

Molecular mechanism of SugR-mediated sugar-dependent expression of the *ldhA* gene encoding L-lactate dehydrogenase in *Corynebacterium glutamicum*

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Abstract This paper reports on the transcriptional regulation mechanism of the *Corynebacterium glutamicum ldhA* gene encoding L-lactate dehydrogenase responsible for production of L-lactate. DNA affinity purification allowed us to identify SugR, a global repressor of genes involved in sugar uptake and glycolysis, as a protein binding to the *ldhA* promoter region. Whereas *ldhA* gene expression and *ldhA* promoter activity were completely repressed during growth of wild-type cells in the absence of sugar, no such repression was observed in *sugR* mutant cells, indicating that SugR represses *ldhA* transcription. Electrophoretic mobility shift assays and DNase I footprint analyses revealed that two direct repeats, centered at position-17 with respect to the transcriptional start point, are required for SugR binding to the *ldhA* promoter and that fructose-1-phosphate (F-1-P) is the strongest negative effector of repressor activity of SugR. Furthermore, the *ldhA* promoter activity during growth on either fructose or sucrose, under which F-1-P is generated, is higher than on glucose, supporting the results of the in vitro binding assays. Thus, *C. glutamicum ldhA* is repressed by SugR in the absence of sugar and the expression level is dependent on the extent of depression, which varies in response to sugars provided.

Keywords *Corynebacterium glutamicum* · Lactate dehydrogenase · Transcriptional regulation

Introduction

In the absence of an external electron acceptor, facultative aerobic bacteria induce fermentative pathways to maintain the cytosolic NADH to NAD⁺ ratio. Fermentation of sugars to lactate is widespread. A variety of bacteria produce lactate during anaerobic growth; in some cases, the L-isomer is made; in others, D-lactate is the product. *Escherichia coli* produces D-lactate (Clark 1989), while *Bacillus subtilis* and many lactobacilli produce L-lactate (Cruz Ramos et al. 2000). Lactate dehydrogenase (LDH) is a cytoplasmic NAD-linked enzyme that converts pyruvate to lactate. During fermentation, LDH is the key enzyme involved in reoxidation of the NADH formed during glycolysis.

Expression of LDH-encoding genes in *E. coli* and *B. subtilis* is under the control of multiple transcriptional regulators. In *E. coli*, fermentative LDH, encoded by the *ldhA* gene, is induced in anaerobically grown cultures (Clark 1989; Mat-Jan et al. 1989). Expression of the *ldhA* gene is affected by both the redox sensor system ArcAB and the carbohydrate metabolism regulators, CsrAB and Mlc (Jiang et al. 2001). However, direct binding of the regulators to the *ldhA* promoter region and the regulatory mechanisms involved have not been investigated. In *B. subtilis*, the LDH-encoding gene, *ldh*, is induced together with the *alsSD* genes, encoding acetolactate synthase and acetolactate decarboxylase, respectively, by ArfM (Cruz Ramos et al. 2000; Marino et al. 2001). ArfM is activated via the redox-sensing regulators, ResDE and Fnr, in response to anaerobiosis (Marino et al. 2001). The induction of the genes is repressed in the presence of nitrate by YdiH (Rex; Cruz Ramos et al. 2000; Larsson et al. 2005; Reents et al. 2006). YdiH is a homolog of Rex, a redox-sensing transcriptional regulator that responds to

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cellular NADH to NAD⁺ ratio in *Streptomyces coelicolor* A3(2) (Brekasis and Paget 2003; Schau et al. 2004).

Corynebacterium glutamicum is a nonpathogenic high-GC Gram-positive bacterium which is widely used in the industrial production of amino acids, e.g., L-glutamate and L-lysine (Kinoshita et al. 1957; Hermann 2003). In *C. glutamicum*, the *ldhA* gene encodes LDH responsible for production of L-lactate (Inui et al. 2004). We have previously shown that both the expression level of the *ldhA* gene and LDH activity are increased in response to low oxygen tension at the onset of the stationary phase of growth on glucose and remain high under oxygen deprivation (Inui et al. 2007). However, the regulatory mechanism of *ldhA* expression in *C. glutamicum* has remained elusive.

In this study, SugR, a repressor of genes involved in the phosphoenolpyruvate:sugar phosphotransferase system (PTS; Engels and Wendisch 2007; Gaigalat et al. 2007; Tanaka et al. 2008), was identified as a protein binding to the promoter region of the *C. glutamicum ldhA* gene. By measuring both *ldhA* mRNA levels and LDH activity in the *sugR* mutant and the wild-type strain, we show that SugR represses *ldhA* expression. This is consistent with an independent recent study identifying the *ldhA* promoter as one of the targets of SugR binding by ChIP-chip analysis (Engels et al. 2008). Moreover, we here report detailed analyses of SugR binding by DNase I footprinting analyses and electrophoretic mobility shift assays (EMSAs) using various mutated *ldhA* promoter fragments. Analyses of

ldhA expression pattern in response to sugars by *lacZ* reporter assays revealed the sugar-dependent regulation mechanism mediated by SugR.

Materials and methods

Bacterial strains and plasmids

The strains and plasmids used in this study are listed in Table 1.

Culture conditions

For genetic manipulation, *E. coli* strains were grown at 37°C in Luria-Bertani medium. *C. glutamicum* strains were grown at 33°C in nutrient-rich medium (A medium; Inui et al. 2007) with 4% glucose. When appropriate, the media were supplemented with antibiotics. The final antibiotic concentrations for *E. coli* were 50 µg of ampicillin per milliliter and 50 µg of kanamycin per milliliter; for *C. glutamicum*, kanamycin (50 µg ml⁻¹) was used. Growth experiments of *C. glutamicum* were performed using A medium containing 1% glucose or acetate as described previously (Toyoda et al. 2008).

DNA manipulations

The oligonucleotides used in this study (Table 2) were obtained from Gene design (Osaka, Japan). Plasmid DNA

Table 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference
Strains		
<i>E. coli</i>		
JM109	<i>recA1 endA1 gyrA96 thi hsdR17 supE44 relA1 Δ(lac-proAB)/F'</i> [<i>traD36 proAB⁺ lacI^f lacZΔM15</i>]	Takara
JM110	<i>dam dcm supE44 hsdR17 tih leu rpsL lacy galK galT ara tonA thr tsx Δ(lac-proAB)/F'</i> [<i>traD36 proAB⁺q lacZΔM15</i>]	(Sambrook et al. 1989)
BL21(DE3)	F ⁻ <i>ompT gal dcm lon hsdS_B(r_B⁻ m_B⁻) λ(DE3)</i>	(Studier and Moffatt 1986)
<i>C. glutamicum</i>		
R	Wild-type strain	(Yukawa et al. 2007)
KT1	R with deletion in <i>sugR</i>	(Toyoda et al. 2008)
KT14	R with <i>PldhA-lacZ</i>	This study
KT15	KT1 with <i>PldhA-lacZ</i>	This study
Plasmids		
pGEM T-Easy	Ap ^r ; TA cloning vector	Promega
pCRA725	Km ^r ; the suicide vector containing the <i>B. subtilis sacB</i> gene	(Inui et al. 2004)
pCRA741	Km ^r ; pCRA725 with a 2.0-kb PCR fragment from strain-specific island 7 and a 3.1-kb PCR fragment containing the <i>E. coli lacZ</i> gene	(Inui et al. 2007)
pCRC614	Km ^r ; pCRA741 with a 214-bp PCR fragment containing the <i>C. glutamicum</i> R <i>ldhA</i> promoter region	This study
pCRC615	Ap ^r ; pGEMT-easy with a 433-bp PCR fragment containing the <i>ldhA</i> promoter region	This study

