The DeoR-type transcriptional regulator SugR acts as a repressor for genes encoding the phosphoenolpyruvate:sugar phosphotransferase system (PTS) in Corynebacterium glutamicum

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Abstract

Background: The major uptake system responsible for the transport of fructose, glucose, and sucrose in Corynebacterium glutamicum ATCC 13032 is the phosphoenolpyruvate:sugar phosphotransferase system (PTS). The genes encoding PTS components, namely ptsI, ptsH, and ptsF belong to the fructose-PTS gene cluster, whereas ptsG and ptsS are located in two separate regions of the C. glutamicum genome. Due to the localization within and adjacent to the fructose-PTS gene cluster, two genes coding for DeoR-type transcriptional regulators, cg2118 and sugR, are putative candidates involved in the transcriptional regulation of the fructose-PTS cluster genes.

Results: Four transcripts of the extended fructose-PTS gene cluster that comprise the genes sugR-cg2116, ptsI, cg2118-fruK-ptsF, and ptsH, respectively, were characterized. In addition, it was shown that transcription of the fructose-PTS gene cluster is enhanced during growth on glucose or fructose when compared to acetate. Subsequently, the two genes sugR and cg2118 encoding for DeoR-type regulators were mutated and PTS gene transcription was found to be strongly enhanced in the presence of acetate only in the sugR deletion mutant. The SugR regulon was further characterized by microarray hybridizations using the sugR mutant and its parental strain, revealing that also the PTS genes ptsG and ptsS belong to this regulon. Binding of purified SugR repressor protein to a 21 bp sequence identified the SugR binding site as an AC-rich motif. The two experimentally identified SugR binding sites in the fructose-PTS gene cluster are located within or downstream of the mapped promoters, typical for transcriptional repressors. Effector studies using electrophoretic mobility shift assays (EMSA) revealed the fructose PTS-specific metabolite fructose-1-phosphate (F-1-P) as a highly efficient, negative effector of the SugR repressor, acting in the micromolar range. Beside F-1-P, other sugar-phosphates like fructose-1,6-bisphosphate (F-1,6-P) and glucose-6-phosphate (G-6-P) also negatively affect SugR-binding, but in millimolar concentrations.

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Conclusion: In *C. glutamicum* ATCC 13032 the DeoR-type regulator SugR acts as a pleiotropic transcriptional repressor of all described PTS genes. Thus, in contrast to most DeoR-type repressors described, SugR is able to act also on the transcription of the distantly located genes *ptsG* and *ptsS* of *C. glutamicum*. Transcriptional repression of the fructose-PTS gene cluster is observed during growth on acetate and transcription is derepressed in the presence of the PTS sugars glucose and fructose. This derepression of the fructose-PTS gene cluster is mainly modulated by the negative effector F-1-P, but reduced sensitivity to the other effectors, F-1,6-P or G-6-P might cause differential transcriptional regulation of genes of the general part of the PTS (*ptsI, ptsG, ptsS*) and associated genes encoding sugar-specific functions.

Background

A sugar transport system which is widespread among various microorganisms is the phosphoenolpyruvate:sugar phosphotransferase system (PTS) [1,2]. The PTS is characterized by the uptake of the carbon source, which is simultaneously phosphorylated resulting in intracellular sugar-phosphate. The transfer of the phosphate group to its substrate consists of five distinct reactions. The first step is the autophosphorylation of enzymeI, where phosphoenolpyruvate acts as the phosphate donor. Secondly, enzymeI transfers the phosphate group to the His-15 residue of the HPr protein. EnzymeI and HPr together are designated the general or central part of the PTS. HPr, in turn, transfers the phosphate group to the substrate-specific enzymeII. In general, enzymeII consists of two hydrophilic domains (IIA and IIB) and one transmembrane domain (IIC). Subsequently, the phosphate group is transferred within the enzymeII complex from domain IIA to domain IIB. The IIC-domain transports the substrate into the cell and the phosphate group is finally transferred from IIB to the substrate and the phosphorylated sugar is released into the cytoplasm [3].

The fast growing, Gram-positive bacterium *Corynebacterium glutamicum* is widely used for the fermentative production of amino acids [4] and the PTS system is the major uptake system responsible for the transport of fructose, glucose, and sucrose in this organism [5,6]. In *C. glutamicum* ATCC 13032 five genes were identified, which encode proteins with known functions in the PTS (Table 1). In detail, *ptsI* encodes the enzyme and *ptsH* the enzyme HPr of the general part of the PTS [7]. Furthermore, *ptsG* encodes the sugar-specific enzymeII involved in the uptake of glucose, *ptsS* is involved in sucrose uptake, and finally *ptsF* is responsible for the uptake of fructose [6,8]. However, physiological results were already obtained for the function of all PTS genes with known function in *C. glutamicum* [7,9]. Additionally, for the general part of the PTS, the activities of enzymeI and HPr were demonstrated in a cell-free assay. PTS-dependent transport and the physiological function of the *ptsG* gene in the uptake of glucose were also investigated [5,10]. Genetically engineered mutations in the putative glucose- and sucrose-specific enzymesII encoded by *ptsG* and *ptsS* revealed impaired growth on glucose and sucrose, respectively, indicating glucose transport by PtsG and sucrose transport by PtsS [8]. Inactivation of the *ptsF* gene resulted in a significantly reduced growth on fructose as a sole carbon source, therewith indicating the function of PtsF in fructose uptake [6,8]. Furthermore, PtsF-defective mutants showed a diminished growth rate compared to the wild type with sucrose as a sole carbon source, and additionally an accumulation of external fructose during exponential growth [6]. Due to the lack of a fructokinase activity in *C. glutamicum* the internally liberated fructose, resulting from the cleavage of sucrose-6-phosphate taken up by PtsS, cannot be metabolized and is exported [11,8]. Therefore, PtsF activity is also necessary to import the external fructose in order to completely metabolize sucrose. Furthermore, Moon et al. [8] identified one enzymeII encoded by cg3365 and cg3366 with unknown substrate specificity.

The transcriptional regulation of genes involved in PTS transport are well studied in a variety of Gram-negative and low-G+C Gram-positive bacteria [12-15]. However, little is known about gene regulation of PTS in *Corynebacteria*. An HPr-kinase/phosphatase activity as well as the essentially conserved serine-residue 46 in the HPr amino acid sequence, which exerts carbon catabolite control in low-G+C Gram-positive bacteria, was not detected in *C. glutamicum* [7,16]. Additionally, genes encoding transcriptional antiterminators of the BgI-G-family like GltT of *B. subtilis* (regulation of the *ptsGHI* operon) or BglG of *E. coli* (regulation of the β-glucoside PTS) are only represented by a pseudogene in the genome of *C. glutamicum* ATCC 13032 (cg3144/NCgl2743) [17]. However, it has to be noted that other *C. glutamicum* isolates apparently have functional copies of antiterminator-like genes as well as additional genes for enzymes II [18]. As another transcriptional regulatory system, in the low G+C Gram-positive bacterium *Streptococcus gordonii* the DeoR-type regulator FruR was characterized, which controls fructose-PTS gene expression in dependence of fructose, sucrose or xylitol [15]. The expression of the PTS genes *ptsF, ptsG*, and *ptsS* in *C. glutamicum* was initially considered to be consti-
the understanding for the expression of all described PTS cluster genes, regulator, which they termed SugR. The transcriptional regulation of the strain by microarray hybridization, these authors found C. glutamicum ptsG, a type transcriptional regulator encoded by the gene sugR.

Wendisch [22] reported the identification of the DeoR-family transcriptional regulator of all PTS genes. Electro- phosphate mobility shift assays with purified SugR protein were employed to determine the SugR binding sites and its effector metabolites.

During the preparation of this manuscript, Engels and Wendisch [22] reported the identification of the DeoR-type transcriptional regulator encoded by the gene sugR in C. glutamicum, acting as a transcriptional repressor of the ptsG gene. By comparing a sugR mutant with its parent strain by microarray hybridization, these authors found that the ptsF and ptsS genes are under the control of this regulator, which they termed SugR. The transcriptional role of SugR was extended in this work to the fructose-PTS cluster genes ptsI, cg2118, fruK, and ptsH and completes the understanding for the expression of all described PTS genes in C. glutamicum in general and the expression of the genes of the fructose-PTS cluster in detail. Furthermore, the effector studies in this work points to a sugar specific regulation of these genes similar to the TrmB regulator of Thermococcus litoralis and Pyrococcus furiosus [23,24].

