were used as carbon and energy sources: 200 mM glucose, 200 mM potassium acetate, 200 mM sodium pyruvate, 200 mM sodium L-lactate, 100 mM ribose, 50 mM fructose plus 100 mM sodium L-lactate, and 50 mM glucose plus 100 mM sodium L-lactate. Media contained 50 μg/ml kanamycin, 50 μg/ml ampicillin, or 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG), when appropriate. For all cloning experiments, *E. coli* DH5α (20) was used as the host and was cultivated aerobically at 37°C.

Recombinant DNA experiments. The enzymes used for recombinant DNA work were obtained from Roche Diagnostics (Mannheim, Germany). The oligonucleotides were obtained from Operon (Cologne, Germany) or MWG (Ebersberg, Germany). Standard methods, including PCR, restriction, or ligation, were carried out as described by Sambrook and Russell (48). Plasmids were isolated from *E. coli* with a QIAprep spin miniprep kit (Qiagen, Hilden, Germany). *E. coli* was transformed by the RbCl method (19), and *C. glutamicum* was transformed by electroporation (55) using the following conditions: 25 μF, 600 Ω, and 2.5 kV/cm (Bio-Rad Gene Pulser Xcell; Bio-Rad Laboratories, Hercules, CA). After electroporation, 4 ml LB medium was immediately added to the sample. After a heat shock at 46°C for 6 min, the cells were incubated at 30°C for 50 min to regenerate before they were plated. DNA sequencing was performed by Agowa GmbH (Berlin, Germany).

Construction of an *lldR* deletion mutant. An in-frame *lldR* (NCgl2814) deletion mutant of *C. glutamicum* was constructed by a two-step homologous recombination procedure as described previously (38). The *lldR* up- and downstream regions (~450 bp each) were amplified using the oligonucleotide pairs *lldR*-A/*lldR*-B and *lldR*-C/*lldR*-D. The PCR products served as templates for crossover PCR performed with oligonucleotides *lldR*-A and *lldR*-D. The resulting ~0.9-kb PCR product was restricted with SphI and XbaI and cloned into SphI/XbaI-restricted plasmid pK19mob*sacB*. After DNA sequence analysis of the resulting plasmid, pk19mob*sacB*-Δ*lldR*, confirmed that the cloned PCR product did not contain mutations, the plasmid was transferred into *C. glutamicum* by electroporation. Screening for the first and second recombination events was performed as described previously (38). Kanamycin-sensitive and sucrose-resistant clones were tested by PCR analysis of chromosomal DNA with the primer pair *lldR*-0/*lldR*-1. Clones that had the desired in-frame deletion of the *lldR* gene, in which all of the nucleotides except the first 6 codons and the last 12 codons were replaced by a 21-bp tag, had an 0.93-kb PCR fragment instead of the 1.6-kb PCR fragment obtained with wild-type DNA.

Overproduction and purification of LldR^{His}. *E. coli* BL21(DE3) carrying plasmid pET16b-*lldR* was grown at 37°C in 500 ml of LB medium with 50 μg/ml ampicillin to an optical density at 600 nm of 0.6 before 1 mM IPTG was added. After cultivation for another 4 h at room temperature, cells were harvested by centrifugation (10 min, 11,325 × *g*, 4°C), washed in 20 ml TNI5 buffer (20 mM Tris-HCl [pH 7.9], 300 mM NaCl, 5% [vol/vol] glycerol, 5 mM imidazole), and stored at −20°C. For preparation of cell extracts, thawed cells were resuspended in 10 ml of TNI5 buffer containing 1 mM diisopropylfluorophosphate and 1 mM phenylmethylsulfonyl fluoride. The cell suspension was passed five times through a French pressure cell (SLM Aminco, Spectronic Instruments, Rochester, NY) at 1,800 lb/in^2. Cell debris and intact cells were removed by centrifugation (20 min, 5,292 × *g*, 4°C). The cell extract was then subjected to ultracentrifugation (1 to 1.5 h, 150,000 × *g*, 4°C). After ultracentrifugation, the supernatant was purified by nickel affinity chromatography using nickel-activated nitrilotriacetic acid-agarose (Novagen, San Diego, CA). The column was washed with TNI175 buffer (which contained 175 mM imidazole). Then the LldR^{His} protein was eluted with TNI400 buffer (which contained 400 mM imidazole). Dominant protein-containing fractions were pooled, and the elution buffer was exchanged against BS buffer (100 mM Tris-HCl, 20% [vol/vol] glycerol, 100 mM KCl, 20 mM $MgCl_2$, 1 mM EDTA; pH 7.5) using PD10 columns.

Quinone-dependent L-lactate dehydrogenase assay. For determination of enzyme activities, exponentially growing cells were harvested by centrifugation (4,500 × *g*, 5 min, 4°C), and crude extracts were prepared as described previously by Stansen et al. (53). The quinone-dependent L-lactate dehydrogenase activity was measured using a spectrophotometric assay mixture containing 100 mM KH_2PO_4 (pH 7.5), 0.05 mM 2,6-dichloroindophenol, and an appropriate amount of crude extract. The assay was started by addition of 20 mM L-lactate at 30°C, and the decrease in absorbance of 2,6-dichloroindophenol (ε_{600} = 20 mM^{-1} cm^{-1}) was determined.

Determination of the transcriptional start site. The transcriptional start site of the NCgl2816-*lldD* operon was determined by random amplification of cDNA ends (RACE)-PCR using a 5′/3′ second-generation RACE kit (Roche, Mannheim, Germany) as recommended by the manufacturer. The primers used were 2816-RT for reverse transcription, 2816-PCR1 for the first PCR, and 2816-PCR2 for the nested PCR.

TABLE 2. Oligonucleotides used in this study

Primer	Sequence (5′→3′)[a]	Use
Overexpression		
310-Kom-for	GCGTCGACAAGGAGATATAGAT**ATG**AGTGTGAAAGCAC ATGAATC (SalI)	Amplification of NCgl2814 (forward)
310-Kom-rev	GCGTCGACCGTGTAGATCTGAAACCGC (SalI)	Amplification of NCgl2814 (reverse)
310-His-for	GCCAT**ATG**AGTGTGAAAGCACATGAATCTGTC (NdeI)	Amplification of NCgl2814 for purification (forward)
310-His-rev	GCGTCGACTTAGGCCTCGGCGGCAG (SalI)	Amplification of NCgl2814 for purification (reverse)
Deletion of NCgl2814		
310-A	GCGCATGCAGGTTCCGCGGATAAACAG (SphI)	Amplification of the left internal fragment (forward)
310-B	**CCCATCCACTAAACTTAAACA**TTCATGTGC TTTCACACTCA TTAG	Amplification of the left internal fragment (reverse)
310-C	**TGTTTAAGTTTAGTGGATGGG**GGCTACTAC GAAGAAACCG	Amplification of the right internal fragment (forward)
310-D	GCTCTAGATTGTTTCGCGGTGAGGC (XbaI)	Amplification of the right internal fragment (reverse)
310-0	TCAAAGCTTTCAACGTGCC	Proof of deletion (forward)
310-1	GCTAGTTCGTCGTCTAGC	Proof of deletion (reverse)
Gel shift		
312-F1	GCCACGTGGAGGATCCTTTGGG (BamHI)	Amplification of fragments F0 and F1 (forward)
312-R3	CTGCCACTCGAGCTCCCCAGC (SacI)	Amplification of fragments F0 and F5 (reverse)
312-R1	CCCATTTAAGCAACAGAGTTAGTTAATC	Amplification of fragment F1 (reverse)
312-F12	GCGTCCGTGGCCGTTTCC	Amplification of fragment F2 (forward)
312-R21	GGCGTGTCACCTTTAATTGTCAATGG	Amplification of fragments F2 and F12 (reverse)
312-F2	GGTAATTGGATTCGACTGTTTTCC	Amplification of fragment F3 (forward)
312-R2	TAAACGGGCTGAAACCGATTGG	Amplification of fragment F3 (reverse)
312-F22	TTACATTCTTGTGGTCTGACCATG	Amplification of fragment F4 (forward)
312-R31	TTTTGATCTACTGCGGTTGTCATG	Amplification of fragments F4, F4WT, F4M1, F4M2, F4M3, F4M4, F4M5, F4M6, and F4M12 (reverse)
312-F3	TCCCGCCGTCCGTTTCAGAGAAGAGG	Amplification of fragment F5 (forward)
0430-for	GAAAGCTCAGAAGAAGGTCCAGAG	Amplification of control fragment 0430 (forward)
0430-rev	GCTGGATGGGATAACGGAGGTC	Amplification of control fragment 0430 (reverse)
312-F4-WT	TTACATTCTTGTGGTCTGACCATGAGGTTGGG	Amplification of fragment F4WT (forward)
312-F4-Mut1	TTACATTCTTGGGTGCTGACCATGAGGTTGGG	Amplification of fragment F4M1 (forward)
312-F4-Mut2	TTACATTCTTGTGGTCTGCACGTGAGGTTGGG	Amplification of fragment F4M2 (forward)
312-F4-Mut12	TTACATTCTTGGGTGCTGCACGTGAGGTTGGG	Amplification of fragment F4M12 (forward)
312-F4-Mut3	TGCAATTCTTGGGTGCTGCACGTGAGGTTGGG	Amplification of fragment F4M3 (forward)
312-F4-Mut4	TTACCAACTTGGGTGCTGCACGTGAGGTTGGG	Amplification of fragment F4M4 (forward)
312-F4-Mut5	TTACCAACTTGGGTGCTGCACGTGAGTGGGGGCCAATCG	Amplification of fragment F4M5 (forward)
312-F4-Mut6	TTACATTCTTGGGTGCTGCACGTGAGGTTTTTCCAATCG GTTC	Amplification of fragment F4M6 (forward)
RACE-PCR		
2816-RT	CGCCACCCATGAGCATCAAGG	Primer for reverse transcription of NCgl2816
2816-PCR1	GATGTGGGCGAAGATGAC	PCR primer 1 for NCgl2816
2816-PCR2	CAGGGAGAAGGATGCGTACG	Nested PCR primer for NCgl2816

[a] Restriction sites are underlined, and the restriction enzymes are indicated in parentheses. The overlapping complementary sequences for crossover PCR and start codons are indicated by bold type.

Gel shift assays. Gel shift assays with $LldR^{His}$ were performed as described previously (58). Briefly, purified $LldR^{His}$ (at concentrations ranging from 0 to 2.4 μM) was mixed with the full-length promoter of NCgl2816-*lldD* fragment F0 or promoter subfragments F1 to F5 in a 20-μl (total volume) mixture that contained 50 mM Tris-HCl, 10% (vol/vol) glycerol, 50 mM KCl, 10 mM $MgCl_2$, and 0.5 mM EDTA (pH 7.5). Then a nontarget promoter fragment was added at a concentration of 40 to 46 nM as a negative control. The full-length promoter of NCgl2816-*lldD* covering the region from position −252 to position 79 relative to the translational start was obtained by performing PCR with the primers listed in Table 2. After incubation for 30 min at room temperature, the samples were separated on a 10% native polyacrylamide gel at room temperature and 170 V (constant voltage) using 1× Tris-borate-EDTA (89 mM Tris base, 89 mM boric acid, 2 mM EDTA; pH 8.3) as the electrophoresis buffer. The gels were subsequently stained with SYBR green I according to the instructions of the supplier (Sigma, Rödermark, Germany) and photographed. To test for possible effectors, the protein was incubated with glucose-6-phosphate, fructose-6-phosphate, fructose-1,6-bisphosphate, phosphoenolpyruvate, pyruvate, L-lactate, D-lactate, and acetyl-coenzyme (acetyl-CoA) (20 mM each) in the binding buffer for 15 min before promoter DNA fragment F0 was added and the mixture was incubated for an additional 30 min. All PCR products used in the gel shift assays were purified with a PCR purification kit (Qiagen, Hilden, Germany) and eluted in 10 mM Tris-HCl (pH 8.5).

DNA microarray analysis. Generation of *C. glutamicum* whole-genome DNA microarrays (56), synthesis of fluorescently labeled cDNA from total RNA, microarray hybridization, washing, and gene expression analysis were performed as described previously (23, 32, 33, 44). Genes that exhibited significantly changed mRNA levels (changed by at least a factor of two; $P \leq 0.05$, as determined by Student's *t* test) were determined in two different DNA microarray experiments performed with RNA isolated from two independent cultures in CgXII minimal medium.

Affinity chromatography. Enrichment of DNA-binding proteins interacting with the upstream regions of NCgl2816-*lldD* was performed as described previously (13). A 331-bp NCgl2816 promoter fragment was amplified by PCR using genomic DNA from *C. glutamicum* and oligonucleotides 312-F1 and 312-R3, one of which (312-F1) was tagged with biotin via a TEG linker (Operon, Cologne, Germany). Proteins that bound nonspecifically were washed off with TGED buffer (50 mM Tris-HCl [pH 7.6], 1 mM dithiothreitol, 10 mM $MgCl_2$, 1 mM EDTA, 10% [vol/vol] glycerol, 10 μM phenylmethylsulfonyl fluoride) containing 400 μg chromosomal DNA, and specifically bound proteins were subsequently eluted with TGED buffer containing 2 M NaCl. The proteins present in the high-salt eluate were separated on 10% sodium dodecyl sulfate (SDS)-polyacrylamide gels and subsequently analyzed by matrix-assisted laser desorption ionization—time of flight (MALDI-TOF) mass spectrometry (see below).

MALDI-TOF mass spectrometry. For peptide mass fingerprinting, a protein band of interest was excised from colloidal Coomassie blue-stained gels and subjected to in-gel digestion with trypsin essentially as described previously (50). Data acquisition and analysis were performed using a Voyager DE-STR mass spectrometer (Applied Biosystems, Weiterstadt, Germany), Voyager Control Panel software (version 5.0), and Voyager Data Explorer software (version 3.5) as described previously (50).

RESULTS

Identification of LldR as a protein binding to the upstream region of the NCgl2816-*lldD* operon. To identify a transcriptional regulator(s) of the L-lactate utilization operon NCgl2816-*lldD*, which appears to be regulated by L-lactate (53), proteins specifically binding to its upstream region were enriched by DNA affinity chromatography. A 331-bp biotinylated promoter DNA probe (positions −251 to 80 relative to the NCgl2816 start codon) was linked to streptavidin-coated magnetic beads and incubated with crude extracts from *C. glutamicum* grown on minimal medium containing L-lactate as the sole carbon source. After washing with TGED buffer containing 400 μg of genomic DNA from *C. glutamicum* as a competitor, specifically bound proteins were eluted with buffer containing 2 M NaCl and identified by peptide mass fingerprint analysis as described previously (4, 13, 32). Among these proteins was a putative transcriptional regulator (Fig. 1A), which was designated LldR and was subsequently shown to regulate the NCgl2816-*lldD* operon. The *lldR* gene (corresponding to NCgl2814) is located close to the NCgl2816-*lldD* operon and is separated only by NCgl2815 encoding a hypothetical protein. As deduced from the gene sequence, LldR consists of 213 amino acids and has a predicted molecular mass of 25.1 kDa, which corresponds well with the apparent mass observed by SDS-polyacrylamide gel electrophoresis (PAGE) (Fig. 1). Its N terminus contains a predicted helix-turn-helix domain for DNA binding, and over its entire length LldR shares similarities with FadR-type regulators, a subfamily of GntR-type regulators (47) that belongs to the cluster of orthologous genes COG2186.

Binding of purified $LldR^{His}$ to the NCgl2816-*lldD* promoter. First, the transcriptional start site of the NCgl2816-*lldD* operon was identified by RACE-PCR. Transcription of the NCgl2816-*lldD* operon starts with a G which is located 73 bp upstream of the ATG start codon. The promoter contains a TACATT motif

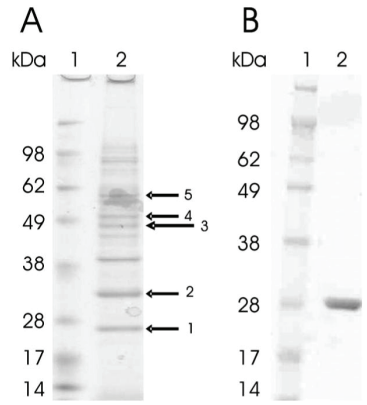

FIG. 1. (A) SDS-PAGE of *C. glutamicum* proteins eluted in a DNA affinity chromatography experiment with TGED buffer containing 2 M NaCl using the NCgl2816-*lldD* promoter as a probe and cell extracts from L-lactate-grown cells of *C. glutamicum* WT (lane 2). MALDI-TOF mass spectrometry of tryptic peptides from the protein bands revealed LldR (arrow 1), RamA (NCgl2472) (arrow 2), a subunit of DNA polymerase I (NCgl1299) (arrow 3), a subunit of DNA polymerase III (NCgl2035) (arrow 4), and a subunit of a putative restriction nuclease (NCgl1705) (arrow 5). The prominent band between arrows 2 and 3 could not be identified. Lane 1 contained protein standards. (B) SDS-PAGE of purified LldR. Lane 2 contained purified LldR with a His tag, and lane 1 contained protein standards.

(from position −11 to position −6) (Fig. 2A), which closely resembles the consensus −10 hexamer TA(C/T)AAT of *C. glutamicum* promoters (41), as well as the hexamer TTGACA (from position −36 to position −31), which is similar to the consensus −35 region (TTGCCA).

To characterize the binding of LldR to the upstream region of NCgl2816-*lldD*, the LldR protein containing an amino-terminal His tag was overproduced in *E. coli* and purified to near homogeneity by nickel chelate chromatography (see Materials and Methods) (Fig. 1B). For gel shift assays, DNA fragments (24 nM) were incubated with various concentrations of the $LldR^{His}$ protein (0 to 2.4 μM) and then separated on 10% polyacrylamide gels. $LldR^{His}$ bound to the upstream region of the NCgl2816-*lldD* operon with high affinity as a 10-fold molar excess of $LldR^{His}$ protein resulted in a complete gel shift (Fig. 2B). Two LldR-DNA complexes having different gel mobilities were observed. In contrast, $LldR^{His}$ did not bind to a negative control DNA, the promoter fragment of NCgl0430 (encoding an uncharacterized putative transcriptional regulator).

Gel shift assays with different and partially overlapping subfragments (72 nM) of the NCgl2816-*lldD* upstream region allowed confinement of the LldR binding site(s) to a region from position −13 to position 41 relative to the transcriptional start site of NCgl2816 (Fig. 2C). Subfragments F1, F2, and F5 were not bound by LldR (11-fold molar excess), whereas an LldR-DNA complex was formed after incubation of LldR with subfragments F3 and F4. These data indicate that the overlapping region of subfragments F3 and F4 (positions −13 to 41) contains the binding site of LldR. By comparing known or putative operator sites of several FadR-type regulators, Rigali et al. (47) postulated that TNGTNNNACNA is the consensus operator motif for FadR-type regulators. This motif with hyphen-

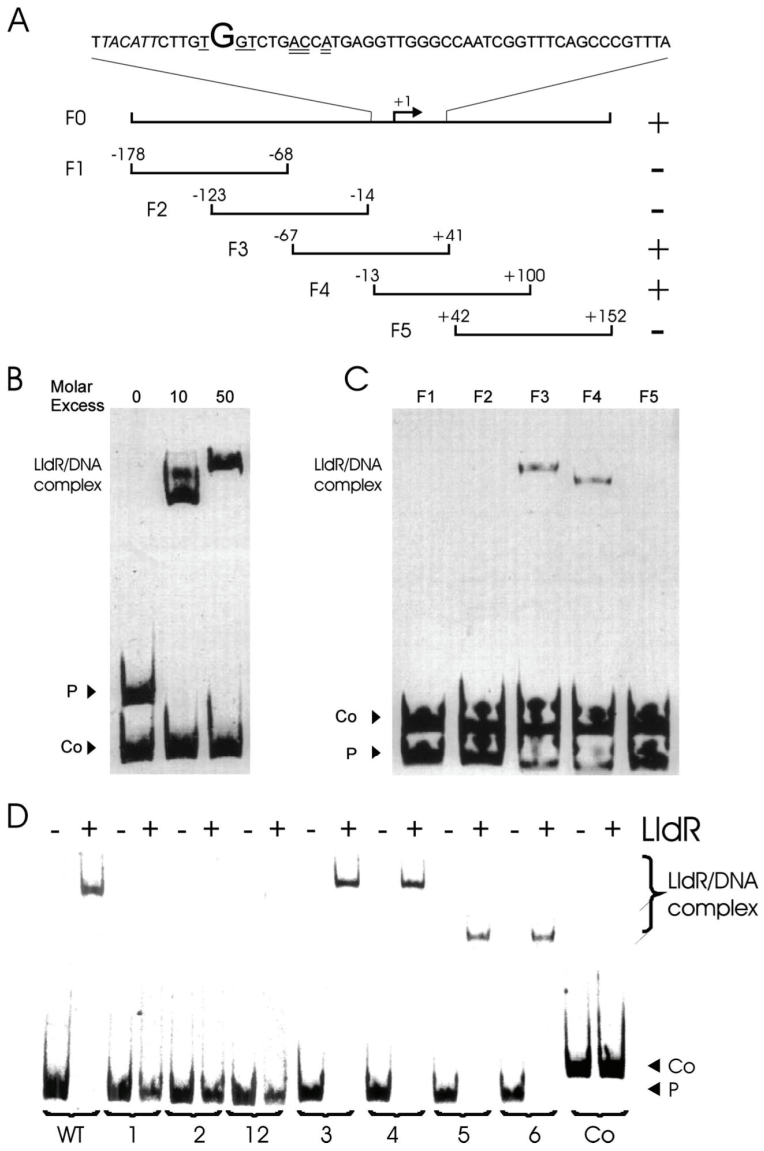

FIG. 2. Binding of LldR to the NCgl2816-*lldD* promoter. (A) DNA fragments used to analyze $LldR^{His}$ binding to the NCgl2816-*lldD* promoter region. The numbers indicate the ends of the fragments relative to the NCgl2816 transcriptional start site (+1). Binding of LldR to the fragments is indicated by a plus sign, and a lack of binding is indicated by a minus sign. Oligonucleotides used for amplification of the six fragments are listed in Table 2. The sequence above the fragments shows the region between position −13 and position 41 relative to the transcriptional start site. The transcriptional start is indicated by a large letter, the −10 region is italicized, and the half-sites of the consensus operator sequence for FadR-type regulators are underlined (single and double underlining). (B) Purified His-tagged LldR protein was used in 0-, 10- and 50-fold molar excess over DNA fragment F0 (P) before separation by native PAGE and SYBR green I staining. A 175-bp fragment of the NCgl0430 (43 nM) promoter served as a negative control DNA fragment (Co). (C) Subfragments F1, F2, F3, F4, and F5 of NCgl2816-*lldD* promoter fragment F0 were incubated with an 11-fold molar excess of purified LldR, separated by PAGE, and stained with SYBR green I. A 190-bp fragment of the NCgl2027 promoter (40 nM) served as a control fragment (Co). (D) Subfragment F4 and derived fragments with different mutations in or near the consensus sequence for FadR-type regulators M1, M2, M12, M3, M4, M5, and M6 (P) were incubated with an 11-fold molar excess of $LldR^{His}$. Lanes WT, wild type; lanes 1, the nucleotides in panel A underlined with one line were changed by PCR from TGT to GTG (fragment M1); lanes 2, the nucleotides in panel A underlined with two lines were changed by PCR from ACA to TAG (fragment M2); lanes 12, all the underlined nucleotides in panel A were changed by PCR (fragment 12). Changes outside the consensus sequence were introduced into fragments M3 (7 bp upstream; TCA → GCA) (lanes 3), M4 (4 bp upstream; ATT → CAA) (lanes 4), M5 (3 bp downstream; GTT → TGG) (lanes 5), and M6 (7 bp downstream; GGG → TTT) (lanes 6). A PCR product from position −178 to position −14 relative to the NCgl2816-*lldD* transcriptional start site (46 nM) served as a negative control (lanes Co). Oligonucleotides used for amplification of the fragments are listed in Table 2.