Table 2 Oligonucleotide primers used in this study

Primer name	Sequence (5'–3') ^a
DNA affinity beads and EMSA	
DNase I footprint	
pldhA RV-bio	Biotin-TCGATCCCCTCCTGATTCC
pldhA FW	GCGAGTGGTGAGCAGAGACG
pldhA FW Cy3	Cy3-TTTAGTGC GGTTTAAGCGTTGC
pldhA RV Cy3	Cy3-TTTCGATCCCCTCCTGATTCC
pldhA FW1 for P1 fragment	CTTTACTTTGTGGATTACGGG
pldhA RVNaeI	ATTGCCGGCGGTTTCTTTTCATTTCGATC
pldhA FW2 for P2 fragment	CATTTTCGGACATAATCGGGC
pldhA FW3 for P3 fragment	TAATTAAGGTGTAACAAAGGAATCCG
pldhA RV1 for P4 fragment	TGTCCGAAAATGTGGTCAGAC
pldhA FW4 for DR-II fragment	TAATCGGGCATAATTAAGGTG
pldhA FW4 mut1	TAAT <u>CCCC</u> ATAATTAAGGTGTAACAAAG
pldhA FW4 mut2	TAATCGGG <u>GTA</u> ATAATTAAGGTGTAACAAAG
pldhA FW4 mut3	TAATCGGGC <u>ATT</u> ATAATTAAGGTGTAACAAAG
pldhA FW4 mut4	TAATCGGGCATAAT <u>ATT</u> AGGTGTAACAAAGG
pldhA RV2 for DR-I fragment	GATTATGTCCGAAAATGTGGTCAGAC
pldhA RV2 mut1	<u>GTA</u> AATGTCCGAAAATGTGGTCAGAC
pldhA RV2 mut2	GATTTACTCCGAAAATGTGGTCAGAC
pldhA RV2 mut3	GATTATG <u>AGG</u> AAAATGTGGTCAGAC
pldhA RV2 mut4	GATTATGTCCG <u>TTT</u> ATGTGGTCAGACCATG
pldhA FW8	ATCCCCGGAAGCTAGCTCTGC
pldhA RV3	GATCAGTGCGTATGCGTATGC
pldhA IRD FW	IRDye700-TTAGTGCGGGTTTAAGCGTTGCCAG
pldhA IRD RV	IRDye700-TCCAACATCTCCTGCGCCAATAAGG
qRT-PCR	
16S FW	TCGATGCAACGCGAAGAAC
16S RV	GAACCGACCACAAGGGAAAAAC
ldhA FW	GAACACGGCGACTGAACCTT
ldhA RV	AGCATGCGGCTAAGCGATA

^a Mutagenized nucleotides are indicated with underlines.

was isolated with the QIAprep spin miniprep kit (Qiagen, Hilden, Germany). Chromosomal DNA was isolated from *C. glutamicum* with Genomic prep (GE Healthcare Bioscience, Piscataway, NJ, USA), modified by using 4 mg ml⁻¹ lysozyme at 37°C for 2 h. Restriction endonucleases were purchased from Takara (Osaka, Japan) and used according to the manufacturer's instructions. Polymerase chain reaction (PCR) was performed using a GeneAmp PCR system (Applied Biosystems, Foster City, CA, USA) using LA Taq polymerase (Takara) or Phusion polymerase (New England Biolabs). The resulting PCR fragments were purified with the QIAquick PCR purification kit (Qiagen). *E. coli* was transformed by the CaCl₂ procedure, as described by Sambrook et al. (1989). *C. glutamicum* was transformed by electroporation as described previously (Vertès et al. 1993). The nucleotide sequence of cloned DNA fragments was determined with Genetic analyzer 3130xl (Applied Biosystems).

DNA affinity purification

DNA affinity purification of proteins binding to the *ldhA* promoter region was performed as described previously (Toyoda et al. 2008). The *ldhA* promoter region between -177 and +89 relative to the transcriptional start point (TSP) of the *ldhA* gene was generated by PCR using primer pldhA RV-bio, which was tagged with biotin, and primer pldhA FW (Table 2). The Dynabeads (Dyna) coupled with the *ldhA* promoter fragment were equilibrated with 600-μl binding buffer (50 mM Tris-HCl, pH 7.5, 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM dithiothreitol (DTT), 100 mM NaCl, 0.05% Triton-X, and 10% (vol/vol) glycerol) and incubated with cell extract for 2 h at room temperature with shaking. Unbound and nonspecifically bound proteins were removed by magnetic separation with a magnet particle concentrator (Dyna) and several washes with 600 μl of binding buffer with 120 mM NaCl. The

proteins bound to the coupled Dynabeads were eluted with 0.2 and 1 M NaCl (40 μ l each). Eluted fractions were collected and subjected to 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) by the technique of Laemmli (1970). Subsequently, the samples were electrophoretically transferred onto a Sequi-Blot polyvinylidene fluoride membrane (Bio-Rad Laboratories) using the TransBlot SD cell (Bio-Rad Laboratories). Proteins on the blot were detected by Coomassie brilliant blue staining. The N-terminal amino acid sequence of the protein bands was analyzed by Edman sequence analysis (APRO Life Science Institute, Inc, Tokushima, Japan).

Quantitative RT-PCR

Total RNA was extracted from *C. glutamicum* cells using the Qiagen RNeasy Mini kit (Qiagen) as described previously (Toyoda et al. 2008). Isolated RNA samples were checked for purity by both agarose gel electrophoresis and spectrophotometric analysis and stored at -80°C . Quantitative reverse-transcriptase PCR (qRT-PCR) was performed using an Applied Biosystems 7500 Fast Real-Time PCR System (Applied Biosystems) and Power SYBR Green PCR Master Mix with MuLV Reverse Transcriptase and RNase Inhibitor of GeneAmp RNA PCR kit (Applied Biosystems) as described previously (Toyoda et al. 2008). Specific primers (Table 2) were designed using the Primer Express Software version 3.0 (Applied Biosystems). The specificity of the amplicons was checked by qRT-PCR dissociation curve analysis.

LDH assay

C. glutamicum cells were harvested by centrifugation and washed with 50 mM Tris-HCl buffer, pH 7.5, and resuspended with buffer containing 50 mM Tris-HCl, pH 7.5, 10 mM MgCl_2 , 1 mM EDTA, 1 mM DTT, and 10% (vol/vol) of glycerol. The cells were mechanically disrupted using zirconia/silica beads as described previously (Toyoda et al. 2008), and the supernatant after centrifugation was used as crude extracts for the enzyme assay. The activity of LDH was determined as described previously (Bunch et al. 1997).