Table 1: Molecular characteristics of PTS and putative PTS associated genes of C. glutamicum ATCC13032

| CDS | NCBI No. | gene name | size [aa] | size [kDa] | Annotation |
|-----|----------|-----------|-----------|------------|------------|
| ext. | cg2115   | NCgi1856  | sugR      | 259        | 27.6       | Transcriptional regulator protein, DeoR-family |
|      | cg2116   | NCgi1857  | -         | 320        | 34.1       | Putative L-phosphofructokinase |
|      | cg2117   | NCgi1858  | ptsI      | 568        | 59.6       | Phosphotransferase system (PTS), enzyme I |
|      | cg2118   | NCgi1859  | -         | 264        | 28.0       | Putative transcriptional regulator protein, DeoR-family |
|      | cg2119   | NCgi1860  | fruK      | 330        | 34.0       | L-phosphofructokinase |
|      | cg2120   | NCgi1861  | ptsF      | 688        | 70.5       | Fructose-specific PTS component, enzyme IIABC |
|      | cg2121   | NCgi1862  | ptsH      | 89         | 9.1        | Phosphocarrier protein HPr, PTS component |
| non clustered | cg1537  | NCgi1305  | ptsG      | 683        | 72.6       | Glucose-specific PTS component, enzyme IIABC |
|      | cg2925   | NCgi2553  | ptsS      | 661        | 69.1       | Sucrose-specific PTS component, enzyme IIABC |
|      | cg3365   | NCgi2933  | -         | 513        | 52.7       | L-ascorbate type PTS component, enzyme IIC |
|      | cg3366   | NCgi2934  | -         | 270        | 29.0       | L-ascorbate type PTS component, enzyme IIA |

1 Coding sequences (CDS) are numbered in reference to the complete genome sequence of C. glutamicum (GenBank accession number BX927147).
2 Numbers in the genome database of the National Center for Biotechnology Information (NCBI No.).

The aim of this study was the characterization of the transcriptional regulation of PTS genes in C. glutamicum. By first analysing gene transcription in the fructose-PTS gene cluster and transcriptional regulation of all known PTS genes, we found a PTS-sugar specific transcriptional regulation of all PTS genes. By deletion mutagenesis of cg2115 (sugR) and expression analysis by real-time-RT-PCR and microarray hybridization, SugR was identified as a pleiotropic transcriptional regulator of all PTS genes. Electrophoretic mobility shift assays with purified SugR protein were employed to determine the SugR binding sites and its effector metabolites.

During the preparation of this manuscript, Engels and Wendisch [22] reported the identification of the DeoR-type transcriptional regulator encoded by the gene sugR in C. glutamicum, acting as a transcriptional repressor of the ptsG gene. By comparing a sugR mutant with its parent strain by microarray hybridization, these authors found that the ptsF and ptsS genes are under the control of this regulator, which they termed SugR. The transcriptional role of SugR was extended in this work to the fructose-PTS cluster genes ptsI, cg2118, fruK, and ptsH and completes the understanding for the expression of all described PTS genes in C. glutamicum in general and the expression of the genes of the fructose-PTS cluster in detail. Furthermore, the effector studies in this work points to a sugar specific regulation of these genes similar to the TrmB regulator of Thermococcus litoralis and Pyrococcus furiosus [23,24].

Results

The extended fructose-PTS gene cluster of C. glutamicum contains four transcriptional units

In the genome of C. glutamicum ATCC 13032 [17], the locations of the five PTS genes ptsI, ptsH, ptsF, ptsG, and ptsS were identified. On this account, the PTS genes can be grouped into the fructose-PTS gene cluster (ptsI, cg2118, fruK, ptsF, and ptsH) and the distantly located glucose- and sucrose-PTS genes ptsG and ptsS, respectively, as well as the hitherto uncharacterized genes cg3365 and cg3366 (Table 1). The genes ptsI, cg2118, fruK, ptsF, and ptsH of the fructose-PTS cluster together with the region encoding sugR and cg2116 form the extended fructose-PTS gene cluster (Table 1).

In order to analyse the transcriptional organization of the extended fructose-PTS gene cluster, mRNA transcripts overlapping adjacent coding regions were investigated by real-time RT-PCR. In these experiments, mRNAs spanning the intergenic regions between sugR and cg2116, cg2118 and fruK, fruK and ptsF, and between ptsF and ptsH, respectively, were detected in the C. glutamicum strain RES167 grown on glucose (data not shown). However, a non-coding region of 188 bp between the coding regions of ptsF...
and ptsH was observed indicating a possible promoter upstream of ptsH. Considering these observations and the localization of ptsI on the opposite strand, it is most likely, that the genes sugR-cg2116 and cg2118-fruK-ptsF form two operons, whereas ptsI is monocistronic. Although a read-through transcription from ptsF to ptsH was detected, the ptsH gene is probably also transcribed monocistronically. The operon predictions by the computer program VIMSS [25] and the predicted positions of transcriptional terminators 3' of cg2116, ptsI and ptsH determined by the computer program TransTerm [26], depicted as stem-loop structures in Fig. 1A, support this view.

Consistent with these interpretations, four transcriptional start sites were localized upstream from the genes sugR, ptsI, cg2118, and ptsH, using the rapid amplification of cDNA ends (RACE) method (Fig. 1). The deduced promoter sequences showed similarities to the sequences of the -10 (Tac/taaT) and -35 (TTTGCC/TTGGCA/TTGCCA) regions of the C. glutamicum consensus promoter [27]. The promoter of the cg2118-fruK-ptsF operon showed the highest similarity to the consensus promoter, whereas the promoters of sugR-cg2116, ptsI, and ptsH were less conserved, especially in the -35 region, (Fig. 1B). Furthermore, it was found that the divergent promoters PptsI and Pcg2118 produce mRNAs overlapping by a complementary sequence of 14 nucleotides.

**Transcription of the fructose-PTS gene cluster is enhanced in C. glutamicum cultures grown on glucose or fructose when compared to cultures grown on acetate**

In order to investigate a carbon source-dependent expression of the genes belonging to the extended fructose-PTS gene cluster, the transcription levels were investigated by RT-PCR experiments. For this purpose, total mRNA was isolated from C. glutamicum RES167, grown in minimal media containing acetate, glucose or fructose as a sole carbon source. Glucose and fructose were employed as PTS sugars requiring the expression of the PTS gene cluster for their uptake, whereas acetate represents a non-PTS carbon source. The relative mRNA amounts of the genes belonging to the extended fructose-PTS cluster (sugR to ptsH) were determined using real-time RT-PCR. The values from the glucose- and fructose-grown cultures were compared to those obtained from cultures grown on acetate (Fig. 2).

The mRNA levels of sugR and cg2116 showed only minor changes during growth on glucose and fructose in comparison to acetate. In contrast to this, all genes of the fruc-

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**Figure 1**

The transcriptional organization of the genes in the extended fructose-PTS cluster of C. glutamicum. A) The extended fructose-PTS gene cluster as well as its transcriptional elements are depicted. Gene overlapping RT-PCR products are indicated by black bars and numbered. Transcriptional terminators predicted by TransTerm [26] are denoted as stem loop structures. Positions of the transcription start sites are indicated by arrows. B) The detailed sequences for the promoters P_sugR, P_ptsI, P_cg2118, and P_ptsH. Transcription starts (+1), the distances to the translation start (in parentheses), and the start codons (underlined) are shown. The deduced-10 and -35 promoter regions are highlighted by dark and light grey boxes, respectively.
tose-PTS gene cluster from cg2117 (ptsI) to cg2120 (ptsH) showed significantly higher mRNA levels during growth on glucose and fructose. The levels of the genes ptsI and ptsH did not differ between cells grown on glucose and fructose. This is in accordance with the need for expression in the presence of both sugars. In contrast to this, mRNA isolated from fructose grown cultures showed significantly higher transcription of cg2118, fruK, and ptsF. The different expression patterns of cg2118, fruK, and ptsF in cultures grown on glucose or fructose as sole carbon sources correlated with the requirement of the cell for a higher expression of the fructose-specific enzymeII (ptsF) and the 1-phosphofructokinase encoded by fruK with fructose as the sole carbon source. These results are a clear indication for a carbon source-dependent transcriptional regulation of the genes of the PTS cluster.

