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The Global Repressor SugR Controls Expression of Genes of Glycolysis and of the L-Lactate Dehydrogenase LdhA 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 the overexpression of *sugR* not only perturbed the 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. Chromatin immunoprecipitation combined with DNA microarray analysis and gel retardation experiments were performed to identify further 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*). The deletion of *sugR* resulted in increased mRNA levels of *eno*, *pyk*, and *ldhA* in acetate medium. Enzyme activity measurements revealed that SugR-mediated repression affects the activities of PfkA, Fba, and LdhA in vivo. As the deletion of *sugR* led to increased LdhA activity under aerobic and under oxygen deprivation conditions, L-lactate production by *C. glutamicum* was determined. The overexpression of *sugR* reduced L-lactate production by about 25%, and *sugR* deletion increased L-lactate formation under oxygen deprivation conditions by threefold. Thus, SugR functions as a global repressor of genes of the PTS, glycolysis, and fermentative L-lactate dehydrogenase in *C. glutamicum*.

Corynebacterium glutamicum, which was isolated as an 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 2 million tons of amino acids per year, mainly L-glutamate and L-lysine (31, 43). A general view of this nonpathogenic bacterium, which has become a model organism for the *Corynebacterineae*, a suborder of *Actinomycetales* that also comprises the genus *Mycobacterium* (54), can be found in two recent monographs (9, 17).

C. glutamicum is able to grow on a variety of sugars, sugar alcohols, and organic acids as sole carbon and energy sources (64). As in many other gram-positive and gram-negative bacteria, the phosphoenolpyruvate-dependent phosphotransferase system (PTS) is the major sugar uptake system (15, 37, 45, 47). 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 coutilizes the carbon sources present in mixtures without showing diauxic growth (64). Glucose as the preferred carbon source has been shown to be cometabolized with acetate (63), L-lactate (55), or fructose (14). When glucose is coutilized with another carbon source, e.g., acetate or fructose (16, 63), its uptake is reduced due to the repression of *ptsG* by the recently identified transcriptional repressor SugR (19). It was shown that SugR not only acts as a repressor 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 genes of the general components of the PTS (*ptsH* and *ptsI*) (21, 59). The binding of SugR to the *ptsG* promoter was found to be negatively affected by millimolar concentrations of fructose-6-phosphate (19), while micromolar concentrations of fructose-1-phosphate and millimolar concentrations of glucose-6-phosphate and fructose-1,6-bisphosphate were shown to negatively affect the binding of SugR to the *ptsI-fruR* intergenic region (21). An 8-bp motif upstream of *ptsG* was suggested to be part of the SugR binding site (19). This motif upstream of *ptsG* is part of a 23-bp AC-rich motif which was shown to be required for the binding of SugR to the *ptsI-fruR* intergenic region and which also is present upstream of the SugR targets *ptsS* and *ptsH* (21). In *Escherichia coli* and *Bacillus subtilis*, regulators like Crp (cyclic AMP [cAMP] receptor protein) and CcpA (catabolite control protein A) not only regulate *pts* genes but also are global regulators of carbon metabolism in these bacteria. CcpA is the master regulator of carbon catabolite regulation in *B. subtilis* (30, 53) 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 repressor gene *gntR*) (29, 44, 53). Activation and repression mediated by CcpA may utilize different conformational changes of the protein (53). Because *ccpA* mutants are unable to activate glycolysis or carbon overflow metabolism, CcpA appears to control a superregulon of glucose catabolism in this organism (61). In *E. coli*,

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristics[a]	Source and/or reference
E. coli strains		
DH5α	F$^-$ *thi-1 endA1 hsdR17*(r$^-$ m$^-$) *supE44* Δ*lacU169* (ϕ80*lacZ*Δ*M15*) *recA1 gyrA96 relA1*	Bethesda Research Laboratories; 28
BL21(DE3)	*ompT hsdS$_B$*(r$_B^-$ m$_B^-$) *gal dcm* (DE3)	Novagen
C. glutamicum strains		
WT	ATCC 13032; wild-type strain, auxotrophic for biotin	ATCC
Δ*sugR* WT	In-frame deletion of the *sugR* gene of WT	19
WT *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 a 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* (*oriV*$_{E.c.}$, *sacB*, *lacZ*α)	50
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}	48
pVWEx1-*sugR*	Kan^r; pVWEx1 with an 807-bp fragment of the *sugR* gene and an artificial RBS	19
pET16b	Amp^r; overproduction of proteins with an N-terminal decahistidine tag in *E. coli* (pBR322 *oriV*$_{E.c.}$, *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

[a] *oriV*$_{E.c.}$, *oriV* from *E. coli*; RBS, ribosome binding site.

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). Chromatin immunoprecipitation combined with DNA microarray analysis (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), and *pckA* (phosphoenolpyruvate carboxykinase) (25, 26).

In this study, it was determined that in addition to regulating *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, 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 the 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, and 120 mM ribose were used. For all cloning purposes, *E. coli* DH5α was used as the host, and for the overproduction of SugR, *E. coli* BL21(DE3) (56) was used. The *E. coli* strains were cultivated aerobically in Luria-Bertani (LB) medium (49) 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, and ligation were carried out according to reference 49. 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, 62). 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*Tag-tagged SugR from the genomic *sugR* locus, the plasmid pK19*mobsacB-sugR*^{Strep} was constructed. Base pairs 349 to 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) (52). The PCR product was subcloned into the pGEM-T vector (Promega, Wisconsin) and was cloned as an EcoRI/BamHI fragment into the pK19*mobsacB* vector (50). *C. glutamicum* was transformed with the resulting plasmid, pK19*mobsacB-sugR*^{Strep}, by electroporation, and the site-specific integration of the plasmid into the *sugR* genomic locus was verified by PCR using the primers *sugR* and M13 (Table 2). As expected, only *C. glutamicum* WT-*sugR*^{Strep} yielded 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) by use of 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 performed as described previously (19). Briefly, purified SugR^{His} (in concentrations ranging from 0 to 3.3 μM) was mixed with various promoter fragments (186 to 967 bp; final concentrations, 7 to 37 nM [see Fig. 2 below]) in a total volume of 20 μl and contained 50 mM Tris-HCl, 10% (vol/vol) glycerol, 50 mM KCl, 10 mM $MgCl_2$, 0.5 mM EDTA, pH 7.5. A 78- or a 398-bp cg2228 promoter fragment (90 or 20 nM, respectively) served as the 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 a constant 170 V by use of 1× 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.