Construction of *ldhA* promoter-*lacZ* fusions

DNA fragments containing the *ldhA* promoter region were generated by PCR using primers pldhA FW1 and pldhA RVNaeI and cloned in front of the *lacZ* gene in pCRA741 (Inui et al. 2007), yielding pCRC614. Direction and sequence of the inserted fragment were confirmed by sequencing. The plasmid pCRC614 was isolated as non-

methylated DNA from *E. coli* JM110 for efficient gene introduction into *C. glutamicum* R chromosome (Vertès et al. 1993) and was subsequently integrated into the chromosome of the wild-type *C. glutamicum* R and *sugR* mutant KT1 (Toyoda et al. 2008) by markerless gene insertion methods, as described previously (Inui et al. 2007), resulting in strains KT14 and KT15, respectively. The sequence of the *ldhA* promoter in *PldhA-lacZ* fusion inserted in the chromosome was confirmed by direct sequencing of PCR fragments which were amplified using primer specific for *lacZ* and primer pldhA FW1 and genomic DNA, extracted from the mutants obtained, as a template.

β -galactosidase assay

β -galactosidase activity in *C. glutamicum* was determined as described previously (Toyoda et al. 2008). Cells permeabilized with toluene were incubated with *o*-nitrophenyl- β -galactoside, and activity was measured in Miller units as previously described (Miller 1972). Assays were carried out in triplicate for each sample, and results are presented as mean \pm standard deviations.

Electrophoretic mobility shift assay

Cy3-labeled DNA fragments used in EMSAs were generated by PCR and purified with PCR purification kit (Qiagen). The *ldhA* promoter fragments P1, P2, and P3 were generated using primers pldhA FW1, pldhA FW2, and pldhA FW3, respectively, together with primer pldhA RV Cy3. DNA fragment P4 was generated using a pair of primers pldhA RV1 and pldhA FW Cy3. DNA fragments DR-I and DR-II, covering the direct repeats in the *ldhA* promoter, were generated using primer pairs pldhA RV2/pldhA FW Cy3 and pldhA FW4/pldhA RV Cy3, respectively. DNA fragments containing mutations in the direct repeat in the DR-I were generated using primer pairs pldhA RV2 mut1/pldhA FW Cy3 to pldhA RV2 mut4/pldhA FW Cy3. DNA fragments containing mutations in the direct repeat in the DR-II were generated with primer pairs pldhA FW4 mut1/pldhA RV Cy3 to pldhA FW4 mut4/pldhA RV Cy3. EMSA with His-tagged SugR was performed as described previously (Toyoda et al. 2008). The resulting DNA-protein complexes were loaded onto a 5% polyacrylamide gel. DNA and DNA-protein complexes were visualized by a Typhoon TRIO Variable Mode Imager (GE Healthcare Bioscience). To test for possible effectors, the protein was incubated with glucose-6-phosphate (G-6-P), fructose-1-phosphate (F-1-P), fructose-6-phosphate (F-6-P), and fructose-1,6-bisphosphate (F-1,6-P) in the binding buffer for 15 min before labeled DNA was added

and the mixture was incubated for an additional 25 min at room temperature.

DNase I footprinting

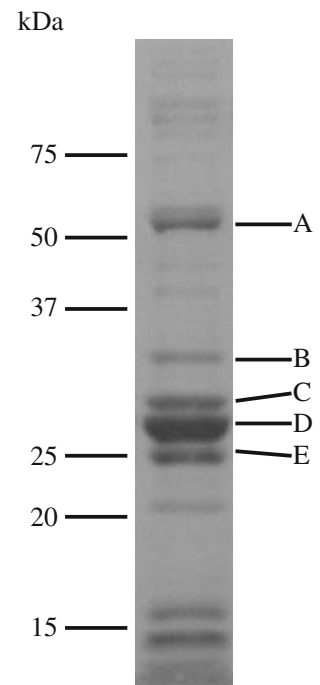
DNase I footprinting was performed as described previously (Toyoda et al. 2009). Labeled DNA probes were obtained by amplification with 5'-IRD700-labeled oligonucleotides. Primer pair *pldhA* IRD FW/*pldhA* RV3 or *pldhA* IRD RV/*pldhA* FW8 was used to generate the coding- or noncoding-strand-labeled DNA fragment. DNA fragment containing the *ldhA* promoter region was amplified by PCR using primer pair *pldhA* RV3/*pldhA* FW8 and cloned into pGEM T-easy, yielding pCRC615. The DNA sequencing reactions were set up using the same IRD-700-labeled primers and pCRC615 as a template and a DYEnamic direct cycle sequencing kit with 7-deaza-dGTP (GE healthcare Bioscience). To test for possible effectors, the protein was preincubated with metabolites as described in the part of electrophoretic mobility shift assay; then, the IRD700-labeled probe was added and the mixture was treated as described above.

Results

Identification of proteins binding to the *ldhA* promoter of *C. glutamicum*

To obtain transcriptional regulators binding to the *ldhA* promoter region, we performed DNA affinity purification with magnetic streptavidin beads as described previously (Toyoda et al. 2008). A 266-bp biotinylated promoter region between position +89 and -177 relative to the TSP of the *ldhA* gene (Inui et al. 2007) was linked to streptavidin-coated magnetic beads and incubated with cell extracts from wild-type *C. glutamicum* R grown in minimal medium containing glucose as the sole carbon source. We identified five proteins by comparing the N-terminal amino acid sequences of the corresponding bands on SDS-PAGE (Fig. 1) with those of all open reading frames of *C. glutamicum* R (Yukawa et al. 2007). The proteins identified included three transcriptional regulators: two DeoR-type transcriptional regulators (CgR0187 and CgR1761) and a GntR-type transcriptional regulator (CgR2816). CgR1761 corresponded to SugR, a repressor of genes involved in PTS in *C. glutamicum* (Engels and Wendisch 2007; Gaigalat et al. 2007; Tanaka et al. 2008). The protein encoded by *cgR_2816* corresponded to LldR, which has been identified as a repressor of the L-lactate utilization operon (Georgi et al. 2008). The protein encoded by *cgR_0187* was a transcriptional regulator which has not yet been characterized to date. In this study, we investigated the role of SugR in regulation of *ldhA* expression.