The sugR gene is involved in the transcriptional regulation of the fructose-PTS cluster containing ptsl, cg2118, fruK, ptsF, and ptsH of C. glutamicum

Due to the classification of sugR and cg2118 as DeoR-type regulators, they are both interesting candidate genes for investigating their involvement in the carbon source-dependent transcriptional regulation of the fructose-PTS cluster genes. Single deletion mutants of sugR and cg2118 were constructed by gene replacement in C. glutamicum RES167 in order to elucidate the possible roles in this regulation (data not shown). These deletions removed 242 of 780 nucleotides from the sugR coding region and 674 of 795 nucleotides from the cg2118 coding region, rendering the respective gene non-functional and resulting in strains LG01 (RES167 ΔsugR) and LG02 (RES167 Δcg2118), respectively.

The possible involvement of sugR or cg2118 in transcriptional regulation of ptsl, the cg2118-fruK-ptsF operon, and ptsH was determined by real-time RT-PCR. For these
experiments, total mRNAs isolated from RES167 and the mutant strains LG01 and LG02 were used. The cells were grown in shaking flasks in liquid minimal media with 2% acetate as sole carbon source. Acetate as the sole carbon source was chosen, because an increased transcription of the fructose-PTS cluster genes could be observed when RES167 was grown on glucose and fructose instead on acetate. Therefore, growth on acetate may cause the transcriptional repression of the fructose-PTS cluster genes. To find out whether the sugR or the cg2118 gene product is involved in the transcriptional regulation of the fructose-PTS cluster genes, the expression in the two deletion mutants LG01 and LG02 was compared to the expression in the RES167 strain when all strains were grown on acetate. The analysis revealed a strongly increased transcription of the fructose-PTS cluster genes ptsI, cg2118, fruK, ptsF, and ptsH in the sugR mutant LG01 (Fig. 3). In contrast to this, the transcription of the fructose-PTS cluster genes in the cg2118 mutant LG02 was only weakly influenced. It is therefore concluded, that the sugR gene product downregulates transcription of the fructose-PTS gene cluster during growth on acetate, whereas cg2118 and its gene product does not seem to be involved in the transcription of the fructose-PTS cluster genes under the applied experimental conditions.

The sugR gene of C. glutamicum is involved in the repression of PTS gene transcription

Comparative microarray hybridization experiments with total mRNA isolated from the cg2115 deletion mutant LG01 and the parental strain RES167 both grown on acetate were conducted in order to explore the complete SugR regulon. In the microarray experiments using the C. glutamicum whole-genome DNA microarray described previously [28], two biological replicates were hybridized, simultaneously applying label-swapping. The results of the microarray experiments comparing the sugR mutant and the RES167 strain revealed 23 genes with increased

Figure 3
Relative gene expressions of C. glutamicum fructose-PTS cluster genes in dependence on the regulatory genes sugR and cg2118. The C. glutamicum strains LG01, LG02, and RES167 were grown in liquid media containing acetate as sole carbon source. By RT-PCR the mRNA levels of the fructose-PTS cluster genes ptsI, cg2118, fruK, ptsF, and ptsH of the mutant strains LG01 and LG02 were compared to those of RES167. Due to the deletion of the cg2118 coding region, the expression of the truncated cg2118 gene in the mutant LG02 could not be determined. Results are means of four measurements from two biological replicates. Standard deviations are indicated by error bars.
and five genes with decreased transcript levels in the sugR mutant. All genes of the fructose-PTS gene cluster were found in the group of genes with induced transcript levels (Fig. 4 and Table 2). Interestingly, the genes ptsG and ptsS, which encode the enzyme II proteins for the uptake of glucose and sucrose, respectively, were also found to have an elevated expression level. To confirm the microarray results, subsequent real-time RT-PCR measurements were conducted, which validated the enhanced transcription of ptsG and ptsS in the sugR mutant with ratios of 16.8 and 101.8 compared to RES167, respectively. Beside the PTS genes, the transcriptional levels of ldh and adhA encoding lactate dehydrogenase and alcohol dehydrogenase, respectively, were found to be significantly increased. Significant increases in transcript levels were also observed for cg2025 and cg2026, encoding hypothetical proteins. Besides ldh and adhA, no further genes with known function in carbon metabolism were found to be significantly induced.

Since Engels and Wendisch [22] also compared global transcript levels by microarray hybridizations in a sugR mutant and its parent strain, the resulting stimulon can be compared to the one obtained in this work. In the intersection of the genes showing elevated transcript levels in both experiments, only the PTS genes mentioned above are apparent. The remaining genes corresponding to each stimulon are different between both studies and might be a result of the different growth conditions used (LB versus minimal media with acetate) or might be results of an indirect effect of the derepression of PTS gene transcription.

The SugR protein of C. glutamicum binds to sequences located upstream of the coding regions of ptsI, cg2118, ptsH, ptsG, and ptsS

To verify the direct involvement of the SugR protein in the regulation of the PTS gene cluster electrophoretic mobility shift assays (EMSA) were performed. For these experiments the SugR protein was purified after overexpression.

Figure 4
Analyses of the transcriptomes of the C. glutamicum sugR mutant LG01 and its parent strain RES167 by microarray hybridizations. The strains were grown in liquid minimal media containing acetate as sole carbon source. Global gene expression of LG01 was compared to that of RES167 using microarray analyses performed on two biological and two technical replicates. Genes found to be significantly differentially expressed (m-value ≥ ± 1) are indicated by green (elevated expression) or red (decreased expression) dots, respectively. Gene names belonging to the fructose-PTS gene cluster are boxed, and the non clustered PTS genes are underlined.
in *E. coli* as an N-terminal translational fusion with a self-cleavable intein tag using the pTYB1 vector [29]. The advantage of this system is that the native protein can be obtained without an additional attached tag sequence. The quality of the purified protein was controlled by 1-dimensional SDS-PAGE and its size was estimated to be ~28 kDa. This observation is in accordance with the annotation of the sugR coding region, which is predicted to encode a 27.6 kDa protein with an N-terminal DeoR-domain comprising a helix-turn-helix (HTH) motif. The identity of SugR was subsequently confirmed by peptide mass fingerprinting using MALDI-TOF mass spectrometry (data not shown).

As binding partners for SugR in the EMSA experiments, overlapping and fluorescently-labeled PCR fragments from the intergenic regions between ptsI and cg2118, as well as the regions upstream from the coding regions of sugR, ptsH, ptsG, ptsS, adhA, cg2025, and cg2026 were generated. As negative controls, internal DNA fragments of the cg2118- and ptsH-coding region were also produced by PCR and incubated with the purified protein to test for non-specific binding of the protein (data not shown).

For the gel retardation experiments 15 pmol of the SugR protein and 0.05 pmol of the fluorescently-labeled PCR fragments were used. It was shown that SugR caused band-shifts in experiments with DNA fragments I1, I2, and I4 located in the ptsI-cg2118 intergenic region (Fig. 5). Furthermore, binding of the purified SugR protein was observed to the DNA fragment H1 which is located upstream of the ptsH coding region, to the fragments G1 and G2 located upstream of the ptsG coding region and to the fragments S1 to S3 derived upstream of the coding region of ptsS.