ChIP-to-chip and transcriptome analysis. From a fresh LB agar plate with *C. glutamicum* WT as the control strain and the *C. glutamicum* WT-*sugR*^{Strep} strain,

TABLE 2. Oligonucleotides used in this study

Name	Sequence (5′ → 3′)	Sequence feature			Use
		Nucleotide in NC003450 (underlined)	Restriction site (bold)	Tag (italics)	
sugR	CG**GAATTC**TAGCTCCAAGCAGTGGTCGATCG	2038164	EcoRI		ChIP-to-chip
sugR-Strep	CG**GGATCC***TTATTTTTCGAACTGCGGGTGGCT CCAAGCGCTT*TCTGCAATCACAACTTCTAC ATCG	2038591	BamHI	*Strep*Tag II	ChIP-to-chip
M13-rev	CACACAGGAAACAGCTATGACCATG				Integration control
P0441-for	GCTAAAAAAGTAGAAATTATCGACC	387199			Gel shift
P0441-rev	GCCGGCTCCGAGTACTAC	387730			Gel shift
P1110-for	GTGATGCCTGCGGTTGCTG	1033597			Gel shift
P1110-rev	CAAATGAGATCACCATGATGACGGC	1034029			Gel shift
P1111-for	CTAAAACTTAACAAGCGCAACCCCC	1034739			Gel shift
P1111-rev	CCGCGGGAGTCGAGAATTTC	1034998			Gel shift
P1142_1143-for	GCGGTGATTCGAGCCGCTAAATCAAG	1060191			Gel shift
P1142_1143-rev	GTGCTGGCTGAGACTAGCTGGC	1059882			Gel shift
P1408_1409-for	GCGACGACGGTGGCAGTGCTGACC	1314735			Gel shift
P1408_1409-rev	CCGGGGCAGTCGCCGCCTGAC	1315095			Gel shift
F4(*ptsG*)-for	GCATAATCTGACAGTGTGTCCGTTTTC	1422834			Gel shift
F4(*ptsG*)-rev	GGCTCCCCCGCAATAGATTTGTGG	1423019			Gel shift
P1773_1774-for	GATCGTGTCCAAGGGTTCTCCTCC	1663548			Gel shift
P1773_1774-rev	GCGTACAGTGAGCGCCTGAAGTTC	1664450			Gel shift
P2115-for	GTCGCTTTTCAGGTTCCCGC	2037511			Gel shift
P2115-rev	CTCAACTGCCGTTAATGAGGCAATC	2037865			Gel shift
P2121-for	TCGGACCTTGACCCGATGTCTGGTC	2045575			Gel shift
P2121-rev	CGTGCGTGCAGGCCAACGGAG	2045811			Gel shift
P2157-for	GGCTTTAGGAGCCTAGTGGC	2073290			Gel shift
P2157-rev	CCAGCAATCACTGCGATAGTGATTAG	2072856			Gel shift
P2291-for	GTTTCAACCATAGGCCTGACCTGGC	2207298			Gel shift
P2291-rev	CTAGCCACCGCTGGGCCTAG	2207043			Gel shift
P3068-for	CCCCGATAGTGTATGTGCTGAC	2955520			Gel shift
P3068-rev	CTTAGCACGATCGAGCATCTCGTTA	2955223			Gel shift
P3169_3170-for	GGTAAGACCGCAGCGTAGCTTTTGG	3054803			Gel shift
P3169_3170-rev	CCTTATTCTTGGTCGGCGCCTCG	3053838			Gel shift
P3219-for	GTTGCCAGGCGAGTGGTGAGC	3113669			Gel shift
P3219-rev	CATCTCCTGCGCCAATGAGGACAATC	3113343			Gel shift
P3366_3367-for	GCTGTGAACGACCCAAAACTCAAACTTAG	3242913			Gel shift
P3366_3367-rev	CGGTCGAGTTCTATGCGTTCTGC	3242640			Gel shift
cg2228_for	GTTCGCTACGTCCGAGTGATCACC	2146341			Negative control for gel shift
cg2228_rev_short	CTCAGGCATGATGATGTCAGGC	2146264			Negative control for gel shift
cg2228_rev	GTCCCCCACGATTGTTTAAAAGTC	2145944			Negative control for gel shift
P1145_for	CGTTTCGCAGTACGGAGCG	1063987			Gel shift
P1145_rev	CCTTCACTTCACCCATGGTGTC	1063566			Gel shift
P2837_for	CGCAGCAATGTGATAACAGAGG	2727080			Gel shift
P2837_rev	GGCACACCATGGGTTTCAAAG	2726526			Gel shift
P3048-for	GATTCCTGCTGGACCTGCAGCG	2938454			Gel shift
P3048-rev	CGAAGCTGCGGTTGACCGTGG	2937840			Gel shift
P2831_2833-for	CGGTGATGTTGTTGTACACATTG	2721953			Gel shift
P2831_2833-rev	CAGCGCCGAACGAATAGC	2721178			Gel shift
P1283_1284-for	CGCCTTTAACAAACCCTGTTGAG	1182041			Gel shift
P1283_1284-rev	GCAGCGAGAGAGATGCAC	1181626			Gel shift
P2795_2796-for	GAGTTTGGCAGCTTCCACG	2684967			Gel shift
P2795_2796-rev	GAACAACACTATTGATTGGACCTTC	2684599			Gel shift
P2262-for	TCCCTGATTTCATCGGAGGG	2176106			Gel shift
P2262-rev	GCGAGAACCACGAGGAC	2175455			Gel shift
P0680_0681-for	GGTCCGCGCTTGGACAGT	600827			Gel shift
P0680_0681-rev	GTGTTATGGGCGATGGCATC	601020			Gel shift
P0645-for	ACCCGCCCTCCATCTGCAT	568268			Gel shift
P0645-rev	GAATGGGCATCCACCGGT	568032			Gel shift

the first preculture, 5 ml of LB medium, was inoculated. After the cells were washed 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/liter protocatechuic acid and 0.2 mg/liter biotin, or in LB medium. As carbon and energy sources, 100 mM glucose, 100 mM fructose, or 50 mM sucrose was used. The main cultures were cultivated to mid-exponential phase (OD_{600} of 4 to 6). Fifty-milliliter portions of these main cultures were harvested by cen-