Fig. 1 SDS-PAGE of *C. glutamicum* proteins binding to the *ldhA* promoter region. Cell extracts of *C. glutamicum* R grown in minimal medium containing 1% glucose was incubated with DNA affinity beads and bound proteins were eluted with 1 M NaCl. The N-terminal amino acid sequence of the protein bands A to E was analyzed; A, putative gluconate kinase (CgR2896); B, DeoR-type transcriptional regulator (CgR0187) and 50S ribosomal protein L2 (CgR0611); C, SugR (CgR1761); D, ROK family sugar kinase (CgR1739); E, LldR (CgR2816). A molecular mass standard is shown to the left of the panel



Inactivation of *sugR* upregulates *ldhA* expression

SugR is a global regulator involved in sugar-dependent gene expression (Engels and Wendisch 2007; Gaigalat et al. 2007; Tanaka et al. 2008; Toyoda et al. 2008). Hence, we compared *ldhA* expression of the *sugR* mutant strain KT1 to that of the wild-type strain during growth on either glucose or acetate. Total RNA was extracted from cells grown in nutrient-rich medium (A medium) containing glucose or acetate at the mid-exponential (3 h) and at the onset of the stationary phase (6 h), and the relative mRNA levels of *ldhA* were determined by qRT-PCR (Fig. 2a). As previously shown (Toyoda et al. 2008), there was no marked difference in growth rate on either glucose or acetate between the KT1 and the wild-type strain. Wild-type *ldhA* mRNA levels on glucose were comparable to those on acetate at the exponential phase (Fig. 2a). Whereas the *ldhA* mRNA levels increased nearly 30-fold at the onset of the stationary phase on glucose, they did not show any changes on acetate (Fig. 2a). Strain KT1 *ldhA* mRNA levels were more than fivefold higher than those in the wild-type strain under all the conditions tested, except at the onset of the stationary phase on glucose, where the *ldhA* mRNA levels in strain KT1 were only slightly higher. The increase in the level of the *ldhA* mRNA in the *sugR* mutant was most noticeable at the onset of the stationary phase on acetate.

The activity of NAD-dependent LDH in the *sugR* mutant KT1 grown in A medium containing either glucose or acetate was compared with that in the wild-type strain (Fig. 2b). The LDH activity was barely detectable in the wild type at the

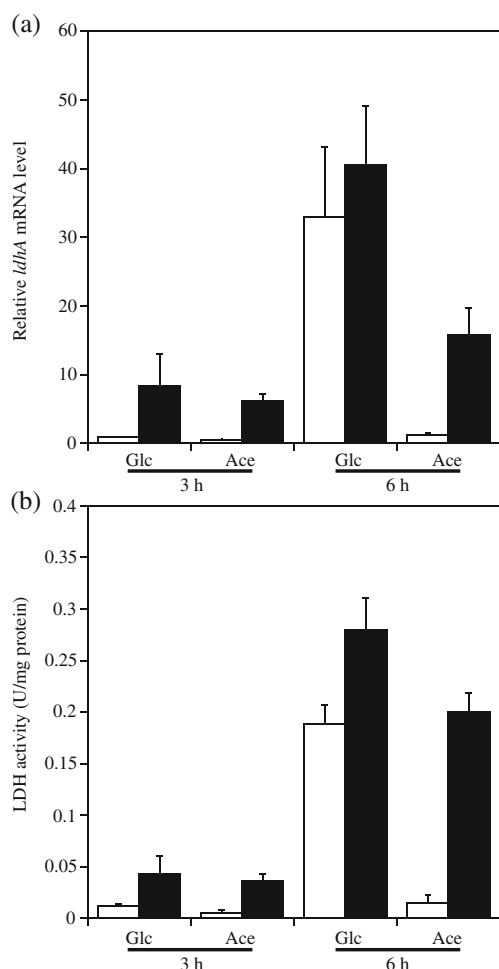


Fig. 2 Effect of deletion of the *sugR* gene on *ldhA* mRNA level (a) and LDH activity (b). **a** The level of *ldhA* mRNA in the wild-type R (white) and the *sugR* mutant KT1 (black) during growth in nutrient-rich A medium with 1% glucose (Glc) or acetate (Ace) at the exponential (3 h) and the stationary (6 h) phases was analyzed by qRT-PCR, and the levels relative to that in the wild type grown in the presence of glucose at 3 h were determined. Mean values from three independent cultures are shown with standard deviations. **b** LDH activity in the wild type (white) and the *sugR* mutant KT1 (black) during growth under the same conditions as described for *ldhA* mRNA level. Mean values from at least three independent cultures are shown with standard deviations

exponential phase of growth on either glucose or acetate. The wild-type LDH activity on glucose increased nearly 15-fold at the onset of the stationary phase, whereas that on acetate did not change. As observed with the level of *ldhA* mRNA, the LDH activity of strain KT1 was higher than that of the wild type under all the conditions examined. These results indicated that *ldhA* expression is dependent on the growth phase and carbon source used and that SugR has a negative effect on *ldhA* expression not only during the exponential phase of growth on glucose but throughout growth on acetate.

Inactivation of *sugR* upregulates *ldhA* promoter activity

To investigate the promoter activity of the *ldhA* gene, we integrated the *ldhA* promoter-*lacZ* translational fusion (*PldhA-lacZ*) into the chromosome of wild-type *C. glutamicum* R and *sugR* mutant strain KT1. The resulting strains were designated KT14 and KT15, respectively. We measured β -galactosidase activity to examine the *PldhA-lacZ* expression in strains KT14 and KT15 grown for 10 h in A medium containing either glucose or acetate (Fig. 3). In the presence of glucose, almost no β -galactosidase activity was detected in strain KT14 at the exponential phase, while the activity was markedly higher at the onset of the stationary phase (6 h; Fig. 3a). In the presence of acetate, no β -galactosidase activity was detected in strain KT14 throughout the cultivation period (Fig. 3b). Strain KT15 exhibited significantly higher β -galactosidase activity than strain KT14 throughout the growth on glucose. In the presence of acetate, the β -galactosidase activity of strain KT15 increased with growth of cells (Fig. 3). The results were consistent with the *ldhA* mRNA level and LDH activity and demonstrated that *ldhA* expression in cells grown on acetate is completely repressed by SugR.

Binding of SugR to the *ldhA* promoter region

To verify the binding of SugR to the *ldhA* promoter region, we performed EMSAs with a DNA fragment containing the region between positions -102 and $+91$ relative to the TSP of *ldhA* and the recombinant SugR protein. The results of EMSAs showed that SugR specifically bound to the *ldhA* promoter (Fig. 4). To identify the SugR binding site in the *ldhA* promoter region, we performed EMSAs with various lengths of the *ldhA* promoter fragments (Fig. 4). SugR was able to bind to fragment P2 but unable to bind to fragments P3 and P4, indicating that the region between positions -31 and -11 relative to the TSP of the *ldhA* gene contains SugR binding site (Fig. 4).