The promoter region of sugR was also tested for SugR binding, but no shifts of the labeled PCR fragments were

| CDS    | NCBI No. | Gene name | m-value | a-value | Annotation                                      |
|--------|----------|-----------|---------|---------|------------------------------------------------|
| cg2026 | -        | -         | 3.53    | 11.68   | Hypothetical protein                            |
| cg2925 | Ncgl2553 | ptsS      | 3.39    | 11.87   | Phosphotransferase system (PTS), sucrase-specific enzyme IIABC component |
| cg2120 | Ncgl1861 | ptsF      | 3.27    | 12.04   | Phosphotransferase system (PTS), fructose-specific enzyme IIABC component |
| cg3107 | Ncgl2709 | adhA      | 3.23    | 13.00   | Alcohol dehydrogenase                           |
| cg2118 | Ncgl1859 | ptsG      | 3.00    | 12.00   | Transcriptional regulator protein, DeoR-family |
| cg1537 | Ncgl1305 | ptsG      | 2.70    | 11.97   | Phosphotransferase system (PTS), glucose-specific enzyme IIABC component |
| cg2119 | Ncgl1860 | fruK      | 2.67    | 11.59   | 1-Phosphofructokinase                           |
| cg2025 | -        | -         | 2.62    | 11.43   | Hypothetical protein                            |
| cg2034 | Ncgl1739 | -         | 2.38    | 11.24   | Hypothetical protein                            |
| cg2117 | Ncgl1858 | ptsI      | 2.13    | 12.28   | Phosphotransferase system (PTS), Enzyme I       |
| cg3219 | Ncgl2810 | idh       | 1.88    | 13.51   | L-Lactate dehydrogenase                         |
| cg2001 | Ncgl1708 | -         | 1.82    | 10.75   | Conserved hypothetical protein                  |
| cg1992 | Ncgl1699 | -         | 1.55    | 11.25   | Hypothetical protein                            |
| cg2121 | Ncgl1862 | ptsH      | 1.39    | 13.62   | Phosphotransferase system (PTS), phosphocarrier protein HPr |
| cg0683 | Ncgl0565 | -         | 1.39    | 11.50   | Putative permease                               |
| cg2030 | Ncgl1735 | -         | 1.31    | 10.61   | Hypothetical protein                            |
| cg1761 | Ncgl1500 | rijS2     | 1.19    | 13.00   | Cysteine desulphydrase                          |
| cg0812 | Ncgl0678 | dtsR1     | 1.18    | 13.13   | Acetyl/propionyl-CoA carboxylase, beta chain    |
| cg3112 | Ncgl2713 | cyZ       | 1.15    | 12.19   | Sulfate transporter                             |
| cg0898 | Ncgl0754 | -         | 1.14    | 11.73   | Pyridoxine biosynthesis enzyme                  |
| cg2033 | Ncgl1738 | -         | 1.12    | 10.89   | Putative secreted protein                       |
| cg2703 | Ncgl2373 | -         | 1.07    | 11.98   | ABC-type putative sugar transporter, permease subunit |
| cg1290 | Ncgl1094 | metE      | 1.00    | 14.45   | S-Methyltetrahydropteroylglutamate-homocysteine methyltransferase |
| cg3096 | Ncgl2698 | -         | -1.00   | 13.42   | Aldehyde dehydrogenase                         |
| cg2911 | Ncgl2539 | -         | -1.00   | 14.12   | ABC-type putative Mn/Zn transporter, substrate-binding lipoprotein |
| cg1055 | Ncgl0888 | menG      | -1.21   | 12.74   | S-Adenosylmethionine:2-demethylmenaquinone methyltransferase |
| cg2320 | Ncgl2034 | -         | -1.25   | 13.24   | Putative transcriptional regulator, ArsR-family |
| cg3057 | Ncgl2664 | -         | -2.10   | 13.37   | Putative secreted protein                       |

1 Coding sequences (CDS) are numbered in reference to the complete genome sequence of *C. glutamicum* (GenBank accession number BX927147)

2 Numbers in the genome database of the National Center for Biotechnology Information (NCBI No.)
detected (Fig. 5). Furthermore, labeled PCR fragments upstream of the cg2025, cg2026, and adhA coding regions were tested in EMSA experiments, but no binding of SugR was observed (data not shown).

The C. glutamicum SugR repressor binds to two 21 bp DNA regions interfering with ptsI and cg2118-fruK-ptsF transcription

As described above, SugR binding was detected to the DNA fragments I2 and I4 located in the intergenic region between ptsI and cg2118. In contrast, no binding to the DNA fragment I3 was observed. These observations helped to identify two genetic regions A and B which are proposed to carry the specific binding sites for SugR (Fig. 6A). Region A is a 59 bp long sub-region of the DNA fragment I2 and region B is a 87 bp long sub-region of I4. DNA sequence comparison of regionA to regionB revealed highly similar sequences of 21 nucleotides in length. To confirm these motifs as putative SugR binding sequences, double-stranded(ds)-oligonucleotides perfectly matching the 21 nucleotides with four flanking basepairs, representing the original genomic sequence on both sides, were used in competitive EMSA experiments and denoted as oligoA and oligoB, respectively (Fig. 6B). Interestingly, both oligonucleotides were able to displace the labeled DNA fragment I2. To further verify the binding sequence of SugR, ds-oligonucleotides with transversions of the four flanking basepairs of oligoA and oligoB, resulted in oligoAt and oligoBt, respectively, which were used in additional EMSA experiments (Fig. 6B). OligoAt and oligoBt, however, showed no differences in the competitive EMSA experiments compared to oligoA and oligoB, defining both 21 bp long binding motifs as sufficient for SugR binding. According to the localization of the two SugR binding motifs in regionA and regionB of the DNA fragments I2 and I4, respectively (Fig. 6A), these two motifs were named motifA and motifB.

Subsequently, the positions of the two 21 bp SugR binding sequences motifA and motifB were mapped in the intergenic region between the coding regions of ptsI and cg2118 (Fig. 7). The two experimentally proven binding motifs in the intergenic region between ptsI and cg2118 are located within P\text{cg2118} and downstream of P\text{ptsI} (motifA), or downstream the P\text{cg2118} (motifB), respectively.

Due to the divergent orientation of the coding regions ptsI...
and cg2118 and the overlapping transcripts from the promoters P\textsubscript{cg2118} and P\textsubscript{ptsI}, binding of SugR to motifA will interfere with transcription from both promoters, whereas the binding of SugR to motifB will only interfere with the transcription from P\textsubscript{cg2118}.

To find additional binding sites of SugR, sequence alignments of motifA and motifB to the upstream coding regions of ptsH, ptsG, and ptsS were conducted. In these alignments, only DNA regions which showed a band-shift in the EMSA experiments were used.

By this, two putative binding motifs were found upstream of the coding regions of ptsH and ptsG and ptsS namely ptsH1, ptsH2, and ptsG1, ptsG2, and ptsS1, ptsS2 respectively (Fig. 8). Concluding, these experiments led to the identification of eight binding motifs. Two validated SugR binding sequences motifA and motifB located in the intergenic region of ptsI and cg2118, as well as six putative
binding motifs located upstream of the coding regions of ptsH, ptsG, and ptsS. Sequence alignments of all eight motifs revealed that the six nucleotides ACAAAAC in the 5’- and the four nucleotides AACA in the 3’-region were well conserved (Fig. 8). Additionally, a predominant occurrence was observed for cytosine and adenine nucleotides at positions 9 to 17 of the consensus motif. The characteristics of the putative consensus motif were determined as an A+C-rich and non-palindromic sequence (Fig. 8).

The effectors fructose-1-phosphate, fructose-1,6-bisphosphate, and glucose-6-phosphate influence the binding activity of the SugR repressor

In order to determine the effector substances influencing the activity of SugR, additional EMSA experiments were carried out. In these experiments, the SugR-I2 complex was incubated with fructose-1-phosphate (F-1-P) which exclusively occurs when fructose is taken up by the fructose-specific PTS. Additionally, glucose-6-phosphate (G-6-P) occurring during the transport of glucose by the glucose-specific PTS component PtsG, fructose-1,6-bisphosphate (F-1,6-P) and fructose-6-phosphate (F-6-P), originating from the consumption of different sugars, as intermediates of glycolysis were used. Furthermore, the non-phosphorylated substrates of PtsF and PtsG, fructose and glucose, respectively, as well as acetate were used. A loss of SugR binding to the DNA fragment I2 was observed to different extents using increasing concentrations of F-1-P, F-1,6-P, and G-6-P (Fig. 9). Using F-1-P as effector substance a clear effect at concentrations equal to and exceeding 10 μM could be observed. In the case of F-1,6-P, significantly higher concentrations of the effector substance (≥ 5 mM) for resolving the SugR-I2 complex had to be applied. Additionally, G-6-P showed significant influence on the SugR-I2 complex only at concentrations above 10 mM. The binding of SugR to the DNA fragment I2 was not influenced by F-6-P concentrations up to 60 mM (Fig. 9). Additionally, no influence was observed on SugR binding using the non-phosphorylated sugars glucose and fructose as well as acetate at concentrations up to 60 mM (data not shown).