trifugation (10 min, 11,325 × *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% (vol/vol) 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 min, 3,500 × *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 of buffer A with one pill of 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 sheared by sonication (two times for 30 s with Branson sonifier W-250 [Danbury, CT] with a pulse length of 40% and an intensity of 1) to give an average fragment size of 200 to 750 bp. Cell debris was removed by centrifugation (20 min, 5,300 × *g*, 4°C) and ultracentrifugation (1 h, 150,000 × *g*, 4°C). The cytosolic fraction was then used for immunoprecipitation and therefore was spiked with 5 μg/ml avidin and incubated for 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% (wt/vol) *Strep*Tactin-Sepharose (IBA GmbH, Göttingen, Germany). After the column was washed 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 1% sodium dodecyl sulfate was added 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% (vol/vol) ethanol, dried, and resuspended in 100 μl EB buffer (10 mM Tris-HCl, pH 8.5; Qiagen, Hilden, Germany). Fluorescent labeling of genomic DNA was performed as described previously (20, 51). Preparation and labeling of RNA for transcriptome analysis, hybridization to the *C. glutamicum* whole-genome microarray, and array scanning were performed as described previously (19). The enrichment factors were the ratios of the hybridization signal from the labeled DNA of the $SugR^{Strep}$-DNA complexes isolated from *C. glutamicum* WT-*sugR*^{Strep} divided by the hybridization signal from the mock experiment with *C. glutamicum* WT.

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 in reference 19. D-Ribose was separated by high-performance liquid chromatography with an organic acid resin column (300 by 8 mm, 10-μm inner diameter, 25 Å; CS Chromatographie Service; Langerwehe, Germany) at 60°C by use of injection volumes of 5 μl, 5 mM H_2SO_4 as the mobile phase, a flow rate of 1.0 ml/min, and an overall run time of 15 min. Substances were detected via a refractive index detector 1200 series (Agilent Technologies, Santa Clara, CA). Concentrations were determined by comparing the sample probes with external standards.

Measurement of enzyme activities. For measurements of enzyme activities, the *C. glutamicum* WT, WTΔ*sugR*, WT(pVWEx1), and WT(pVWEx1-*sugR*) strains were cultivated in LB medium to OD_{600}s of 2 to 3.5. The cells were harvested by centrifugation (10 min, 4°C, 3,220 × *g*), washed twice in 100 mM Tris-HCl, pH 7.3, plus 10% (vol/vol) glycerol, and stored at −70°C until use. For the preparation of cell extracts, the cell pellet was resuspended in 500 μl of the washing buffer and the cells were mechanically disrupted by bead beating three times for 20 s with 0.5 g of zirconia-silica beads (diameter, 0.1 mm; Roth, Karlsruhe, Germany) by use of a Silamat S5 (Vivadent, Ellwangen, Germany). After centrifugation (45 min, 4°C, 12,100 × *g*), the supernatant was used immediately for the enzyme assay. Protein concentrations were determined with the Bradford assay kit (Bio-Rad Laboratories, Hercules, Canada) with bovine serum albumin used as the standard.

Determination of the specific activity of the 6-phosphofructokinase PfkA (EC 2.7.1.11) in crude extracts was performed as described previously (57). The two different enzyme tests described in reference 57 were named test A (coupling to pyruvate kinase/lactate dehydrogenase) and test B (coupling to aldolase, triosephosphate isomerase, and glycerol-3-phosphate dehydrogenase) in this study. Both assay mixtures (500-μl total volumes) contained 100 mM Tris-HCl, pH 7.5, 0.2 mM NADH, 10 mM $MgCl_2$, 1 mM ATP, and 20 to 75 μl of crude extract. In addition, the mixture for test A contained 2.75 U/ml NAD-dependent L-lactate dehydrogenase and 2.2 U/ml pyruvate kinase, and the mixture for test B contained 0.4 U/ml aldolase, 3.06 U/ml α-glycerol-3-phosphate dehydrogenase, and 0.033 U/ml triosephosphate isomerase. The reaction was started by the addition of 4 mM fructose-6-phosphate, and the increase in absorption at 340 nm [$\varepsilon_{340\ nm}$(NADH) = 6.3 mM^{-1} cm^{-1}] was monitored at 30°C for 5 to 30 min using a Shimadzu UV-1202 spectrophotometer (Shimadzu, Duisburg, Germany).

Determination of the specific activity of the fructose-1,6-bisphosphate aldolase Fba (EC 4.1.2.13) in crude extracts was performed as described in reference 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-glycerol-3-phosphate dehydrogenase, 0.018 U/ml triosephosphate isomerase, and 20 to 50 μl of crude extract. The reaction was started by the addition of 2 mM fructose-1,6-bisphosphate, and the increase in absorption at 340 nm was monitored at 30°C for 5 to 10 min using a Shimadzu UV-1202 spectrophotometer (Shimadzu, Duisburg, Germany).

Determination of the specific activity of the NAD-dependent L-lactate dehydrogenase LdhA (EC 1.1.1.27) in crude extracts was performed as described previously (8). The assay mixture (500-μl total volume) contained 20 mM MOPS (morpholinepropanesulfonic acid), pH 7.0, 0.2 mM NADH, and 1 to 20 μl of crude extract. The reaction was started by the addition of 30 mM pyruvate, and the increase in absorption at 340 nm was monitored at 30°C for 5 min using a Shimadzu UV-1202 spectrophotometer (Shimadzu, Duisburg, Germany).

Determination of the specific activity of the pyruvate kinase Pyk (EC 2.7.1.40) in crude extracts was performed as described previously (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 to 20 μl of crude extract. The reaction was started by the addition of 240 mM phosphoenolpyruvate, and the increase in absorption at 340 nm was monitored at 30°C for 5 to 30 min using a Shimadzu UV-1202 spectrophotometer (Shimadzu, Duisburg, Germany).

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

Transketolase was assayed as described previously (58). The reaction mixture contained 50 mM Tris-HCl buffer, pH 7.5, 0.24 mM NADH, 0.01 mM thiamine pyrophosphate, 1.0 mM $MgCl_2$, 0.5 mM xylulose-5-phosphate, 0.5 mM ribulose-5-phosphate, and 20 μg of a mixture of triosephosphate isomerase and glycerol-3-phosphate dehydrogenase in a total volume of 0.5 ml. The reaction was started by the addition of the enzyme, and the decrease in NADH was monitored at 340 nm.