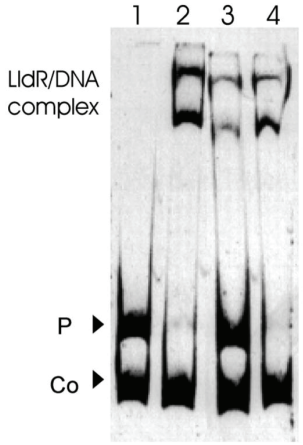

FIG. 3. Binding of LldR in the presence of D-lactate or L-lactate. The 331-bp F0 fragment (24 nM) of the NCgl2816-*lldD* promoter was incubated without protein (lane 1) or with a 20-fold molar excess of purified LldR in the absence of an effector (lane 2), in the presence of 40 mM L-lactate (lane 3), or in the presence of 40 mM D-lactate (line 4). A 175-bp promoter fragment of NCgl0430 (43 nM) served as a negative control (Co).

ated dyad symmetry is present in the LldR-binding region at positions −1 to 10 (Fig. 2A). To test whether the putative consensus operator motif plays a role in binding of LldR to the NCgl2816-*lldD* promoter, we performed gel shift assays with subfragment F4 and three derived variants containing mutations in the left and/or right putative operator half-sites. In mutant M1, the left three conserved nucleotides of the inverted repeat (Fig. 2A) were changed from TNGT to GNTG, while in mutant M2 the right three conserved nucleotides of the inverted repeat (Fig. 2A) were changed from ACNA to CANG. Mutant M12 had these changes in both half-sites (Fig. 2D). In gel shift assays, wild-type subfragment F4 was completely shifted by LldR at a 10-fold molar excess, whereas the mutations in both half-sites of the putative consensus motif for FadR-type regulators described above abolished the formation of an LldR-DNA complex (Fig. 2D). Mutations outside this motif (mutants M3 to M6) did not affect binding of LldR (Fig. 2D, lanes 3 to 6). The data reveal that LldR binds to the motif TGGTCTGACCA in the promoter region of the NCgl2816-*lldD* operon and that both the TNGT nucleotides at positions −1, 2, and 3 and the ACNA nucleotides at positions 7, 8, and 10 are essential for this interaction.

L-Lactate prevents binding of LldR^{His} to the NCgl2816-*lldD* promoter region. As expression of the NCgl2816-*lldD* operon is maximal when L-lactate is present in the medium and as the binding affinity of FadR-type regulators can be modulated by an effector molecule, whether binding of LldR to the NCgl2816-*lldD* promoter region was affected by intermediates of the central carbon metabolism was tested. To do this, the purified LldR^{His} protein was incubated with the putative effectors at a concentration of 20 mM for 15 min before addition of NCgl2816-*lldD* promoter fragment F0 (24 nM), and after further incubation for 30 min free DNA and protein-DNA complexes were separated on 10% nondenaturing polyacrylamide gels. The presence of 20 mM phosphoenolpyruvate, glucose-6-phosphate, fructose-6-phosphate, fructose-1,6-bisphosphate, pyruvate, or acetyl-CoA had no effect on the affinity of binding of LldR^{His} to promoter fragment F0 (data not shown). However, 40 mM L-lactate (and to a lesser extent 20 mM L-lactate [data not shown]) prevented binding of LldR^{His} to the DNA region upstream of NCgl2816-*lldD* (Fig. 3, lane 3), while 40 mM D-lactate did not prevent this binding (lane 4). Thus, L-lactate could be identified as an inducer of LldR.

Effects of inactivation and overexpression of *lldR* on growth and LldD activity. For functional analysis of the *lldR* gene, an in-frame deletion mutant was constructed by two-step homologous recombination. In the resulting mutant, WTΔ*lldR*, the whole *lldR* coding region except the 6 5′-terminal codons and 12 3′-terminal codons was replaced by a 21-bp tag (see Materials and Methods). For IPTG-inducible overexpression of the *lldR* gene, the gene was cloned into the *E. coli*/*C. glutamicum* shuttle vector pVWEx1. There were no significant differences in growth rate and biomass formation between *C. glutamicum* strains WT(pVWEx1), WTΔ*lldR*(pVWEx1), and WTΔ*lldR*(pVWEx1-*lldR*) in minimal medium containing glucose, pyruvate, acetate, or ribose as the sole carbon source (Fig. 4 and data not shown).

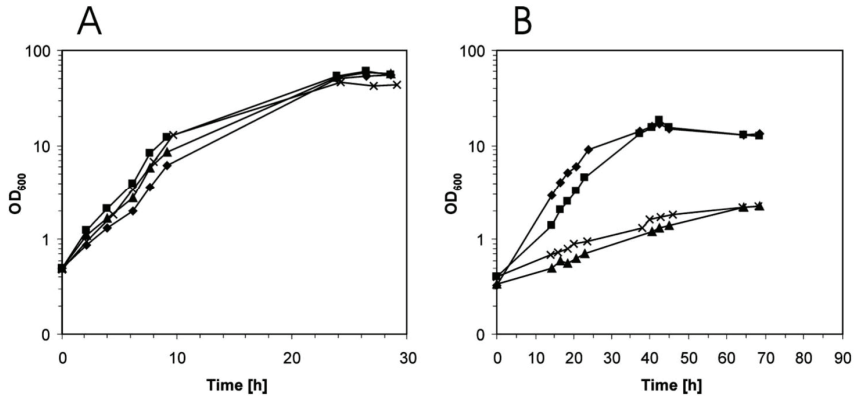

FIG. 4. Growth of *C. glutamicum* strains WT(pVWEx1) (■), WTΔ*lldR*(pVWEx1) (◆), WT(pVWEx1-*lldR*) (×), and WTΔ*lldR*(pVWEx1-*lldR*) (▲) on minimal medium with 200 mM glucose (A) or 200 mM sodium L-lactate (B). IPTG (1 mM) was added immediately after inoculation.

TABLE 3. Specific activities of the quinone-dependent L-lactate dehydrogenase in *C. glutamicum* strains WT(pVWEx1), WTΔ*lldR*(pVWEx1), and WTΔ*lldR*(pVWEx1-*lldR*)

Carbon source(s)	Sp act of quinone-dependent L-lactate dehydrogenase LldD (μmol min^{-1} mg^{-1})[a]		
	WT (pVWEx1)	WTΔ*lldR* (pVWEx1)	WTΔ*lldR* (pVWEx1-*lldR*)
L-Lactate	0.14	0.08	<0.01
Glucose + L-lactate	0.15	0.12	0.01
Glucose	0.01	0.13	0.01
Pyruvate	0.01	0.10	0.01
Acetate	0.01	0.16	<0.01
Ribose	0.02	0.14	<0.01

[a] All data are mean values of at least two determinations for at least two independent cultures with errors of <14%. The cultures contained 1 mM IPTG.

However, when 200 mM L-lactate was the sole carbon source, the growth of *C. glutamicum* WTΔ*lldR*(pVWEx1-*lldR*) and WT (pVWEx1-*lldR*) was perturbed as the growth rates (0.04 and 0.03 h^{-1}, respectively) and biomass formation were reduced compared to those of *C. glutamicum* WT(pVWEx1) and WTΔ*lldR*(pVWEx1) (growth rates, 0.10 and 0.12 h^{-1}, respectively) (Fig. 4). A lag phase in lactate medium was observed for WT(pVWEx1) but not for WTΔ*lldR*(pVWEx1), which is consistent with the view that in the wild type some time is required for induction of the NCgl2816-*lldD* operon, while the operon is always derepressed in the *lldR* deletion mutant.

The specific activity of the quinone-dependent L-lactate dehydrogenase LldD was determined using crude extracts of *C. glutamicum* WT(pVWEx1), WTΔ*lldR*(pVWEx1), and WTΔ*lldR* (pVWEx1-*lldR*) grown on minimal medium with L-lactate, L-lactate plus glucose, glucose, pyruvate, acetate, or ribose. On media lacking L-lactate, the specific activities of LldD were low (0.01 to 0.02 μmol min^{-1} mg [dry weight]$^{-1}$) in *C. glutamicum* WT (pVWEx1), while they were 6- to 15-fold higher during growth on 200 mM L-lactate and 50 mM glucose plus 100 mM L-lactate (0.13 and 0.15 μmol min^{-1} mg^{-1}, respectively) (Table 3). In the strain lacking *lldR*, the specific activities of LldD were high on all media tested [7- to 16-fold higher than the specific activity in *C. glutamicum* WT(pVWEx1) on media lacking L-lactate] (Table 3). The finding that the specific activities of LldD were slightly higher in *C. glutamicum* WT(pVWEx1) grown on L-lactate and on glucose plus L-lactate than in the strain lacking *lldR* might indicate that an additional regulator(s) is involved (Table 3). Overexpression of *lldR* led to very low specific activities of LldD on all carbon sources tested even in the presence of L-lactate (Table 3).

DNA microarray analysis of the transcriptomes of *C. glutamicum* WT(pVWEx1), WTΔ*lldR*(pVWEx1), and WTΔ*lldR*(pVWEx1-*lldR*). In order to determine the effects of LldR on global gene expression, whole-genome DNA microarrays of *C. glutamicum* (56) were used to compare the mRNA levels of strains WT (pVWEx1), WTΔ*lldR*(pVWEx1), and WTΔ*lldR*(pVWEx1-*lldR*). In the absence of LldR, only the genes of the L-lactate-utilizing NCgl2816-*lldD* operon showed significantly increased mRNA levels (NCgl2816, 8.8-fold increased; and *lldD*, 6.8-fold increased). On the other hand, overexpression of *lldR* led to twofold decreases in the mRNA levels of NCgl2715 and *ldhA*, as well as to strongly decreased mRNA levels of *lldD* and NCgl2816 (25- and 11-fold decreased levels, respectively, compared to the control). However, as only *lldD* and NCgl2816 showed increased mRNA levels in the absence of LldR and decreased mRNA levels when *lldR* was overexpressed, LldR likely regulates only the NCgl2816-*lldD* operon for L-lactate utilization.

DISCUSSION

In this study we showed that the *C. glutamicum* protein LldR, which belongs to the FadR subfamily of GntR family regulators, represses expression of the NCgl2816-*lldD* operon. Homologs of LldR from *C. glutamicum* are encoded in the genomes of other *Corynebacterineae*, like *C. glutamicum* R (97% sequence identity; cgR_2816), *Corynebacterium efficiens* (76% sequence identity; CE2757), *Corynebacterium diphtheriae* (38% sequence identity; DIP0011), *Rhodococcus* sp. (42% sequence identity; RHA1_ro03478), and *Mycobacterium smegmatis* (42% sequence identity; MSMEG_0895), while other mycobacterial genomes apparently lack homologous genes. In addition to *Corynebacterineae*, LldR homologs also occur in species of other suborders of the *Actinomycetales*, like *Arthrobacter aurescens* (33% sequence identity; AAur_3797), *Saccharopolyspora erythraea* (37% sequence identity; SACE_3508), and *Nocardioides* sp. (37% sequence identity; Noca_2132). There is also considerable sequence identity between LldR from *C. glutamicum* and proteins from distantly related species, including clostridia like *Clostridium perfringens* (33% sequence identity; CPR_0301) or *Caulobacter crescentus* (32% sequence identity; CC_2813). LldR from *C. glutamicum* shares only 19 and 22% sequence identity with the proteins for which the regulator family and subfamily were named, GntR, the gluconate-responsive repressor of the gluconate operon of *Bacillus subtilis* (15), and FadR, the acyl-CoA-responsive regulator of fatty acid degradation and biosynthesis of *E. coli* (7, 40), respectively.

LldR (formerly LctR) from *E. coli*, a putative regulator of the L-lactate utilization operon of this bacterium (10), and LldR from *C. glutamicum* share 25% identical amino acids over the entire length, 42% identical amino acids in the N-terminal helix-turn-helix DNA-binding domain, and 26% identical amino acids in the first half of the C-terminal domain (amino acids 97 to 164 in *C. glutamicum* and amino acids 100 to 167 in *E. coli*), which typically is important for ligand binding in FadR-type regulators. A regulatory role for LldR from *E. coli* has been inferred only indirectly as anaerobic expression of an *lldD-lacZ* fusion was elevated when multiple copies of the region upstream of *lldP* were present (34). Binding of LldR from *E. coli* to the promoter region of the *lldPRD* operon has not been demonstrated experimentally, but it was postulated to involve a sequence similar to the binding site of PdhR from *E. coli* (46) and similar to the consensus sequence for FadR-type regulators, TNGTNNNACNA (47). Alternatively, PdhR, rather than LldR, could bind to this sequence and regulate *lldPRD* in response to pyruvate availability (34). The binding site of LldR of *C. glutamicum* could be identified experimentally by gel shift assays and mutational analysis. When binding of LldR from *C. glutamicum* to the promoter of NCgl2816-*lldD* was assayed, two LldR-DNA complexes were observed (Fig. 2). The LldR-DNA complex with higher gel mobility was dominant at lower molar excess of LldR. This might have been due either to a second binding event (although a sequence similar

TABLE 4. Genes showing at least twofold-altered mRNA levels in transcriptome comparisons of *C. glutamicum* WT(pVWEx1) with WTΔ*lldR*(pVWEx1) and WTΔ*lldR*(pVWEx1-*lldR*)

Gene (identifier)	Annotation	mRNA level relative to WT(pVWEx1)	
		WTΔ*lldR*(pVWEx1)[a]	WTΔ*lldR*(pVWEx1-*lldR*)[b]
NCgl2715	Sulfate adenyltransferase subunit	1.1	0.47
NCgl2737	Putative membrane protease subunit	0.28	0.25
NCgl2810 (*ldhA*)	LdhA, NAD^+-dependent L-lactate dehydrogenase	1.0	0.45
NCgl2814 (*lldR*)	LldR, repressor of the NCgl2816-*lldD* operon	0.15	24.9
NCgl2816	Transporter	8.8	0.04
NCgl2817 (*lldD*)	LldD, quinone-dependent L-lactate dehydrogenase	6.8	0.09

[a] The relative mRNA levels of strains WTΔ*lldR*(pVWEx1) and WT(pVWEx1) were compared during exponential growth on minimal medium containing 100 mM ribose.
[b] The relative mRNA levels of strains WTΔ*lldR*(pVWEx1-*lldR*) and WT(pVWEx1) were compared during exponential growth on minimal medium containing 50 mM fructose plus 100 mM L-lactate.

to the identified binding site could not be found) or to binding of a higher-order multimer of LldR (e.g., LldR tetramer rather than LldR dimer). The different gel mobilities of subfragments F3 and F4 (Fig. 2C) might be due to a small difference in length (subfragment F4 is 5 bp longer) and/or to the position of the LldR binding site within the fragments (more to the center in subfragment F3). The sequence motif upstream of NCgl2816, $^{-1}$TGGTCTGACCA^{10}, shows hyphenated dyad symmetry containing the two half-sites, TNGT and ACNA, of the consensus sequence for FadR-type regulators. Mutational analysis revealed that both half-sites are essential for binding of LldR to the NCgl2816-*lldD* promoter, while mutations outside this motif did not affect LldR binding. The motif overlaps the transcriptional start site of the NCgl2816-*lldD* operon, which is consistent with a repression mechanism involving interference with the RNA polymerase-promoter interaction.

The inducer of the *C. glutamicum* NCgl2816-*lldD* operon could be identified as L-lactate as this compound prevents binding of LldR to the NCgl2816-*lldD* promoter in vitro at a concentration of 40 mM, while, for comparison, 1 mM pyruvate abolished binding of the FadR-like regulator PdhR from *E. coli* to the promoter of the *pdhR-aceEF-lpd* operon (46). However, detection of intracellular L-lactate concentrations of 32 to 39 mM in glucose-grown *C. glutamicum* ATCC 17965 cells (42) indicates that L-lactate affects LldR function at physiologically relevant concentrations. Besides L-lactate antagonizing repression by LldR, effectors for only two other transcriptional regulators of carbon metabolism are known in *C. glutamicum*: fructose-6-phosphate inhibits repression of the PTS genes *ptsG*, *ptsS*, and *ptsF* by SugR (14), and cyclic AMP is required for repression of the malate synthase gene *aceB* by GlxR (29). Regulation of the NCgl2816-*lldD* operon by a mechanism other than L-lactate via LldR (e.g., by oxygen availability or pH) has not been studied yet, but it was observed that in long-term lactic acid-adapted *C. glutamicum* cells grown in continuous culture at pH 5.7, the mRNA levels of NCgl2816 and *lldD* were not changed compared to the levels in continuous cultures at pH 7.5 (25). The finding that RamA binds to the promoter of the NCgl2816-*lldD* operon (Fig. 1) suggests that RamA represses or activates this operon. Whether RamA is indeed involved in regulation of NCgl2816-*lldD* remains to be studied.

In *C. glutamicum*, L-lactate may accumulate in the medium at concentrations up to >200 mM under oxygen deprivation conditions (22). Transient accumulation of L-lactate in the medium can be observed during growth on glucose (45) and to a greater extent during growth on fructose (9, 45) even under fully aerobic conditions. L-Lactate occurs as a by-product during L-lysine production on glucose, fructose, and sucrose (27, 28), as well as during glutamate production (53). Cells grown on L-lactate showed altered mRNA levels for other genes (e.g., the isocitrate lyase gene *aceA* and the phosphotransacetylase gene *pta*) in addition to the NCgl2816-*lldD* operon compared with pyruvate-grown cells (53). L-Lactate was also shown to stimulate S-layer formation of *C. glutamicum* strain ATCC 14067 (52). These expression differences are likely due to control by the regulators of carbon metabolism RamA (6) and RamB (16), as shown previously for *aceA*, *pta*, and the S-layer protein gene *cspB* (21). As only NCgl2816 and *lldD* showed >6-fold-higher mRNA levels when *lldR* was deleted, as well as >10-fold-lower mRNA levels when *lldR* was overexpressed (Table 4), LldR appears to be a specific regulator of the NCgl2816-*lldD* operon. According to the current model for regulation of the L-lactate utilization operon NCgl2816-*lldD* in *C. glutamicum*, LldR binds to its operator sequence, $^{-1}$T GGTCTGACCA^{10}, upstream of NCgl2816 and represses transcription of NCgl2816-*lldD*. In the presence of L-lactate, L-lactate binds to LldR, preventing repression of NCgl2816-*lldD* by LldR. Thus, transcription of the NCgl2816-*lldD* operon is controlled by L-lactate availability.

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SHORT CONTRIBUTION

V. Engels • V.F. Wendisch

sugR deletion improves L-lysine production in *Corynebacterium glutamicum*

Abstract *Corynebacterium glutamicum* is used for the production of lysine from glucose (starch hydrolysate) and sucrose or fructose containing molasses. The DeoR-type transcriptional regulator SugR is a global repressor of *ptsG*, *ptsS* and *ptsF* expression, encoding the glucose, fructose and sucrose specific enzyme II permeases of the PTS system in *C. glutamicum*, respectively, as well as of genes for enzymes in glycolyis, the pentose phosphate pathway and the fermentative L-lactate dehydrogenase. Therefore, it was tested how deletion of *sugR* affected lysine production by a *C. glutamicum* DM1729, a genetically defined lysine-producing derivative of *C. glutamicum* wild type. On the one hand, deletion of sugR led to a 20%, 50% and 70% increased L-lysine yield on glucose, fructose and sucrose containing media, but on the other hand a long lag-phase combined with a reduced growth rate and sugar uptake rate afterwards was observed for DM1729Δ*sugR* as well. Deletion of *sugR* in the wild type background neither changed biomass yields nor caused long lag-phases, although the growth rates were slightly reduced. This is the first example of engineering amino acid production by *C. glutamicum* by deletion of a global regulatory gene.

Introduction

As an essential amino acid, L-lysine is not synthesized in animals, thus it must be ingested as lysine or lysine-containing proteins. In 1957 Kinoshita and co-workers first isolated *Corynebacterium glutamicum* (formerly *Micrococcus glutamicus*) due to its ability to excrete L-glutamate under biotin-limiting conditions (Kinoshita et al., 1957; Abe et al., 1967). Nowadays, *C. glutamicum*, a predominantly aerobic, biotin-auxotrophic Gram-positive bacterium, is one of the biotechnologically most important bacterial species with an industrial production of about 1.5 million tons of L-glutamate per year and 0.85 million tons of L-lysine per year (Hermann, 2003; Leuchtenberger et al., 2005). Due to its prominent industrial importance, *C. glutamicum* has become a model organism for the *Corynebacterineae*, a suborder of the *Actinomycetales*, which also comprises the genus *Mycobacterium* (Stackebrandt et al., 1997). A general view of this non-pathogenic bacterium, which has been studied extensively, can be found in two recent monographs (Eggeling and Bott, 2005; Burkovski, 2008).

A large number of molecular targets essential for efficient lysine production has been recognized in different areas, since all genes involved in L-lysine biosynthesis have been identified (Ishino et al., 1987; Yeh et al., 1988; Kalinowski et al., 1990; Cremer et al., 1991; Kalinowski et al., 1991; Wehrmann et al., 1994; Hartmann et al., 2003): the lysine biosynthetic pathway, pathways forming by-products which should be as low as possible, pathways which ensure the optimal carbon precursor supply for production and regeneration of the cofactor NADPH (Eggeling, 1994; Sahm et al., 2000; Hermann, 2003; Pfefferle et al., 2003). The overexpression of the pyruvate carboxylase encoded by *pyc* is one example for increasing the carbon precursor supply which results in improved lysine formation in *C. glutamicum* (Peters-Wendisch et al., 2001). *pyc* overexpression resulted in approximately 50% higher lysine accumulation in the culture supernatant indicating that the anaplerotic pyruvate carboxylase reaction is one major bottleneck for amino acid production in *C. glutamicum* (Peters-Wendisch et al., 2001). The key enzyme of the split pathway for lysine synthesis is the aspartokinase encoded by *lysC* (Schrumpf et al., 1991). This enzyme shows a cumulative feedback inhibition by lysine and threonine but it was also shown that overexpression of *lysC* and *lysC* alleles coding for a aspartokinase that is not feedback-

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inhibited by lysine both improved lysine production in *C. glutamicum* (Shiio and Miyajima, 1969; Thierbach et al., 1990; Cremer et al., 1991; Kalinowski et al., 1991; Schrumpf et al., 1992). Moreover, the reduction of side product formation was shown to improve lysine production. This was achieved due to the introducing of *hom* alleles resulting in a restricted homoserine dehydrogenase enzyme. In these *hom* mutants the aspartokinase was not feedback-inhibited due to the low threonine concentrations and thus lysine production was enhanced (Follettie et al., 1988; Eikmanns et al., 1991).