Discussion
The C. glutamicum regulatory gene sugR is involved in the repression of the PTS genes

In an initial study four putative transcripts were identified by RT-PCR covering sugR and cg2118, which are co-transcribed, ptsI and ptsH, which are transcribed monocistronically, and the cg2118-fruk-ptsF operon. Coherent with this was the experimental identification of four promoters upstream of the coding regions of sugR, ptsI, cg2118, and ptsH.

In the C. glutamicum strain RES167 the transcript levels of ptsI, the cg2118-fruk-ptsF operon and of ptsH were considerably higher on the PTS sugars glucose and fructose in comparison to the non-PTS carbon source acetate. It is interesting to note, that the transcript levels of these five genes were moderately induced in glucose-grown cells, whereas in fructose-grown cells the transcript level of the cg2118-fruk-ptsF operon, specifically necessary for the transport and metabolism of fructose, was much stronger induced than that of the general PTS genes ptsI and ptsH. This indicates a differential activation of transcription for the general and the fructose-specific part of the fructose-PTS gene cluster.
Microarray hybridizations comparing global transcript levels in the *C. glutamicum* sugR mutant LG01 and its parent strain, both grown on acetate, helped to confine the genes belonging to the stimulon of the LG01 mutant. For this, it was interesting to note that together with the genes of the fructose-PTS cluster, the distantly located PTS genes *ptsG* and *ptsS* were also found to be derepressed in the sugR mutant. These results were subsequently verified by using the more sensitive real-time RT-PCR technique verifying the expansion of the putative SugR regulon to the non-clustered PTS genes *ptsG* and *ptsS*.

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### Table 1: Predicted SugR Binding Sites

| Motif Name | Upstream of CDS | Proven Binding Sequences | Predicted Binding Sequences | Distance to TL [bp] |
|------------|-----------------|--------------------------|-----------------------------|---------------------|
| A          | *ptsI* / *cg2118* | **ACAAAACAAAACACATATCAACA** | **ACAAAACAAAACACATATCAACA** | N<sub>84/323</sub> |
| B          | *ptsI* / *cg2118* | **ACAAAACAAAACACATATCAACA** | **ACAAAACAAAACACATATCAACA** | N<sub>309/98</sub> |
| ptsH1      | *ptsH*           | **ACAAATGTCTCAGTAGACTAAA** |                            | N<sub>126</sub>    |
| ptsH2      | *ptsH*           | **GGTAGCGTGGCAAAACGAACA**  |                            | N<sub>144</sub>    |
| ptsG1      | *ptsG*           | **ACATATTCCAGATTTACCT**    |                            | N<sub>335</sub>    |
| ptsG2      | *ptsG*           | **GCAGGTCCGACATATTCACAA**  |                            | N<sub>344</sub>    |
| ptsS1      | *ptsS*           | **ACAAAACATTCCATGCTGATAA** |                            | N<sub>49</sub>     |
| ptsS2      | *ptsS*           | **TTGATCGGACACCAATAACA**   |                            | N<sub>66</sub>     |

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**Figure 8**

Comparison of experimentally determined and predicted SugR binding sites located upstream of the coding regions of *ptsI*, *cg2118*, *ptsH*, *ptsG*, and *ptsS* and construction of a consensus sequence. A) Predicted binding motifs were determined by sequence comparisons of proven motif A and motif B to the DNA fragments showing positive SugR binding in the EMSA studies. Proven and predicted motifs are separated by a horizontal, dashed line. Boxed letters in the experimentally proven motifs A and B and the putative SugR binding sequences located upstream the coding regions of *ptsH*, *ptsG*, and *ptsS* denote identical nucleotides in all sequences. Distances to the according translation starts (TL) are indicated. B) A frequency plot of the deduced consensus sequence of all motifs is constructed by means of the WebLogo tool. The overall height of each stack of letters indicates the sequence conservation at each position of the 21-bp motif, whereas the height of each symbol within the stack reflects the relative frequency of the corresponding nucleotide at that position.
In a parallel study, Engels and Wendisch [22] analyzed the same regulatory genes by constructing mutants and analyzing effects on the regulation of PTS genes. They initially focused on transcriptional regulation of \( \textit{ptsG} \) and showed that this gene was transcriptionally regulated by the product of the \( \textit{sugR} \) gene. By using microarray analysis to compare the global transcription levels of the \( \textit{sugR} \) mutant to the parental strain grown on LB medium, they also ensured that \( \textit{ptsS} \) and the genes \( \textit{cg2118} \) and \( \textit{ptsF} \) are under the same transcriptional control. However, in their study the gene carrying the regulatory function was named \( \textit{sugR} \).

By our microarray results, we were able to extend the \( \textit{SugR} \) regulon by adding \( \textit{fruK} \) and the genes encoding the general part of the PTS, \( \textit{ptsI} \) and \( \textit{ptsH} \). Hence, this work shows that all functionally characterized PTS genes are under the control of the \( \textit{SugR} \) regulator.

In our microarray analysis, differential regulation of the genes \( \textit{cg3365} \) and \( \textit{cg3366} \), putatively encoding a PTS component of a hitherto unknown function, was not detected. Therefore, these genes might not be a part of the \( \textit{SugR} \) regulon. In contrast to this, elevated transcript levels were detected for the genes \( \textit{cg2025} \) and \( \textit{cg2026} \), encoding proteins of unknown function, and \( \textit{adhA} \), putatively encoding a Zn-dependent alcohol dehydrogenase. But, EMSA studies only confirmed the \textit{in vitro} binding of \( \textit{SugR} \) upstream to the according coding regions of the PTS genes. The significant expression changes of \( \textit{cg2025} \), \( \textit{cg2026} \), and \( \textit{adhA} \) are therefore most likely indirect effects of the \( \textit{sugR} \) mutation.

An autoregulation of \( \textit{SugR} \) is unlikely, because real-time RT-PCR experiments showed only marginal deviations in the transcriptional regulation of the \( \textit{sugR-cg2116} \) operon in the \( \textit{sugR} \) mutant in comparison with its parent strain. Coherent with this, binding of purified \( \textit{SugR} \) protein upstream of the coding sequence of \( \textit{sugR} \) was not detected.

**The DeoR-type regulator \( \textit{SugR} \) acts as a repressor on transcription of all PTS genes in \( \textit{C. glutamicum} \)**

In electrophoretic mobility shift assays (EMSA), binding of the native, purified \( \textit{SugR} \) protein to DNA fragments located upstream of the coding sequences of \( \textit{ptsI} \), \( \textit{ptsH} \), \( \textit{ptsG} \), \( \textit{ptsS} \), and the \( \textit{cg2118-fruK-ptsF} \) operon was detected. This result substantiated the identification of these genes as part of the \( \textit{SugR} \) regulon. Binding of \( \textit{SugR} \) to its target genes occurs in the absence of effectors and transcription is negatively affected as described above. Therefore, \( \textit{SugR} \) acts as repressor of transcription on PTS genes.

The results obtained in this work correlate well with the current knowledge, where DeoR-type regulators of Gram-positive bacteria often act as repressor of sugar-specific PTS genes responsible for the uptake and metabolism of sugars like fructose (\textit{Lactococcus lactis} [13]; \textit{Streptococcus gordonii} [15]), lactose (\textit{L. lactis} [30]; \textit{Staphylococcus aureus} [31]), and sorbose (\textit{L. casei} [32]). In \textit{Bacillus subtilis} DeoR-
type regulators are also involved in the transcriptional regulation of deoxyribonucleoside degradation [33]. The function of DeoR-type regulators was further investigated in the Gram-negative organisms E. coli and Salmonella enterica where they have transcriptional influences on non-PTS operons involved in the metabolism of deoxyribose [34,35]. A common feature of the described DeoR-type regulators is the transcriptional control restricted to neighboring genes. Solely the DeoR-type regulator DeoT of E. coli has potential target genes of various metabolic pathways distributed in diverse genetic loci and was designated as a master regulator [36]. In accordance to the distribution of the PTS genes in the C. glutamicum genome, the SugR repressor can be designated as a pleiotropic regulator of the PTS genes.

