Complex Regulation of the Phosphoenolpyruvate Carboxykinase Gene pck and Characterization of Its GntR-Type Regulator IolR as a Repressor of myo-Inositol Utilization Genes in Corynebacterium glutamicum

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DNA affinity chromatography with the promoter region of the Corynebacterium glutamicum pck gene, encoding phosphoenolpyruvate carboxykinase, led to the isolation of four transcriptional regulators, i.e., RamA, GntR1, GntR2, and IolR. Determination of the phosphoenolpyruvate carboxykinase activity of the ΔramA, ΔgntR1 ΔgntR2, and ΔiolR deletion mutants indicated that RamA represses pck during growth on glucose about 2-fold, whereas GntR1, GntR2, and IolR activate pck expression about 2-fold irrespective of whether glucose or acetate served as the carbon source. The DNA binding sites of the four regulators in the pck promoter region were identified and their positions correlated with the predicted functions as repressor or activators. The iolR gene is located upstream and in a divergent orientation with respect to a iol gene cluster, encoding proteins involved in myo-inositol uptake and degradation. Comparative DNA microarray analysis of the ΔiolR mutant and the parental wild-type strain revealed strongly (>100-fold) elevated mRNA levels of the iol genes in the mutant, indicating that the primary function of IolR is the repression of the iol genes. IolR binding sites were identified in the promoter regions of iolC, iolT1, and iolR. IolR therefore is presumably subject to negative autoregulation. A consensus DNA binding motif (5'-KGWCHTRA-3') which corresponds well to those of other GntR-type regulators of the HutC family was identified. Taken together, our results disclose a complex regulation of the pck gene in C. glutamicum and identify IolR as an efficient repressor of genes involved in myo-inositol catabolism of this organism.

Corynebacterium glutamicum is a facultative anaerobic Gram-positive soil bacterium of the order Corynebacteriales (1) that grows on a variety of carbon sources and has been used for more than 50 years for large-scale production of l-amino acids (2, 3). Recent metabolic engineering studies have shown that C. glutamicum is also capable of producing a variety of other commercially interesting compounds, e.g., other l-amino acids (4), d-amino acids (5), organic acids such as succinate (6–9), diamines such as cadaverine (10, 11) or putrescine (12), biofuels such as ethanol or isobutanol (13–15), and proteins (16–18).

For growth on organic acids such as acetate, gluconeogenic reactions are necessary in order to provide the cells with hexose and pentose sugars (19). Depending on the expression levels of the phosphoenolpyruvate (PEP)-pyruvate-oxaloacetate node of a given organism, PEP carboxykinase or malic enzyme and/or oxaloacetate decarboxylase in combination with PEP synthetase catalyzed the conversion of C4 intermediates from the tricarboxylic acid (TCA) cycle into PEP (20), the direct precursor for gluconeogenesis. C. glutamicum possesses a rather complex PEP-pyruvate-oxaloacetate node (Fig. 1) compared to other model organisms such as Escherichia coli and Bacillus subtilis and is equipped with PEP carboxykinase as well as with malic enzyme and oxaloacetate decarboxylase (20). Although the presence of a PEP synthetase was previously proposed for some strains (21, 22), the inability of a defined PEP carboxykinase-negative mutant of C. glutamicum to grow on acetate or lactate (23) argues against a functional PEP synthetase in C. glutamicum and a gluconeogenic function of malic enzyme or oxaloacetate decarboxylase. The validity of this finding is additionally supported by the fact that a pyruvate carboxylase-negative strain was unable to grow on lactate (24). In a PEP synthetase-possessing strain, this enzyme in combination with PEP carboxykinase should have overcome the anaplerotic deficiency caused by the absence of pyruvate carboxylase (20).

PEP carboxykinase catalyzes the reversible conversion between oxaloacetate and PEP (25): oxaloacetate + ATP/GTP ⇌ PEP + CO2 + ADP/GDP. While microbial enzymes often use ATP as a phosphate donor, the C. glutamicum PEP carboxykinase has been shown to be highly specific for GTP (26–29) and thus represents a notable exception. The kinetic analysis of the purified enzyme revealed ATP to inhibit PEP carboxykinase activity in the oxaloacetate-forming reaction (28), indicating that the enzyme mainly functions in gluconeogenesis and not in anaplerosis under physiological conditions. Since PEP carboxykinase of C. glutamicum also shows significant activity in cells grown on glucose (23, 28) and due to the fact that optimization of the cellular oxaloacetate concentration is crucial, especially for improved l-lysine produc-
tion (30), deletion of the pck gene in a \textit{L-lysine}-producing strain resulted in an increase in \textit{L-lysine} productivity by 20% (23). Therefore, besides the activities of pyruvate carboxylase and PEP carboxylase, PEP carboxykinase activity is an important target to modulate the net carbon flux toward oxaloacetate and thus increase precursor supply for \textit{L-lysine} production.