Transaldolase was assayed as described previously (58). The reaction mixture contained 50 mM Tris-HCl buffer, pH 7.5, 5 mM EDTA, 0.5 mM erythrose-4-phosphate, 4.0 mM fructose-6-phosphate, 0.2 mM NADH, and 20 μg of a mixture of triosephosphate isomerase and glycerol-3-phosphate dehydrogenase in a total volume of 0.5 ml. The reaction was started by the addition of the enzyme, and the decrease in NADH was monitored at 340 nm.

L-Lactic acid production. For L-lactic acid production, the *C. glutamicum* WT and WT Δ*sugR* strains along with strains WT(pVWEx1) and WT(pVWEx1-*sugR*) were cultivated aerobically at 30°C for about 16 h in 100 ml LB complex medium with 4% (wt/vol) glucose as the carbon source. When appropriate, media were supplemented with 50 μg/ml kanamycin. The precultures were harvested by centrifugation (5 min, 11,325 × *g*, 4°C), cells were washed in CgXII medium (pH 7.2) without any carbon source, and 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/liter protocatechuic acid, and 0.2 mg/liter biotin; with 30 mM potassium nitrate as the electron acceptor; with 1 μg/ml resazurin as the oxygen indicator; and when appropriate with 50 μg/ml kanamycin and 1 mM isopropyl-β-D-thiogalactopyranoside (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 with gentle shaking for 3 hours.

RESULTS

Effect of *sugR* overexpression on growth on fructose, sucrose, or ribose. SugR binds to promoters of PTS genes and was previously shown to affect the utilization of the PTS substrate glucose in vivo (19). To test whether SugR also affects the utilization of the PTS substrates sucrose and fructose in vivo, the growth of a *C. glutamicum* strain overexpressing *sugR* was compared to that of 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 fruc-

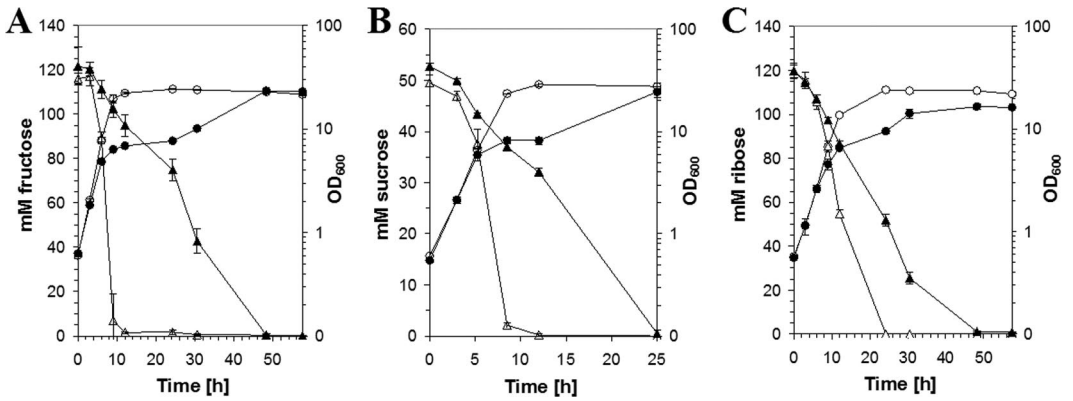

FIG. 1. Role of SugR for growth of *C. glutamicum* on minimal medium with fructose, sucrose, or ribose. Growth of *C. glutamicum* WT-(pVWEx1) (open symbols) and WT(pVWEx1-*sugR*) (filled symbols) on CgXII minimal medium containing 100 mM fructose (A), 50 mM sucrose (B), or 120 mM ribose (C). The cultures were induced 3 hours after inoculation by the addition of 1 mM IPTG. The optical densities (circles) and the fructose, sucrose, and ribose concentrations (triangles) are indicated.

tose. Moreover, the strain overexpressing *sugR* utilized the added fructose with an uptake rate that was decreased approximately twofold in comparison to what was seen for the control strain (Fig. 1A). The overexpression of *sugR* also perturbed the growth of *C. glutamicum* on sucrose minimal medium, and the sucrose uptake rate was reduced about 13% compared to that of the control strain (Fig. 1B). The more pronounced effect of *sugR* overexpression on fructose utilization might be due to the fact that SugR represses the *fruR-fruK-ptsF* operon, encoding both the fructose-specific PTS component and 1-phosphofructokinase, while SugR represses only *ptsS* and not the sucrose-6-phosphate hydrolase gene *scrB* (18). Taken together, the overexpression of *sugR* had a negative influence on the utilization of all known PTS substrates (glucose, fructose, and sucrose) by *C. glutamicum*.

To test whether the control of substrate utilization by SugR is limited to PTS substrates, the empty vector 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 the sole carbon and energy source (Fig. 1C). *C. glutamicum* WT(pVWEx1-*sugR*) grew slower on ribose minimal medium than the control strain WT-(pVWEx1) and formed less biomass (Fig. 1C). 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 the 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 ChIP-to-chip analysis (34) was applied. The *C. glutamicum* WT-*sugR*^{Strep} strain, which produces an affinity-tagged SugR protein instead of native SugR, was constructed in order to facilitate the purification of SugR^{Strep}-DNA complexes formed in vivo. A vector containing the 3′ end of the *sugR* gene extended by codons for the 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 the *C. glutamicum* WT-*sugR*^{Strep} strain and in the control *C. glutamicum* WT strain were cross-linked 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 the reversal of the cross-links, the coprecipitated DNA was purified, fluorescently labeled, 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 values of ≤ 0.05) and those genes or genomic regions which were 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. Enrichment of the known SugR target genes *ptsG* and *ptsH* as well as *sugR* itself and the gene for the fourth EII permease in *C. glutamicum*, possibly transporting an as-yet-unknown substrate (cg3366, *sgcA*), could be shown by the ChIP-to-chip experiments. 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. The ChIP-to-chip analysis identified a number of genes encoding enzymes of the central carbon metabolism as candidate SugR target genes, i.e., the genes for trehalose phosphatase (*otsB*, cg2909), 6-phosphofructokinase (*pfkA* [lies divergent to cg1408]), fructose-1,6-bisphosphate aldolase (cg3068, *fba*), enolase (cg1111, *eno*), pyruvate kinase (cg2291, *pyk*), fermentative NAD-dependent L-lactate dehydrogenase (cg3219, *ldhA*), which is crucial for anaerobic L-lactate production (32), dihydrolipoamide dehydrogenase (cg0441, *lpd*), and the genomic region between the genes for polyprenyltransferase and transketolase (cg1774, *tkt*).