Georgi et al. investigated the effect of malic enzyme (encoded by *malE*) and fructose-1,6-bisphosphatase (encoded by *fbp*) on lysine production in *C. glutamicum* (Georgi et al., 2005). Whereas overexpression of *malE* did not improve lysine production on glucose, fructose, glucose plus fructose or sucrose, the overexpression of *fbp* increased the lysine yield on sucrose by a factor of two but not on fructose (Georgi et al., 2005). Additionally, the authors could show that overexpression of *fbp* led to a near-complete utilization of sucrose while considerable concentrations of sucrose remained in the medium in the control strain. Transcriptional regulation of genes required for uptake of glucose, fructose and sucrose was unknown at that time but was postulated to be concerted (Krömer et al., 2004).

The DeoR-type transcriptional regulator SugR has recently been identified in our group as repressor of the PTS permeases specific for the uptake of glucose, fructose and sucrose in *C. glutamicum* (Engels and Wendisch, 2007). By combined analysis of the transcriptome, the metabolome and the fluxome in *C. glutamicum* during lysine production it was shown by Krömer and co-workers that expression of *ptsG* was strongly increased after switch from growth to lysine production, but was gradually decreased during the process by approximately 60% (Krömer et al., 2004). Thus, the aim of this work was the analysis of the effect of a *sugR* deletion in the background of a lysine production *C. glutamicum* strain DM1729 with respect to a possible increased lysine yield on the PTS sugars glucose, fructose and sucrose.

Materials and methods

Bacterial strains, media and growth conditions

The bacterial strains and the plasmids, their relevant characteristics and sources used in this study are listed in Table 1. The *C. glutamicum* type strain ATCC13032 was used as wild type (WT) and ATCC13032 DM1729 (Kalinowski et al., 2003; Georgi et al., 2005; B. Bathe, Degussa AG) was used as DM1729. For growth of *C. glutamicum* strains, the preculture, 60 ml Luria-Bertani (LB) medium (Sambrook and Russell, 2001), was inoculated from a fresh LB agar plate. After washing the cells in medium without carbon source, the main cultures were inoculated to an optical density at 600 nm (OD_{600}) of 0.5 in 60 ml CgXII minimal medium (Keilhauer et al., 1993), which contained 0.03 g/l protecatechuic acid and 0.2 mg/l biotin. As carbon and energy sources 100 mM glucose, 100 mM fructose, 50 mM sucrose, 150 mM potassium acetate plus 50 mM glucose were used. Precultures and main cultures were incubated at 30°C and 120 rpm on a rotary shaker in 500 ml baffled shake flasks. For all cloning purposes, *Escherichia coli* DH5α was used as host. The *E. coli* strains were cultivated aerobically in LB medium at 37°C.

Recombinant DNA work

The enzymes for recombinant DNA work were obtained from Roche Diagnostics (Mannheim, Germany). The oligonucleotides were obtained from Operon (Cologne, Germany) and are listed in Table 1. Standard methods like restriction were carried out according to (Sambrook and Russell, 2001). Plasmids from *E. coli* were isolated with the QIAprep spin miniprep kit (Qiagen, Hilden, Germany). *C. glutamicum* was transformed by electroporation (van der Rest et al., 1999; Engels and Wendisch, 2007).

Construction of *C. glutamicum* DM1729Δ*sugR*

The *in-frame sugR* deletion mutants of *C. glutamicum* DM1729 were constructed via a two-step homologous recombination procedure as described previously (Niebisch and Bott, 2001; Engels and Wendisch, 2007). Transfer of the plasmid pK19*mobsacB*-Δ*sugR* into *C. glutamicum* DM1729 by electroporation and screening for the right mutants was performed as described previously (Niebisch and Bott, 2001; Engels and Wendisch, 2007). Of 5 clones tested by PCR (primer pair *sugR*-k-for/*sugR*-k-rev), 1 showed the wild type situation and 4 had the desired *in-frame* deletion of the *sugR* gene.

Determination of glucose, fructose, sucrose and acetate concentrations

Potassium acetate, D-glucose and D-fructose as well as sucrose were quantified enzymatically with the acetic acid kit, the D-glucose/D-fructose kit or the sucrose/D-glucose kit (R-Biopharm, Darmstadt, Germany) according to the manufacturers' instructions as described previously (Engels and Wendisch, 2007).

Determination of L-lysine in the supernatants

To quantify L-lysine in the culture supernatant, cells were removed from the culture samples (1 ml) by centrifugation for 5' at 16100 x *g* and filtration (Spartan 13/0.2 RC filter unit, Schleicher & Schuell, Dassel, Germany). Aliquots were then used directly for reverse-phase high-performance liquid chromatography (RP-HPLC) or stored at –20°C. L-lysine concentrations were determined by automatic precolumn derivatization with ortho-phtaldialdehyde (OPA)

Table 1 Bacterial strains, plasmids and oligonucleotides used in this study

Strain, plasmid or oligonucleotide	Relevant characteristics or sequence[a]	References or purpose
***E. coli* strains**		
DH5α	F⁻*thi*-1 *endA*1 *hsdr*17(r⁻, m⁻) *supE*44 Δ*lacU*169 (ϕ80*lacZ*ΔM15) *recA*1 *gyrA*96 *relA*1	Bethesda Research Laboratories; (Hanahan, 1983)
***C. glutamicum* strains**		
WT	ATCC13032; wild-type strain, auxotrophic for biotin	ATCC
DM1729	*pyc*^{P458S}, *hom*^{V59A}, *lysC*^{T311I}; derived from the *C. glutamicum* wild-type strain ATCC 13032 auxotrophic for biotin	B. Bathe, Degussa (Georgi et al., 2005)
Δ*sugR*	*in-frame* deletion of the *sugR* gene of WT	(Engels and Wendisch, 2007)
DM1729Δ*sugR*	DM1729 with an *in-frame* deletion of the *sugR* gene	This study
Plasmids		
pk19*mobsacB*-Δ*sugR*	Kan^{R}, pk19*mobsacB* with the deletion construct of the gene *sugR*	(Engels and Wendisch, 2007)
Oligonucleotides		
sugR-k-for	5'-GTTCGTCGCGGCAATGATTGACG-3' (2037351)	(Engels and Wendisch, 2007), control of *sugR* deletion
sugR-k-rev	5'-CTCACCACATCCACAAACCACGC-3' (2039042)	

[a] nucleotide underlined is the first nucleotide in NC003450 which is given in parentheses.

and RP-HPLC (Agilent Technologies 1100 LC System; Aglient Technologies, Waldbronn, Germany) with fluorimetric detection of the amino acid-inositol-derivatives (excitation at 230 nm; emission at 450 nm) as described previosly (Jones and Gilligan, 1983; Schrumpf et al., 1991). Hypersil OSD 5 µm columns were used (precolumn, 40 x 4 mm; column 120 x 4 mm; Chromatographie Service GmbH, Langerwehe, Germany). The substances were eluted according their hydrophobicity with a flow rate of 0.35 ml/min in the first minute and 0.6 ml/min in the following 15 min at 40°C with a buffer gradient consisted of 0.1 M sodium acetate, pH 7.2 (with 0.03% sodium azide), as the polar phase and methanol as the nonpolar phase. The identification and quantification was performed by comparison with an external standard.

Results

Effect of *sugR* deletion on growth and glucose, sucrose, fructose and acetate utilization

In our first publication we could show that the DeoR-type regulator SugR acts as transcriptional regulator of the PTS permeases specific for the uptake of glucose, fructose and sucrose, respectivly (Engels and Wendisch, 2007). Additional studies revealed that SugR is a pleiotropic regulator which obviously regulates (1) PTS sugar uptake, (2) the flux through the glycolysis and (3) pentose phosphate pathway genes. These facts offer three possible explanations for improved lysine formation due to a *sugR* deletion and in addition it was previously reported that the sugar uptake might be a limiting factor (Kiefer et al., 2004; Georgi et al., 2005). Therefore, it was tested whether a *sugR* deletion in *C. glutamicum* DM1729, which was derived from the sequenced wild type *C. glutamicum* ATCC13032 (Kalinowski et al., 2003) by three allelic exchanges (*pyc*^{P458S}, *hom*^{V59A}, *lysC*^{T311I}), led to higher lysine yields on the PTS sugars glucose, fructose and sucrose as well as on glucose plus acetate. The *C. glutamicum* strains DM1729 and DM1729Δ*sugR* were cultivated on CgXII minimal medium with 100 mM glucose, 100 mM fructose and 50 mM sucrose as well as on 50 mM glucose plus 150 mM potassium acetate as sole carbon and energy sources. Unexpectedly, the DM1729Δ*sugR* deletion mutant showed strong growth defect on the PTS

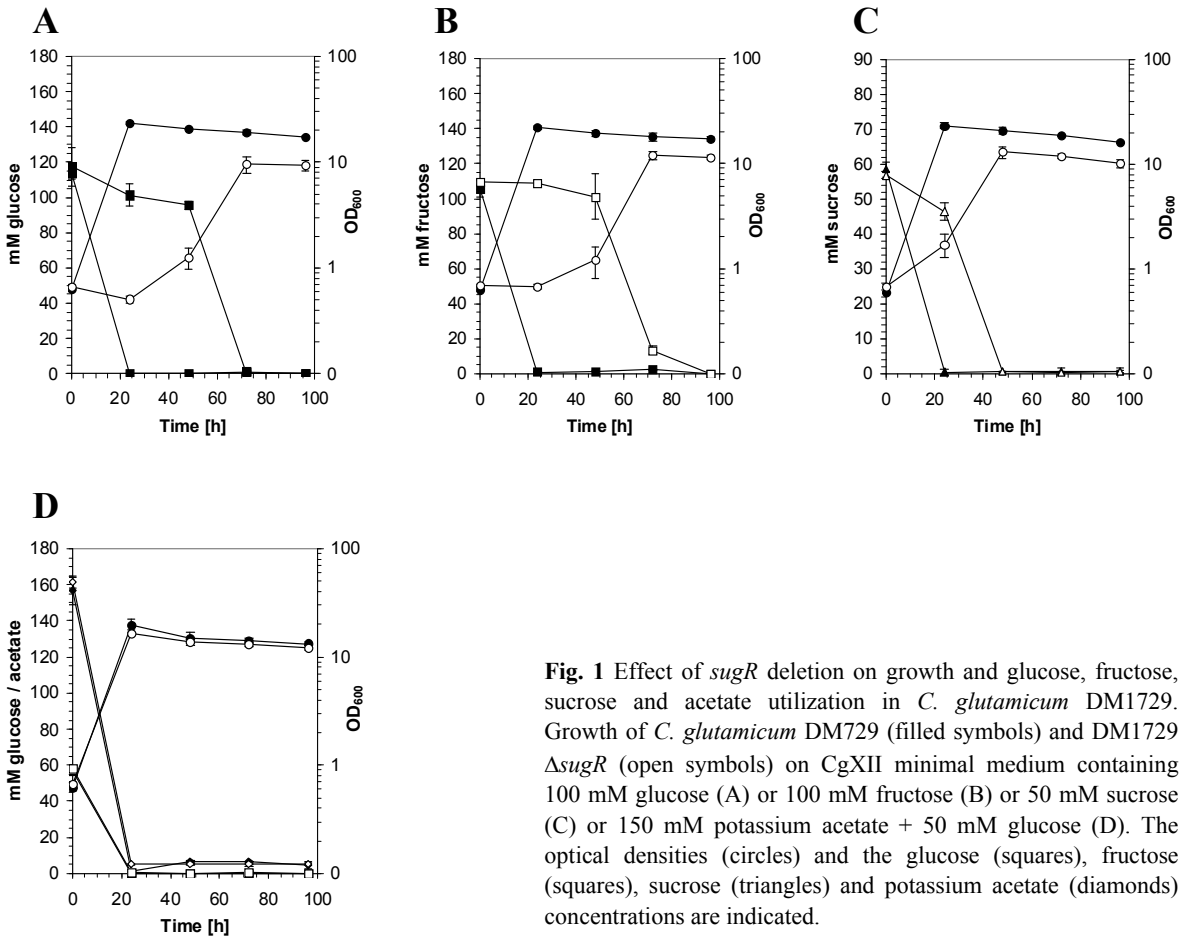

Fig. 1 Effect of *sugR* deletion on growth and glucose, fructose, sucrose and acetate utilization in *C. glutamicum* DM1729. Growth of *C. glutamicum* DM729 (filled symbols) and DM1729 Δ*sugR* (open symbols) on CgXII minimal medium containing 100 mM glucose (A) or 100 mM fructose (B) or 50 mM sucrose (C) or 150 mM potassium acetate + 50 mM glucose (D). The optical densities (circles) and the glucose (squares), fructose (squares), sucrose (triangles) and potassium acetate (diamonds) concentrations are indicated.

sugars glucose, fructose and sucrose, respectively (Fig. 1 A/B/C). In contrast to the parental strain DM1729 on glucose, fructose and sucrose minimal medium, the Δ*sugR* deletion mutant showed a lag-phase of about 24 hours and a significantly decreased growth rate afterwards. Moreover, the mutant utilized the sugars glucose, fructose and sucrose with a decreased uptake rate in comparison to DM1729 (Fig. 1 A/B/C). Although the carbon sources were utilized completely in both strains, the biomass formed by the Δ*sugR* deletion mutant was only approximately half of the biomass formed by the parental strain (7.8 ± 0.5 compared to 17.1 ± 0.2 gDW l^{-1} on glucose, 9.6 ± 0.6 compared to 17.7 ± 0.3 gDW l^{-1} on fructose and 10.2 ± 0.2 compared to 17.0 ± 0.1 gDW l^{-1} on sucrose, respectively). In contrast to these results, the addition of acetate on glucose plus acetate minimal medium led to almost equal growth rates, sugar consumption rates and biomass formed in both strains DM1729 and DM1729Δ*sugR* (Fig. 1 D).

In previous experiments with the Δ*sugR* mutant, which was derived from WT, this strong growth perturbation on the corresponding carbon sources was not observed (Engels and Wendisch, 2007). In these experiments a second preculture (which was equivalent to the main culture) after the first LB culture was inoculated and incubated over night at 30°C with which the main culture was then inoculated (Engels and Wendisch, 2007). To look whether these effects are due to the second preculture used in the former growth experiments but not these experiments presented here, the former experiments were repeated under the same conditions as described in Materials and methods. The experiments with the second preculture over night indicated that the *sugR* deletion mutant also showed slightly perturbed growth on fructose and sucrose minimal medium compared to the WT control (0.37 ± 0.01 compared to 0.42 ± 0.03 h^{-1} on fructose, 0.39 ± 0.02 compared to 0.46 ± 0.01 h^{-1} on sucrose), but no differences were observed on glucose minimal medium (0.40 ± 0.00 compared to 0.40 ± 0.01 h^{-1}). No lag-phase and the same final optical density arose in the Δ*sugR* mutant in comparison to the WT on each PTS sugar (data not shown).

Taken together, a deletion of the transcriptional regulator SugR led to slightly decreased growth rates in the WT background on the PTS sugars fructose and

sucrose, but to a severe growth defect in the strain background of the lysine producing *C. glutamicum* strain DM1729 on glucose, fructose and sucrose minimal medium.

sugR deletion and L-lysine production on different PTS sugars

To quantify L-lysine in the culture supernatant, culture samples (1 ml) taken from different time points during growth of *C. glutamicum* strains DM1729 and DM1729Δ*sugR* on different media were used for reverse-phase high-performance liquid chromatography (RP-HPLC). Lysine yields of DM1729 (black columns) and DM1729Δ*sugR* (white columns) on CgXII minimal medium containing 100 mM glucose or 100 mM fructose or 50 mM sucrose or 150 mM potassium acetate plus 50 mM glucose were shown in Figure 2. In these experiments it was demonstrated that due to the *sugR* deletion the lysine yield was increased about 20% on glucose minimal medium (0.12 ± 0.01 in comparison to 0.10 ± 0.01 mol-C/mol-C, respectively), about 50% increased on fructose minimal medium (0.13 ± 0.00 in comparison to 0.09 ± 0.00 mol-C/mol-C, respectively) and approximately 70% increased on sucrose minimal medium (0.14 ± 0.00 in comparison to 0.08 ± 0.01 mol-C/mol-C, respectively) in comparison to the parental strain DM1729 (Fig. 2). Nearly equal amounts, about 5% more lysine in the parental strain, of L-lysine were obtained in DM1729 and DM1729Δ*sugR* on glucose plus acetate mixture (0.11 ± 0.00 in comparison to 0.12 ± 0.00 mol-C/mol-C, respectively) (Fig. 2). By comparing the mM L-lysine produced by the strains DM1729 and DM1729Δ*sugR* over the cultivation time on the different carbon sources (data not shown), one can see that both strains produced lysine with comparable rates as they grew and utilized the carbon sources. Thus, it is not surprising that both strains reached the maximal lysine concentration on glucose-acetate mixture already after 24 hours of cultivation (data not shown). This fits to the data from Figure 1: after 24 hours both strains reached the final optical density and the glucose as well as the acetate were utilized completely. On glucose, fructose and sucrose minimal medium, the DM1729Δ*sugR* deletion mutant reached the maximal L-lysine concentration after 72 hours (glucose and fructose) and 48 hours (sucrose), respectively (data not shown). As shown in Figure 1, the maximal optical density of DM1729Δ*sugR* on these media was achieved at the same time points and the sugars have been utilized completely, too.

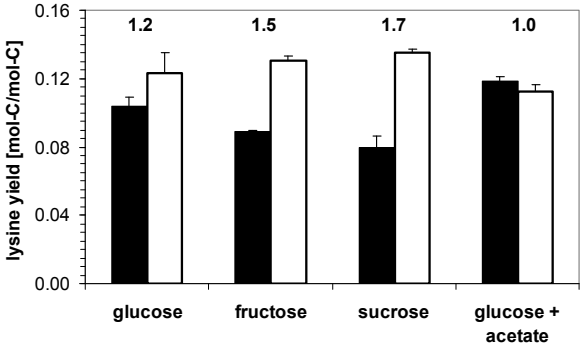

Fig. 2 Lysine yields of *C. glutamicum* strains DM1729 (black columns) and DM1729 Δ*sugR* (white columns) on CgXII minimal medium containing 100 mM glucose or 100 mM fructose or 50 mM sucrose or 150 mM potassium acetate + 50 mM glucose. Yields are expressed as mol carbon (mol-C) of lysine formed per mol-C of carbon source utilized after 96 hours of cultivation and are mean values of two to three independent cultivations. The numbers above the bars indicate the ratio of lysine yields between the deletion mutant DM1729Δ*sugR* and the parental strain DM1729.

Discussion

C. glutamicum plays an important role in microbial production of amino acids, especially for L-glutamate and L-lysine. During the long process of strain development and since the genome sequence of *C. glutamicum* is available (Kalinowski et al., 2003) various molecular targets have been identified. Introduced mutant alleles in the *C. glutamicum* WT of three of these target genes, the aspartokinase (*lysC*) (Cremer et al., 1991), the homoserine dehydrogenase (*hom*) (Follettie et al., 1988) and the pyruvate carboxylase (*pyc*) (Peters-Wendisch et al., 2001), resulted in the strain DM1729 with an increased flux towards lysine biosynthesis, reduced by-product formation and a better precursor supply and thus, with higher lysine yields (Ohnishi et al., 2002).

In our present study, we could show that the deletion of the DeoR-type transcriptional regulator SugR improves lysine production during growth on the PTS sugars glucose, fructose and sucrose in *C. glutamicum* but not on glucose-acetate mixture (Fig. 2). Uptake of the preferred carbon source glucose via the phosphoenolpyruvate-dependent phospho-transferase system (PTS) is reduced during co-utilization of glucose with acetate, sucrose or fructose as compared to growth on glucose as sole carbon source (Wendisch et al., 2000) and *ptsG* transcription was observed to be induced by switching from growth to lysine production and the expression gradually decreased during further cultivation (Krömer et al.,

2004). Resently, we identified SugR as transcriptional regulator of *ptsG*, *ptsS* and *ptsF* expression (coding for the glucose-, fructose- and sucrose-specific enzyme II permeases of the PTS system, cg1537, cg2120 and cg2925, respectively) in *C. glutamicum* (Engels and Wendisch, 2007). On glucose-acetate mixture it was shown that the glucose uptake rate was increased in the *sugR* deletion mutant in comparison to the WT, thus in the absence of SugR, glucose and acetate are co-utilized, but the proportion between glucose and acetate is shifted towards glucose (Engels and Wendisch, 2007). Commensurate with this view is that the lysine yield in the production strain DM1729 on glucose alone was decreased compared to growth on glucose plus acetate. On glucose-acetate mixture the glyoxylate cycle provides the oxaloacetate for lysine production (Wendisch et al., 2000), thus, glycolysis is not that drain compared to growth on glucose alone (see below). According to this, in DM1729 grown on glucose-acetate mixture enough fructose-6-phosphate is present to prevent SugR from binding and expression of the target genes is activated. This observation is in agreement with the result that both strains DM1729 and DM1729Δ*sugR* produce equal amounts of lysine on glucose-acetate mixture and this is even as much as the Δ*sugR* mutant produced on glucose alone (Fig. 2).

An increased flux towards lysine was also achieved by the overexpression of *dapA*, encoding the dihydrodipicolinate synthase in *C. glutamicum* (Eggeling et al., 1998). *dapA* overexpression resulted in an increased flux towards lysine and a decreased flux towards threonine (Eggeling et al., 1998). As carbon precursors for lysine production oxaloacetate or aspartate or pyruvate are used. *C. glutamicum* possesses both anaplerotic enzymes phospho-enolpyruvate carboxylase (encoded by *ppc*) and pyruvate carboxylase (encoded by *pyc*). The overexpression of *pyc* resulted in a ~50% lysine accumulation, whereas a minor role for *ppc* was demonstrated (Cremer et al., 1991; Gubler et al., 1994; Peters-Wendisch et al., 1997; Peters-Wendisch et al., 2001). Previously reported potential targets to improve lysine production with *C. glutamicum* on fructose were supposed to be the modification of the flux through the fructose-PTS and the amplification of fructose-1,6-bisphosphatase to increase the flux through the pentose phosphate pathway (Kiefer et al., 2004). Since SugR acts as repressor of the fructose-PTS, *ptsF* should be derepressed in a *sugR* deletion strain leading to a better lysine production. However, lysine yields were unchanged on glucose and/or fructose minimal medium but were two fold increased on sucrose minimal medium due to the overexpression of fructose-1,6-bisphosphatase in the lysine production strain DM1730 (Georgi et al., 2005). As lysine production is characterized by a high NADPH supply where 4 mol of NADPH are used for the production of 1 mol lysine (Marx et al., 1997), the NADPH supply appears to be one of the major bottlenecks for lysine production on fructose and sucrose. Measurements of the intracellular concentration of fructose-1,6-bisphosphate (Georgi et al., 2005) indicated that the higher lysine yields resulted from a higher NADPH supply due to the decrease of fructose-1,6-bisphosphate in sucrose-grown cells which inhibits the 6-phosphogluconate dehydrogenase encoded by *gnd* (cg1643) in *C. glutamicum* (Georgi et al., 2005). Thus, the 6-phosphogluconate dehydrogenase and in general the enhancement of the flux through the pentose phosphate pathway were suggested to be important targets to improve lysine production in this organism (Georgi et al., 2005). The growth defect observed in DM1729Δ*sugR* grown on the PTS sugars glucose, fructose and sucrose (Fig. 1) was decimated in WTΔ*sugR* under the same growth conditions. Only the growth rate was slightly decreased, but neither the final optical density was reduced, not the severe lag-phase was observed in this comparison. The only differences between the *C. glutamicum* WT and the lysine production strain DM1729 are the three allelic exchanges in *hom*, *pyc* and *lysC*. These allelic exchanges provide the subtraction of pyruvate for the lysine biosynthesis. Thus, glycolysis seems to drain and as consequence the fructose-1,6-bisphosphate concentration, which inhibits the 6-phosphogluconate dehydrogenase, decreases. The 6-phosphogluconate dehydrogenase is derepressed and the flux through the pentose phosphate pathway is increased which leads in consequence to a higher lysine yield.