Expression of the PEP carboxykinase gene \textit{pck} (sometimes also named \textit{peckA}) is controlled in different ways in different microorganisms. In most bacteria studied so far, \textit{pck} is expressed depending on the carbon source in the growth medium, with expression being low during growth with glycolytic substrates and higher during growth with gluconeogenic substrates. In \textit{E. coli}, the \textit{pck}A gene as well as the genes encoding enzymes of the alternative C4-decarboxylating route, i.e., \textit{maeb} (malic enzyme), \textit{scf} (malic enzyme), and \textit{ppsA} (PEP synthase), are subject to glucose repression (31–35). For \textit{pck}A, glucose repression is relieved via (i) cyclic AMP (cAMP) receptor protein (CRP)-dependent activation in the presence of elevated cytoplasmic cAMP levels in response to glucose starvation (33) and (ii) activation by the catabolite repressor/activator Cra (formerly known as FruR) (36). In \textit{B. subtilis}, \textit{peck}A transcription is also repressed in the presence of glucose but is independent of the presence of CcpA, the major transcriptional catabolite repressor of this organism. Instead, the transcriptional regulator CcpN, which is a repressor of \textit{peck}A expression and directs carbon flow between glycolysis and gluconeogenesis (37), could be identified.

In \textit{C. glutamicum}, carbon source-dependent expression of the \textit{pck} gene was suggested by the two- and 3-fold-higher specific PEP carboxykinase activities in acetate- and lactate-grown cells, respectively, compared to glucose-grown cells (23). DNA microarray and quantitative reverse transcription-PCR experiments confirmed an acetate-dependent transcriptional regulation of the \textit{pck} gene (38). In \textit{C. glutamicum} ATCC 13032, a growth phase-dependent regulation of \textit{pck} gene expression was not observed by either enzyme activity (25) or quantitative protein measurements (39), whereas in \textit{C. glutamicum} R, growth-phase-dependent differences in the \textit{pck} transcript level on glucose were reported, with maxima in the exponential and early stationary phases (40). The presence of a binding site for the CRP homologue GlxR in the \textit{pck} promoter region and the fact that GlxR binds to the \textit{pck} promoter in a cAMP-dependent manner \textit{in vitro} (40, 41) indicate an involvement of GlxR in regulation of \textit{pck} transcription. \textit{In silico} analysis of the \textit{pck} promoter also revealed binding sites for the regulator of acetate metabolism RamA (42). DNA microarray analysis indicated that RamA represses \textit{pck} transcription during growth on glucose, as the \textit{pck} mRNA level was increased in a RamA-negative strain, and a direct interaction between RamA and the \textit{pck} promoter region could be shown (43).

In the present work, DNA affinity chromatography with the \textit{pck} promoter region was used to search for additional regulators of \textit{pck} expression. In addition to RamA, we identified the two functionally redundant transcriptional regulators, GntR1 and GntR2 (44), and another GntR-type regulator, Cg0196. The relevance of these regulators for \textit{pck} expression under glycolytic and gluconeogenic conditions was investigated. Additionally, we analyzed the regulon of Cg0196 by transcriptome analysis of a \Delta c0196 mutant and by DNA binding studies with purified Cg0196. Our results clearly showed that the primary function of Cg0196 is the transcriptional control of a variety of iol genes involved in myo-inositol metabolism (45). Therefore, we designated Cg0196 IolR and the corresponding gene \textit{iolR}.