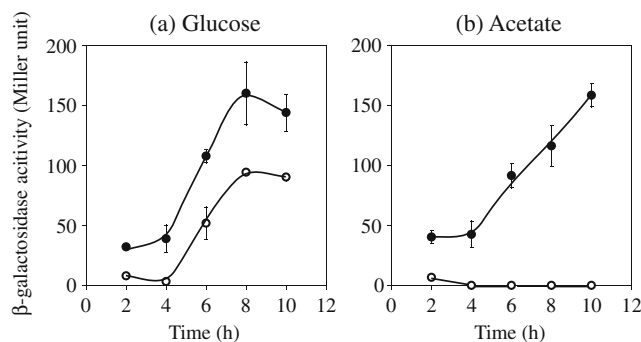


Fig. 3 The *ldhA* promoter activity of strain KT14 (wild type, white circles) and strain KT15 (*sugR* mutant, black circles) during growth on A medium with 1% of glucose (a) and acetate (b). The activity is mean values from at least three independent cultivations with standard deviations

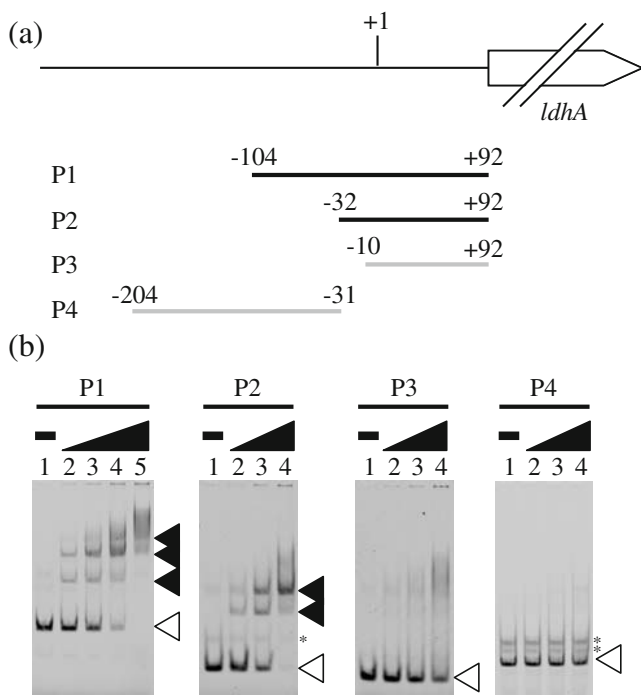


Fig. 4 Binding of SugR to the *ldhA* promoter region. **a** DNA fragments (P1–P4) used in the electrophoretic mobility shift assays are indicated with bars. The DNA fragments binding and not binding to SugR are indicated with black and gray bars, respectively. The *ldhA* gene is indicated with the box arrow and the position with respect to the transcriptional start point (+1) of *ldhA* is indicated. **b** EMSA using the *ldhA* promoter fragments and SugR protein. The DNA fragments used in the EMSAs are indicated at the top of the gels. Each well contained 10 nM of DNA fragment. Lanes 1 to 4 show EMSAs using 0, 100, 200, and 400 nM of SugR protein, respectively. Lane 5 in the panel of the fragment P1 shows an EMSA using 800 nM of SugR and the fragment P1. The free DNA probe and SugR–DNA complexes are indicated with white and black arrowheads, respectively. Nonspecific bands are indicated with asterisks

To determine the binding sites of SugR, DNase I footprinting assays were performed. Each of the strands of a DNA fragment encompassing the region from positions –203 to +146 with respect to the TSP was labeled, incubated with an increasing quantity of SugR, and then hydrolyzed by DNase I. The results showed that the protected region on the coding strand extended from positions –38 to +26 relative to the TSP of the *ldhA* gene while protected region on the noncoding strand extended from –41 to +23 (Fig. 5). In the protected region, we found two 10-bp direct repeats (5'-CGGNCATAATCGGNCA TAAT-3') between positions –26 and –7 (Fig. 6). Relevance of the motif to the SugR binding was tested by mutational analyses using EMSAs (Fig. 6). Mutations in GGNCAT of the direct repeats almost completely inhibited SugR binding to DNA (Fig. 6). In contrast, mutations in AAT of the direct repeats and three bases outside the direct repeat had almost no effect on binding of SugR. These results showed that CGGNCAT of the two direct repeats is essential for the

binding of SugR to the *ldhA* promoter. Whereas DNA fragments containing the two direct repeats generated two SugR–DNA complexes upon binding (Fig. 4, fragment P2), deletion of one of the direct repeats resulted in single shifted band (Fig. 6, fragments DR-I and DR-II). This suggested that multiple SugR proteins bind to the direct repeats.

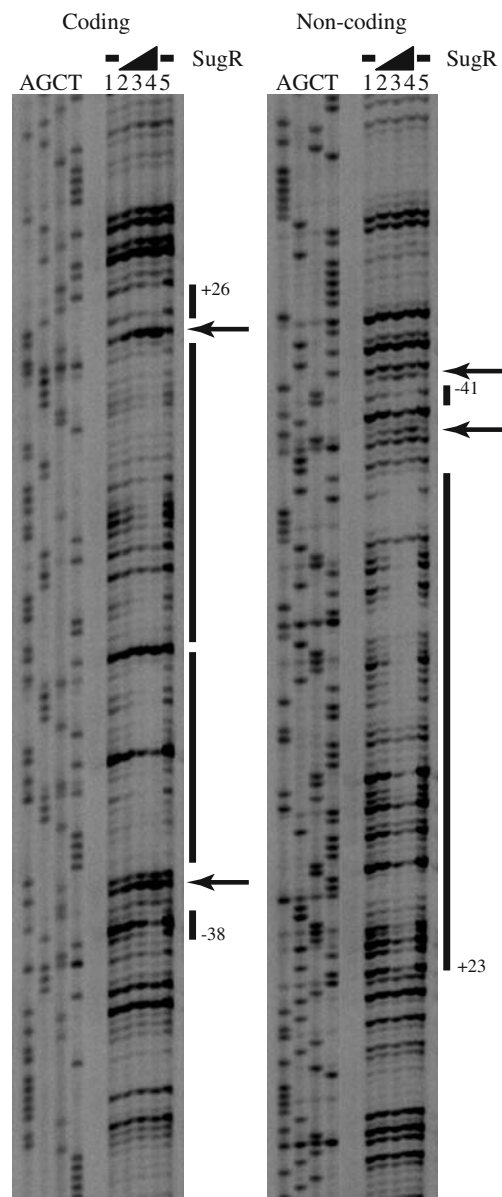


Fig. 5 DNase I footprinting analysis of the interaction between SugR and the *ldhA* promoter regions examined on the coding and the noncoding strands. A DNA fragment (10 nM) was incubated with increasing amounts of SugR: lanes 1 and 5, no protein; lane 2, 200 nM; lane 3, 400 nM; lane 4, 800 nM. Protected regions are indicated by bars and hypersensitive sites are indicated by arrows. Positions with respect to the TSP of *ldhA* are indicated. The DNA sequencing reactions were set up using the same labeled primer and plasmid pCRC615