SugR regulates not only the PTS genes but appears to be a pleiotropic regulator of further genes of fundamental pathways, e.g. the enolase (*eno*), the fructose-1,6-bisphosphate aldolase (*fba*), the pyruvate kinase (*pyk*), the 6-phosphofructokinase (*pfkA*), the NAD-dependent L-lactate dehydrogenase (*ldhA*), and the transketolase (*tkt*), a key enzyme of the pentose phosphate pathway (which is part of the *tkt-tal-zwf-opcA-pgl* operon also encoding the pentose phosphate pathway enzymes transaldolase, glucose-6-phosphate dehydrogenase and 6-phosphogluconolactonase; (Yokota and Lindley, 2005; Engels and Wendisch unpublished data). Thus, SugR seems not only to control the glycolytic flux straight forward to pyruvate which serves as precursor for L-lysine and L-lactate production in *C. glutamicum* but also seems to control the flux through the pentose phosphate pathway leading to a better NADPH supply and is therefore a good candidate for improving L-lysine production with *C. glutamicum*. Flux measurements in *C.*

glutamicum DM1729Δ*sugR* in comparison to DM1729 would confirm this hypothesis.

Only in the strain backgound of DM1729 not in the WT and only on glucose, fructose and sucrose and not on glucose plus acetate the *sugR* deletion leads to a lag-phase of approximately 24 hours (Fig. 1 and data not shown). A second consequence of *dapA* overexpression mentioned above is a drastic growth rate reduction which was restored to some extent by the addition of homoserine and could therefore be due to the limiting availability of some amino acids (Eggeling et al., 1998). Recently, the deletion of the *aceE* gene, encoding the E1p enzyme of the pyruvate dehydrogenase complex (PDHC), in the *C. glutamicum* DM1729 background was shown to reduce growth on glucose and acetate containing media (Blombach et al., 2007). This effect was not found in the WTΔ*aceE* under the same conditions. Moreover, the DM1729Δ*aceE* strain showed a 50% lower substrate-specific biomass yield but also a 40% higher substrate-specific L-lysine yield (Blombach et al., 2007). Thus, *aceE* deletion in a comparable manner as *sugR* deletion combined with the allelic exchanges introduced into *C. glutamicum* DM1729 are obviously unfavourable for growth but, however, advantageous for L-lysine production in this context (Blombach et al., 2007; and this study). If the growth defect in DM1729Δ*sugR* can also be restored to some extend by the addition of missing precursors for other essential reactions as shown for *dapA* overexpression and *aceE* deletion, too (Blombach et al., 2007), has to be investigated by further experiments.

In summary, we could show that the deletion of the transcriptional regulator SugR in the lysine production strain DM1729 on the one hand led to a 20%, 50% and 70% increased L-lysine yield on glucose, fructose and sucrose containing media, respectively. However, this deletion led on the other hand to a 24 hours lag-phase combined with a reduced growth rate and sugar uptake rate afterwards. Nevertheless, SugR appears to be a good candidate to improve lysine formation on PTS sugars.

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Functional and biochemical characterization of ScrB (Cg2927) as sucrose-6-phosphate hydrolase essential for sucrose utilization by *Corynebacterium glutamicum*

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Corynebacterium glutamicum, PTS, sucrose utilization, sucrose-6-phosphate hydrolase, ScrB

Abstract

Corynebacterium glutamicum can grow on a variety of carbohydrates of which glucose, fructose as well as sucrose are taken up and are phosphorylated by the phosphoenolpyruvate-dependent phosphotransferase system (PTS). Here, we show that cg2927 (*scrB*) encodes sucrose-6-phosphate hydrolase. The purified His-tagged protein hydrolysed sucrose-6-phosphate and sucrose, but not sucrose-6'-phosphate. The K_m value for sucrose was 190 mM while the K_m for sucrose-6-phosphate was much lower, i.e. 0.04 mM. The enzyme showed maximal activity at a pH of 6.5 and a temperature of 45°C. Sucrose-6-phosphate hydrolase activity was stimulated by $MgSO_4$ and fructose-6-phosphate and was inhibited by $MnCl_2$, $CaCl_2$, $CuSO_4$ and $ZnSO_4$. A *scrB* deletion mutant could not grow on sucrose as sole carbon source. In addition, growth of the *scrB* deletion mutant was severely disturbed when sucrose was present in addition to glucose, fructose or acetate suggesting that higher intracellular concentrations of sucrose-6-phosphate are toxic for *C. glutamicum*. The sucrose-6-phosphate hydrolase activity was increased two to three fold on fructose or sucrose containing media. Sucrose induction of *scrB* was not due to SugR, which controlled the sucrose-specific PTS gene *ptsS* localized downstream of *scrB*.

Introduction

Corynebacterium glutamicum is a gram-positive, aerobic soil bacterium which plays an important role in the large scale biotechnological production of amino acids, mainly of L-glutamate and L-lysine (Hermann, 2003; Leuchtenberger *et al.*, 2005). This species has become a model organism for the *Corynebacterineae*, a suborder of the *Actinomycetales*, which also comprises *Nocardiaceae* and *Mycobacteriaceae* (Stackebrandt *et al.*, 1997).

Amino acids are produced from glucose (starch hydrolysate) and from sucrose- or fructose-containing molasses. In *C. glutamicum* glucose, fructose and sucrose uptake is mediated by the PTS, the phosphoenolpyruvate-dependent phosphotransferase system with its substrate-specific enzyme II permeases EII^{Glc} (*ptsG*, cg1537), EII^{Fru} (*ptsF*, cg2120), and EII^{Suc} (*ptsS*, cg2925), respectively (Mori & Shiio, 1987; Dominguez & Lindley, 1996; Parche *et al.*, 2001; Kiefer *et al.*, 2002; Moon *et al.*, 2005). The phosphoryl group transfer from phospho*enol*pyruvate (PEP) proceeds via the general PTS components EI (*ptsI*, cg2117) and HPr (*ptsH*, cg2121) to the sugar-specific permeases, which phosphorylate and transport their cognate substrates into the cell (Lengeler *et al.*, 1994; Kotrba *et al.*, 2001; Parche *et al.*, 2001). It was shown previously that EII^{Suc} is required for growth on sucrose as sole carbon source (Moon *et al.*, 2005). It is believed that sucrose uptake by EII^{Suc} leads to sucrose-6-phosphate which subsequently is hydrolysed to glucose-6-phosphate and fructose (Shiio *et al.*, 1990; Kalinowski *et al.*, 2003). Unlike many sucrose-utilizing bacteria, *C. glutamicum* cannot phosphorylate fructose intracellularly. Utilization of intracellular free fructose by *C. glutamicum* involves temporary efflux out of the cell, subsequent re-uptake and phosphorylation by the fructose-PTS to yield fructose-1-phosphate and phosphorylation by FruK to yield the glycolytic intermediate fructose-1,6-

Table 1. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	References
***E. coli* strains**		
DH5α	F⁻*thi*-1 *endA*1 *hsdr*17(r^-, m^-) *supE*44 Δ*lacU*169 (ϕ80*lacZ*ΔM15) *recA*1 *gyrA*96 *relA*1	Bethesda Research Laboratories; Hanahan, 1983
BL21(DE3)	*ompT hsdS*$_B$($r_B^-m_B^-$) *gal dcm* (DE3)	Novagen
***C. glutamicum* strains**		
ATCC 13032	wild-type strain auxotrophic for biotin	ATCC
Δ*scrB*	*in-frame* deletion of the *scrB* gene	This study
Δ*sugR*	*in-frame* deletion of the *sugR* gene	Engels & Wendisch, 2007
Plasmids		
pGEM-T	Amp^R, PCR cloning vector (P_{T7}, P_{SP6})	Promega, WI, USA
pGEM-T-Δ*scrB*	Amp^R, pGEM-T with deletion fragment of 1048 bp of *scrB*	This study
pGEM-T-ScrB^{His}	Amp^R, pGEM-T with a 1302 bp fragment of the gene *scrB*	This study
pk19*mobsacB*	Kan^R, *E. coli*/*C. glutamicum* shuttle-vector for the construction of insertion and deletion mutants in *C. glutamicum*, (pK18 *oriV*$_{E.c.}$, *sacB*, *lacZα*)	Schäfer *et al.*, 1994
pk19*mobsacB*-Δ*scrB*	Kan^R, pk19*mobsacB* with the deletion construct of the gene *scrB*	This study
pVWEx1	Kan^R, P_{tac}, *lacI*^{q}	Peters-Wendisch *et al.*, 2001
pVWEx1-*scrB*	Kan^R, pVWEx1 with a 1302 bp fragment of the *scrB* gene and an artificial RBS	This study
pVWEx1-*sugR*	Kan^R, pVWEx1 with a 807 bp fragment of the *sugR* gene and an artificial RBS	Engels & Wendisch, 2007
pET16b	Amp^R, overproduction of proteins with a N-terminal decahistidine tag in *E. coli* (pBR322 *oriV*$_{E.c.}$, *PT7*, *lacI*)	Novagen
pET16b-ScrB^{His}	Amp^R, pET16b with a 1302 bp fragment of the gene *scrB*	This study

bisphosphate (Dominguez & Lindley, 1996; Dominguez *et al.*, 1998).

The role of the sucrose- and fructose-specific PTS for sucrose metabolism has been studied to some detail (Moon *et al.*, 2005), while sucrose-6-phosphate hydrolase has not been characterized biochemically. In the genome of *C. glutamicum*, two genes code for putative sucrose-6-phosphate hydrolases, cg2927 and cg1267. Cg2927 lies directly upstream of and in the same transcriptional orientation as *ptsS* (Moon *et al.*, 2005) suggesting that this gene encodes the sucrose-6-phosphate hydrolase. Due to the biotechnological importance of sucrose as carbon source of *C. glutamicum*, the cg2927 gene product was characterized and shown to catalyze sucrose-6-phosphate hydrolysis and to be required for sucrose utilization.

Material and methods

Bacterial strains, media, and growth conditions

The strains and plasmids used are listed in Table 1. The oligonucleotides were obtained from Operon (Cologne, Germany) or MWG (Ebersberg, Germany) and are listed in Table S1. The *C. glutamicum* type strain ATCC13032 (Kalinowski *et al.*, 2003) was used as wild type (WT). Growth experiments were performed using CgXII minimal medium as described previously (Engels & Wendisch, 2007). As carbon and energy sources 100 mM glucose, 50 mM sucrose, 50 mM glucose or fructose plus 25 mM sucrose or 150 mM potassium acetate plus 25 mM sucrose were used. When appropriate, the medium was supplemented with kanamycin (50 µg/ml) or ampicillin (50 µg/ml). Growth of *C. glutamicum* was followed by measuring the optical density at 600 nm. For all cloning purposes, *Escherichia coli* DH5α was used as host and *E. coli* BL21(DE3) was used for overproduction of ScrB.

Construction of *scrB in-frame* deletion mutants

The *in-frame scrB* deletion mutants of *C. glutamicum* were constructed via a two-step homologous recombination procedure as described previously (Niebisch & Bott, 2001; Engels & Wendisch, 2007). The *scrB* up- and downstream regions were amplified using primer pairs *scrB*-A/*scrB*-B and *scrB*-C/*scrB*-D.

Subcloning of the subsequent cross-over PCR into the pGEM-T vector (Promega, WI, USA), afterwards cloning as EcoRI-/BamHI-fragment into pK19*mobsacB* vector (Schäfer *et al.*, 1994), transfer of the resulting plasmid pK19*mobsacB*-Δ*scrB* into *C. glutamicum* by electroporation and screening for the correct mutants was performed as described previously (Niebisch & Bott, 2001; Engels & Wendisch, 2007). Of 33 clones tested by PCR (primer pair *scrB*-kv/*scrB*-kr), 15 were wild type and 18 had the desired *in-frame* deletion of the *scrB* gene.

Homologous overexpression of *scrB* from *C. glutamicum*

For homologous overexpression of *scrB*, *scrB* was amplified from genomic DNA of *C. glutamicum* WT using primers *scrB*-for and ScrBShift-rev and cloned into the expression vector pVWEx1 (Peters-Wendisch *et al.*, 2001). The constructed vector pVWEx1-*scrB* allows the IPTG-inducible expression of *scrB* in *C. glutamicum.*

Preparation of total RNA and primer extension analysis

Harvesting exponentially growing cells, RNA isolation and non-radioactive primer extension analysis were performed as described previously (Engels & Wendisch, 2007). The template for primer extension analysis for *ptsS* was generated by PCR amplification with the primers *ptsS*-PrimEx-for and *ptsS*-PrimEx-rev. Sequencing reactions and primer extension products were analyzed as described previously (Engels & Wendisch, 2007). The transcriptional start site of *ptsS* was determined using two different oligonucleotides (*ptsS*-30*, *ptsS*-90*).

Determination of glucose, fructose, sucrose and acetate concentrations

Acetic acid, D-glucose and D-fructose as well as sucrose were quantified enzymatically with the acetic acid kit, the D-glucose/D-fructose kit or the sucrose/D-glucose kit (R-Biopharm, Darmstadt, Germany) according to the manufacturers' instructions as described previously (Engels & Wendisch, 2007).

Preparation of cell-free crude extracts and measurement of ScrB activity in crude extracts

For determination of ScrB activity in cell-free crude extracts, exponentially growing cells were harvested and the cell pellet from 50 ml culture volume was washed in 40 ml 0.1 M MES (pH 6.0) buffer, centrifuged (5', 5422 x *g*, 4°C), resuspended in 1 ml of the same buffer and disrupted by sonication (9', cycle 0.5, amplitude 55%, on ice; sonication processor UP200s, Hielscher Ultrasonics GmbH, Stuttgart, Germany). After centrifugation (1 h, 13000 x *g*, 4°C) the supernatant was used for measuring ScrB activity. Shortly, the assay (1 ml mixture with 0.4 mM sucrose as substrate and 0.25-20 µl crude extract) was performed with the D-glucose/D-fructose kit (R-Biopharm, Darmstadt, Germany) according the manufacturers' recommendations measuring photometrically the reducing sugar glucose released by the formation of NADPH at 340 nm and 30°C.

Overproduction and purification of ScrB

Overproduction and purification of ScrB carrying an N-terminal decahistidine tag was carried out as described previously for SugR (Engels & Wendisch, 2007). Shortly, the *scrB* coding region was amplified using the oligonucleotides ScrB*Shift*-for and ScrB*Shift*-rev and cloned into the pET16b expression vector. The plasmid was transferred into *E. coli* BL21(DE3) and the resulting strain *E. coli* BL21(DE3)(pET16b-ScrB^{His}) was grown in 2 l LB medium (Sambrook *et al.*, 1989) for 4 hours after induction with 0.5 mM IPTG. ScrB was purified by nickel affinity chromatography (Ni-NTA, Novagen, San Diego, USA). After washing with TNI50 buffer (20 mM Tris-HCl, pH 7.9, 300 mM NaCl, 5% (v/v) glycerol, 50 mM imidazol), the ScrB^{His} protein was eluted with TNI400 buffer (which contained 400 mM imidazol). Fractions with the highest protein content were pooled, and the elution buffer was exchanged against 0.1 M MES buffer (pH 6.0) using PD10 columns.

Protein estimation, sucrose-6-phosphate synthesis and enzyme assay

Protein concentrations were determined with the Bradford assay kit (Bio-Rad Laboratories, Hercules, Canada) using bovine serum albumin as standard. Sucrose-6-phosphate was prepared by incubating a permeabilized cell suspension of *C. glutamicum* Δ*scrB* (which were grown in LB complex medium with 50 mM sucrose as carbon source and prepared as described previously (Peters-Wendisch *et al.*, 1997) with sucrose and phospho*enol*pyruvate as described for *S. mutans* (St Martin & Wittenberger, 1979). Enzymatic activity was determined discontinuously at 30°C with 0.1 M MES buffer (pH 6.5) and 0.125-0.8 M sucrose or 0.2-200 mM sucrose-6'-phosphate (Sigma, Taufkirchen, Germany) or 7-18 µM sucrose-6-phosphate as substrate and 20 µl of purified ScrB protein, measuring time-dependently the reducing sugar glucose released with the D-glucose/D-fructose kit (R-Biopharm, Darmstadt, Germany) as described above. Michaelis-Menten constant (K_m) and Maximum velocity (V_{max}) were determined as function of the sucrose or sucrose-6-phosphate concentration. To determine the temperature optimum of ScrB, sucrose-6-phosphate hydrolase was assayed at pH 6.5 in the temperature range from 20-70°C. ScrB acitivity was tested in a pH between 5.0 and 8.0 obtained by adding 0.1 M MES buffer (pH 5.0-8.0) to the reaction mixture. The activation or inhibition of the enzyme was tested by the addition of one of the following compounds: $MgSO_4$, $MnCl_2$, $CaCl_2$, $CuSO_4$, $ZnSO_4$, EDTA, DTT (each 5 mM), fructose-6-phosphate (10 mM), and fructose (100 mM).

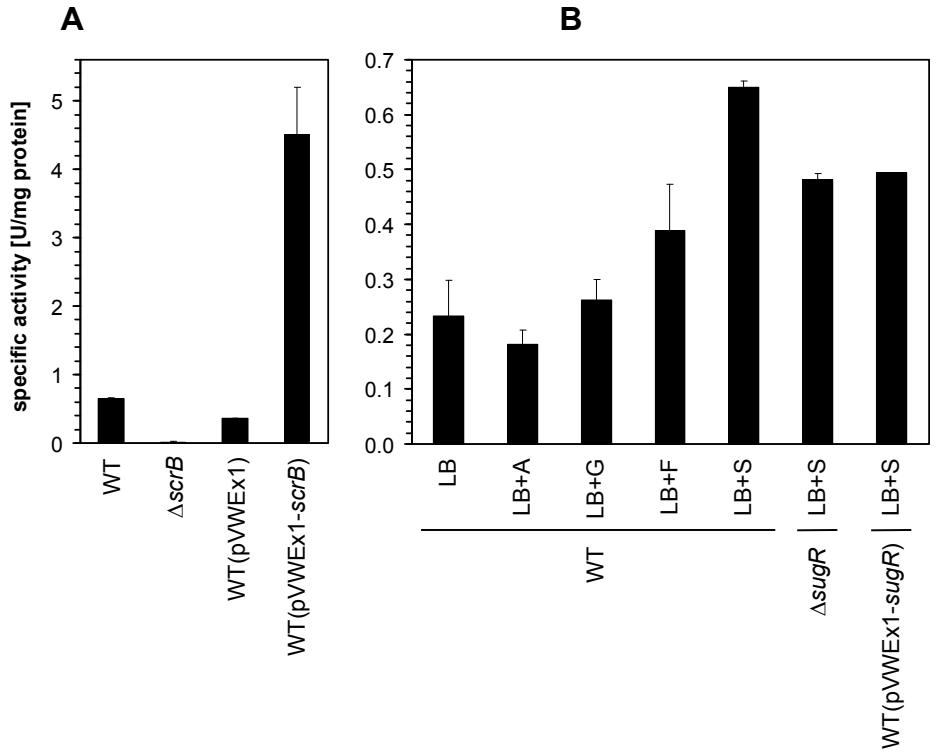

Fig. 1. ScrB activities of different *C. glutamicum* strains during growth on LB with and without added carbon sources. (A) ScrB activity measured in crude extracts from *C. glutamicum* WT, Δ*scrB*, WT(pVWEx1) and WT(pVWEx1-*scrB*) on LB with 50 mM sucrose. (B) ScrB activity measured in crude extracts from *C. glutamicum* WT on LB medium without any addition, with 300 mM potassium acetate (LB+A), with 100 mM glucose (LB+G), with 100 mM fructose (LB+F) or with 50 mM sucrose (LB+S) as well as from *C. glutamicum* Δ*sugR* and from WT(pVWEx1-*sugR*) on LB with 50 mM sucrose. The values represent averages and experimental errors from at least two independent cultivations.

Results and discussion

Biochemical characterization of the sucrose-6-phosphate hydrolase from *C. glutamicum*

(i) ScrB activity in crude extracts. The *C. glutamicum* genome contains two putative candidate genes coding for sucrose-6-phosphate hydrolases (cg1267 and cg2927). As cg2927 lies directly upstream of and in the same transcriptional orientation as *ptsS* (Kalinowski *et al.*, 2003), it was determined whether the cg2927 gene product carries sucrose-6-phosphate hydrolase activity. Cg2927, which was named *scrB*, was expressed from vector pVWEx1 and an *in-frame* deletion mutant was constructed. Sucrose-6-phosphate hydrolase activity was measured in crude extracts of WT, Δ*scrB*, WT(pVWEx1) and WT(pVWEx1-*scrB*) during growth on LB complex medium containing 50 mM sucrose (Fig. 1A). Overexpression of *scrB* resulted in a 12 fold increased activity as compared to the empty vector control, while no sucrose-6-phosphate hydrolase activity could be detected in the *scrB* deletion mutant (≤0.02 U/mg, Fig. 1A). These results indicate that *scrB*/cg2927 encodes sucrose-6-phosphate hydrolase. Moreover, the absence of sucrose-6-phosphate hydrolase activity in the *scrB* deletion mutant supports the assumption that there is no functional homologue of ScrB active in *C. glutamicum* under the tested conditions although the cg1267 gene product shows 26% sequence identity to ScrB.

(ii) Kinetic parameters and optimum conditions. For enzyme characterization with respect to kinetic parameters and optimal conditions, ScrB protein containing an aminoterminal decahistidine tag was overproduced in *E. coli* BL21(DE3) and purified to apparent homogeneity by affinity chromatography as described in Material and methods.

Because substrate amounts of sucrose-6-phosphate are not commercially available, the assay for the sucrose-6-phosphate hydrolase was performed with sucrose as substrate as described for sucrose-6-phosphate hydrolases from other sources (Lunsford & Macrina, 1986; Martin & Russell, 1987). The apparent K_m and V_{max} values of the purified ScrB protein for sucrose were estimated to be about 190 mM and 1.2 U/mg (Fig. 2A). Sucrose-6-phosphate hydrolase from *Streptococcus mutans* and invertase from *Rhodotorula glutinis* showed Michaelis konstants of 180 mM and 227 mM for sucrose as substrate, respectively (Lunsford & Macrina, 1986; Rubio *et al.*, 2002).