**MATERIALS AND METHODS**

**Bacteria, plasmids, and culture conditions.** Bacterial strains and plasmids as well as their relevant characteristics and sources are listed in Table 1. Plasmid \textit{pK19mobsacB-DiolR}1 was obtained as follows: the up- and downstream regions of \textit{iolR} were amplified by PCR using the oligonucleotide pairs \textit{iolR_DF1}_for/\textit{iolR_DF1}_rev and \textit{iolR_DF2}_for/\textit{iolR_DF2}_rev (see Table S1 in the supplemental material) and chromosomal DNA of the \textit{C. glutamicum} wild-type (WT) strain as the template. The resulting PCR fragments were then used as the templates for overlap extension PCR with the oligonucleotides \textit{iolR_DF1}_for and \textit{iolR_DF2}_rev. The resulting 1.51-kb PCR product was cut with HindIII and BamHI and cloned into \textit{pK19mobsacB} cut with the same enzymes. DNA sequencing revealed point mutations in a distinct area in the upstream region of \textit{iolR}. Therefore, a 5’-shortened 1.197-bp fragment was cut out of \textit{pK19mobsacB-DiolR}2 via the use of Sall and BamHI and cloned again into \textit{pK19mobsacB} to obtain \textit{pK19mobsacB-DiolR2} without any unintended mutations. For construction of plasmid \textit{PET16b-iolR}, the \textit{iolR} coding region was amplified from chromosomal DNA of \textit{C. glutamicum} WT using the oligonucleotides \textit{iolR_PET16b_for} and \textit{iolR_PET16b_rev}. The resulting PCR fragment was digested with Ndel and BamHI and cloned into \textit{PET16b} cut with the same enzymes. The IolR protein encoded by \textit{PET16b-iolR} contains an N-terminal extension of 21 amino acids, including a decahistidine tag.

**Trypsin-yeast extract extract medium (46) \((2 \times \text{TY})\) was used for cultivation of \textit{C. glutamicum} and \textit{E. coli} DH5\textalpha{} and Terrific Broth (TB) medium (47) for growth of \textit{E. coli} BL21(DE3). For growth of \textit{C. glutamicum} on glucose, \textit{myo}-inositol, and potassium acetate (at the concentrations indicated in Results), CGXII minimal medium (48) was used. When appropriate, the medium contained kanamycin (50 \(\mu\text{g} \text{ml}^{-1}\) for strains carrying plasmids; 25 \(\mu\text{g} \text{ml}^{-1}\) for the \textit{pK19mobsacB-DiolR2} integration mutant) or ampicillin (100 \(\mu\text{g} \text{ml}^{-1}\)). \textit{C. glutamicum} was grown aerobically at 30°C and \textit{E. coli} at 37°C as 60-ml cultures in 500-ml baffled Erlenmeyer flasks on a rotary shaker at 120 rpm. Growth of the bacteria was followed by measuring the optical density at 600 nm (OD\textsubscript{600}). For screening applications, \textit{C. glutamicum} was grown as 800-\mu{l} cultures in 48-well baffled microtiter plates (Flowerplates) at 80% humidity and 1,200 rpm using a BioLector system.

**FIG 1** The phosphoenolpyruvate (PEP)-pyruvate-oxaloacetate node in \textit{C. glutamicum}. Abbreviations: AK, acetate kinase; CS, citrate synthase; MDH, malate dehydrogenase; ME, malic enzyme; MQO, malatequinone oxidoreductase; ODx, oxaloacetate decarboxylase; PCx, pyruvate carboxylase; PDHC, pyruvate dehydrogenase complex; PEPC\textit{x}, PEP carboxykinase; PEPC\textit{x}, PEP carboxylase; PK, pyruvate kinase; PQO, pyruvate:quinone oxidoreductase; PTA, phosphotransacetylase; PTS, phosphotransferase system.
**Table 1** Strains and plasmids used in this study

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<td>F&lt;sup&gt;−&lt;/sup&gt; acquired ΔlacZYA-argFΔ-U169 deoR recA1 endA1 hsdR17 (rK&lt;sup&gt;−&lt;/sup&gt; m&lt;sup&gt;K&lt;/sup&gt; &lt;sup&gt;−&lt;/sup&gt;) phoA supE44 thi-1 gyrA96 relA1 lacI&lt;sup&gt;−&lt;/sup&gt;Δ23 (lacI lacUV5-T7 gene 1 ind1 sam7 nin5))</td>
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<td>F&lt;sup&gt;−&lt;/sup&gt;Δdecarboxylase F&lt;sup&gt;−&lt;/sup&gt;ΔaroD-araBAD Δ(mfd-tdh-lacX74) hsdR17 (rK&lt;sup&gt;−&lt;/sup&gt; m&lt;sup&gt;K&lt;/sup&gt; &lt;sup&gt;−&lt;/sup&gt;) (DE3 [lacI lacUV5-T7 gene 1 ind1 sam7 nin5])</td>
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<sup>a</sup>Ap<sup>+</sup>, ampicillin; Km<sup>+</sup>, kanamycin; ATCC, American Type Culture Collection.
The samples were loaded onto a native polyacrylamide gel (5% to 15%) with 40 to 100 ng DNA fragments using either the gel shift buffers described previously for RamA (55) and GntR1/GntR2 (44) or 50 mM Tris-HCl [pH 7.4], 50 mM NaCl, 5% [vol/vol] glycerol). After 20 min incubation at room temperature, the DNA was exchanged with TG buffer (50 mM Tris-HCl [pH 7.4], 5% [vol/vol] glycerol). Protein concentrations were determined as described above, and the purified proteins were stored at −20°C until use.