Of the candidate SugR target genes identified by ChIP-to-chip analysis (Table 3), only PTS genes (*ptsG*, *ptsI*/*fruR-fruK-ptsF*, and *ptsS*) were found to be differentially expressed in a comparison between the WT and WT Δ*sugR* strains during growth in LB medium (19). As the absence of SugR showed the greatest effect on *ptsG* expression during growth in acetate

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

Gene ID[b]	Gene name, gene product, and/or deduced function	ChIP-to-chip enrichment factor with:			
		Glucose	Fructose	Sucrose	LB
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
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
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 ($P \leq 0.05$). DNA enriched in SugR^{Strep}-DNA complexes by a factor of two or more was considered as a candidate target bound by SugR in vivo. 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 are listed.

[b] P, enrichment factors based on intergenic regions, of which few were present on the DNA microarray.

medium (19), the gene expressions of the WT and WT Δ*sugR* strains were compared during growth in acetate minimal medium (Table 4). Statistically significant gene expression changes of a factor of three or more were observed for four genes (cg1673, cg2425, cg3368, and cg2071) showing low mRNA levels for the WT Δ*sugR* strain compared to the WT. High mRNA levels in the WT Δ*sugR* strain compared to the WT were determined for 24 genes: *ptsG*, *ptsH*, *ptsI*, *fruR-fruK-ptsF*, *fruK2*, *ptsS*, *ldhA*, *pyk*, *eno*, *pyc*, *lldD*, *dtsR*, *sufD*, *metE*, *fas-IB*, *rplO*, *rpsQ*, *pepB*, *ssuB*, cg1112, cg1977, and cg2430. Thus, evidence from ChIP-to-chip and transcriptome analysis suggests that, besides repressing the PTS genes, SugR also represses *pyk*, *eno*, and *ldhA*.

Gel retardation analysis of candidate SugR target genes. In order to test for a 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. Of the candidate SugR target genes deduced from ChIP-to-chip analysis, primarily genes encoding enzymes of central carbon metabolism were chosen for gel retardation analysis. In addition, the succinyl-coenzyme A (CoA) synthetase gene *sucC* and the phosphotransacetylase gene *pta* were included, as our previous transcriptome analysis suggested control by SugR (19). Complete gel shifts were observed at a 30-fold molar excess of SugR for *sgcA*, *ptsH*, *eno*, and *terC* and at a 60-fold excess for *pyk*, *ldhA*, *ptsG*, *fba*, *pfkA*, and the intergenic regions between cg2795-cg2796 and *aroE1-lipT* (Fig. 2). At a 90-fold excess of SugR, almost complete gel shifts were observed for *ramA-cysK*, *lpd*, *ctaB-tkt*, *sugR*, *ftsY*, *alr*, and *cytP* (Fig. 2). SugR binding to the promoter fragments of the phosphoenolpyruvate carboxykinase gene (*pck*), the succinyl-CoA synthetase (*sucC*) and the fumarase (*fum*) genes, and the phosphotransacetylase gene (*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 MEME software (http://meme.sdsc.edu/) (4). Two putative SugR binding motives were identified in each promoter fragment with the consensus sequence shown in Fig. 3B. The binding sites have in common that they are located at or near the transcriptional start sites, suggesting that SugR acts as a repressor of those genes.

Specific activities of NAD-dependent L-lactate dehydrogenase, 6-phosphofructokinase, fructose-1,6-bisphosphate aldolase, transketolase, transaldolase, pyruvate kinase, and fumarase in *C. glutamicum* WT and WT Δ*sugR* strains and 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 for strains lacking *sugR* or overexpressing *sugR* because SugR was found to bind to the promoter regions of their genes. Transketolase and transaldolase activities were

TABLE 4. Genes whose average mRNA ratios were altered by a factor of three or more[a] in acetate minimal medium for *C. glutamicum* WT compared with the Δ*sugR* mutant

Gene ID[b]	Gene name, gene product, and/or deduced function	mRNA ratio (Δ*sugR* mutant/WT)[c]
cg2925	*ptsS*, sucrose-specific enzyme II of the PTS	50.7
cg2119	*pfkB*, 1-phosphofructokinase protein	47.8
cg2120	*ptsF*, fructose-specific enzyme II of the PTS	32.3
cg2118	*fruK*, DeoR-type transcriptional regulator	26.9
cg2117	*ptsI*, enzyme I of the PTS	11.1
cg2121	*ptsH*, phosphocarrier protein HPr	9.0
cg3219	*ldhA*, NAD-dependent l-lactate dehydrogenase	6.7
cg0812	*dtsR1*, acetyl/propionyl-CoA carboxylase beta chain	6.6
cg0957	*fas-IB*, fatty acid synthase	5.9
cg2116	Putative phosphofructokinase	5.4
cg1290	*metE*, 5-methyltetrahydropteroyltriglutamate-homocysteine methyltransferase	4.8
cg3218	*pyk*, pyruvate kinase	4.3
cg1763	*sufD*, components of an uncharacterized iron-regulated ABC-type transporter	3.6
cg1112	Septum formation initiator, secreted protein	3.6
cg0791	*pyc*, pyruvate carboxylase	3.5
cg0634	*rplO*, 50S ribosomal protein L15	3.5
cg2430	Hypothetical protein	3.5
cg1111	*eno*, enolase	3.3
cg3227	*lldD*, quinone-dependent l-lactate dehydrogenase	3.1
cg2419	*pepB*, leucyl aminopeptidase	3.1
cg1379	*ssuB*, aliphatic sulfonates ATP-binding ABC transporter protein	3.1
cg0604	*rpsQ*, 30S ribosomal protein S17	3.0
cg1977	Putative secreted protein	3.0
cg1537	*ptsG*, glucose-specific enzyme II of the PTS	3.0
cg1673	*ppmN*, polyprenol-phosphate-mannose synthase	0.3
cg2425	Predicted permease	0.3
cg3368	ABC transporter permease protein	0.2
cg2071	*int2′*, putative phage integrase (N-terminal fragment)	0.2
cg2115	*sugR*, transcriptional regulator of sugar metabolism	0.1