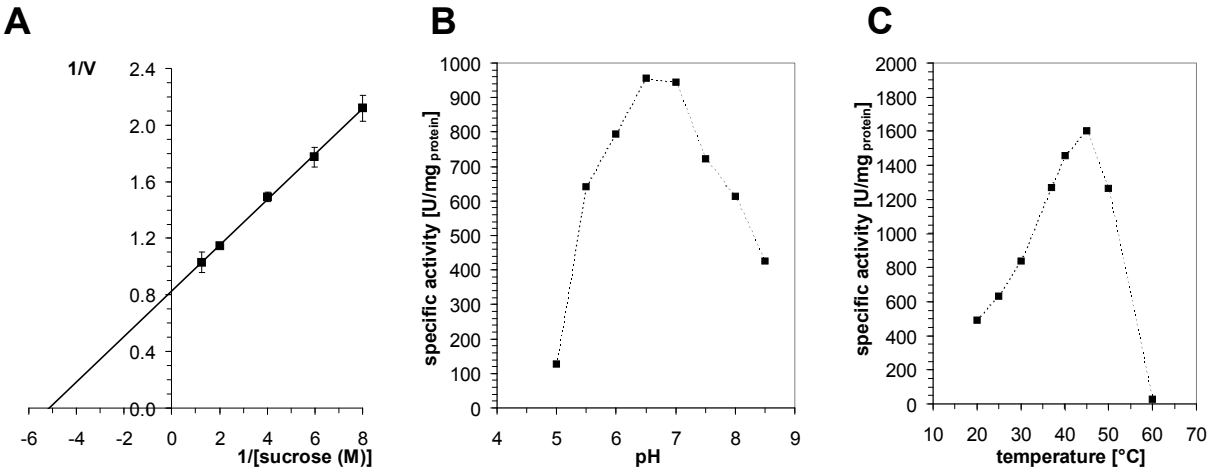

Fig. 2. Kinetic parameters and optimal conditions for ScrB activity from *C. glutamicum*. (A) Lineweaver-Burke plot for determinations of K_m and V_{max} with different concentrations of sucrose as substrate. (B) Effect of pH on ScrB activity. (C) Effect of temperature on ScrB activity. Experimental details are described in the text.

Using sucrose-6-phosphate prepared from permeabilized *C. glutamicum* Δ*scrB* cells, K_m of ScrB for sucrose-6-phosphate was estimated to be in µM range (0.04 mM), which is also the case for ScrB from *S. mutans* (0.08 mM) (Lunsford & Macrina, 1986). Thus, as sucrose-6-phosphate is much more effective than sucrose as substrate (about 5000 fold) it is the natural substrate of ScrB. Interestingly, sucrose-6'-phosphate was not accepted as substrate mutant (≤0.02 U/mg with 0.2-200 mM sucrose-6'-phosphate), indicating that ScrB detects its substrates sucrose or sucrose-6-phosphate at the fructose end. This was also shown for the hydrolases of *Aspergillus niger* and *R. glutinis*, which were able to hydrolyze sucrose and raffinose, but not maltose or trehalose and thus seem to attack sucrose from the fructose end (Rubio & Maldonado, 1995; Rubio *et al.*, 2002).

The optimum pH was between 6.5 and 7.0 for ScrB activity at 30°C (Fig. 2B), which is the optimal temperature for *C. glutamicum* growth. At a pH of 5.0 the ScrB activity was only 13% and at pH 8.5 only 45% of the activity measurable at pH 6.5 (Fig. 2B). For *Lactobacillus reuteri* the optimal pH is at 6.0 and the enzyme kept 60-50% of its optimal activity at pH 7.0-8.0, but almost no activity below pH 3.0 (Cuezzo de Ginés *et al.*, 2000), whereas ScrB from *Bifidobacterium infantis* has its optimal pH at 6.0 with a wide pH range from 4.5 (50% activity remained) to 7.5 (40-50% activity remained) (Warchol *et al.*, 2002). Measurements of the enzyme activity under different temperatures showed that the optimal temperature for ScrB activity in *C. glutamicum* is 45°C (Fig. 2C). Thus, the optimal temperature for ScrB activity is above the optimal temperature for growth as has been observed for the hydrolases from *B. infantis* (Warchol *et al.*, 2002) and *A. niger* (Rubio & Maldonado, 1995).

(iii) Effect of divalent cations, DTT, fructose, and fructose-6-phosphate on ScrB activity. Table 2 shows that the ScrB activity was stimulated about 20% by 5 mM $MgSO_4$ while the addition of other divalent cations reduced the activity slightly ($MnCl_2$ and $CaCl_2$) or severely ($CuSO_4$ and $ZnSO_4$). Similarly, ScrB from *Bifidobacterium lactis* was markedly (56%) inhibited by the addition of 1 mM Zn^{2+} (Janer *et al.*, 2004). ScrB activity from *C. glutamicum* was neither influenced by EDTA nor by DTT. As DTT is a sulfhydryl reducing compound, these results suggest that disulfide bonds are not necessary to preserve the sucrose-6-phosphate hydrolase activity from *C. glutamicum*, in contrast to the findings for the hydrolases from *L. reuteri* and *A. niger*, where 16% or 0% relative activity remained after addition of 1 mM DTT or 6 mM *p*-chlormercuribenzoate (PCMB), respectively (Rubio & Maldonado, 1995; Cuezzo de Ginés *et al.*, 2000). The activity of ScrB from *C. glutamicum* was reduced to approximately 70% by 100 mM fructose which indicates that ScrB is feedback-inhibited by fructose, the product of sucrose-6-phosphate hydrolysis. Interestingly, fructose-6-phosphate, which was shown to inhibit invertase from

Table 2. Effectors of ScrB from *C. glutamicum*. The relative activity is given as percentage of control with no additions.

Compound	Concentration [mM]	Relative activity (%)
Control	-	100
$MgSO_4$	5	120
$MnSO_4$	5	74
$CaCl_2$	5	90
$ZnSO_4$	5	2
$CuSO_4$	5	0
EDTA	5	93
DTT	5	104
Fructose	100	69
Fructose-6-phosphate	10	153

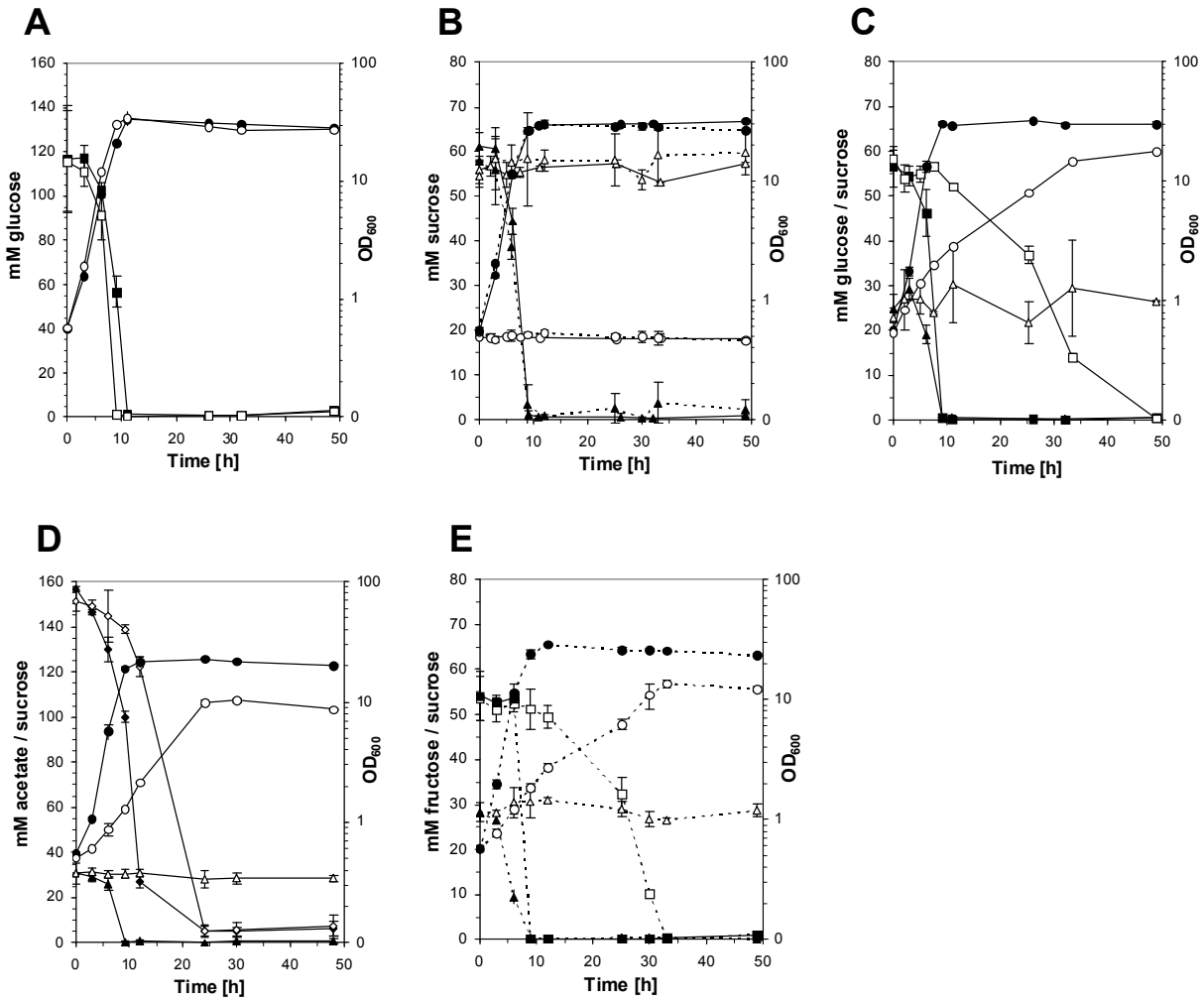

Fig. 3. Role of ScrB for growth of *C. glutamicum* on glucose- or sucrose- or glucose + sucrose-containing or on fructose plus sucrose- or acetate plus sucrose-containing media. Growth of *C. glutamicum* WT (filled symbols), Δ*scrB* (open symbols), Δ*scrB*(pVWEx1) (open symbols, dotted lines) and Δ*scrB*(pVWEx1-*scrB*) (filled symbols, dotted lines) on CgXII minimal medium containing 100 mM glucose (A) or 50 mM sucrose (B) or 50 mM glucose plus 25 mM sucrose (C) or 150 mM potassium acetate plus 25 mM sucrose (D) or 50 mM fructose plus 25 mM sucrose (E). The optical densities (circles) and the glucose (squares), fructose (squares), sucrose (triangles) and potassium acetate (diamonds) concentrations are indicated. When necessary, the cultures were induced direct after inoculation by addition of 1 mM IPTG.

Lilium auratum (Singh & Knox, 1984) stimulated ScrB from *C. glutamicum* as ScrB activity was increased by about 50% due to addition of 10 mM fructose-6-phosphate.

Characterization of the role of sucrose-6-phosphate hydrolase for growth of *C. glutamicum* on sucrose as sole carbon source and on sucrose-containing carbon source mixtures

The lack of detectable sucrose-6-phosphate hydrolase activity in Δ*scrB* (Fig. 1A) suggested that this strain would not be able to utilize sucrose. Accordingly, it was shown that the *in-frame* deletion of *scrB* resulted in a *C. glutamicum* strain which was unable to grow on sucrose as sole carbon and energy source (Fig. 3B). Plasmid-borne expression of *scrB* in the *scrB* deletion mutant complemented the growth phenotype as *C. glutamicum* Δ*scrB*(pVWEx1-*scrB*) grew as well as *C. glutamicum* WT on sucrose as sole carbon source (Fig. 3B). Thus, the *scrB* deletion mutant described here showed the same growth phenotype with sucrose as sole carbon source as *ptsS* mutants (Parche *et al.*, 2001; Moon *et al.*, 2005). One study discussed that disruption of *scrB* results in a sucrose-negative phenotype of a related *C. glutamicum* strain, but no experimental data were shown (Moon et al., 2005). In *Clostridium beijerinckii*, it was shown that a *scrB* disruption strain was unable to grow on sucrose as sole carbon source (Reid *et al.*, 1999). Growth of *C. glutamicum* Δ*scrB* on glucose as sole carbon source was similar to *C. glutamicum* WT, although the growth rate and the glucose uptake rate of *C.*

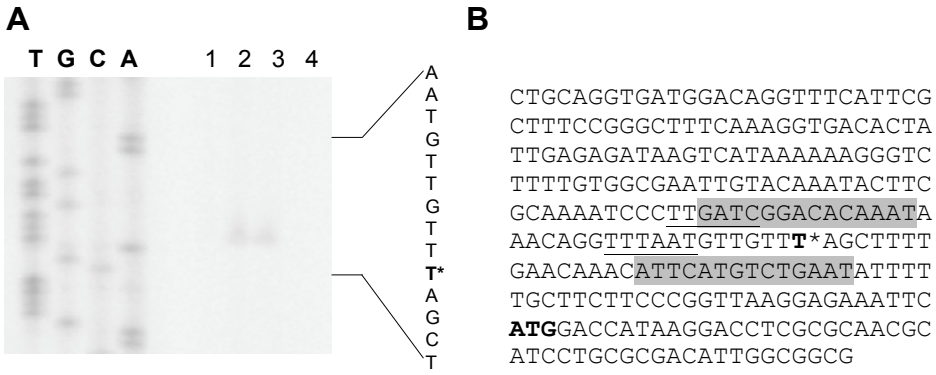

Fig. 4. Expression analysis and determination of the transcriptional start site of the *C. glutamicum ptsS* gene. (A) For primer extension analysis, 10 µg of total RNA isolated from *C. glutamicum* WT grown on LB medium (lane 1) and on CgXII minimal medium containing 50 mM sucrose (lane 2), as well as from *C. glutamicum* WT(pVWEx1) (lane 3) and *C. glutamicum* WT(pVWEx1-*sugR*) (lane 4) grown on sucrose were used. The transcriptional start site is indicated by an asterisk. The corresponding sequencing reactions (lanes T, G, C, A) were performed using the same IRD800-labeled oligonucleotides that was used in the primer extension reactions and PCR products which covered the region of the corresponding transcriptional start site as template DNA. (B) The intergenic region sequence between *scrB* (*cg2927*) and *ptsS* (*cg2925*). The start codon of *ptsS* is indicated in bold, the transcriptional start site is shown in bold and marked with an asterisk, the putative -10 and -35 regions are underlined and the putative SugR binding motifs are highlighted in grey.

glutamicum Δ*scrB* appeared to be slightly higher than those of *C. glutamicum* WT (120 compared to 108 nmol mg^{-1} min^{-1}) (Fig. 3A).

On substrate mixtures containing sucrose, growth of *C. glutamicum* strains lacking sucrose-6-phosphate hydrolase was severely perturbed (Fig. 3). On a glucose-sucrose mixture, *C. glutamicum* Δ*scrB* showed a growth rate reduced to one fifth of that of WT (µ = 0.09 ± 0.01 compared to 0.46 ± 0.01 h^{-1}) and a three fold reduced glucose uptake rate (60 compared to 22 nmol mg^{-1} min^{-1}). As only *C. glutamicum* WT could utilize sucrose (31 nmol mg^{-1} min^{-1}), *C. glutamicum* Δ*scrB* formed less biomass than *C. glutamicum* WT (17.1 ± 0.7 as compared to 29.5 ± 1.3 gDW l^{-1}) (Fig. 3C). It can be inferred that sucrose-6-phosphate accumulating intracellularly as consequence of uptake and phosphorylation of sucrose by EII^{Suc} is toxic for *C. glutamicum*. Similarly, the observation that a *S. mutans* mutant lacking sucrose-phosphate hydrolyzing activity showed perturbed growth on mannitol when sucrose was added to the culture medium was explained by toxicity of high intracellular concentrations of sucrose phosphate (Englesberg *et al.*, 1962; St Martin & Wittenberger, 1979).

The negative effect of sucrose on growth of *C. glutamicum* strains lacking sucrose-6-phosphate hydrolase was not restricted to growth on glucose-sucrose mixtures, but also occurred on fructose-sucrose and acetate-sucrose mixtures (Fig. 3). *C. glutamicum* Δ*scrB*(pVWEx1) formed less biomass on a fructose-sucrose mixture than *C. glutamicum* Δ*scrB*(pVWEx1-*scrB*), grew slower (µ = 0.10 ± 0.01 compared to 0.42 ± 0.00 h^{-1}) and showed a reduced fructose uptake rate (31 as compared to 117 nmol mg^{-1} min^{-1}) (Fig. 3E). Moreover, growth of *C. glutamicum* Δ*scrB* on an acetate-sucrose mixture was slower (µ = 0.13 ± 0.00 compared to 0.47 ± 0.01 h^{-1}) and yielded two fold less biomass than that of *C. glutamicum* WT. The acetate uptake rate was reduced from 202 to 138 nmol mg^{-1} min^{-1} (Fig. 3D). Thus, in the absence of sucrose-6-phosphate hydrolase sucrose perturbs growth of *C. glutamicum* on several carbon sources.

Influence of the carbon source on sucrose-6-phosphate hydrolase activities

To test whether ScrB levels are influenced by the carbon source, the specific activity of sucrose-6-phosphate hydrolase was determined in cell-free extracts of *C. glutamicum* WT under different growth conditions (Fig. 1B). Whereas sucrose-6-phosphate hydrolase activities were similar on LB with or without acetate or glucose, growth on LB medium with added fructose or sucrose was characterized by two to three fold higher sucrose-6-phosphate hydrolase activities (Fig. 1B). This indicated that ScrB appears to be regulated by sucrose and fructose.

The localization of the sucrose-6-phosphate hydrolase gene *scrB* directly upstream of the gene for the sucrose-specific PTS, *ptsS*, suggested co-ordinated transcriptional control of these genes. We have previously shown that sucrose-induction of *ptsS* depends on SugR, which binds upstream of *ptsS* (Engels & Wendisch, 2007). DNA microarray analysis revealed that *ptsS* RNA levels were increased 17 fold in a *sugR* deletion mutant, while *scrB* mRNA levels did not change significantly (0.7 fold; Engels & Wendisch, 2007), which was also observed in an independent study (Gaigalat *et al.*, 2007). By primer extension analysis the transcriptional start site upstream of *ptsS* was determined and *ptsS* mRNA levels during growth

on LB and on sucrose minimal medium were compared. The transcriptional start site is located 62 bp upstream of the ATG start codon of *ptsS* and analysis of the promoter region revealed a sequence (5'-TTTAAT-3') similar to the consensus -10 sequence motif of *C. glutamicum* promoters (Pátek *et al.*, 2003). While hardly any *ptsS* primer extension product could be detected during growth of *C. glutamicum* WT on LB medium, on sucrose minimal medium higher quantities of a single primer extension product were detected using two independent primers for *C. glutamicum* WT and WT(pVWEx1) (Fig. 4, lanes 2 and 3). Overexpression of *sugR* reduced *ptsS* expression on sucrose as *ptsS* primer extension product levels detected for *C. glutamicum* WT(pVWEx1-*sugR*) on sucrose were as low as those detected for *C. glutamicum* WT on LB medium (Fig. 4, lane 4).

When sucrose-6-phosphate hydrolase activities were compared in cell-free crude extracts from *C. glutamicum* strains Δ*sugR* and WT(pVWEx1-*sugR*) during growth in the presence of sucrose (Fig. 1B), comparable activities could be observed indicating that SugR does not regulate *scrB*. Thus, control of *ptsS* and *scrB* with respect to the carbon source sucrose is clearly different. In other bacteria, the genes for the sucrose-specific PTS and for sucrose-6-phosphate hydrolase, which are part of an operon in *Clostridium acetobutylicum*, *C. beijerinckii* and *Bacillus subtilis* and represent two transcriptional units in *Staphylococcus xylosus* (Jankovic & Brückner, 2007), are under sucrose-specific control by repressor proteins (ScrR) and/or are under antitermination-mediated control (ScrT, SacT) (Kunst *et al.*, 1974; Reid *et al.*, 1999; Tangney & Mitchell, 2000; Stülke & Hillen, 2000). In *S. mutans*, however, *ptsS* is sucrose-inducible, but sucrose-6-phosphate hydrolase is synthesized constitutively (St Martin & Wittenberger, 1979), reminiscent of the results obtained here for *C. glutamicum*.

Taken together, it was shown that *C. glutamicum* cg2927, named *scrB*, encodes sucrose-6-phosphate hydrolase. The enzyme showed a optimum pH between 6.5 and 7.0, a temperature optimum at 45°C and accepts sucrose and sucrose-6-phosphate with a 5000 fold lower K_m, while no activity was observed with sucrose-6'-phosphate. A *scrB* deletion mutant, which lacked detectable sucrose-6-phosphate hydrolase activity, was not able to grow on sucrose as sole carbon and energy source and showed perturbed growth on carbon mixtures containing sucrose.

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Supplementary material

The following supplementary material is available for this article:

Table S1. Oligonucleotides used in this study

4 Discussion

In this work the control of carbohydrate uptake and utilization in *C. glutamicum* was intensively studied and several transcriptional regulators have been identified, namely the two functionally equivalent regulators GntR1 and GntR2, the FadR-type regulator LldR (discussed in 4.2) and in particular the DeoR-type global transcriptional regulator SugR.

4.1 Regulation of carbon metabolism in *C. glutamicum* as compared to other bacteria like *E. coli* or *B. subtilis*

SugR from *C. glutamicum*: identification and properties. In *C. glutamicum* four genes code for transcriptional regulators of the DeoR family: Cg0139, Cg0146, SugR (Cg2115; this study and (Gaigalat *et al.*, 2007)) and FruR (Cg2118; (Tanaka *et al.*, 2008a)). Alignments according to the amino acid sequence similarities showed that in *C. jeikeium* K411 only a single transcriptional regulator of the DeoR family exists (encoded by jk1107) (Brune *et al.*, 2005), which shares 54% similarity on amino acid sequence with SugR from *C. glutamicum* and ≤30% to the others. SugR from *C. glutamicum* consists an N-terminal DeoR-type helix-turn-helix motif (PFAM: PF08220) which is responsible for DNA-binding. The C-terminal part of DeoR-type transcriptional regulators can contain an effector-binding domain and/or an oligomerization domain (van Rooijen and de Vos, 1990). DeoR-type transcriptional regulators act in most instances as transcriptional repressors in sugar metabolism (Mortensen *et al.*, 1989; Zeng and Saxild, 1999). This family of prokaryotic regulators is named after *E. coli* DeoR which represses the initiation of transcription of the *deo* operon consisting of four structural genes encoding ribonucleoside and deoxyribonucleoside catabolizing enzymes (Mortensen *et al.*, 1989). DeoR of *B. subtilis* represses the transcription of the *dra-nupC-pdp* operon, which is important for utilization of desoxyribonucleosides and deoxyribose (Zeng *et al.*, 2000). The phospho*enol*pyruvate-dependent phosphotransferase system (PTS) is characterized by the uptake and concomitant phosphorylation of carbon sources resulting in intracellular sugar phosphates (Deutscher *et al.*, 2006; Parche *et al.*, 2001; Postma *et al.*, 1993; Stülke and Hillen, 2000). The general PTS components EI and HPr (Kotrba *et al.*, 2001) as well as the sugar specific EII permeases (Moon *et al.*, 2005) were functionally characterized to some detail in *C. glutamicum*. But only a few studies investigated the regulation of sugar transport systems on the molecular level to date (Moon *et al.*, 2005; Parche *et al.*, 2001). In *B. subtilis,* PTS-specific regulation is mediated by the

phosphorylation state of the histidine containing protein HPr. In the presence of glucose, HPr is phosphorylated at the conserved Ser46 (Blencke *et al.*, 2003) and HPr-Ser-P acts as cofactor of the catabolite control protein A CcpA (Stülke and Hillen, 1999). HPr from *C. glutamicum* lacks the conserved Ser at position 46 (Parche *et al.*, 2001). Moreover, neither a homolog of HPr kinase nor a functional equivalent of *B. subtilis* CcpA were found in *C. glutamicum* and no HPr serine kinase activity could be measured (Parche *et al.*, 2001). The gene encoding the regulator SugR lies in the vicinity of the genes coding for the general PTS components in the genome of *C. glutamicum*, therefore, the function of SugR in the regulation of sugar uptake and consumption was analyzed in this work.