**EMSA.** For testing the binding of RamA, GntR1, GntR2, and IolR to promoter DNA fragments of putative target genes, various concentrations of purified His-tagged versions of the corresponding proteins were mixed with 40 to 100 ng DNA fragments using either the gel shift buffers described previously for RamA (55) and GntR1/GntR2 (44) or 50 mM Tris-HCl (pH 7.4) with 10% (vol/vol) glycerol for IolR. After 20 min incubation at room temperature, the samples were loaded onto a native polyacrylamide gel (5% to 15%) with 20 mM imidazol containing 20 mM imidazol was used for binding and a 20-mL linear gradient of buffer TNG with 50 to 500 mM imidazol was used for elution. Fractions containing IolR-His6, collected at 439 mM imidazol, were pooled and concentrated in a Microcon YM-10 unit (Merck Millpore, Darmstadt, Germany), and the buffer was exchanged with TG buffer (50 mM Tris-HCl [pH 7.4], 5% [vol/vol] glycerol). Protein concentrations were determined as described above, and the purified proteins were stored at −20°C until use.

**Microarray data accession number.** The microarray data were deposited in the GEO database with the accession number GSE44812.

**RESULTS**

**Isolation of transcriptional regulatory proteins binding to the pck promoter region.** Previous studies have shown carbon source-dependent transcriptional regulation of the pck gene encoding PEP carboxykinase of *C. glutamicum* with up to 10-fold-higher transcript levels during growth on gluconeogenic carbon sources compared to growth on glucose (38, 40). Two transcriptional regulators, GlxR and RamA, were reported to be involved in regulation of *pck* transcription (40, 41, 43). However, in the course of characterizing the transcriptional regulator RamA, we speculated about additional regulatory proteins involved in the control of *pck* expression (43). To identify such proteins, we performed DNA affinity chromatography using the immobilized *pck* promoter region. For this purpose, 488-bp biotinylated *pck* promoter fragments (extending from 413 bp upstream to 75 bp downstream of the *pck* start codon) were bound to streptavidin-coated magnetic beads and were incubated with crude extracts obtained from *C. glutamicum* WT cells grown either in minimal medium with 4% (wt/vol) glucose or in minimal medium with 2% (wt/vol) potassium acetate. Cells were harvested in the exponential-growth phase and, for glucose-grown cells, additionally in the stationary phase. Nonspecifically bound proteins were removed by several low-salt washing steps (0.1 M NaCl) with subsequent magnetic separation. Specifically bound proteins were eluted with buffers containing 0.3 M, 0.5 M, and 1 M NaCl and subjected to SDS-PAGE and Coomassie staining. No striking differences could be observed between either the samples from the different growth phases or the samples from the different carbon sources (data not shown).

In Fig. 2, an SDS-polyacrylamide gel with the proteins enriched from cell extracts of a *C. glutamicum* culture growing exponentially on glucose is shown. The five protein bands labeled a to e were isolated and subjected to peptide mass fingerprinting after tryptic digestion. Comparison of the mass lists with the nonredundant NCBI database resulted in the identification of six *C. glutamicum* proteins with a significant MOWSE score, including
IolR can be classified into the HutC subfamily of GntR-type regulators and a length of the C-terminal domain of about 150 amino acids. IolR contains the UbiC transcription regulator-associated (UTRA) domain. According to the secondary structure analysis by DSSP (61) and the crystal structure of residues 105 to 253 (40), the protein isolated by DNA affinity chromatography had a predicted molecular mass of 27.9 kDa, which corresponds well to the mass of the protein isolated by DNA affinity chromatography with a 488-bp biotin-labeled pck promoter fragment immobilized on Streptavidin-coated magnetic beads. Bound proteins were eluted with a buffer containing 0.3 M NaCl (lanes 1 and 2), 0.5 M NaCl (lanes 3 and 4), or 1 M NaCl (lanes 5 and 6). The protein bands labeled a to e were identified by peptide mass as polyribonuclotide phosphorylase GpsI (Cg1266), RamA (Cg2831), IolR (Cg0196), DNA polymerase I PolA (Cg1525), and GntR1 (Cg2783) and GntR2 (Cg1935). Lane M, molecular mass standard.