[a] $P < 0.05$.
[b] Gene numbering, gene designations, and descriptions of gene products are for NC006958.
[c] The mRNA ratios represent average values obtained from three DNA microarray experiments performed with RNA isolated from three independent cultures in acetate minimal medium.

determined, as SugR bound upstream of the putative *tkt-tal-zwf-opcA-devB* operon. Fumarase activity was also determined, although SugR binding was very weak (or absent). As shown in Fig. 4, the activities of the tested enzymes were comparable in *C. glutamicum* WT and WT(pVWEx1). In the *C. glutamicum* WT Δ*sugR* strain, the LdhA, Fba, PfkA, and Pyk activities were increased 8.1-fold, 1.6-fold, 1.2-fold, and 1.3-fold, respectively, compared to what was seen for the WT (Fig. 4A to 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 for the empty vector control (Fig. 4A to C). The specific activity of Pyk was not significantly changed due to the overexpression of *sugR* (Fig. 4D), indicating that the role of SugR for *pyk* expression is not absolutely clear. The specific activities of transketolase, transaldolase, and fumarase were comparable for all strains tested (Fig. 4 and data not shown). Taken together, the results indicate that SugR acts as a repressor of the genes for NAD-dependent L-lactate dehydrogenase, 6-phosphofructokinase, and fructose-1,6-bisphosphate aldolase and affects their activities in vivo.

Influence of deletion and overexpression of *sugR* on L-lactate formation by *C. glutamicum*. The formation of L-lactate, which is secreted into the medium during anaerobiosis or as a by-product during glutamate production (37, 55), requires *ldhA* (32), which was shown to be repressed by SugR (Fig. 2 and 4A). To test the physiological relevance of *ldhA* control by SugR, levels of L-lactate formation by the *C. glutamicum* WT and WT Δ*sugR* strains and WT(pVWEx1) and WT(pVWEx1-*sugR*) were compared during aerobic growth on glucose and under oxygen deprivation conditions. During the aerobic growth on glucose of the *C. glutamicum* WT, WT Δ*sugR*, and WT(pVWEx1) strains, L-lactate transiently accumulated to maximal concentrations of 19 ± 2 mM, 24 ± 3 mM, and 23 ± 2 mM, respectively, after 12 h, while during the cultivation of WT(pVWEx1-*sugR*), L-lactate could not be detected (<0.5 mM [data not shown]). Under oxygen deprivation conditions, the WT Δ*sugR* deletion mutant showed a threefold-increased L-lactate formation compared to the WT, whereas the strain overexpressing *sugR* formed approximately 30% less L-lactate than the control (Fig. 5A). The specific activity of fermentative NAD-dependent L-lactate dehydrogenase was about twofold lower for WT(pVWEx1) than for WT(pVWEx1-*sugR*) and about threefold higher for the WT Δ*sugR* strain than for the WT (Fig. 5B). Thus, SugR repression of *ldhA* is important for aerobic and 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, 59). The ChIP-to-chip and gel retardation analysis shown here revealed that SugR represses not only PTS genes but also a number of further genes, which mainly encode enzymes of the central carbon metabolism. It was shown here that the genes encoding 6-phosphofructokinase (*pfkA*), fructose-1,6-bisphosphate aldolase (*fba*), and NAD-dependent L-lactate dehydrogenase (*ldhA*) belong to the SugR regulon (Table 3; Fig. 2). Thus, SugR coordinately controls the expression of genes for the uptake of carbohydrates via the PTS and for their further metabolism in the central pathways of glycolysis (*pfkA*, *fba*, and *ldhA*). The genes *pfkA*, *fba*, and *ldhA* are not known targets of the carbon regulators AcnR (represses aconitase gene *acn* [41]), GntR1 and GntR2 (repress gluconate utilization genes *gntP*, *gntK*, and *gnd* and activate *ptsG* and *ptsS* [10]), 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]). 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 dehydrogenase (2, 7, 24). The occurrence of RamB binding motifs suggests that *ptsG*, the tricarboxylic acid (TCA) cycle genes *gltA* and *acn*, and the phosphoenolpyruvate carboxykinase and malic enzyme genes *pck* and *malE* are regulated by RamB (3). RamA activates *adhA*, *aceA*, *aceB*, *pta-ack*, and *ramB* (2, 3, 11,