The SugR regulon. The DeoR-type transcriptional regulator SugR (Cg2115) was identified as repressor of *ptsG*, *ptsS* and *fruR-fruK-ptsF* expression in *C. glutamicum* (Engels and Wendisch, 2007) encoding the PTS permeases specific for glucose and sucrose uptake and a repressor of the fructose-PTS (Tanaka *et al.*, 2008a), the 1-phosphofructokinase and the permease specific for fructose uptake, respectively. In a parallel study, Gaigalat *et al.* (2007) reported about SugR as pleiotropic regulator of all *pts* genes in *C. glutamicum*. The regulation of the general PTS components by SugR was also revealed for *C. glutamicum* R, a strain providing high lactate yields (Tanaka *et al.*, 2008a). In addition, SugR not only controls PTS-genes, but also a number of further genes, mainly encoding enzymes of central carbon metabolism in *C. glutamicum*. The SugR regulon therefore comprises at least 17 genes in 14 transcription units. The genes encoding e.g. 6-phosphofructokinase (*pfkA*), fructose-1,6-bisphosphate aldolase (*fba*), enolase (*eno*), pyruvate kinase (*pyk*), NAD-dependent L-lactate dehydrogenase (*ldhA*) and transketolase (*tkt*) were identified as SugR targets by ChIP-to-chip analysis and gel retardation experiments (section 3.3; Table 3, Figure 2). Enzymatic activity measeurements revealed that SugR-mediated repression affects the activities of PfkA, LdhA, Pyk and Fba *in vivo*, whereas maximum SugR control is on LdhA activity. Thus, SugR coordinately controls expression of genes for carbohydrate uptake via the PTS and for their further metabolism (with the exception of *scrB* encoding the sucrose-6-phosphate hydrolase, see below). Among them are genes encoding enzymes of glycolysis and the pentose phosphate pathway as well as the NAD-dependent L-lactate dehydrogenase (discussed in section 4.2). In contrast, fewer targets are regulated by the carbon regulators AcnR (represses aconitase gene *acn*; (Krug *et al.*, 2005)), GntR1 and GntR2 (see below) and GlxR (represses *gntP, gntK* and isocitrate lyase and malate synthase genes *aceA* and *aceB*; (Kim *et al.*, 2004; Letek *et al.*, 2006)).

Concerning the number of regulated genes, the SugR regulon resembles the regulons of RamA und RamB (Arndt and Eikmanns, 2008; Wendisch, 2006). Figure 4.1 shows those genes/pathways regulated by SugR in orange in comparison to those genes/pathways under RamA/RamB control in light blue. RamB was shown to repress its own gene *ramB* as well as

the glyoxylate pathway genes *aceA* and *aceB*, the acetate activation operon *pta-ack* and the alcohol dehydrogenase gene *adhA* and to activate *aceE*, which encodes subunit E1 of pyruvate dehydrogenase (Arndt and Eikmanns, 2007; Blombach *et al.*, 2007; Gerstmeir *et al.*, 2004). The occurrence of RamB binding motifs suggests that the TCA cycle genes *gltA* and *acn,* and the PEP carboxykinase and malic enzyme genes *pck* and *malE* are also regulated by RamB (Arndt and Eikmanns, 2008). RamA activates *adhA, aceA, aceB, pta-ack* and *ramB* (Arndt and Eikmanns, 2007, 2008; Cramer *et al.*, 2006; Cramer and Eikmanns, 2007) and, in addition, likely regulates the TCA cycle genes *sdhCAB* for succinate dehydrogenase and *acn*, as well as *pck* and *malE* (Arndt and Eikmanns, 2008). It is noteworthy that genes for enzymes of glycolysis and the pentose phosphate pathway were not found to be regulated by either RamA or RamB, while SugR regulates *pfkA, fba, eno, pyk, ldhA* and *tkt* (see above). In a first approximation, regulation of the central carbon metabolism in *C. glutamicum* comprises control of the glyoxylate and TCA cycle genes by the global regulators RamA and RamB on the one side (Figure 4.1; light blue) and control of glycolysis and pentose phosphate pathway genes by SugR on the other side (Figure 4.1; orange). As *C. glutamicum,* in contrast to *E. coli* and *B. subtilis*, generally coutilizes carbon sources present in substrate mixtures, the carbon regulators SugR, RamA and RamB primarily fine-tune central metabolic pathways for simultaneous substrate utilization. SugR is the first transcriptional regulator described to date for genes of the glycolysis in *C. glutamicum*, e.g. *pyk*, *fba* or *eno*. In this regard, it is interesting to note that ChIP-to-chip analysis revealed binding of SugR to some targets, mainly PTS-genes (e.g. *ptsG* or *ptsH,* but also *pyk* and *tkt*), primarily in the absence of PTS-sugars, while binding to other targets (e.g. *eno, ldhA* or *fba*) was readily detected during growth in the absence or presence of sucrose, fructose or glucose. The physiological relevance of these observations remains to be studied. In contrast to the global regulation by SugR (uptake of PTS sugars; central metabolism: glycolysis and pentose phosphate pathway; LdhA: L-lactate formation → Figure 4.1 orange) and RamA/RamB (glucose uptake; acetate-specific pathways: acetate-activiation; TCA cycle; glyoxylate cycle → Figure 4.1 light blue), the uptake and utilization of other carbon sources is regulated by dedicated regulators as discussed in the following parts (GntR1/GntR2: gluconate metabolism; LldR: L-lactate uptake). But none of these three global regulators SugR, RamA and RamB has been shown to regulate genes for transporters of non-PTS (Arndt and Eikmanns, 2008) as indicated in Figure 4.1. Thus, this observation supports the assumption that SugR control is of prime importance for *C. glutamicum*.

In *E. coli* and *B. subtilis*, regulators like CRP and CcpA are also not only regulating *pts* genes but are global regulators of the carbon metabolism in these organisms. CcpA, a member of the LacI/GalR family of transcriptional regulators, is the master regulator of carbon catabolite regulation in *B. subtilis* (Henkin *et al.*, 1991; Sprehe *et al.*, 2007) and

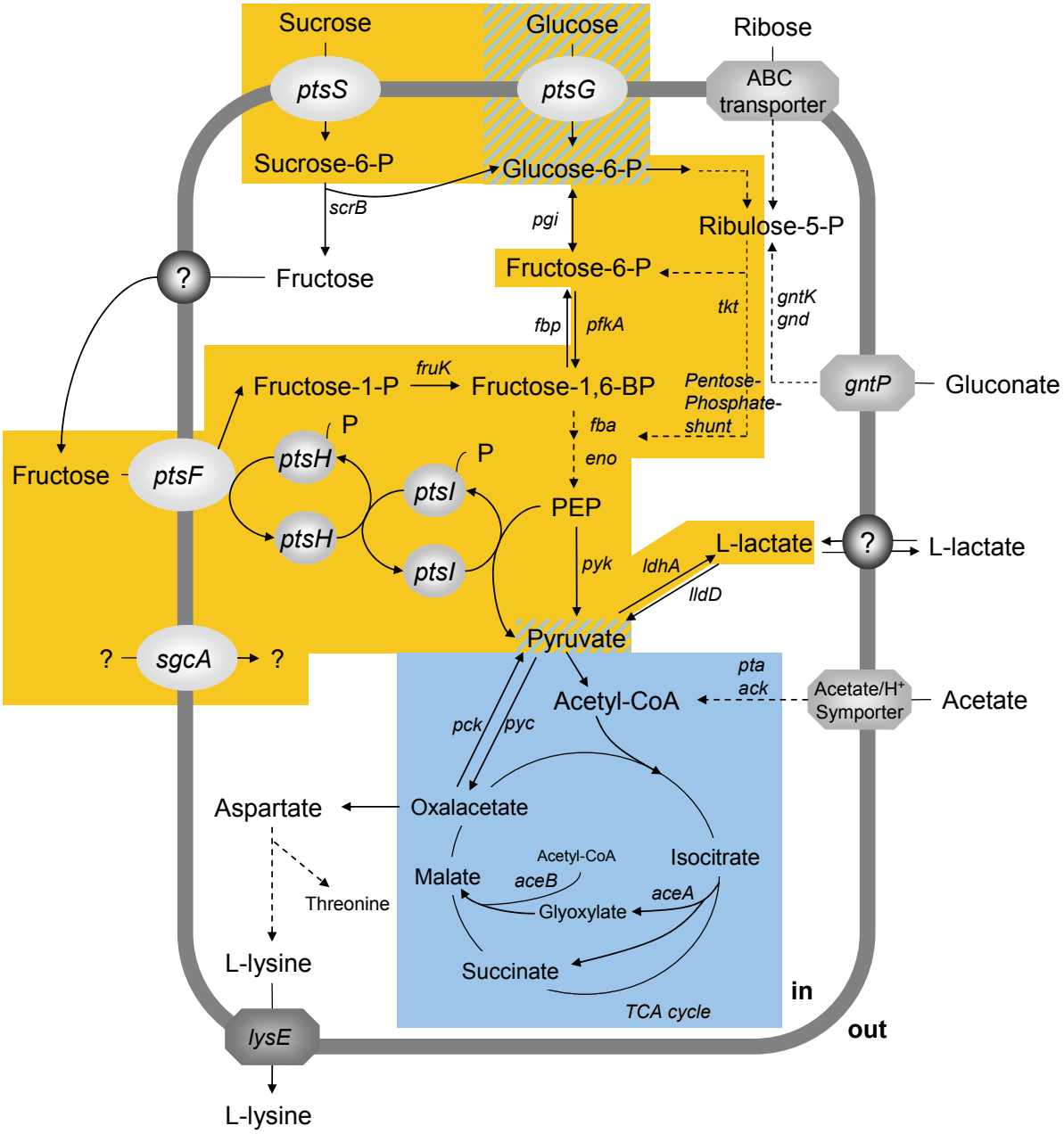

Figure 4.1. Transcriptional regulation of carbohydrate uptake and utilization by the global regulators SugR and RamA/RamB in *C. glutamicum*. The gene names of transport proteins and enzymes are given (see introduction Figure 2.1). SugR regulon is shaded in orange, RamA/RamB regulon is shaded in light blue and those genes regulated by SugR and RamB are shaded in both colours.

regulates more than 300 genes by either activation (e.g. α-acetolactate synthase *alsS*, acetate kinase *ackA*) or repression (e.g. *gntR*) (Henkin, 1996; Miwa *et al.*, 1997; Sprehe *et al.*, 2007). It was suggested that *ccpA* mutants are unable to activate glycolysis or carbon overflow metabolism and CcpA might be a key regulator molecule, controlling a superregulon

of glucose catabolism in this organism by repression of genes of the TCA cycle and for utilization of carbon sources other than glucose (Deutscher *et al.*, 2002; Tobisch *et al.*, 1999). The enzymes required for the interconversion of triosephosphates in *B. subtilis* are encoded in the *gapA-pgk-tpi-pgm-eno* operon, called *gapA* operon (Tobisch *et al.*, 1999). Ludwig *et al.* (2001) have shown that the level of derepression varies with the carbon source, e.g. carbohydrates catabolized via the upper part of the glycolysis such as glucose or fructose or carbohydrates catabolized via the pentose phosphate pathway such as gluconate induce the *gapA* operon. Two different regulators control the expression of the *gapA* operon, the global regulator CcpA and the regulator CggR (Doan and Aymerich, 2003; Fillinger *et al.*, 2000; Ludwig *et al.*, 2001). *In vitro* experiments showed that fructose-1,6-bisphosphate acts as inhibitor of CggR DNA binding (Doan and Aymerich, 2003), but also an anabolic effector derived from amino acid metabolism is predicted (Ludwig *et al.*, 2001). Fructose-1,6-bisphosphate is also the major signaling molecule in CcpA-dependent catabolite repression and activation in *B. subtilis* (Deutscher *et al.*, 2002; Stülke and Hillen, 2000) and thus, is the integration point between both CcpA and CggR regulatory networks (Doan and Aymerich, 2003). Apart from the catabolic repression of genes by CcpA, it appears that *B. subtilis* has two different regulatory pathways activating two distinct subsets of genes in response to the availibility of glycolytic substrates using the same effector molecule, i.e. CcpA-dependent activation of carbon overflow metabolism genes (*ackA* or *pta*) and CggR-dependent derepression of central glycolytic genes *gapA pgk tpi pgm* and *eno* (Doan and Aymerich, 2003). Moreover, recent results indicated that activation and repression mediated by CcpA may utilize different conformational changes of the protein (Sprehe *et al.*, 2007). In *E. coli* CRP, one of the global regulators known to regulate >50% of the cell's transcription units, which encode enzymes of many different pathways including glycolysis and the TCA cycle (Grainger *et al.*, 2005). Chip-to-chip and transcriptome analysis using DNA microarrays showed that CRP binds to dozens of promoter regions in the *E. coli* chromosome, e.g. the promoter regions of *rbsD* (D-ribose high affinity transport system), *gnt* (gluconate transporter), *aceA* and *aceB* (isocitrate lyase, malate synthase), *gnd* (6-phosphogluconate dehydrogenase) or *pckA* (phospho*enol*pyruvate carboxykinase) (Gosset *et al.*, 2004; Grainger *et al.*, 2005). In both *E. coli* and *B. subtilis*, carbon catabolite control is a dominant regulatory mechanism responsible for diauxic growth phenomena.

The binding site of SugR and the effector of SugR-binding. Sequence comparisons of the promoter regions bound by the global repressor SugR in *C. glutamicum* identified a conserved sequence motif (NN**TCRRACA**N**W**N**W**N; section 3.3, Table 4) that is found two times in the promoter regions of all verified SugR targets and coincides with the sequences upstream of *ptsG* and in the intergenic region between *ptsI* and cg2118 (Gaigalat *et al.*, 2007) shown by mutational analysis to be essential for SugR binding. *In vitro* evidence

suggests that a $TG(T)_{2-5}G$ sequence might in addition be involved in SugR binding in *C. glutamicum* R, a strain providing high lactate yields (Tanaka *et al.*, 2008b). DeoR from *E. coli* for instance binds to a 16 bp palindromic sequence 5'-TGTTAGAA·TTCTAACA-3' in either two of the three operator sites O_1, O_2 and O_E forming a single or double DNA loop (Mortensen *et al.*, 1989). FruR from *Lactococcus lactis* potentially binds to four repeating non-palindromic sequences upstream of the fructose-PTS gene cluster (Barrière *et al.*, 2005). Since the locations and the relative orientations between the two sequence motifs within a given promoter region vary it is currently not known how different orientations and locations of the binding sequence motifs affect the action of the SugR from *C. glutamicum in vivo* and if SugR occurs in different multimeric forms as described for DeoR of *E. coli*.

Fructose-6-phosphate was the only (from all tested) intermediate of the central carbon metabolism that interfered with SugR-binding in this work. Thus, fructose-6-phosphate concentrations were measured in the ATCC13032 WT on six different carbon sources (unpublished data), namely the PTS sugars glucose, fructose and sucrose and the gluconeogenic carbon sources pyruvate, acetate and citrate (Figure 4.2B). A comparison to previous results obtained by measuring the *ptsG*'-'*cat* fusion expression on the same media (Figure 4.2A) clearly points out that the repression of SugR is maximal when fructose-6-phosphate concentration is low. Thus, the observed expression levels of the *ptsG*'-'*cat* fusion in *C. glutamicum* WT and the *sugR* deletion mutant during growth on sugars or gluconeogenic carbon sources can be explained by fructose-6-phosphate dependent regulation by SugR. Although fructose-6-phosphate is the ideal intracellular substance for sensing the presence of all PTS substrates, also fructose-1-phosphate as well as fructose-1,6-bisphosphate and glucose-6-phosphate interfered with SugR-binding in another study (Gaigalat *et al.*, 2007). Effector molecules for DeoR-type regulators are generally phosphorylated intermediates of the relevant metabolic pathways as shown for DeoR of *E. coli* and *B. subtilis* (Mortensen *et al.*, 1989; Zeng *et al.*, 2000). But also non-phosphorylated inducers have been described, e.g. opine for AccR from *Agrobacterium tumefaciens* (Beck von Bodman *et al.*, 1992) or fucose for FucR from *Bacteroides thetaiotaomicron* (Hooper *et al.*, 1999). Fructose-1-phosphate as well as fructose-1,6-bisphosphate and glucose-6-phosphate interfered with SugR-binding to the *fruR-fruK-ptsF* promoter region (Gaigalat *et al.*, 2007), whereas fructose-6-phosphate was shown to interfere with SugR-binding to the *ptsG* promoter region in this study. A varying binding specificity of the SugR protein to different DNA promoter regions in dependency of the identified effectors is suggested and could be verified by real-time interaction studies using e.g. a BIAcore biosensor. Such studies revealed the complex stability of muscle aldolase with FBPase in the presence of several glycolytic intermediates, e.g. fructose-6-phosphate or

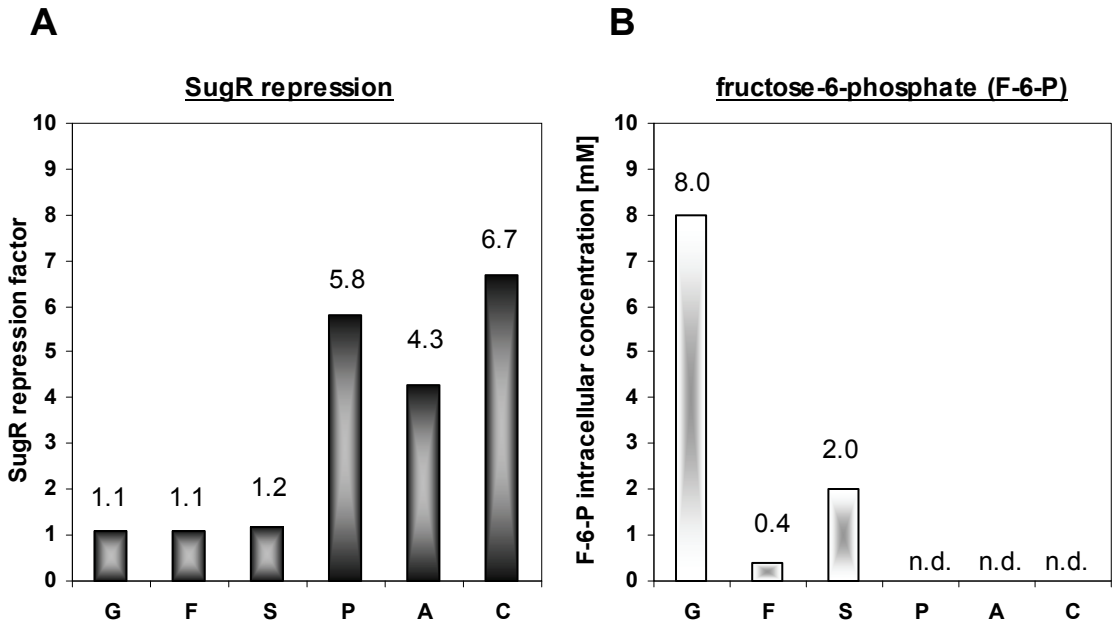

Figure 4.2. (A) Ratio of *ptsG* expressions between the deletion mutant Δ*sugR* and the wild type from the *ptsG*'-'*cat* fusion experiments in *C. glutamicum* WT(pET2-*ptsG*) and *C. glutamicum* Δ*sugR*(pET2-*ptsG*) on 100 mM glucose (G), 100 mM fructose (F), 50 mM sucrose (S), 200 mM Na-pyruvate (P), 300 mM K-acetate (A) and 100 mM Na_3-citrate + 100 mM $MgCl_2$ (C) shown in the result section 3.1. (B) Intracellular concentrations of fructose-6-phosphate during growth of *C. glutamicum* ATCC13032 WT on the same media as indicated under (A). All data are mean values of at least two determinations of at least two independent cultivations.

dihydroxyacetone phosphate (Rakus *et al.*, 2003). Examples where different triggers affect different actions of the same transcriptional regulator are the TyrR protein of *E. coli* and the transcriptional regulator TrmB from *Pyrococcus furiosus*. In *E. coli,* genetic regulation of aromatic amino acid biosynthesis and uptake is effected by the TyrR protein, which acts via ligand-mediated repression or activation (Wilson *et al.*, 1995). Tyrosine, but also phenylalanine and tryptophan, modulate transcriptional regulation by TyrR (Pittard, 1996 2005). In the presence of ATP and tyrosine or of ATP and high concentrations of phenylalanine, TyrR forms hexamers and acts as repressor, whereas it exists as a dimer in the presence of tryptophan and low concentrations of phenylalanine (Pittard, 1996; Polen *et al.*, 2005; Wilson *et al.*, 1995). TrmB of the hyperthermophilic archaea *P. furiosus* is the transcriptional repressor of the gene cluster of the trehalose/maltose ABC transporter (the TM system) with trehalose and maltose as inducers and of the maltodextrin ABC transporter genes (the MD system) with maltotriose and sucrose as inducers (Lee *et al.*, 2005; Lee *et al.*, 2007). In addition, glucose which was bound by TrmB acted as corepressor for both systems (Lee *et al.*, 2008). Thus, this regulator is able to bind different sugars likely causing different

conformational changes of TrmB. This enables TrmB to interact with different promoter regions which represents a simple mechanism for selecting the usage of one carbon source over the other (Lee *et al.*, 2008).

Regulation of sucrose uptake and initial utilization in *C. glutamicum*. The sucrose-6-phosphate hydrolase (ScrB) catalyzes the initial step in sucrose metabolism and its gene lies directly upstream and in the same transcriptional orientation as *ptsS* (Moon *et al.*, 2005). It was shown that SugR does not regulate *scrB* in *C. glutamicum* (Figure 2.1 and section 3.6). Biochemical characterization of ScrB enzyme and analysis of SugR-dependent control of *scrB* expression showed that control of *ptsS* and *scrB* with respect to the carbon source sucrose is clearly different. Sucrose induction of *ptsS* depends on SugR, while sucrose induction of *scrB* did not require SugR since ScrB activities were comparable in the Δ*sugR* mutant and the *sugR* overexpressing strain WT(pVWEx1-*sugR*) during growth in the presence of sucrose (section 3.6; Fig. 1B). In other bacteria, the genes for the sucrose-specific PTS and for the sucrose-6-phosphate hydrolase, which for instance are part of an operon in *B. subtilis,* but represent two transcriptional units in *Staphylococcus xylosus* (Jankovic and Brückner, 2007), are under sucrose-specific control by repressor proteins (ScrR) which are encoded in the vicinity of the two genes in the genome and/or are under antitermination-mediated control (ScrT, SacT) (Kunst *et al.*, 1974; Reid *et al.*, 1999; Tangney and Mitchell, 2000); (Stülke and Hillen, 2000). In *C. glutamicum,* however, no transcriptional regulator is encoded in the genomic region of *ptsS* and *scrB*. Thus, sucrose-induction of *scrB* is currently not understood. The group of Tanaka *et al.* investigated the effect of the second DeoR-type transcriptional regulator FruR encoded in the *fruR-fruK-ptsF* operon only with regard to the regulation of *pts* genes but not on *scrB* (Tanaka *et al.*, 2008a). Thus, FruR is a possible candidate of *scrB* regulation in *C. glutamicum*.