In vitro binding of RamA, GntR1, GntR2, and IolR to the pck promoter. In silico analysis of the C. glutamicum pck promoter used for DNA affinity chromatography indicated the presence of one GntR1/GntR2 and five RamA binding sites. The latter are located at positions $-4$ to $-7$, $-84$ to $-90$, $-150$ to $-156$, $-197$ to $-202$, and $-247$ to $-252$ relative to the TSS of pck (40), respectively, and totally match the described RamA consensus motifs A/C-$G_{4-6}$-C/T and A-C$_{4-5}$-A/G/T (42, 43). The potential GntR1/GntR2 binding site is located at position $-326$ to $-340$ relative to the TSS of pck and matches the described consensus motif WWtgA/T (42, 43). To confirm binding of RamA and GntR1/GntR2 to the predicted binding sites and to identify the IolR binding site within the pck promoter fragment, a second series of EMSAs was performed with a set of six different subfragments of P$_{pck}$ 1 (P$_{pck}$ 2 to P$_{pck}$ 7). With RamA-His$_6$, distinct retardations were observed with all fragments containing at least one predicted RamA binding site, indicating that all of these sites are able to bind RamA. In the case of GntR1 and GntR2, only those fragments containing the predicted binding site (P$_{pck}$ 2 and P$_{pck}$ 4) were retarded, whereas the other fragments showed no interaction, supporting the notion of the functionality of the predicted motif (Fig. 3).

In the case of IolR, no preliminary information was available on the position of the DNA binding site. The EMSAs with the subfragments revealed binding to fragments P$_{pck}$ 2 and P$_{pck}$ 4, but not to P$_{pck}$ 3 and P$_{pck}$ 5, indicating the IolR binding site to be located within fragment P$_{pck}$ 4, which covers bases $-376$ to $-239$ relative to the TSS of the pck gene (Fig. 3). To exactly localize the binding site of IolR, another set of subfragments (P$_{pck}$ 8 to P$_{pck}$ 13) was analyzed and shifts were observed with fragments P$_{pck}$ 9, P$_{pck}$ 11, and P$_{pck}$ 13 (Fig. 3). Therefore, the IolR binding site was assumed to be located at position $-294$ to $-272$ within the pck promoter region. This finding was substantiated by showing that IolR-His$_{10}$ binds to a 23-9 bp double-stranded (ds) oligonucleotide (P$_{pck}$ O1; 5’-TCCGAGTGGTTAAGGGTGTGTT-3’) representing the overlapping region between fragments P$_{pck}$ 11 and P$_{pck}$ 13 (Fig. 3).

Impact of ramA, gntR1 and or gntR2, and iolR gene deletion on PEP carboxykinase activity. To test the role of the transcriptional regulators RamA, GntR1, GntR2, and IolR in pck gene expression, we compared the specific PEP carboxykinase activity of cell extracts of C. glutamicum WT, ΔramA, ΔgntR1, ΔgntR2, ΔgntR1 ΔgntR2, and ΔiolR strains (Fig. 4). The cells were grown either with 2% (wt/vol) glucose or with 1% (wt/vol) potassium acetate as the sole carbon source and harvested in the exponential-growth phase. In accordance with previous results (23), the WT strain showed about 2-fold-higher PEP carboxykinase activity
FIG 3 Binding of RamA, GntR1, GntR2, and IolR to the pkc promoter region. (A) DNA fragments (P_{10} to P_{13}) used to determine the location of the RamA, GntR1, GntR2, and IolR binding site(s) in the pkc promoter are shown. The transcription start site (TSS) of the pkc gene is indicated by an arrow labeled +1. Black bars indicate the DNA fragments used for electrophoretic mobility shift assays (EMSAs). The numbers given to the right show the positions of the fragment ends relative to the TSS. The GlxR, RamA, GntR1/GntR2, and IolR binding sites are indicated with checkered, white, black, and striped boxes, respectively. (B) Results of EMSAs using different fragments of the pkc promoter region. The numbers given to the right show the positions of the fragment ends relative to the TSS. Free DNA and DNA-protein complexes are indicated with white and black arrowheads, respectively. Nonspecific bands are indicated with asterisks.