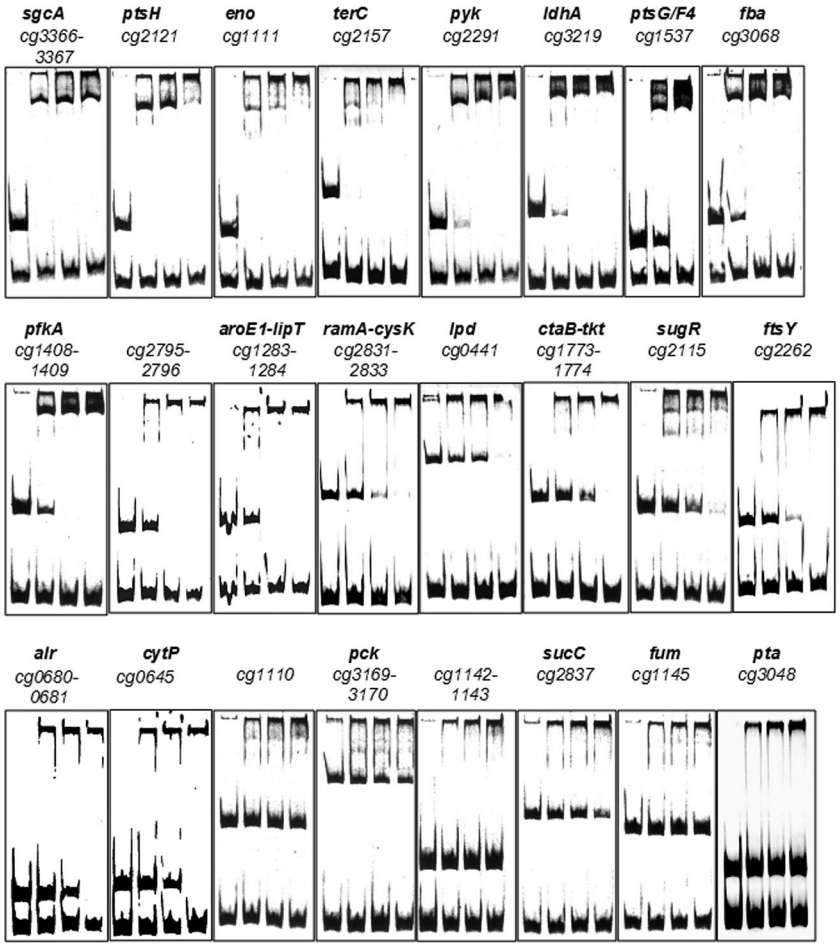

FIG. 2. Binding of SugR^{His} to candidate target genes. DNA fragments (186 to 967 bp; final concentration, 7 to 37 nM) covering the promoter regions of further putative target genes of SugR were incubated for 30 min at room temperature without SugR^{His} protein (first lanes) or with a 30-fold (second lanes), 60-fold (third lanes), or 90-fold (fourth lanes) molar excess of purified SugR^{His} protein before separation by native polyacrylamide gel electrophoresis (10%) and staining with SYBR green I. In the case of the *ptsG/F4* fragment, the first lane is without SugR^{His} protein and the second and third lanes are with 30- and 60-fold molar excesses of purified SugR^{His} protein, respectively. A 78- or 398-bp cg2228 promoter fragment (90 or 20 nM, respectively) served as the negative control. Oligonucleotides used for the amplification of these fragments via PCR are listed in Table 2.

12) and, in addition, likely regulates the TCA cycle genes *sdhCAB* for succinate dehydrogenase and *acn*, as well as *pck* and *malE*, as RamA binding site motifs are found in the promoter regions of these genes (3).

The regulons of the carbon catabolite regulators cAMP receptor protein-cAMP complex 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 the cAMP receptor protein-cAMP complex (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 (13). 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 coutilizes 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 (Fig. 3B) is found two times in the promoter regions of all identified putative SugR targets and coincides with 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 (Fig. 3A). In vitro evidence suggests that a $TG(T)_{2\text{-}5}G$ sequence might additionally be involved in SugR binding in *C. glutamicum* R, a related strain providing high lactate yields (60). 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 two of the three operator sites, namely, 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 nonpalindromic sequences upstream of the fructose-PTS gene cluster (5). Currently, it is not known how the different orientations and locations of the binding sequence motifs affect the action of the SugR from *C. glutamicum* in vivo and if SugR

A

gene name/gene ID	putative SugR binding site	strand	shift	position/location	
ptsG (cg1537)	GGTCGGACATATTC	+	++	-82 to -69	TS
	AAACGGACACACTG	-		-101 to -114	
ptsS (cg2925)	GATCGGACACAAAT	+	++	-33 to -20	TS
	ATTCAGACATGAAT	-		+30 to +17	
ptsH (cg2121)	GGTCGGACATTGTT	+	+++	-116 to -153	TL
	AGTGAGACATTGTT	-		-113 to -126	
ptsI/fruR-fruK-ptsF (cg2117-cg2120)	ATTCAAACATTTGG	+	++	-343 to -330	TL
	CATCAGCCACAGTA	+		-425 to -412	
pyk (cg2291)	GAACAGACAATGTT	-	++	-82 to -95	TL
	AAAAAGACTAAATT	-		-126 to -113	
ldhA (cg3219)	GGTCAGACCAAGTG	-	++	-126 to -139	TL
	TTTCGGACATAATC	+		-121 to -108	
sgcA (cg3366)	GGTCAAACATAAAG	-	+++	-38 to -51	TL
	GTACAAACATAAGT	-		+10 to -4	
eno (cg1111)	GATCAGACATAATC	-	+++	-166 to -179	TL
	TTTCAGCCACTGTG	-		+10 to -4	
cg1408-pfkA (cg1408-cg1409)	GGTCGGACACATTT	-	++	-172 to -185	TL
	GGTCAGAGACAGTT	+		-89 to -76	
	GCTCGGACAATGTC	+		-144 to -131	
sugR (cg2115)	ATTCGGACATATTT	+	+	-45 to -32	TL
	AGTGAGACAATGGG	+		-65 to -52	
fba (cg3068)	AATCAGACATTGTG	+	++	-108 to -95	TL
	GATCAAACATTGAT	-		-115 to -128	
terC (cg2157)	AATCGGACATGCTC	-	+++	-291 to -304	TL
	GTTCGCACAGTTTG	+		-334 to -331	
ctaB-tkt (cg1173-cg1174)	ACTCTGACAGAAGC	+	+	-733 to -719	TL
	TTTCAAACCATTTG	+		-627 to -613	
ramA-cysK (cg2831-cg2833)	GGAAGTACACTGTA	+	+	-8 to -21	TS
	GTGCCAACATATTG	+		-55 to -68	
cytP (cg0645)	ATTCAAACTTCACT	+	+	-41 to -54	TL
	CTTCGGCCACACAC	+		-19 to -32	
alr (cg0680-0681)	GGTGGCACAATAGT	+	+	-6 to -19	TL
	GTTTGGACAGGTGT	+		-116 to -119	
ftsY (cg2262)	CTTCTGACCTGCTC	+	+	-12 to -25	TL
	GGTCAAACCTGCCA	+		-138 to -151	
cg2795-cg2796	GGATGGACATTTTT	+	++	-18 to -31	TL
	ATTCCAAACCCCAA	+		-45 to -57	
lpd (cg0441)	AGTCGCACAGGGCC	+	+	-320 to -333	TL
	TTTCCCACAGCATC	+		-179 to -192	
aroE1-lipT (cg1283-cg1284)	AACCGCACACAAAT	+	++	-104 to -117	TL
	TCCCACACAATCAG	+		-45 to -58	

B

FIG. 3. SugR binding sites in the DNA fragments verified by band shift analysis. (A) The SugR binding sites shown in this figure were identified by a motif search using the MEME software (http://meme.sdsc.edu/) (4) and the promoter fragments used in the gel shift analysis. The column labeled "shift" indicates whether a complete gel shift occurred at a 30-fold (+++), 60-fold (++), or 90-fold (+) excess of SugR 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. (B) A frequency plot of the deduced consensus sequence conservation at each position of the 14-bp motif, where the height of each symbol within the stack reflects the relative frequency of the corresponding nucleotide at that position.

occurs in different multimeric forms, as described for DeoR of *E. coli*.