The SugR binding site comprises a 21-base pair AC-rich motif

The sequence comparisons of the SugR binding motifs led to a putative consensus sequence, which consists of two conserved non-palindromic flanking regions and an AC-rich center region. The DeoR regulators described for E. coli (DeoR), B. subtilis (DeoR), and Salmonella enterica (DeoQ) [34,37,35] are recognizing palindromic sequences, as well as the the DeoR-type regulator which is responsible for the transcriptional regulation of the fructose-PTS gene cluster in Streptococcus gordonii (FruR) [15]. In addition the DeoR-type regulator of the fructose-PTS gene cluster of Lactococcus lactis (FruR) potentially binds to four repeating non-palindromic sequences [13]. The putative consensus sequence of the SugR binding site, however, neither shows significant similarity to the known palindromic binding sequences, nor to the non-palindromic sequences of DeoR-type repressors mentioned above.

In their parallel study, Engels and Wendisch [22] studied the binding of SugR (SugR), modified and purified by an aminoterminal decahistidine tag, to the ptsG upstream region. By comparing the results obtained from EMSA experiments using overlapping DNA fragments, they confined the binding site to a 23 bp sequence in which they found an 8 nt motif (5'-GTCGGACA-3') which is partly conserved in the upstream regions of ptsS and cg2118. Although the possibility remains that the SugR binding site is shorter than the 21 nucleotides as predicted here, the 8nt possibly involved in the binding of SugR as defined by Engels and Wendisch [22] is included in a putative 21 nt sequence (ptsG2) postulated in this work.

The two experimentally determined SugR binding motifs A and B are located in the intergenic region between ptsl and cg2118 and are therefore of special interest. On one hand, motif A is overlapping the promoter region of P$_{cg2118}$ and located downstream of P$_{ptsl}$ on the other hand motif B is located downstream the promoter P$_{cg2118}$. The binding within or downstream the promoter region is common to transcriptional repressors [38]. Therefore, the binding of SugR in the operator region of ptsl and cg2118 apparently results in the repression of the transcription of the divergently orientated genes. Even though the SugR binding sites upstream of the ptsG coding region, detected in the work of Engels et al. [22] and this work are located upstream the promoter P$_{ptsG}$, a spacing of 30 bp to the 35th nucleotide may be sufficient for transcriptional repression by SugR. However, the maximal repression of thedeo operon in E. coli is produced by DeoR binding to three operator sequences which are overlapping and located upstream of the transcription start sites [34], resulting in cooperative binding of different regulatory sequences leading to gene repression [39]. Such behaviour is putatively not present in C. glutamicum since a SugR binding to DNA fragments located between transcription and translation start (fragments G3 to G5; Fig. 5) was not observed. However, further binding sites located in the coding region of ptsG were not ruled out by experimental approaches and therefore the repression of ptsG transcription by SugR remains unclear.

Fructose-1-phosphate and other sugar phosphates act as effectors releasing SugR from its binding sites

The strong effect of F-1-P on SugR binding to the ptsl-cg2118 intergenic region is very interesting, since F-1-P is a metabolite mainly occurring during the uptake of fructose mediated by PtsF. Therefore, F-1-P is an ideal intracellular substance for sensing the presence of external fructose and efficiently induces expression of the genes encoding the general and fructose-specific parts of the PTS, ptsl, ptsI, and ptsF, respectively. Once external fructose is consumed, the F-1-P concentration is reduced by the activity of 1-phosphofructokinase encoded by fruK converting F-1-P to the less effective metabolite F-1,6-P. Although, the internal concentration of F-1-P in C. glutamicum grown on glucose or fructose is unknown, the internal concentrations of the effector substances G-6-P and F-1,6-P to the amounts used for the EMSA-studies suggests that SugR is negatively influenced by theses effectors in vivo. During the consumption of the F-1,6-P pool, the regulatory system might return to a maximal repressed state. The effector substance F-1-P was also shown to specifically affect the global regulator Cra of E. coli [40,41]. It is furthermore described as the main effector for the FruR repressors of the fructose operon in Streptococcus gordonii [15] and Spiroplasma citri [14].

Besides the intermediate occurring during the consumption of glucose and fructose, namely F-1,6-P, the product
of glucose uptake via the PTS, namely G-6-P, is apparently able to affect SugR binding activity and to derepress transcription of the PTS genes. This would be necessary at least for the genes of the general part of the PTS (ptsI, ptsH) and for the distantly located ptsG gene. However, much higher concentrations of F-1,6-P or G-6-P are necessary for resolving the DNA-SugR protein complex. These results were confirmed by the RT-PCR data for the wildtype, where the transcription of the fructose-PTS cluster genes of cells grown on glucose did not reach the induction levels of cells grown on fructose, indicating higher activity of SugR on glucose then on fructose and resulting in higher induction levels of the PTS cluster genes on fructose.

As a striking difference, Engels and Wendisch [22] identified F-6-P as the only effector of SugR (SugR) when bound to the ptsG upstream region. F-6-P is the successive metabolic intermediate produced from G-6-P by the phosphoglucone isomerase. Therefore, F-6-P is a suitable candidate for the cell to determine whether glucose is externally available.

In the experiments performed with the ptsI-cg2118 intergenic region, no effect on SugR binding was observed for F-6-P up to concentrations exceeding 20 mM, which were applied for full resolution of the complex between SugR (SugR) and the ptsG upstream region in the study of Engels and Wendisch [22]. However, internal concentrations of 13 mM or 3 mM and 1 mM or 2 mM F-6-P were determined for C. glutamicum grown on glucose and fructose, respectively [42,19], ranging below the amounts used for the EMSA studies here.

In addition, the divergent promoters P_{ptsI} and P_{cg2118} are coordinately regulated as shown by the mRNA measurements in the sugR mutant compared to the parental strain. However, divergently transcribed genes were already described, e.g. for the C. glutamicum genes aceA and aceB [43]. The corresponding promoters partly overlap by their -10 region and regulation of the divergent genes aceA and aceB is obtained by binding of the regulator RamB to one binding site located in the intergenic region [44]. Hence, switching between the repressive and the activated state is coordinately triggered. Nevertheless, the aceA and aceB promoters are only overlapping by their promoter sequence. The promoters P_{ptsI} and P_{cg2118} are producing transcripts, which are complementary by 14 nucleotides, whereby the overlapping mRNA transcripts do not influence the regulatory coupling of the divergent genes ptsI and cg2118, but represent a hence unique promoter structure in C. glutamicum.