FIG 4 PEP carboxykinase activity of cell extracts obtained from the indicated C. glutamicum strains grown in CGXII minimal medium supplemented with either 2% (wt/vol) glucose (black bars) or 1% (wt/vol) potassium acetate (white bars). The ΔramA mutant is not able to grow on acetate as the sole carbon and energy source. Mean values and standard deviations of the results of three independent cultures are shown.

during growth with acetate (0.35 ± 0.02 U/mg protein) compared to growth with glucose (0.15 ± 0.01 U/mg protein). As expected, C. glutamicum ΔramA was not able to grow on acetate as the sole carbon source (42). With glucose as the carbon source, the PEP carboxykinase activity of the ΔramA mutant (0.34 ± 0.03 U/mg protein) was comparable to that of the WT during growth with acetate, confirming RamA to be a repressor of pkc transcription during growth with glucose. As expected from the functional redundancy of GntR1 and GntR2 (44), the PEP carboxykinase activities of the C. glutamicum ΔgntR1 and ΔgntR2 strains (0.18 ± 0.02 and 0.17 ± 0.02 U/mg protein on glucose; 0.39 ± 0.02 and 0.37 ± 0.02 U/mg protein on acetate) did not significantly differ from the activities of the WT under these conditions. However, deletion of both gntR1 and gntR2 resulted in less than half of the PEP carboxykinase activity seen with the WT, both on glucose (0.07 ± 0.01 U/mg protein) and on acetate (0.17 ± 0.01 U/mg protein). Therefore, we propose identification of GntR1 and GntR2 as activators of pkc transcription. Similar to the results observed for the ΔgntR1 ΔgntR2 double mutant, the PEP carboxykinase activity of the iolR-negative strain was also reduced more than 2-fold compared to that of the WT, both on glucose (0.06 ± 0.01 U/mg protein) and on acetate (0.17 ± 0.01 U/mg protein). Consequently, also IolR presumably functions as an activator of pkc transcription.

Transcriptome analysis of the ΔiolR mutant strain. To identify additional genes that are potentially regulated by IolR, the transcriptome profile of the iolR deletion strain of C. glutamicum was compared to that of the WT strain using DNA microarray analysis. A relatively small number (22) of genes exhibited an at least 3-fold change in their transcript levels (Table 2). Among those genes with a lower mRNA level in the ΔiolR mutant were gapX, encoding the NADP-dependent glyceraldehyde-3-phosphate dehydrogenase, aceA, encoding isocitrate lyase, and, consistent with the lowered PEP carboxykinase activity seen with the WT, both on glucose (0.06 ± 0.01 U/mg protein) and on acetate (0.17 ± 0.01 U/mg protein), the ΔiolR mutant also exhibited significantly lower transcript levels in the ΔiolR mutant and also with the in vitro binding of IolR to the pkc promoter region (see above), the pkc gene. With the threshold reduced to 2 (data not shown), aceB, encoding malate synthase, mez, encoding malic enzyme, sucC, encoding the β-subunit of succinyl-coenzyme A (CoA) synthetase, and ptsS, encoding the sugar-specific IIABC component of the phosphotransferase system, also exhibited significantly lower transcript levels in the ΔiolR mutant. Interestingly, with gapX, pck, mez, aceA, and aceB, expression of a prominent set of genes encoding enzymes linked to gluconeogenesis was negatively affected by the deletion of iolR.
TABLE 2 Transcriptome comparison of the C. glutamicum ΔiolR mutant and the wild-type strain using DNA microarrays

| cg locus tag | NCgI locus tag | Known or predicted function of the gene product | Gene   | mRNA ratio
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*Transcript levels are log2 values of the mean of the results of three independent DNA microarray experiments starting from independent cultures grown in CGXII minimal medium with 4% (wt/vol) glucose. All genes listed showed at least a 3-fold change in mRNA levels (increased or decreased) in at least two of the three replicates with 