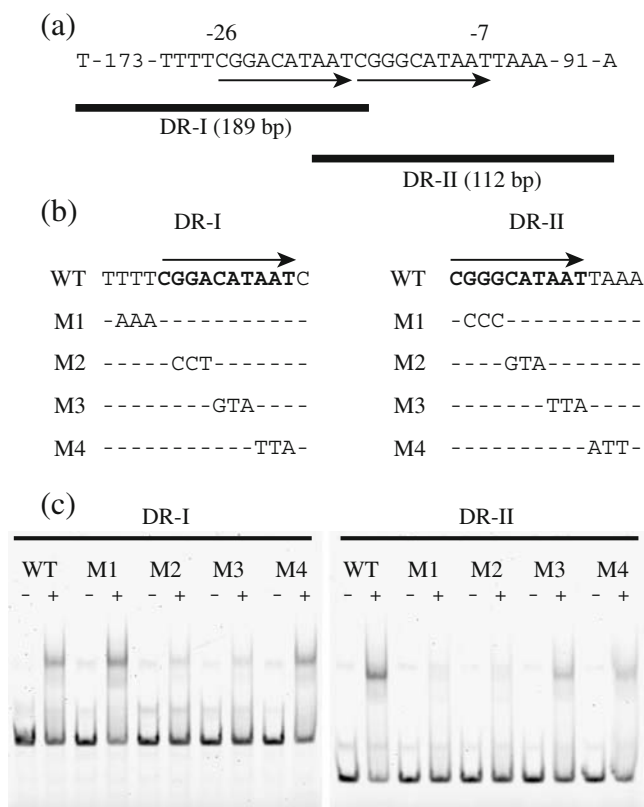


Fig. 6 SugR binds to the two direct repeats in the *ldhA* promoter. **a** Nucleotide sequence of the two direct repeats is shown. *Arrows* indicate the direct repeats. The position with respect to the TSP of the *ldhA* gene is shown above the sequence. DNA fragments, DR-I and DR-II, used in EMSAs, are shown with *bold bars*. **b** Mutations introduced in the DR-I and DR-II fragments are listed below the wild-type sequence. The direct repeats included in the fragments are indicated with *bold letters* and *arrows*. **c** EMSA using the DR-I, DR-II, and their derivatives containing mutations and SugR protein. The DNA fragments used in the EMSAs are indicated at the top of the gels. Each well contained 10 nM of DNA fragment. *Lanes* labeled with a *minus sign* contains no SugR and those labeled with a *plus sign* contains 800 nM of SugR

Fructose-1-phosphate and fructose-1,6-bisphosphate have negative effects on binding of SugR to the *ldhA* promoter

As binding of SugR to the target promoters is inhibited by sugar metabolites (Engels and Wendisch 2007; Gaigalat et al. 2007; Toyoda et al. 2008), we investigated effects of the metabolites on SugR binding to the *ldhA* promoter by EMSAs (Fig. 7). F-1-P inhibited binding of SugR at 40 μ M. F-1,6-P had an inhibitory effect on SugR binding above 20 mM. F-6-P and G-6-P, at up to 40 mM, had no effect on binding of SugR.

DNase I footprinting experiments were then used to confirm these results. SugR was preincubated with different concentrations of F-1-P or other molecules to be tested prior to addition of the DNA-labeled probe. These experi-

ments showed that SugR lost its ability to protect its target sequence with increasing F-1-P concentration (Fig. 8), whereas high concentrations of F-6-P or G-6-P did not interfere with the binding activity of SugR. The results provided confirmation that F-1-P acts as the negative effector molecule on SugR binding to the *ldhA* promoter.

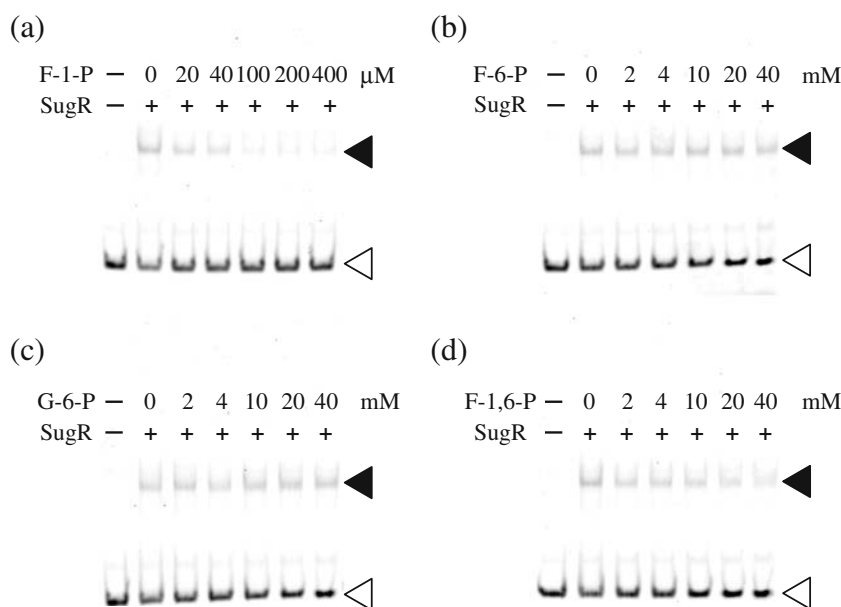
ldhA promoter activity was enhanced in the presence of fructose and sucrose

The strongest effector of SugR, F-1-P, is generated upon uptake of fructose by PtsF, fructose-specific component of PTS, during growth on fructose and sucrose but is not generated during glucose metabolism. To gain insight into effects of the sugar metabolites on SugR activity *in vivo*, *PldhA-lacZ* expression in the genetic background of the wild-type strain was examined during growth on fructose and sucrose. The β -galactosidase activity of the fructose-grown cells was higher than that of the glucose-grown cells at the exponential phase. The activity of both the fructose- and glucose-grown cells similarly increased at the onset of the stationary phase, and subsequently the activity of the fructose-grown cells decreased to the same level as that of the glucose-grown cells at the stationary phase. The *PldhA-lacZ* expression level of the sucrose-grown cells was comparable to that of the fructose-grown cells throughout the cultivation period. The enhanced promoter activity in fructose- or sucrose-grown cells was comparable with that in the *sugR* mutant strain KT15 grown on glucose throughout the growth (Figs. 9 and 3). These results suggest that repressor activity of SugR is completely lost due to F-1-P generated during the growth in the presence of fructose or sucrose but not in the presence of glucose.

Discussion

In this study, we identified SugR as a protein binding to the *ldhA* promoter and examined the involvement of the protein in the regulation of *ldhA* expression in response to sugar. Analyses of the LDH activity, the *ldhA* mRNA level, and the *ldhA* promoter-*lacZ* fusion revealed that *ldhA* expression is markedly induced at the onset of the stationary phase only in the presence of sugar, while the induction is completely repressed in the absence of sugar (Fig. 3). The results indicate that the presence of sugar is essential for the induction of *ldhA* expression, although, as previously shown (Inui et al. 2007), the expression is also controlled by oxygen tension. Our observation that upregulation of the *ldhA* mRNA level by disruption of the *sugR* gene in the absence

Fig. 7 Effects of sugar intermediate concentrations on SugR binding to the *ldhA* promoter. EMSA using 10-nM DNA fragment containing the *ldhA* promoter (fragment DR-II in Fig. 6) and 400 nM SugR in the presence of **a** fructose-1-phosphate (*F-1-P*), **b** fructose-6-phosphate (*F-6-P*), **c** glucose-6-phosphate (*G-6-P*), and **d** fructose-1,6-bisphosphate (*F-1,6-P*) was conducted. The free DNA probe and SugR–DNA complexes are indicated with white and black arrowheads, respectively