As mentioned above, binding of SugR to motifB only interferes with cg2118-fruK-ptsF transcription providing the opportunity for the cell to differentially regulate expression of the fructose-specific part, and the ptsI and ptsH gene of the general part of the PTS as observed for fructose and glucose grown cells. In detail, the expression of the PTS gene cluster in C. glutamicum grown on acetate was compared to the one on fructose and glucose, respectively, indicates that the complete gene cluster is derepressed in the presence of glucose. In the presence of fructose, however, the expression of the fructose-specific genes fruK and ptsF is significantly higher than the expression of ptsI and ptsH. Therefore, the level of the transcription from P_{cg2118} is somehow further adjusted by the actual carbon source, whereas the transcription from P_{ptsI} and P_{ptsH} remains unaffected. At the moment it is not clear how this differential regulation, affecting the transcription from P_{cg2118} is exerted. It is possible that the SugR-DNA complexes react differentially to different effectors as it is shown for the SugR (SugR) binding site upstream of the coding region of ptsG [22]. This differential behaviour might be interpreted in a sense that SugR is able to form complexes with individual binding sites that have different sensitivities to sugar phosphates. A sugar specific regulation of different genes was also observed in the hyperthermophilic archaean Pyrococcus furiosus, where the transcriptional regulator of the trehalose/maltose and the maltodextrin ABC transporter TrmB is inactivated by the effectors maltose and trehalose or maltotriose and sucrose, respectively [23,24]. However, the regulation of different genes by recognition of varying effectors could be an adequate explanation for the differing effectors determined by Engels [22] and this work. Furthermore, it can not be excluded that other regulators may play a role in the differential expression of the PTS gene cluster. The function of the second Deor-type regulator gene located in the PTS gene cluster cg2118 in transcriptional regulation of the PTS gene cluster remained unclear. The deletion mutant of cg2118, however, showed no influence on transcription of the PTS genes under the tested conditions, therefore leaving the potential targets of Cg2118 unknown. However, a sugR/cg2118 double mutant (strain LG03) was constructed and investigated with RT-PCR compared to the sugR single mutant, but no further induction of the transcription levels were observed for the fructose PTS cluster genes when both strains were grown on fructose or acetate (data not shown). Furthermore, the Cg2118 protein was purified by the IMPACT-method described and EMSA-studies were carried out with the intergenic region of ptsI/cg2118 and the upstream region of ptsH. Binding of Cg2118 to the corresponding DNA-fragments was not detected (data not shown). Further microarray experiments with the deletion mutant of cg2118 will be a subject of further studies and may reveal the function and the regulon of Cg2118.
Conclusion
The detailed investigation of the transcriptional regulation revealed the DeoR-type regulator SugR to be responsible for the repression of the fructose-PTS cluster genes ptsI, cg2118, fruK, ptsF, and ptsH, as well as of the distantly located PTS-genes ptsG and ptsS. These results were confirmed by the determined promoter and SugR-binding sequences, extending the knowledge on PTS-dependent transcriptional regulation.

The main negative effector of SugR with regard to the fructose-PTS genes (F-1-P), was different to that identified in the study concentrating on the regulation of ptsG (F-6-P) by Engels and Wendisch [22]. In combination, these results indicated that SugR is able to regulate genes in dependence on the presence of different effectors, reflecting a hitherto unknown regulatory mechanism in Corynebacteria.

It remains to be assessed whether the arrangement and sequence composition of the SugR-binding sites are responsible for the recognition of different effectors by SugR. Further experiments dealing with the mutational analyses of the SugR-binding sites could clarify this issue.

Methods
Strains and media
Bacterial strains and plasmids used in this study are listed in Table 3. All C. glutamicum strains were cultivated in shaking flasks at 30°C with 150 rpm in minimal medium MM1. The growth medium contained 2% (w/v) carbon source dependent on the test setup (glucose, fructose, and sodium-acetate), 1% (NH₄)₂SO₄, 0.3% urea, 0.1% K,HPO₄; pH 7.2. After autoclaving the following substrates were added: 50 μM MnSO₄ × 1H₂O and 50 gl-1 NaCl.

DNA isolation, transfer and manipulation
E. coli DH5α MCR was used for routine recombinant DNA experiments. Plasmid DNA of E. coli was isolated by means of the GenElute bacterial DNA isolation kit (Sigma Aldrich, Steinheim Germany). DNA-modification, analysis by gel-electrophoresis, and ligation were applied as standard procedures [45]. Restriction endonucleases and T4 DNA ligase were obtained from Amersham-Pharmacia (Freiburg, Germany) and Roche (Mannheim, Germany). Plasmid DNA was introduced into E. coli and C. glutamicum strains by electroporation as described previously [47,48] employing the Bio-Rad Gene Pulser system (Bio-Rad, Munich, Germany).

Construction of the C. glutamicum deletion mutant strains
The plasmids pLMJ1 and pLMJ2 carrying deletion fragments of the genes sugR and cg2118 were constructed using the GeneSOEing method based on the PCR-mediated recombination as described by Horton et al. [49] (Table 3). Artificial Muni- and BamHI-sites for sugR and BglII- and EcoRI-sites for cg2118 were added by 5’-primer extension on both ends of the deletion fragment. Subsequently the resulting fragments were cleaved by the corresponding restriction enzymes and cloned into the pK18mobsacB vector. The resulting plasmids pLMJ1, pLMJ2 were transferred by electroporation into C. glutamicum RES167 [47] and therefore used to introduce the deletions by homologous recombination into C. glutamicum [50]. Thus the C. glutamicum mutant strains LG01, LG02, and LG03 carrying deletions of the genes sugR, cg2118, and a sugR/cg2118 double deletion were obtained.

The sugR, cg2118, and sugR/cg2118 deletion mutant strains were subsequently verified by PCR using additional primers positioned outside of the deletion construct as described by Rückert et al. [51].

Genetic construction, expression and purification of heterologous expressed Intein-coupled protein SugR
The SugR protein was purified by a translational fusion to intein and subsequent affinity purification performed by the IMPACT-CN system (New England Biolabs, USA), which allows the purification of the target protein without any remaining tag by thiol-induced self-cleavage of the intein. The sugR expression plasmids (pLG11) was constructed by PCR amplification of a 777 bp fragment, including the complete sugR gene, except the stopcodon extension at 72°C whereas amplification time was chosen corresponding to fragment length and speed of the polymerase. The cycle was repeated 34 times, followed by a final extension step for 7 min at 72°C. Purification of the PCR products was carried out by using a PCR Purification Spin kit (Qiagen, Hilden, Germany).

PCR techniques
PCR experiments were performed by using the DNA Engine Dyad thermocycler (PTC-0220) from MJ research Inc. (Watertown, Mass.). Oligonucleotides were obtained from Operon (Qiagen, Germany). Amplification of DNA was performed with Phusion Hot Start DNA polymerase (New England Biolabs, USA), which features proof-reading activity. Initial denaturation of the template DNA was carried out at 96°C for 4 min. The PCR cycle started with denaturation for 30 s, followed by annealing at primer dependent temperature at Tₘ (2AT + 4GC) [46], and
to verify C-terminal fusion of the desired protein with a 55 kDa intein-tag of the pTYB1 vector (New England Biolabs, USA). At the 5' end an artificial Ndel-site and at the 3' end of the fragment an artificial Sapl-site was added by 5' primer extension. As recommended by the vendor the Ndel-site contains an ATG sequence for translation initiation and the Sapl-site places the C-terminus of the target protein immediately adjacent to the intein cleavage site and results in the purification of a target protein without any extra vector-derived residues at its C-terminus. After cleavage with Sapl and Ndel the fragment was directed cloned into the expression vector pTYB1 that was cleaved and results in the purification of a target protein without any extra vector-derived residues at its C-terminus.

E. coli ER2566 containing plG1 for expression and purification resulted in the mutant strains LG21 and LG31, respectively.

E. coli ER2566 containing plG1 used for heterologous expression of SugR was grown as a preculture o/N in 10 ml LB media with 100 μg ml⁻¹ ampicillin at 37°C. The main culture with a volume of 250 ml was inoculated with a cell density of 0.1 x 10⁸ cells x ml⁻¹ and grown to an o.D. of 0.5 to 0.8 at 37°C. The expression of the proteins was achieved by IPTG (0.5 mM) induction of the lac-promoter and lowering the culture temperature to 16°C in order to optimize T7 RNA-polymerase transcription. The culture was grown o/N and all cells were pelleted by 6450 × g for 15 min at 4°C. The pellet was then resuspended in 25 ml lysis buffer (20 mM Na₂HPO₄, 500 mM NaCl, 1 mM EDTA, 0.1 % TritonX-100, 20 μM PMSF, 0.1 mM TCEP, pH 8.0) and the cells were disrupted by French press procedure at medium speed with 2000 psi repeating 3 times. The debris was pelleted at 6450 × g for 30 min at 4°C. The supernatant containing the crude protein extract was collected for IMPACT-CN purification (New England Biolabs, USA).