On the other hand, an up to 445-fold increase in mRNA level could be observed for 16 genes in the mutant (Table 2). Twelve of them, mostly exhibiting a more than 100-fold increase in their mRNA level, are located in a large gene cluster encoding proteins for myo-inositol uptake and degradation (see Fig. S1 in the supplemental material) (45). A residual subset of three genes, gntP, gntK, and gntR2, code for glucconate permease, gluconate kinase, and one of the functionally redundant repressors of glucconate metabolism, respectively (44). To analyze whether iolC, iolT1, gntP, gntK, gntR2, aceA, aceB, mez, and gapX are directly regulated by IolR, binding of purified IolR-His6 to the corresponding promoter regions was tested in EMSAs. Obvious retardation could be observed only with the intergenic region between iolR and iolC and the promoter region of iolT1 (see below and data not shown).

Identification of IolR binding sites within the promoter regions of iolC, iolR, and iolT1 and prediction of a conserved IolR binding motif.

The iolC and iolT1 genes are part of a so-called C. glutamicum iol cluster (45), whose genes were named according to the iol cluster of B. subtilis (62). The iolC gene (cg0197) was annotated as 2-deoxy-5-keto-gluconokinase and is the first of 10 genes that are most likely cotranscribed as an operon. The iolT1 gene (cg0223) encoding myo-inositol transporter 1 is located further downstream in a divergent orientation. The iolR gene is located immediately upstream of iolC in the opposite orientation. The TSSs of the respective genes (see Table S2 in the supplemental material) were derived from a RNAseq library generated in the course of another project at the Institute for Genome Research and Systems Biology, University of Bielefeld (unpublished data). For iolR, two neighboring A residues located 136 and 137 bp upstream of the start codon were identified as TSSs. In the case of iolT1, an A residue located 85 bp upstream of the start codon and another A residue located 113 bp upstream of the start codon were found to be the TSSs. For both iolC and iolT, the TSSs closer to the start codon gave rise to a larger amount of transcripts and are labeled +1 in Fig. 5. For iolC, the TSS was located 5 bp downstream of the proposed start codon. Therefore, a reannotation of the iolC coding sequence was required in which the translational start site was shifted from position 168976 to position 169039 according to the NCBI RefSeq NC_006958.1. This reannotation is based on the occurrence of a putative Shine-Dalgarno sequence upstream of the ATG start codon and is consistent with the annotation in the sequence entry NCBI RefSeq NC_003450.3 as well as with the sequence used for crystallization of IolC (MMDB accession no. 86522; PDB accession no. 3PL2). The TSS was located 58 bp upstream of the start codon at position 160039.

Binding of IolR-His6 to the promoter region of iolT1 and to the intergenic region between iolR and iolC was tested in gel shift experiments by using a 353-bp fragment of the iolT1 promoter region and a 497-bp fragment covering the region between iolR and iolC. In the case of iolT1, a single protein-DNA complex was formed, whereas in the case of the iolR-iolC region, two distinct protein-DNA complexes were observed (Fig. 5). These results suggested the presence of at least one IolR binding site within the iolT1 promoter and of at least two IolR binding sites in the iolR-iolC intergenic region. To exactly localize the binding sites, subfragments of various sizes were used in a second series of EMSAs (Fig. 5). Within the iolT1 promoter region, a specific interaction was detected with fragments PiolT1 3 and PiolT1 4 (Fig. 5A and B). The slight interaction with fragment PiolT1 5 was abolished when 20 bp was removed from its 5‘ end (PiolT1 6), indicating that the IolR binding site is located between positions −114 and −3 with respect to the TSS of iolT1. A set of seven 30-bp ds-oligonu-
cleotides (PiolT1O1 to PiolT1O7) was used to identify the IolR binding site(s) within this region. At a 5-fold molar excess of IolR-His10, only oligonucleotides PiolT1O5 and PiolT1O6 shifted, suggesting the presence of two IolR binding sites between position 62 and position 18 relative to the TSS of PiolT1 that is closer to the start codon. At least three IolR binding sites were predicted within the intergenic region between iolR and iolC due to IolR-His10-mediated retardation of the fragments PiolRC3, PiolRC5, PiolRC6, and PiolRC9 (Fig. 5C and D). To localize the binding sites within these fragments, a set of 13 30-bp ds-oligonucleotides (PiolRCO1 to PiolRCO13) was used (Fig. 5D). At a 5-fold molar excess of IolR-His10 interaction was detected with oligonucleotides PiolRCO2, PiolRCO5, PiolRCO9, and PiolRCO10, spanning positions +108 to +79, +3 to −27, and −58 to −102 relative to the TSS of iolT and with oligonucleotides PiolRCO11, PiolRCO12, and PiolRCO13, spanning positions −44 to +17 relative to the TSS of iolC. These results suggest the presence of at least five IolR binding sites within the intergenic region between iolR and iolC.