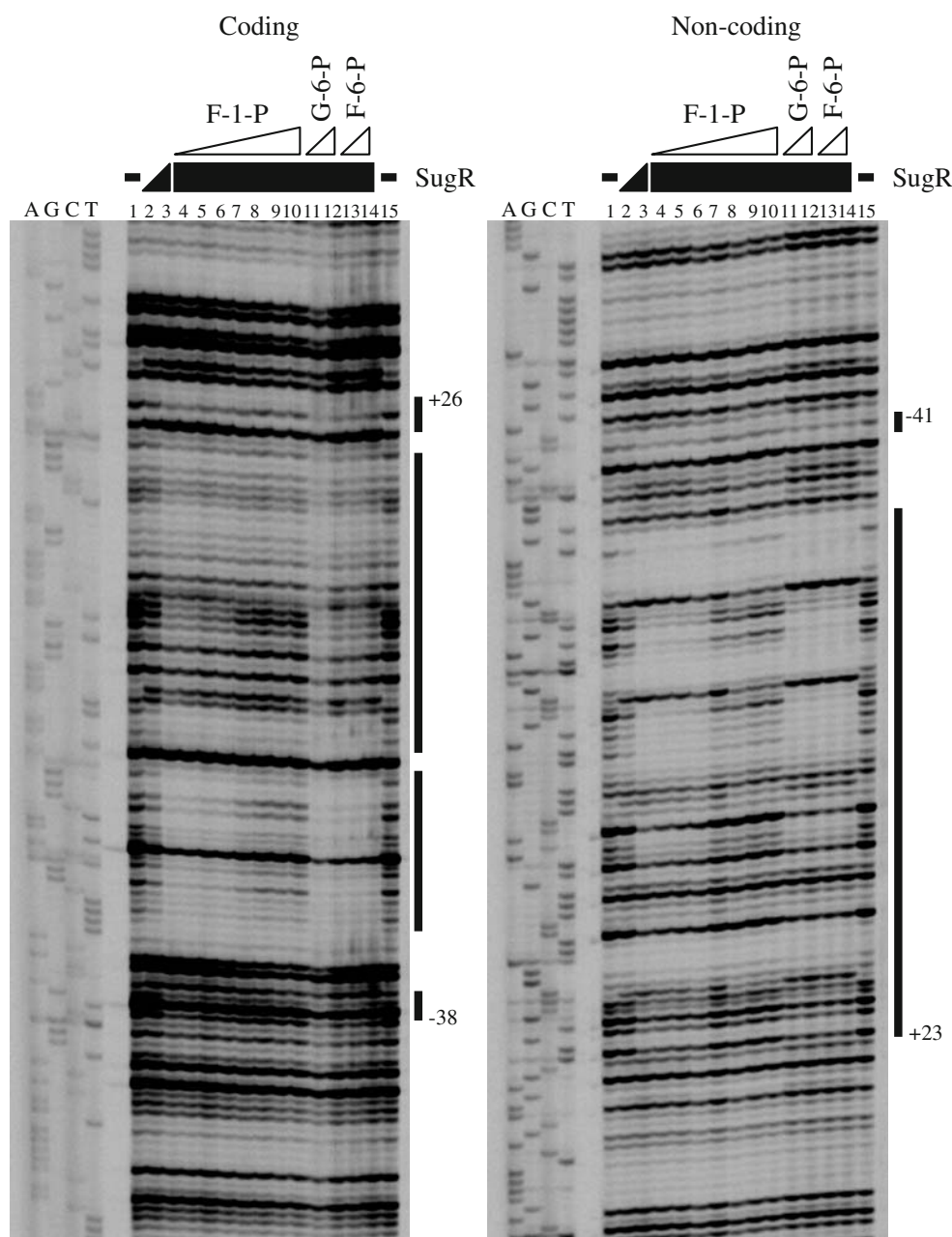


of sugar is consistent with recent studies of SugR regulon defined by transcriptome analyses (Gaigalat et al. 2007) and by ChIP-chip analyses (Engels et al. 2008). These findings indicate that SugR acts as a transcriptional repressor of *ldhA*, in addition to PTS sugar uptake genes and glycolytic genes (Engels and Wendisch 2007; Gaigalat et al. 2007; Engels et al. 2008; Tanaka et al. 2008; Toyoda et al. 2008), and that the presence of sugar alleviates the repression to induce the target genes coordinately (Fig. 10). The coordinated regulation will allow the cells to efficiently utilize sugar in response to environmental conditions. As demonstrated in this study, detailed characteristics of SugR binding to the *ldhA* promoter in vitro and expression patterns of the *ldhA* promoter-*lacZ* fusion in vivo provided further experimental evidence to elucidate the sugar response mechanism of *ldhA* transcription mediated by SugR.

Repressor activity of SugR is controlled by sugar phosphates, including F-1-P and F-1,6-P, while effects of the molecules on binding of SugR are different among the target promoters studied. As in the case of the *gapA* promoter, F-1-P and F-1,6-P have a negative effect on SugR binding to the *ldhA* promoter, and the effect of F-1-P is much stronger than that of F-1,6-P (Fig. 7; Toyoda et al. 2008). F-6-P and G-6-P have no effect on SugR binding to the promoters of *gapA* and *ldhA* (Fig. 7; Toyoda et al. 2008). In addition to F-1-P and F-1,6-P, G-6-P also has a negative effect on SugR binding to the intergenic region between *ptsI* and *ptsF* operon (Gaigalat et al. 2007). As for the *ptsG* promoter, only F-6-P inhibits SugR binding (Engels and Wendisch 2007). Although cause of the difference in the effects is not clear, the distinct effects

may modulate sugar-dependent global regulation mediated by SugR. F-1-P is generated upon uptake of fructose by PtsF, fructose-specific component of PTS. F-1-P is also generated during growth on sucrose in *C. glutamicum* because *C. glutamicum* requires PtsF to metabolize sucrose completely due to the absence of fructokinase activity to phosphorylate fructose liberated from sucrose (Dominguez and Lindley 1996). On the other hand, glucose is phosphorylated to G-6-P upon uptake by PtsG and subsequently metabolized through glycolysis to F-6-P and F-1,6-P. The *ldhA* promoter activity during growth on either fructose or sucrose was higher than that on glucose (Fig. 9), supporting the result that F-1-P acts as the strongest negative effector of SugR in vitro. During growth on fructose, the *ldhA* promoter is active even at the exponential phase where almost no promoter activity is detected during growth on glucose (Fig. 9). L-lactate production has been observed even under fully aerobic conditions during growth on fructose in *C. glutamicum* (Péquignot et al. 1997; Dominguez et al. 1998; Kiefer et al. 2002). This could be attributed in part to inactivation of repressor activity of SugR by F-1-P. In many bacteria, expression of fermentative LDH is induced under oxygen limitation in the absence of external electron acceptor. Although sugars are essential for growth under fermentative conditions, studies on sugar response of *ldhA* expression are limited. In *E. coli*, carbohydrate metabolism regulators, CsrAB and Mlc, are indirectly involved in regulation of *ldhA* (Jiang et al. 2001), although direct regulators are not known so far. In *Streptococcus thermophilus* and *Streptococcus bovis*, CcpA, a global regulator involved in catabolite repression

Fig. 8 Effect of fructose-1-phosphate (F-1-P), glucose-6-phosphate (G-6-P), and fructose-6-phosphate (F-6-P) on SugR binding to the *ldhA* promoter region. The experiment was performed as described in the legend for Fig. 5 for DNase I footprinting on the labeled coding and noncoding strands. Varying amounts of the metabolites to be tested were incubated with SugR before addition of labeled DNA probe. Lanes 1 and 15, no protein; lane 2, 200 nM of SugR; lanes 3–14, 400 nM of SugR; lanes 4–10, with 0.025, 0.05, 0.1, 0.25, 0.5, 1.0, and 2.5 mM of F-1-P; lanes 11 and 12, with 5 and 20 mM of G-6-P; lanes 13 and 14, with 5 and 20 mM of F-6-P. Protected regions are indicated by bars. Positions with respect to the TSP of *ldhA* are indicated



in low-GC Gram-positive bacteria, is involved in upregulation of *ldh* in response to sugar provided (van den Bogaard et al. 2000; Asanuma et al. 2004).