Complex regulation of *ptsG* by SugR and other transcriptional regulators. Expression of *ptsG* is under the control of several transcriptional regulators: SugR, RamB, GntR1 and GntR2. The role of SugR and GntR1 as well as GntR2 thereby is clear but remains to be clarified for RamA and RamB. In collaboration with Julia Frunzke the two functionally redundant regulators GntR1 and GntR2 have been identified in *C. glutamicum*, which coordinately control gluconate catabolism and glucose uptake, presumably in dependency of the intracellular concentration of gluconate and glucono-δ-lactone (result section 3.2). Whereas the negative control of genes involved in gluconate metabolism by GntR-type regulators has previously been demonstrated e.g. in *E. coli* (Izu *et al.*, 1997; Peekhaus and Conway, 1998; Porco *et al.*, 1997) or *B. subtilis* (Fujita and Miwa, 1989; Miwa and Fujita, 1988; Reizer *et al.*, 1991), the simultaneous positive control by these regulators on *ptsG* was a novel aspect. Besides its negative influence on *ptsG* expression mediated by GntR1 and GntR2 in the presence of gluconate, gluconate appears to have also a positive effect on *ptsG*

expression: in the *ptsG'-'cat* fusion assays, expression of *ptsG* in the Δ*gntR1*Δ*gntR2* mutant was two fold higher on gluconate or glucose plus gluconate than on glucose alone. This is the first example of gluconate-dependent transcriptional control of PTS genes. The observed differences *ptsG'-'cat* fusion assays on the different carbon sources measured in this study (Frunzke *et al.*, 2008) might be caused by the SugR protein, which represses *ptsG* and other PTS genes during growth on gluconeogenic carbon sources. In this work SugR activity was shown to be controlled by fructose-6-phosphate, thus linking regulation of glucose uptake to the first glycolytic intermediate after the branch-point to the pentose phosphate pathway. When gluconate is catabolized via the pentose phosphate pathway, it enters glycolysis at the level of fructose-6-phosphate and glyceraldehyde-3-phosphate (Figure 2.1). Therefore, it seems possible that the intracellular fructose-6-phosphate concentration is increased in the presence of gluconate and repression of *ptsG* by SugR is diminished. This explanation might be confirmed or disproved by analysis of *ptsG* expression in a Δ*gntR1*Δ*gntR2*Δ*sugR* triple mutant and measurement of fructose-6-phosphate concentrations on gluconate minimal medium. Gerstmeir *et al.* (Gerstmeir *et al.*, 2004) suggested a putative binding site for the global regulator RamB also in the *ptsG* promoter region. In *C. glutamicum*, repression of genes of acetate metabolism by RamB was only observed during growth on glucose, i.e. in the absence of acetate (Gerstmeir *et al.*, 2004). As the putative binding sites of RamB are located downstream of the transcriptional start site of *ptsG*, RamB might function as a negative regulator of *ptsG* besides SugR (Figure 4.1 blue and orange striped). It is noteworthy that expression of the transcriptional fusion containing the *ptsG* promoter fragment 4, which lacks the putative RamB binding sites, was about two fold higher than expression of the full-length *ptsG* promoter fusion. This would likely stand for a repression of *ptsG* by the factor of two due to RamB binding.

In other bacteria, e.g. *E. coli*, regulation of *ptsG* is well understood and complex (Bettenbrock *et al.*, 2006; Gosset *et al.*, 2004; Kimata *et al.*, 2001; Lee *et al.*, 2000; Plumbridge, 1998, 1999; Ryu and Garges, 1994; Zheng *et al.*, 2004). In *E. coli* cAMP receptor protein (CRP) and Mlc are known to be the major regulators of *ptsHIcrr* (coding for enzyme I, HPr and $EIIA^{Glc}$) and *ptsG* (coding for $EIICB^{Glc}$) in response to the availability of carbon sources (Gosset *et al.*, 2004; Plumbridge, 1999). Transcription of *ptsG* is stimulated by cAMP·CRP and is repressed by Mlc (Gosset *et al.*, 2004; Kimata *et al.*, 2001; Plumbridge, 1998, 1999; Ryu and Garges, 1994; Zheng *et al.*, 2004). CRP is activated by binding cAMP and the intracellular concentration of cAMP which is synthesized from ATP in turn is dependent on the activity of the adenylate cyclase encoded by *cyaA* (Brückner and Titgemeyer, 2002). The activity of CyaA however is up to the phosphorylation state of the EII permeases which depends on the presence or absence of PTS substrates. Mlc binds upstream of *ptsG* and represses its transcription in the absence of glucose. During growth on

glucose, EIICB^{Glc} occurs in its unphosphorylated form due to glucose uptake and binds to Mlc, which results in derepression of *ptsG* (Bettenbrock *et al.*, 2006; Lee *et al.*, 2000). FIS, a nucleoid-associated protein, facilitates rapid adaptation of *E. coli* to different carbon and energy sources through the formation of nucleoprotein complexes either with cAMP·CRP in the presence of glucose or with Mlc in the absence of glucose (Shin *et al.*, 2003). Moreover, *ptsG* transcription appears to be inhibited by the response regulator ArcA, which is phosphorylated and activated by the histidine kinase ArcB under reducing conditions. Phosphorylated ArcA binds to the CRP-binding site of *ptsG*, thus interfering with CRP activation of *ptsG* transcription (Jeong *et al.*, 2004). In *B. subtilis*, *ptsGHI* transcription terminates in the absence of glucose due to the inactivation of the RNA-binding antitermination protein GlcT by phosphorylation by the phosphorylated EII^{Glc}. In the presence of the inducer glucose, phosphorylated EII^{Glc} transports and phosphorylates the incoming glucose. GlcT protein is now able to bind the ribonucleic antiterminator or RAT sequence of the nascent *ptsGHI* operon RNA and prevents premature termination of transcription (Schilling *et al.*, 2004; Schmalisch *et al.*, 2003; Stülke *et al.*, 1997). It was found that the upstream region of *ptsG* from *C. diphtheriae* contains a RAT-like sequence and a GlcT-like protein (DIP1150) is encoded in its genome (Parche *et al.*, 2001). However, the *C. glutamicum* genome does not code for a functional GlcT-like protein, as the putative 197 amino acid protein encoded by cg3144 showing the highest sequence similarity to amino acids 93-275 of *C. diphtheriae* DIP1150, lacks the 92 N-terminal amino acids of *C. diphtheriae* DIP1150 including the RNA binding domain (Hartmann *et al.*, 2003). In addition, the region upstream of *C. glutamicum ptsG* is lacking a RAT-like sequence (Parche *et al.*, 2001).

Regulation of the fructose utilization operon *fruR-fruK-ptsF*. In order to metabolize fructose, external fructose is taken up via the fructose-specific PTS (*ptsF*) as fructose-1-phosphate and converted into fructose-1,6-bisphosphate by the 1-phosphofructokinase (*fruK*). Both genes are part of the *fruR-fruK-ptsF* operon in contrast to *ptsS* and *scrB* discussed above. The second DeoR-type transcriptional regulator encoded within this operon in *C. glutamicum*, namely FruR (Cg2118), was recently shown to alleviate the induction of *ptsI*, *ptsH* and the *fructose-pts* by fructose (Tanaka *et al.*, 2008a). The effector of FruR binding was not directly experimentally proven, but it is likely that fructose-1-phosphate as first direct intermediate of fructose metabolism interferes with FruR binding, which is also the case for FruR of *Lactococcus lactis* (Barrière *et al.*, 2005). Supporting this hypothesis is that fructose is the most effective inducing sugar but also sucrose induced the expression of *pts* genes tested (Tanaka *et al.*, 2008a). Previous experiments showed that expression of *ptsF*, *ptsH* and *ptsI* is induced by the addition of fructose compared to glucose which was not observed for *ptsG* expression (Tanaka *et al.*, 2008b). In addition, the results indicated that

the induction of *ptsF*, *ptsH* and *ptsI* by fructose is independently from SugR due to same relative mRNA expression levels in the WT and the *sugR* deletion mutant of these genes on fructose-acetate mixture. The authors suggested that the sucrose effect is caused by the emerging fructose. Since *C. glutamicum* cannot phosphorylate fructose intracellularly, utilization involves temporary efflux out of the cell, subsequent re-uptake and phosphorylation by the fructose-PTS to yield fructose-1-phosphate (Dominguez and Lindley, 1996; Dominguez *et al.*, 1998).

Control of the non-PTS sugar uptake systems. The physiological significance of SugR control of non-PTS genes became obvious by the facts that *sugR* overexpression perturbed utilization of ribose and that deletion of *sugR* resulted in increased L-lactate formation under oxygen-deprivation conditions (section 4.2). However, SugR was not found to be involved in the regulation of the ribose operon (*rbsACBD*) encoding the subunits of an ABC transport system (Wendisch, 2003). Growth of *C. glutamicum* on ribose requires pyruvate kinase activity. A Δ*pyk* mutant was not able to grow on ribose as sole carbon and energy source (Netzer *et al.*, 2004a) and as SugR was found to bind upstream of *pyk*, growth perturbation due to overexpression of *sugR* can be explained by repression of *pyk* by SugR. In addition, SugR repression of the transketolase gene *tkt*, which is important for growth on ribose, likely augments growth perturbation on ribose by *sugR* overexpression.

As there is no hint concerning a SugR-dependent control of lactate uptake systems, SugR, however, is involved in L-lactate formation by the NAD-dependent L-lactate dehydrogenase in *C. glutamicum* as mentioned above. This topic is focussed in the next section in detail.

4.2 Regulation of L-lactate metabolism in *C. glutamicum* by the global regulator SugR and the FadR-type regulator LldR

L-lactate is both a metabolic product as well as a carbon substrate for growth. *C. glutamicum* can grow aerobically on L-lactate as sole carbon and energy source (Cocaign and et al., 1993; Gourdon *et al.*, 2000; Stansen *et al.*, 2005). Growth of *C. glutamicum* on L-lactate requires an L-lactate uptake system, which has not been definitely identified yet, and a quinone-dependent L-lactate dehydrogenase LldD (cg3227; EC 1.1.2.3), which is a peripheral membrane protein catalyzing oxidation of L-lactate to the central metabolite pyruvate (Bott and Niebisch, 2003; Schluesener *et al.*, 2005; Stansen *et al.*, 2005). Both genes, the gene for the putative permease (cg3226) and *lldD*, are transcribed in an operon in *C. glutamicum* (Stansen *et al.*, 2005). In *E. coli*, the L-lactate utilization operon encodes besides LldD a L-lactate permease (LldP) and a putative lactate regulator protein LldR (Dong *et al.*, 1993). However, a putative regulatory gene is not encoded in the *C. glutamicum*

cg3226-*lldD* operon. L-lactate is taken up by *E. coli* either by the L-lactate permease LldP or by the glycolate permease GlcA (Nunez *et al.*, 2002). Expression of the cg3226-*lldD* operon from *C. glutamicum* was shown to be regulated by the carbon source and is maximal in the presence of L-lactate (Stansen *et al.*, 2005). When L-lactate was present in the medium, 17 fold higher mRNA levels of cg3226 and *lldD* as well as a 10 fold higher specific activity of the quinone-dependent L-lactate dehydrogenase were observed (Stansen *et al.*, 2005).

On the other hand, *C. glutamicum* is able to secrete L-lactate into the medium, e.g. as by-product during glutamate and lysine production (Kiefer *et al.*, 2002; Stansen *et al.*, 2005) or under oxygen-deprivation conditions (Inui *et al.*, 2004). L-lactate formation in *C. glutamicum* requires the NAD-dependent L-lactate dehydrogenase LdhA (EC 1.1.1.27; (Inui *et al.*, 2004)) which converts pyruvate into L-lactate. *ldhA* mRNA levels were increased about 9 fold under oxygen deprivation conditions (Inui *et al.*, 2007) as expected for fermentative enzymes. However, the regulatory mechanism for anaerobic induction or aerobic repression of *ldhA* in *C. glutamicum* is currently unknown. The peripheral membrane protein LldD uses quinone as electron acceptor, whereas the soluble NAD-dependent L-lactate dehydrogenase LdhA is presumably used for reoxidation of NADH under conditions where NADH oxidation by the respiratory chain is limiting (Bott and Niebisch, 2003). As described above, under these conditions L-lactate is formed as a fermentation product.

Transcription of the *lld* operon of *E. coli* is repressed by the two-component signal transduction system ArcA/ArcB during anaerobiosis, is activated by the pyruvate dehydrogenase repressor PdhR, and might be repressed by LldR encoded within the lactate utilization operon (Dong *et al.*, 1993; Quail and Guest, 1995). In a collaboration with Tobias Georgi it was shown that the cg3226-*lldD* operon in *C. glutamicum* is repressed by the FadR-type transcriptional regulator LldR (Cg3224) in the absence of its effector L-lactate (Georgi *et al.*, 2008). Homologs of LldR from *C. glutamicum* are encoded in the genomes of other *Corynebacterineae* like *C. glutamicum* R (97% sequence identity, cg_R2816), *C. efficiens* (76%, CE2757), *C. diphtheriae* (38%, DIP0011) and *Mycobacterium smegmatis* (42%, MSMEG_0895), while other mycobacterial genomes apparently lack homologous genes (Georgi *et al.*, 2008). The binding site of LldR of *C. glutamicum* could be identified experimentally by gel retardation experiments and mutational analysis (section 3.4; Figure 2). The sequence motif upstream of cg3226, $^{-1}$TGGTCTGACCA^{+10}, shows hyphenated dyad symmetry containing the two half sites, TNGT and ACNA, of the consensus sequence for FadR-type regulators (Rigali *et al.*, 2002). Mutational analysis revealed that both half-sites are essential for binding of LldR to the cg3226-*lldD* promoter. As the motif overlaps with the transcriptional start site of the cg3226-*lldD* operon, this likely represents a repression mechanism due to interference with RNA polymerase-promoter recognition. The inducer of the *C. glutamicum* cg3226-*lldD* operon could be identified as L-lactate as it prevents binding

of LldR to the cg3226-*lldD* promoter *in vitro* at 40 mM, while for comparison 1 mM pyruvate abolished binding of the FadR-like regulator PdhR from *E. coli* to the promoter of the *pdhR-aceEF-lpd* operon (Quail and Guest, 1995). L-lactate effects LldR function at physiologically relevant concentrations as indicated by measurements of intracellular L-lactate concentrations of 32 to 39 mM in glucose-grown *C. glutamicum* ATCC17965 cells (Pequignot *et al.*, 1997). Regulation of the cg3226-*lldD* operon other than by L-lactate via LldR, e.g. by oxygen availability or pH, has not been studied yet, but it was observed that in long-term lactic acid adapted *C. glutamicum* cells grown in continuous culture at pH 5.7, mRNA levels of cg3226 and *lldD* were not changed in comparison to continuous cultures at pH 7.5 (Jakob *et al.*, 2007). The finding that RamA binds to the promoter of the cg3226-*lldD* operon (Georgi, 2006) suggests that RamA represses or activates this operon. Further investigations on this topic revealed that RamA is required for L-lactate utilization in the presence of glucose (Georgi, 2006). A deletion of the *ramA* gene in the *C. glutamicum* had no influence on growth on fructose plus L-lactate containing media. In contrast, on glucose/L-lactate mixture the *C. glutamicum* WT co-utilized glucose and L-lactate, a *ramA* deletion mutant showed a sequential consumption of glucose before L-lactate (Georgi, 2006). Moreover, a Δ*ramA* mutant was not able to grow on L-lactate as sole carbon and energy source (Georgi, 2006). Thus, RamA is an activator essential for the expression of L-lactate utilization genes in *C. glutamicum* and its binding is likely inhibited by fructose or an intermediate of fructose metabolism as the consumption of L-lactate was fructose-specific inhibited on fructose/L-lactate mixtures. According to the current model for regulation of the L-lactate utilization operon cg3226-*lldD* in *C. glutamicum* LldR binds to its operator sequence upstream of cg3226 and represses transcription of cg3226-*lldD*. In the presence of L-lactate, L-lactate binds to LldR preventing repression of cg3226-*lldD* by LldR. Thus, LldR control ensures that the L-lactate utilization operon cg3226-*lldD* is expressed only when L-lactate is present (Figure 4.3).

Microbial production of L-lactate is traditionally attributed to the lactic acid bacteria, e.g. *Saccharomyces cerevisiae* (Adachi *et al.*, 1998) and *E. coli* (Chang *et al.*, 1999). In *E. coli* two independent regulatory systems, the Fnr modulon which encodes proteins that are involved in cellular adaption to growth in anoxic environments (Unden and Schirawski, 1997; Unden *et al.*, 2002) and the ArcA/ArcB two component signal transduction system that regulates gene expression in response to redox conditions (Liu and De Wulf, 2004; Perrenoud and Sauer, 2005), have been identified that serve major roles in modulating expression of the pathways for carbon and electron flow in response to oxygen availability (Gunsalus and Park, 1994). Both can respond independently to a shift between aerobic and anaerobic conditions and can act as activator or repressor of gene expression. Whereas Fnr

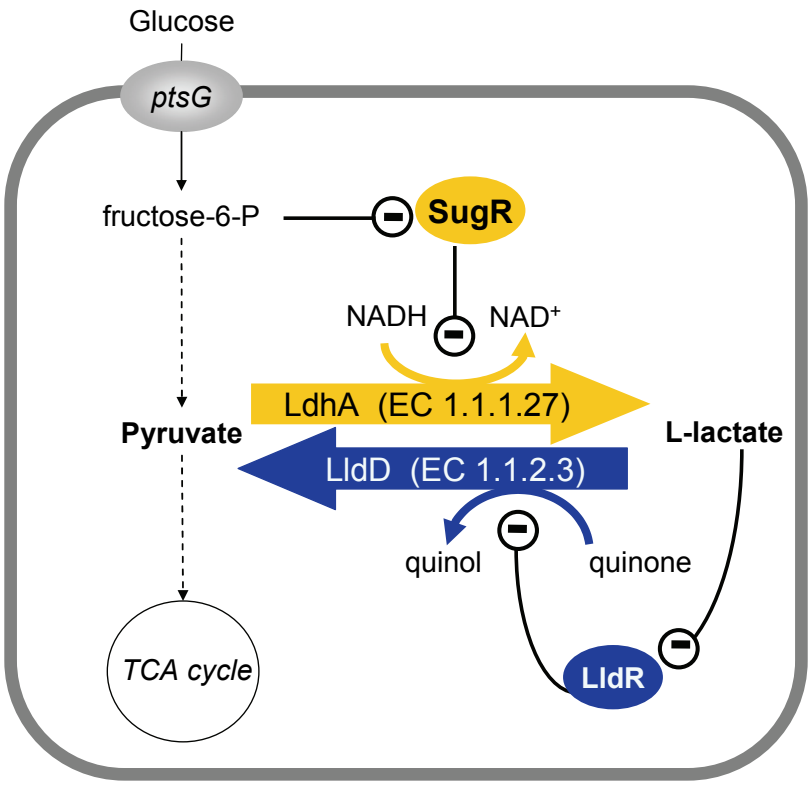

Figure 4.3. Model of regulation of the L-lactate metabolism in *C. glutamicum* by SugR and LldR.

is active only under anaerobic conditions, ArcA/ArcB functions under both aerobic and anaerobic conditions (Gunsalus and Park, 1994). These two regulatory elements act in combination to coordinate the pathways for carbon flow from pyruvate to the various end products of aerobic and anaerobic metabolism, to electron flow via the respiratory pathways. However, a Fnr homologue in *C. glutamicum* has not been identified (Nishimura *et al.*, 2007). Those proteins with the highest similarity (26% and 20%, respectively) in *C. glutamicum* to Fnr from *E. coli* on protein level are cg1327, a bacterial regulator of the crp-family, and GlxR, which is involved in regulation of the glyoxylate pathway in *C. glutamicum* (Kim *et al.*, 2004). An observed regulatory effect of Mlc on *ldhA* in *E. coli* appeared to be indirect because binding of Mlc to the *ldhA* promoter region could not be demonstrated *in vitro* (Jiang *et al.*, 2001). Moreover, the authors had the idea that increasing sugar metabolism indirectly induces *ldhA* (Jiang *et al.*, 2001). In *B. subtilis*, it was shown that YdiH functions as repressor of the *ldh* gene (Larsson *et al.*, 2005). It is proposed that YdiH serves as redox sensor, being itself regulated by cellular differences in the free levels of NAD^+ and NADH (Larsson *et al.*, 2005). During fermentation, LDH is the key enzyme involved in reoxidation of NADH formed by glycolysis (Cruz Ramos *et al.*, 2000). Repression of transcription of *ldh* by nitrate, which can be used as electron acceptor for anaerobic growth of *B. subtilis*, is therefore mediated by

YdiH due to nitrate-respiration-dependent NADH oxidation (Cruz Ramos *et al.*, 2000; Larsson *et al.*, 2005). In this work, it was shown in *C. glutamicum* that *ldhA* is a target of SugR and that SugR represses *ldhA* due to its binding to the corresponding promoter region as described above and in section 3.3 (Fig. 2, Table 3, Fig. 3A). In the absence of SugR, LdhA activities were about eight fold higher than in *C. glutamicum* WT under aerobic conditions. Also shown in this work was that under oxygen-deprivation conditions L-lactate formation increased three fold on glucose minimal medium. Several glycolytic intermediates (glucose-6-phosphate, fructose-6-phosphate, fructose-1,6-bisphosphate or fructose-1-phosphate) were identified to interfere with SugR binding to its target promoters ((Gaigalat *et al.*, 2007) and this study). Under aerobic conditions SugR control of *ldhA* therefore ensures that *ldhA* expression is maximal when supply of carbohydrate growth substrates entering glycolysis is sufficient and therewith fructose-6-phosphate (or glucose-6-phosphate or fructose-1,6-bisphosphate) concentrations are high (Figure 4.3). Nevertheless, there has to be also an anaerobic control of carbon flow and respiration in *C. glutamicum* as shown above for other organisms.