A 14 ml Protino column (Macherey and Nagel, Germany) was prepared with 7 ml of chitin beads and topped with a polycarbonate filter. Subsequently the packed column was equilibrated by washing with ten column volumes of precooled (4°C) column buffer (20 mM Na₂HPO₄, 500 mM NaCl, 1 mM EDTA, pH 8.0). Afterwards the column is ready for sample loading at 0.5–1 ml min⁻¹. Again the column was washed with ten column volumes of column buffer which can be adjusted up to 1 M NaCl. The cleavage of the intein is induced by washing with three column volumes of cleavage buffer which can be adjusted up to 1 M NaCl. The cleavage buffer (20 mM Na₂HPO₄, 500 mM NaCl, 1 mM EDTA, 50 mM DTT, pH 8.0) sealing the column to prevent drying and subsequent incubation at 4°C for 16 h. The elution of the recombinant protein can be performed by washing the column with 1–1.5 volumes of column buffer. In this case the elution volume of 7.5 ml was applied to the column and the flowthrough was collected for the subsequent concentration step with Amicon Ultra-4 centrifugal filter device with a MW cutoff of 5 kDa (Millipore, USA) down to an approximative volume of 250 μl. The concentrated elution fraction was washed...
twice with washing buffer (20 mM Na₂HPO₄, 10 mM NaCl, pH 8.0) again to a total volume of 250 μl. Different aliquots of the purification procedure can be collected (e.g. crude protein extract, flow through after the binding, and washing fractions) and tested with the purified protein by 1D-SDS-PAGE.

Separation of cytoplasmic proteins of C. glutamicum RES167

One-dimensional denaturing sodium dodecyl sulfate polyacrylamide gel electrophoresis (1D-SDS-PAGE) to separate proteins was used as described by Laemmli et al. [52] with a 4% stacking gel and a 12.5% resolving gel. Samples were denatured in the presence of 2% SDS and 4% mercaptoethanol in Tris-HCl buffer (60 mM, pH 6.8) by heating to 100°C for 5 min. Apparent molecular weights were derived from the relative mobility of standard proteins as given in the Fermentas Protein Ladder (Fermentas Life Sciences GmbH, St. Leon-Roth).

Subsequently SugR identification was obtained by peptide mass fingerprint analysis [53] utilizing the Bruker Ultraflex MALDI-TOF mass spectrometer (Bruker Daltonic, Bremen, Germany). Therefore, protein spots, which should be identified, were excised from the Coomassie stained gel and digested with a modified Trypsin enzyme (Promega, Mannheim, Germany). The protocol for tryptic digest and the settings of the MALDI-TOF-MS were described previously [52]. Peptide fingerprints thus obtained were compared with in silico generated tryptic fingerprints derived from the C. glutamicum ATCC 13032 genome data [17] by using the MASCOT software (MATRIX Science Ltd., London, UK; [54]). Best hits identified the corresponding genes of the proteins analyzed.

Operator binding assays by electrophoretic mobility shift assay (EMSA) with purified SugR

EMSA studies were performed with a set of Cy3-labeled PCR products, which were amplified using appropriate Cy3-labeled 20 mers (Operon, Germany). Unlabeled oligonucleotides representing putative binding sequences in front of sugR, ptsl/cg2118, and ptsH were annealed with the corresponding complementary oligonucleotides under standard conditions [47]. The resulting double-stranded oligonucleotides were purified by means of Qiagen MinElute columns and used in EMSA displacement experiments.

During all EMSA studies 15 pmol of purified SugR protein was added to 4 μl reaction buffer (1 mM MgCl₂, 0.5 mM EDTA, 0.5 mM DTT, 100 mM NaCl, 10 mM Tris, 20% glycerin, pH 7.5). Subsequently, 3 μl 87.9% glycerine, 0.05 pmol of Cy3-labeled PCR product and H₂O was added to get a final volume of 15 μl. The assay was incubated at RT for 5 min and then separated with a 2% agarose gel prepared in gel buffer (40 mM Tris, 10 mM sodium acetate, 1 mM EDTA, pH 7.8). A voltage of 14 Vcm⁻¹ was applied for 35 min. The gel was then scanned with the Typhoon 8600 Variable Mode Imager (Amersham Biosciences Europe, Germany). During effector screening studies, the purified SugR protein was incubated at RT for 10 min with acetate, glucose, fructose, fructose-1-phosphate, fructose-1,6-bisphosphate, fructose-6-phosphate, or glucose-6-phosphate prior to the addition of the Cy3-labeled PCR product. During EMSA competitive experiments, the purified SugR protein was added to reaction buffer, mixed with unlabeled competitor oligonucleotide and incubated at RT for 5 min prior to adding the labeled PCR fragments. After addition of the Cy3-labeled PCR product, the assay was incubated for additional 5 min before separating by gel electrophoresis.

Total RNA Isolation from C. glutamicum cultures

Cultures were grown to the logarithmic growth phase (O.D.600 = 10) and 1 × 10⁹ cells were harvested by centrifugation at 16,000 × g for 15s. The supernatant was removed by pipetting and the pellet immediately frozen in liquid nitrogen. The frozen cells were resuspended in 800 μl RTL-buffer (Rneasy Mini Kit, Qiagen) and instantly disrupted by means of the Ribolyser instrument (Hybaid, Heidelberg, Germany). Disruption was performed by three time intervals of 30s at speed-level 6.5 with intermediary cooling of the probes on ice for 1 min. Preparation of total RNA from C. glutamicum cells was performed as described by Hüser et al. [55].

Determination of transcription starts by 5'-RACE

Total RNA was isolated as described above. Primers binding downstream of the annotated translation starts of sugR, ptsl, cg2118, and ptsH were used along with 1.5 μg of total RNA for cDNA synthesis. The cDNA was then modified and amplified using the 5’RACE Kit (Roche Diagnostics) according to the supplier’s protocol. Resulting PCR products were ligated into the vector pCR2.1 by applying the TOPO TA cloning system and chemically competent E. coli TOP10 cells (Invitrogen). Sequencing of cloned RACE products was carried out by IIT Biotech (Bielefeld, Germany).

Relative quantification of mRNA levels using real-time RT-PCR

RT-PCR primers were designed to amplify an intergenic region of the analyzed gene of about 150–200 bp length with the Primer Design 4.2 software (Sci Ed Software) and were purchased from Operon (Qiagen, Germany). The real-time RT-PCR experiment was performed using the LightCycler instrument (Roche, Germany) in combination with the QuantiTect SYBR Green RT-PCR Kit (Qiagen, Germany) mixed with the specific primers and 300 ng of sample RNA.
The RT-PCR program consists of 3 segments starting with the reverse transcription at 50°C for 20 min, followed by the initial activation of the HotStar Taq DNA polymerase at 95°C for 15 min. Thirdly the RT-PCR step was performed 55 times in cycles of 95°C for 15s, 55°C for 30s, and 72°C for 20s. The melting curve was recorded over a range of 65 to 95°C with a heating slope of 0.1°C pers at continuous fluorescence measurement. The crossing point for each gene and condition was calculated by the data analysis method provided by the LightCycler software, using the maximum increase or acceleration of fluorescence.

Microarray experiments and analysis
Total RNA isolated from C. glutamicum was also used for global transcriptional analyses. The cDNA synthesis and array hybridization were performed as described by Hüser et al. [55]. Data analysis was performed with the Imagen Gene V6.0 and EMMA V2.2 [56] software packages. Evaluation of the hybridization experiment was done as described by Rey et al. [57] using an m-value cutoff of ±1, which corresponds to expression changes equal or greater than 2 fold. Furthermore, m-values were considered as significant if the Student’s t-test resulted in a p-value ≤ 0.05. The microarrays used represent all 3002 coding regions of C. glutamicum RES167 as 70 mer oligonucleotides.

Bioinformatic tools used to analyse nucleotide sequences
For interpreting the data of the C. glutamicum ATCC 13032 genome project the automated sequence investigation program GenDB [58] was used. Sequence comparisons were carried out by using the ClustalX software [59].

Authors’ contributions
LG carried out the experimental work and drafted the manuscript. JPS participated during the microarray analyses. MH constructed the cg2118 deletion mutant. SM participated during experimental work and illustration design. AT participated in supervision. AP aided in coordination and participated in supervision. JK conceived of the study and participated in coordination and supervision. All authors read and approved the final manuscript.

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