DNase I footprinting analyses and EMSAs show that the 10-bp direct repeats, located between positions -7 and -26 with respect to the TSP, are required and enough for SugR binding to the *ldhA* promoter region (Figs. 4, 5, and 6). Based on the consensus sequence, TC(A/G)(A/G)ACA, proposed by comparing the promoter sequences of the target genes, two putative SugR binding sites are suggested to be located in the *ldhA* promoter region between positions

-35 and -42 and between -21 and -28 with respect to the TSP (Engels et al. 2008). However, SugR did not bind to the distal site (between positions -35 and -42), while the proximal site is included in the direct repeats. Each half site of the direct repeats (CGGNCATAAT) was able to bind to SugR (Fig. 6). In addition, a part of the direct repeats (ATCGGGCATAAT), which was not proposed to be the binding site by Engels et al. (2008), is perfectly conserved in the upstream region of *ldhA* in *C. glutamicum* ATCC 13032 (NC_003450), *Corynebacterium efficiens* YS-314 (NC_004369), and *Corynebacterium diphtheriae* NCTC

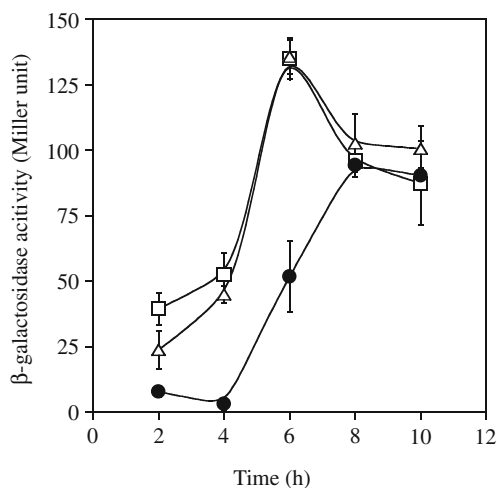


Fig. 9 The *ldhA* promoter activity of strain KT14 during growth on A medium with 1% of glucose (filled circles), fructose (open squares), and sucrose (open triangles). The activity is mean values from at least three independent cultivations with standard deviations

13129 (NC_002935), indicating the importance of the sequence in control of the promoter activity in these organisms. The mutational analyses using EMSA show that the sequence, GGNCAT, in the direct repeats is essential for SugR binding (Fig. 6). This is consistent with the consensus sequence proposed (Engels et al. 2008). The location of the SugR binding sites indicates that SugR represses *ldhA* expression by preventing RNA polymerase from binding to the promoter region. It should be noted that the SugR binding site is not conserved in the *ldhA* promoter region of *Corynebacterium jeikeium* K411 (NC_007164). This might be linked to the absence of components of PTS in its genome (Tauch et al. 2005).

To avoid wasting NADH by LDH, *ldhA* expression should be controlled in response not only to sugar by SugR but also to redox balance in the cell by unknown transcriptional regulators. This might be supported by the fact that increase in the *ldhA* promoter activity at the onset of the stationary phase was observed in the *sugR* mutant as well as in the wild type (Fig. 3). In *B. subtilis*, transcriptional regulators controlling anaerobic respiration and fermentation (ResDE, Fnr, ArfM, and Rex) are involved in *ldh* expression (Cruz Ramos et al. 2000; Larsson et al. 2005; Wang et al. 2008). In *E. coli*, a redox sensor system, ArcAB, is also involved in *ldhA* expression (Jiang et al. 2001). Although open reading frames encoding putative transcriptional regulators and two-component systems are found in the genome of *C. glutamicum*, those involved in a redox-sensing mechanism have not been reported so far. In addition to SugR, we obtained two other transcriptional

regulators, CgR0187 and CgR2816 (Fig. 1), but these regulators have no relevance to the redox-sensing systems of *E. coli* and *B. subtilis*. Independently, GlxR, a CRP-type transcriptional regulator controlling various physiological reactions (Kim et al. 2004; Letek et al. 2006; Jungwirth et al. 2008), was recently shown to bind to the *ldhA* promoter region at position -62 with respect to the TSP in vitro (Kohl et al. 2008). It is suggested that GlxR plays a positive role on the *ldhA* expression due to the location of the binding site (Kohl et al. 2008). These findings indicate that regulation of *ldhA* is under the control of complex transcriptional regulatory network.

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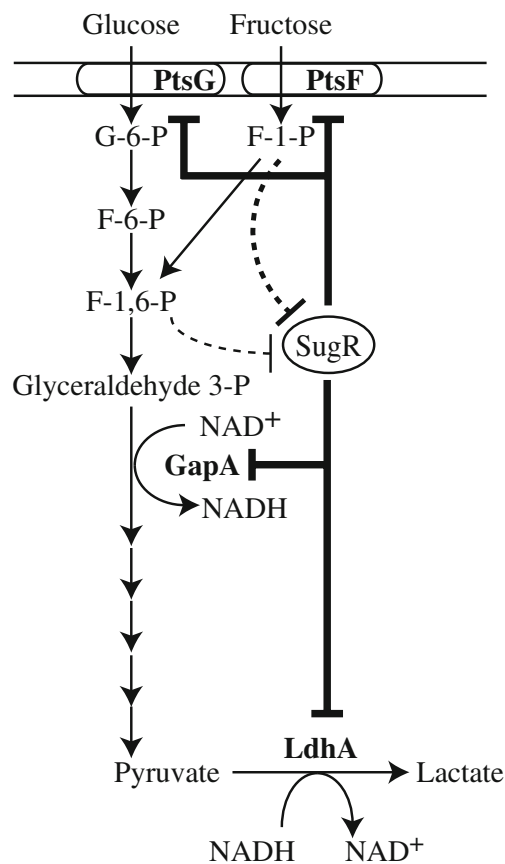


Fig. 10 Model of regulation of sugar uptake (PtsG and PtsF), GAPDH (GapA), and LDH (LdhA) by SugR. Uptake of glucose and fructose and the glycolytic pathway are depicted. Bold blunted lines indicate repression of gene expression by SugR. Dashed curved lines with bars indicate negative effect of sugar phosphates on repressor activity of SugR

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