As there is apparently no Fnr homologue encoded in the *C. glutamicum* genome, one could speculate about other homologues of regulatory genes involved in anaerobic control like in *B. subtilis* or *E. coli* that were mentioned above. ArcA/ArcB functions under both aerobic and anaerobic conditions. The *arcA* mutant had pleiotropic effects on expression of many other aerobic function enzymes including TCA cycle enzymes, fatty acid degradation enzymes, ubiquinone oxidase and isocitrate lyase of the glyoxylate pathway (Iuchi and Lin, 1988). The sensor kinase ArcB and the ArcA response regulator of the two-component signal transduction system in *E. coli* share weak similarity (34% and 28% sequence identity, respectively) to the CgtSR4 two component system of *C. glutamicum*. Genome-wide transcriptome analysis using DNA microarray indicated that the CgtSR4 two-component system is involved in the response to two different types of stress, i.e. phosphate starvation and oxidative stress (Wessel, 2003). Four proteins which are involved in protection of the cell against oxidative stress, i.e. KatA (catalase), Dps (starvation-inducible DNA-binding protein), SufC and SufD (Fe-S cluster assembly proteins), were enriched in a ∆*cgtS4* mutant in comparison to the wild type (Wingens, 2004). In addition, all four genes were recently shown to be a part of the DtxR regulon in *C. glutamicum* (Wennerhold and Bott, 2006). DtxR obviously is the master regulator of iron metabolism in *C. glutamicum*, coordinately regulating genes involved in iron uptake, iron storage and [Fe-S] cluster biosynthesis (Frunzke, 2007; Wennerhold and Bott, 2006). The genome of *C. glutamicum* encodes 13 two-component systems of which three, the MtrAB, the PhoRS and the CgtSR11 two-component systems have been characterized in some detail (Brocker and Bott, 2006; Frunzke, 2007; Kocan *et al.*, 2006; Möker *et al.*, 2004; Schaaf and Bott, 2007). The CgtSR11 two-component system

appears to be involved in heme homeostasis (Frunzke, 2007), whereas the PhoRS two-component system is involved in the phosphate starvation response (Kocan *et al.*, 2006; Schaaf and Bott, 2007) and the MtrAB two-component system appears to be involved in a general stress response in *C. glutamicum* including osmoprotection (Brocker and Bott, 2006; Möker *et al.*, 2004). In *E. coli* and *Salmonella typhimurium* oxidative stress response is mediated by the positive regulatory protein OxyR (Farr and Kogoma, 1991; Tartaglia *et al.*, 1989). In *S. typhimurium* e.g. peroxide stress proteins and heat-shock proteins are under OxyR control. The regulation by OxyR operates primarily at the transcriptional level and OxyR appeared to be both, the sensor and the transducer of an oxidative stress signal which is H_2O_2 itself (Farr and Kogoma, 1991; Storz *et al.*, 1990). The gene cg2109 in *C. glutamicum* encodes a hydrogen peroxide sensing regulator of the LysR-family (OxyR) which shows 37% sequence identity to OxyR from *E. coli*. It would be interesting to investigate the role of this regulator in *C. glutamicum* under anaerobic conditions. Recent studies showed that *C. glutamicum* grows anaerobically using nitrate as terminal electron acceptor (Nishimura *et al.*, 2007; Takeno *et al.*, 2007). In *E. coli*, the nitrate reductase complex (NarGHI) together with a polypeptide required for the assembly of the complex (NarJ) are encoded in the *narGHIJ* operon. *narK* encodes a nitrate/nitrite transporter and is located upatream and in the same orientation as the operon but constitutes a distinct transcriptional unit (Clegg *et al.*, 2002). Expression of *narGHIJ* and *narK* is activated by the Fnr protein in response to anaerobiosis (Bonnefoy and Demoss, 1994). In *C. glutamicum*, a novel transcriptional regulator ArnR was identified as repressor of the *narKGHIJ* operon under anaerobic conditions (Nishimura *et al.*, 2008). ArnR possesses an oxygen-sensing cofactor, such as a Fe-S cluster, which is likely involved in the control of ArnR activity in response to the oxygen/redox state in the cell (Nishimura *et al.*, 2008). As the ArnR regulator is structurally distinct from any Fnr-type regulators that have been characterized in other bacteria (Reents *et al.*, 2006), the results also revealed that regulation of nitrate reduction in *C. glutamicum* is performed by other factors acting in a Fnr-like manner, too (Nishimura *et al.*, 2008). At the present, the mechanism of nitrate-dependent regulation of the *narKGHIJ* operon remains unclear.

4.3 Improvement of L-lysine production with *C. glutamicum*

The biotechnological production of for instance chemicals from renewable plant biomass has become and further becomes more and more important in view of the continous increasing oil prices. Microorganisms have a long tradition in the white biotechnology, which is a young item but not a new discipline. Since millenia microorganisms are of use in the fermentation of

sugar-containing foodstuff into alcohols by the help of yeasts or the lactic acid fermentation using *Lactobacilli* strains. The soil bacterium *C. glutamicum* plays an important role in microbial production of amino acids, especially of L-glutamate and L-lysine. During the long process of strain development by metabolic engineering and since the genome sequence of *C. glutamicum* is available (Kalinowski *et al.*, 2003) various molecular targets have been identified to improve lysine yields with various production strains existing.

The key enzyme of the split lysine biosynthetic pathway in *C. glutamicum* is the aspartate kinase encoded by *lysC* as enzyme studies revealed that only the single aspartate kinase is feedback-inhibited but the activities of the other five enzymes that convert aspartate via the diaminopimelate dehydrogenase pathway to lysine (Figure 2.2) are neither inhibited nor repressed by any amino acid (Cremer *et al.*, 1988; Cremer *et al.*, 1991). Overexpression of *lysC* (Cremer *et al.*, 1991) or mutant *lysC* alleles (e.g. *lysC*, T311I) which award resistance to the aspartate kinase to feedback inhibition by lysine and/or threonine, improved lysine production by *C. glutamicum* (Kalinowski *et al.*, 1991; Shiio and Miyajima, 1969). The use of *dapA* overexpression, encoding the dihydrodipicolinate synthase in *C. glutamicum*, resulted in an increased flux towards lysine of approximately 20% (Table 4.1) in the lysine producer MH20-22B and a decreased flux towards threonine (Eggeling *et al.*, 1998). This strain MH20-22B (Schrumpf *et al.*, 1992) has been shown to possess feedback-resistant aspartate kinase (Kalinowski *et al.*, 1991) and high lysine export activity (Bröer *et al.*, 1993). A translocator specifically exporting L-lysine from the cell is encoded by *lysE* in the genome of *C. glutamicum*, the synthesis of which is controlled by the regulator protein encoded by *lysG* (Vrljic *et al.*, 1996). To avoid by-product formation in terms of threonine, *hom* alleles (*hom*, V59A) were introduced in the *C. glutamicum* wild type that caused a decreased homoserine dehydrogenase activity (Follettie *et al.*, 1988). As consequence the flow towards lysine is increased and simultanously the feedback inhibition by threonine is decreased. Another route to improve lysine production is to increase the carbon precursor supply, in particular oxaloacetate or aspartate. *C. glutamicum* possesses both phospho*enol*pyruvate carboxylase (*ppc*) and pyruvate carboxylase (*pyc*) as anaplerotic enzymes, which provide oxaloacetate from PEP or pyruvate. Whereas phospho*enol*pyruvate carboxylase seems to play a minor role as anaplerotic enzyme (Cremer *et al.*, 1991; Gubler *et al.*, 1994; Peters-Wendisch *et al.*, 1997), Peters-Wendisch *et al.* (2001) showed that overexpression of the *pyc* gene resulted in approximately 50% higher lysine accumulation in the culture supernatants and that inactivation of *pyc* led to a decrease of about 60% (Table 4.1). Thus, the anaplerotic pyruvate carboxylase reaction was identified as a major bottleneck for amino acid production with *C. glutamicum* (Peters-Wendisch *et al.*, 2001). Oxaloacetate availability can be increased by deletion of *pckA*, the gene for phospho*enol*pyruvate carboxykinase which decarboxylates oxaloacetate to PEP (Riedel *et al.*, 2001). It was shown that overexpression and deletion of

pck in the lysine producer MH20-22B led to a 20% lower and higher lysine accumulation, respectively (Riedel *et al.*, 2001). The biosynthesis of lysine requires the carbon precursors pyruvate and oxaloacetate in a 1:1 ratio (Gubler *et al.*, 1994). As the simultanous action of PTS and pyruvate kinase leads to an almost complete conversion of glucose to pyruvate, while the simultanous action of PTS and phospho*enol*pyruvate carboxylase yields pyruvate and oxaloacetate in equimolar amounts (Gubler *et al.*, 1994), inactivation of the pyruvate kinase *pyk* gene was expected to improve lysine production. However, the deletion of *pyk* decreased the lysine yield about 40% in the lysine producer *C. lactofermentum* 21799 (Gubler *et al.*, 1994) and deletion of *pyk* in *C. glutamicum* lysC^{fbr} decreased lysine production by 6% (Becker *et al.*, 2008). In contrast, in certain strains of *Corynebacterium glutamicum ssp. flavum* pyruvate kinase mutants resulted in an increased lysine production (Ozaki and Shiio, 1983; Shiio *et al.*, 1990). Recently, the deletion of the *aceE* gene encoding the E1p enzyme of the pyruvate dehydrogenase complex (PDHC) in the *C. glutamicum* DM1729 background was shown to improve lysine production (Blombach *et al.*, 2007) since the substrate-specific L-lysine yield on glucose and acetate containing media was increased by 40% (0.13 versus 0.09 mol-C/mol-C) in comparison to the parent strain (Blombach *et al.*, 2007). Lysine production is characterized by a high NADPH supply where 4 mol of NADPH are used for the production of 1 mol lysine (Marx *et al.*, 1997). Thus, the NADPH supply is one of the major bottlenecks for lysine production on glucose and in particular on fructose and sucrose. By redirection of the carbon flux through the pentose phosphate pathway with two NADPH generating enzymatic reactions due to blocking the entry of carbon into glycolysis, an alternative metabolic engineering strategy was used (Marx *et al.*, 2003). The phosphoglucose isomerase encoded by *pgi* is the first enzyme specific for glycolysis. A *pgi* null mutant was shown not only to increase L-lysine formation by a factor of 1.7 but also to reduce by-product formation drastically (Marx *et al.*, 2003). The results supported the hypothesis that increased intracellular NADPH supply by increased flux through the pentose phosphate pathway is beneficial for lysine formation. Georgi *et al.* (2005) could show that overexpression of the *malE* gene encoding the NADPH-dependent malic enzyme in *C. glutamicum* did not improve L-lysine production on any tested carbon source. However, lysine yields were also unchanged due to the overexpression of the fructose-1,6-bisphosphatase gene in the lysine production strain DM1730 on fructose minimal medium but were increased two fold on sucrose minimal medium (Table 4.1) (Georgi *et al.*, 2005). Measurements of the intracellular concentration of fructose-1,6-bisphosphate (Georgi *et al.*, 2005) indicated that the higher lysine yields resulted from a higher NADPH supply due to the decrease of fructose-1,6-bisphosphate in sucrose-grown cells which inhibits a key enzyme of the pentose phosphate pathway, namely the 6-phosphogluconate dehydrogenase encoded by *gnd* (cg1643) in *C. glutamicum*. Thus, the authors suggested that the 6-phosphogluconate

Table 4.1. A recapitulatory selection of targets for a possible improvement of L-lysine biosynthesis in *C. glutamicum* strains with feedback-resistant aspartokinase and their *de facto* impact on lysine yields.

Target Gene name, function	**Impact on L-lysine yield**	**Reference**
Lysine biosynthesis		
dapA, dihydrodipicolinate synthase	20% increased lysine accumulation due to overexpression of *dapA* in MH20-22B	(Eggeling *et al.*, 1998)
Precursor supply		
pyc, pyruvate carboxylase	50% increased lysine accumulation due to overexpression of *pyc*; 60% decreased accumulation due to *pyc* deletion in DG52-5	(Peters-Wendisch *et al.*, 2001)
pck, phospho*enol*pyruvate carboxykinase	20% higher and lower lysine accumulation due to *pck* inactivation and overexpression in MH20-22B	(Riedel *et al.*, 2001)
pyk, pyruvate kinase	40% decreased lysine production due to *pyk* deletion in *C. lactofermentum* 21799	(Gubler *et al.*, 1994)
	Slightly decreased (6%) lysine yield due to *pyk* deletion in ATCC13032 lysC^{fbr}	(Becker *et al.*, 2008)
aceE, E1p subunit of the pyruvate dehydrogenase complex	40% increased lysine yield due to *aceE* deletion in DM1729	(Blombach *et al.*, 2007)
NADPH regneration		
pgi, phosphoglucose isomerase	1.7 fold increase in lysine concentration due to *pgi* mutation in DSM5715	(Marx *et al.*, 2003)
fbp, fructose-1,6-bisphosphatase	2 fold increased lysine yield due to *fbp* overexpression in DM1730 on sucrose	(Georgi *et al.*, 2005)
gnd, 6-phosphogluconate dehydrogenase	15% increased production due to Ser-361→Phe mutation in *gnd* in the strain AHP-3 (equivalent to DM1729)	(Ohnishi *et al.*, 2005)
Gene regulation		
sugR, transcriptional regulator	20%, 50%, 70% increased lysine yield on glucose, fructose, sucrose, respectively due to *sugR* deletion in DM1729	(unpublished results)

dehydrogenase is an important target to improve lysine production in this organism (Georgi *et al.*, 2005). The already known three useful mutations (*pyc*^{P458S}, *hom*^{V59A}, *lysC*^{T311I}) in *C. glutamicum* DM1729 on relevant downstream pathways brought Ohnishi *et al.* (2005) to focus on the 6-phosphogluconate dehydrogenase of pentose phosphate pathway. A Ser-361→Phe mutation in the *gnd* gene and subsequent introduction into the strain APH-3, which is equivalent to DM1729, led to approximately 15% increased L-lysine production (Table 4.1). Enzymatic analysis revealed that the mutant enzyme is less sensitive than the wild type enzyme to allosteric inhibition by intracellular metabolites, such as fructose-1,6-bisphosphate, ATP or NADPH (Ohnishi *et al.*, 2005). The use of DNA microarrays for the identification of new target genes improving lysine production was shown to be successful (Sindelar and Wendisch, 2007). Lysine production could be improved by about 40% through overproduction of cg1015 encoding a putative methyltransferase or the *amtA-ocd-soxA* operon encoding an ammonium transporter (Siewe *et al.*, 1996), a predicted ornithine cyclodeaminase and a putative sarcosine oxidase (Sindelar and Wendisch, 2007).

In this PhD work, the transcriptional regulator SugR was shown to be a good target of improvement of lysine production in *C. glutamicum*. However, SugR does not regulate genes of the L-lysine biosynthetic pathway and bypaths, i.e. *lysC*, *dapA* or *hom*, which were described above to improve lysine production. The fact that SugR controls expression of the *pts* genes and of genes of glycolysis and the pentose phosphate pathway offers three possible explanations for improved lysine production due to deletion of *sugR*, namely (1) uptake of the carbon source, (2) glycolytic flux and (3) NADPH generation in the pentose phosphate pathway. SugR was identified as repressor of *ptsG*, *ptsF* and *ptsS* in *C. glutamicum* and the overexpression of SugR led to perturbed growth on glucose-, fructose- and sucose-containing media and reduced uptake rates of these sugars. Thus, a better sugar uptake via the PTS due to *sugR* deletion was expected to improve L-lysine rates with *C. glutamicum*. It was shown that the deletion of *sugR* improves lysine yields on the PTS sugars glucose, fructose and sucrose in *C. glutamicum* (section 3.6; Figure 2). SugR has been shown to regulate not only the *pts* genes but appears to be a pleiotropic regulator of further genes of fundamental pathways, e.g. enolase, fructose-1,6-bisphosphate aldolase, 6-phosphofructokinase and NAD-dependent L-lactate dehydrogenase. In addition two already described targets which were shown to improve lysine production were among the recently identified SugR targets: the pyruvate kinase and transketolase, a key enzyme of the pentose phosphate pathway (section 3.3). Thus, SugR also seems to control the glycolytic flux straight forward to pyruvate which serves as precursor for L-lysine and L-lactate production in *C. glutamicum*. According to this, the superior precursor supply likely improved L-lactate production three fold in the *sugR* deletion mutant in comparison to the WT. Moreover, SugR also seems to control the flux through the pentose phosphate pathway as SugR^{His} protein

bound to the promoter region of *tkt*, which has thus far not been identified as possible target. The transketolase is part of the *tkt-tal-zwf-opcA-devB* operon encoding also transaldolase, glucose-6-phosphate dehydrogenase, and 6-phosphogluconolactonase. Introduced mutant alleles in the *zwf* gene improved the flux through the pentose phosphate pathway and resulted in an increased lysine formation (Ando *et al.*, 2002). Thus, *sugR* deletion likely increased the flux through the pentose phosphate pathway, leading to a better NADPH supply and is therefore a good candidate for improving L-lysine production with *C. glutamicum*. Flux measurements in *C. glutamicum* DM1729Δ*sugR* in comparison to DM1729 would confirm this hypothesis.

The growth defect observed in DM1729Δ*sugR* grown on the PTS sugars glucose, fructose and sucrose was only marginal with respect to growth rate in WTΔ*sugR* under the same growth conditions. This could be explained by the fact that in the lysine production strain DM1729 the allelic exchanges in *hom, pyc* and *lysC* provide the subtraction of pyruvate for the lysine biosynthesis. Thus, glycolysis seems to drain and as consequence the fructose-1,6-bisphosphate concentration, which inhibits the 6-phosphogluconate dehydrogenase, decreases, the enzyme is derepressed and the flux through the pentose phosphate pathway is increased which leads in consequence to a higher lysine yield. The combination of acetate and glucose led to (i) equal amounts of L-lysine in DM1729 and DM1729Δ*sugR* and (ii) no lag-phase. On glucose-acetate mixture it was shown that the glucose uptake rate was increased in the *sugR* deletion mutant in comparison to the WT, thus in the absence of SugR, glucose and acetate are coutilized, but the proportion between glucose and acetate is shifted towards glucose. Commensurate with this view is that the lysine yield in the production strain DM1729 on glucose was decreased compared to growth on glucose plus acetate. On glucose-acetate mixture the glyoxylate cycle provides the oxaloacetate for lysine production (Wendisch *et al.*, 2000), thus, glycolysis is not that drain compared to growth on glucose alone. According to this, in DM1729 grown on glucose-acetate mixture enough fructose-6-phosphate is present to prevent SugR from binding and expression of the target genes is activated. This observation is in agreement with the result that both strains DM1729 and DM1729Δ*sugR* produce equal amounts of lysine on glucose-acetate mixture and this is even as much as the Δ*sugR* deletion mutant produced on glucose alone. Only in the strain backgound of DM1729 not in the WT and only on glucose, fructose and sucrose and not on glucose plus acetate the *sugR* deletion leads to a lag-phase of approximately 24 hours (section 3.6; Figure 1). Recently, it was shown that the deletion of the *aceE* gene in the *C. glutamicum* DM1729 background also reduced the growth rates (Blombach *et al.*, 2007). This effect was not found in the WTΔ*aceE* under the same conditions. As mentioned above, the DM1729Δ*aceE* strain showed nevertheless a more than 40% higher substrate-specific L-lysine yield (Blombach *et al.*, 2007). Thus, *aceE* deletion in

the same manner as *sugR* deletion combined with the allelic exchanges introduced into *C. glutamicum* DM1729 are obviously unfavourable for growth but lead to higher lysine yields under the tested conditions ((Blombach *et al.*, 2007); and this study). Similarly, it was shown that *dapA* overexpression resulted in a drastic growth rate reduction, too, which was restored to some extent by the addition of homoserine and could therefore be due to the limiting availability of amino acids of the aspartate family (Eggeling *et al.*, 1998). It is known that the dihydrodipicolinate synthase is not regulated at the genetic level and is not controlled in its catalytic activity (Cremer *et al.*, 1988). In contrast, the homoserine dehydrogenase is controlled in its synthesis (repression by methionine) and allosterically inhibited in response to threonine and isoleucine concentrations (Miyajima *et al.*, 1968; Miyajima and Shiio, 1970). Therefore, one would expect that at reduced growth rates the threonine concentrations are too low that homoserine dehydrogenase is not inhibited and homoserine becomes not limited. However, due to *dapA* overexpression this is obviously not the case (Eggeling *et al.*, 1998). If the growth defect in DM1729Δ*sugR* can also be restored to some extend by the addition of missing precursors for other essential reactions as shown for *dapA* overexpression and *aceE* deletion, too, has to be investigated by further experiments.

4.4 Conclusions and Perspectives

The identification and characterization of the roles of the transcriptional regulators SugR, GntR1 and GntR2 as well as LldR in *C. glutamicum* led to a more defined knowledge of control of carbohydrate uptake and utilization in this industrially important organism. The results showed that the carbon metabolism of *C. glutamicum* is subject to a complex regulation at transcriptional level. However, a variety of questions remain to be answered. For instance, lots of effectors or metabolites of transcriptional regulators such as RamA, RamB and AcnR have not been identified to date but are a key for understanding the function of all the regulators involved in this system. Additionally, the identification of further transcriptional regulators of the carbon metabolism is an interesting aspect to replenish the knowledge of the exact control mechanisms. In the genome of *C. glutamicum* 18 genes are suggested to be involved in the control of carbohydrate metabolism (Brinkrolf *et al.*, 2007), including the genes encoding the known regulators AcnR, RamA and RamB, GlxR, SugR and several other regulators of still unknown function, e.g. Cg3388 or Cg0146, one of the two DeoR-type transcriptional regulators besides SugR and FruR. Moreover, the linkages between carbon, nitrogen, phosphorus and sulfur metabolisms have to be identified and characterized. Carbon and nitrogen metabolism are connected by the formation of glutamate from 2-oxoglutarate, one of the key intermediates of the TCA cycle (Commichau *et al.*, 2006).

For *C. glutamicum* detailed information of transport and nitrogen regulation is available on molecular level (Burkovski, 2003a, b). Recent studies on the adaption of *C. glutamicum* to ammonium limitation by Silberbach and co-workers showed that the expression of *ptsG* as well as of *fba* was moderately increased in response to ammonium limitation (Silberbach *et al.*, 2005). In contrast, expression of *sugR* (SugR was shown to directly regulate both genes) was decreased two fold. Since we have no direct hint of an involvement of SugR in nitrogen metabolism, these observations would nevertheless be an interesting topic of futher investigations.

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6 Appendix

Supplementary material – ScrB

Table S1. Oligonucleotides used in this study

Name	**Sequence (5' → 3')**	nt underlined = nt in NC003450	sequence in **bold**, ***italics***	sequence in *italics*
scrB-A	GC**GAATTC**GGTCTACAGCCCAGATGGTGC	2813751	**EcoRI**	-
scrB-B	*CCCATCCACTAAACTTAAACA*GTGCATAGCCCCACACACTAC	2813242	**-**	*Linker*
scrB-C	*TGTTTAAGTTTAGTGGATGGG*TTCCGGGCTTTCAAAGGTGACAC	2811993	**-**	*Linker*
scrB-D	GC**GGATCC**CGGCTGAACCAGTTGGCGTTG	2811494	**BamHI**	-
scrB-k-for	CCATTCGTAAAGAGTTCACTGACC	2813797	**-**	-
scrB-k-rev	GAATCAGCGGGACGAAAATGTCC	2811453	**-**	-
scrB-for	GGTTCC**TCTAGA***AAGGAGATATAGAT***A**TGTGTGGGGCTATGCACACAG	2813258	**XbaI, *nt exchange G → A***	*RBS*
ScrB*Shift*-for	GGTTCC**CATATG**TGTGGGGCTATGCACACAG	2813258	**NdeI**	*nt exchange G → A*
ScrB*Shift*-rev	GGTTCC**GGATCC**CTTATCTCTCAATAGTGTCACC	2811957	**BamHI**	-
ptsS-30*	*[IRD800]*CGCAGGATGCGTTGCGCGAGG	2811773	**-**	marked with *IRD800*
ptsS-90*	*[IRD800]*GCGTAAACGCGTTGCACAGTGTGC	2811715	**-**	see above
ptsS-PrimEx-for	CCGAGTAGACATCCGAGGCAATATTTCCC	2812162	**-**	-
ptsS-PrimEx-rev	CCGCCAATACCTTCACAGCACGGC	2811474	**-**	-

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Erklärung

Die hier vorgelegte Dissertation habe ich eigenständig und ohne unerlaubte Hilfe angefertigt. Ich versichere, dass ich keine anderen als die angegebenen Quellen und Hilfsmittel benutzt, sowie Zitate kenntlich gemacht habe. Die Dissertation wurde in der vorgelegten oder in ähnlicher Form noch bei keiner anderen Institution eingereicht. Ich habe bisher keine erfolglosen Promotionsversuche unternommen.

Elsdorf, den 28.05.2008 (Verena Engels geb. Werner)