SugR control of PTS genes is physiologically relevant, as the utilization of glucose (19), fructose, or sucrose (Fig. 1) is negatively affected by the overexpression of *sugR*, while the deletion of *sugR* resulted in increased glucose uptake during growth on glucose-acetate mixtures (19). In addition, the physiological significance of the SugR control of non-PTS genes

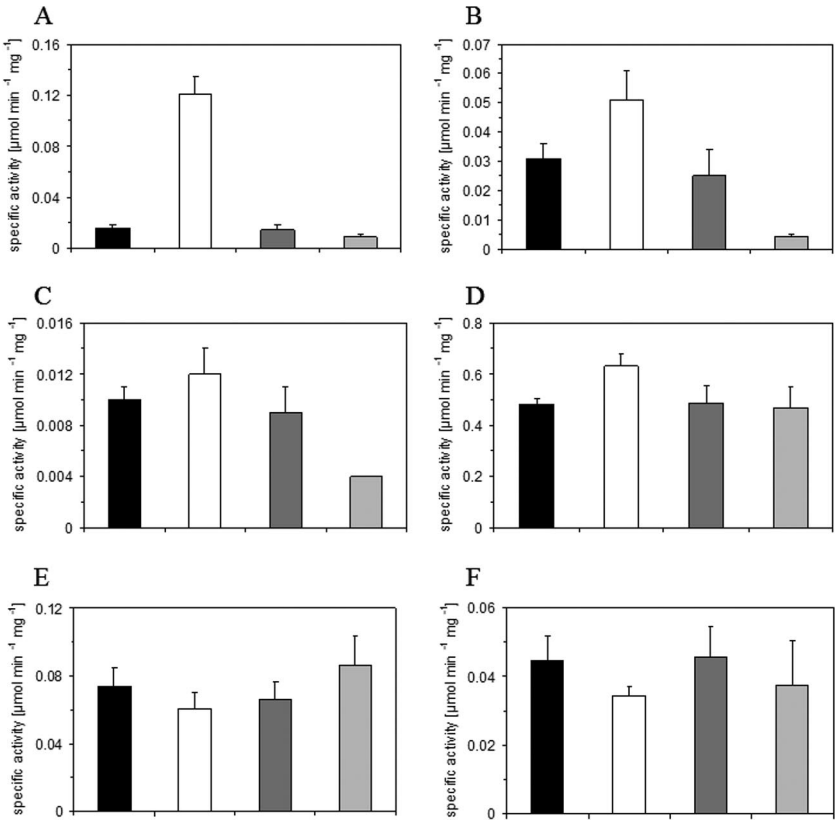

FIG. 4. Specific activities of NAD-dependent L-lactate dehydrogenase (LdhA) (A), fructose-1,6-bisphosphate aldolase (Fba) (B), 6-phosphofructokinase (PfkA) (C), pyruvate kinase (Pyk) (D), transketolase (Tkt) (E), and transaldolase (Tal) (F) in the *C. glutamicum* WT (black bars), WTΔ*sugR* (white bars), WT(pVWEx1) (dark gray bars), and WT(pVWEx1-*sugR*) (light gray bars) strains during aerobic growth on LB. All data are arithmetic means with absolute errors of at least four determinations from one or two independent cultivations.

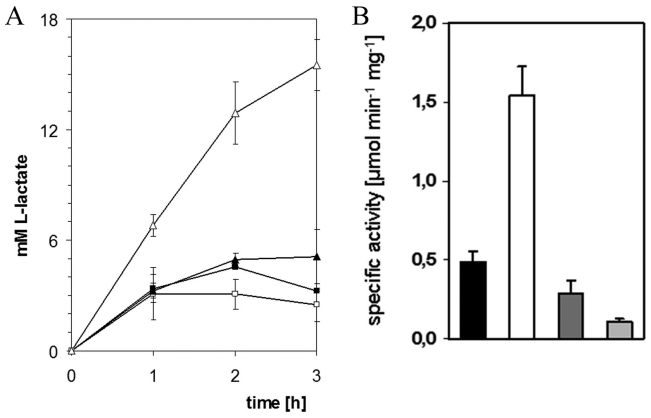

FIG. 5. Production of L-lactate (A) and specific activities of NAD-dependent L-lactate dehydrogenase LdhA (B) during growth on glucose under oxygen deprivation conditions. (A) Concentrations of L-lactate produced by *C. glutamicum* WT (closed triangles), the WT Δ*sugR* mutant (open triangles), WT(pVWEx1) (closed squares), and WT(pVWEx1-*sugR*) (open squares) are indicated. The data represent averages of two independent cultivations. (B) NAD-dependent L-lactate dehydrogenase (LdhA) in the *C. glutamicum* WT (black bars), WTΔ*sugR* (white bars), WT(pVWEx1) (dark gray bars), and WT(pVWEx1-*sugR*) (light gray bars) strains during growth on glucose under oxygen deprivation conditions. All data are arithmetic means with absolute errors of at least four determinations from three independent cultivations.

became obvious by the facts that *sugR* overexpression perturbed the utilization of ribose (Fig. 1) and that the deletion of *sugR* resulted in increased L-lactate formation under oxygen deprivation conditions (Fig. 5). L-Lactate is both a metabolic product and a carbon substrate for growth. The growth of *C. glutamicum* on L-lactate requires quinone-dependent L-lactate dehydrogenase LldD (cg3227; EC 1.1.2.3) (55). 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 a by-product during glutamate and lysine production (37, 55) or under oxygen deprivation conditions (32). L-Lactate formation requires the NAD-dependent L-lactate dehydrogenase LdhA (32) and *ldhA* mRNA levels increased about ninefold under oxygen deprivation conditions (33), as expected for fermentative enzymes. However, the regulatory mechanism for the 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* (Table 3; Fig. 2 and 4A). In the absence of SugR, LdhA activities were about eightfold higher than in *C. glutamicum* WT (Fig. 4A), which was associated with a threefold-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, and

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 the supply of carbohydrate growth substrates entering glycolysis is sufficient.

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