The DNA regions narrowed down as IolR binding sites were analyzed for similarities by the online tool MEME, which resulted in the identification of the consensus motif KGWCHTRACA (K = G or T, W = A or T, H = A or C or T, R = A or G) (Fig. 6). The double shift observed for probe O5 in panel D of Fig. 5 might have been due to a weakly conserved second binding site whose occupation by IolR is enhanced by the well-conserved binding site. The relevance of the identified consensus motif in the binding region of PiolRCO11 was tested by performing EMSAs with DNA fragments in which four nucleotides of the proposed motif were sequentially exchanged. As shown in Fig. 7A, by mutating the half-sites of the highly conserved regions within the palindromic sequence, i.e., TTGT and ACAA, binding of IolR was strongly impaired, whereas no negative impact on binding could be observed by mutation of the center and the neighboring zones. Ac-
ment shifted with higher affinity than the wild-type (WT) fragment; +, mutated fragment shifted like the WT fragment; o, mutated fragment hardly shifted; −, mutated fragment not shifted at all. Bases indicated with white characters shaded in black are important for IolR binding.

According to the MEME result, the IolR binding site within the pck promoter offers the least conserved region of all fragments (Fig. 6A). To elucidate the role of the sequences ATGT and TGTT, which are positioned at the highly conserved regions of the proposed IolR binding motif in the pck promoter, we performed EMSAs with mutated fragments. As shown in Fig. 7B, exchange of TGTT to ACAA, which matches the proposed consensus motif, enhanced binding of IolR-His10, whereas mutations of one or both sites to sequences inconsistent with the consensus motif led to a complete inhibition of IolR-His10 binding.

**Phenotypic consequences of iolR deficiency in combination with pck overexpression.** During cultivation of the iolR deletion mutant for the measurement of PEP carboxykinase activities, we noticed a growth rate that was slightly lower than that of the WT in acetate minimal medium. To further elucidate the phenotypic consequences of IolR deficiency, the growth behavior of the ΔiolR mutant was compared to that of the WT in CGXII minimal medium with different carbon and energy sources. No difference was observed with 2% (wt/vol) glucose as the sole carbon and energy source. The reference WT strain and the ΔiolR (pEK0) strains had PEP carboxykinase activities of 0.15 ± 0.02 and 0.06 ± 0.00 U/mg protein during growth on glucose; 2.42 ± 0.15 and 1.64 ± 0.28 U/mg protein, respectively, during growth with acetate. The reference WT (pEK0) and ΔiolR (pEK0) strains had PEP carboxykinase activities of 0.15 ± 0.02 and 0.06 ± 0.00 U/mg protein during growth on glucose and of 0.38 ± 0.02 and 0.19 ± 0.06 U/mg protein during growth with acetate. Overexpression of pck did not improve growth on acetate of the ΔiolR (pEK0-pckB) strain compared to the reference ΔiolR (pEK0) strain, as the strains showed comparable growth rates of 0.16 ± 0.04 h⁻¹ and 0.16 ± 0.02 h⁻¹, respectively. Both ΔiolR strains grew slower than the WT (pEK0) and WT (pEK0-pckB) strains, which had growth rates on acetate of 0.25 ± 0.01 h⁻¹ and 0.24 ± 0.02 h⁻¹, respectively (Fig. 8B). Interestingly, during growth with myo-inositol, the ΔiolR (pEK0-pckB) strain revealed a prolonged lag phase of about 10 h and a reduced

FIG 7 Mutational analysis of the putative IolR binding motifs within the promoter regions of iolC (A) and pck (B). The importance of the predicted DNA sequence motif (Fig. 6B) for IolR binding was tested in electrophoretic mobility shift assays with DNA fragments in which 4 nucleotides in or next to the proposed motif were exchanged. According to the results of the shifts, the fragments were divided into three categories as follows: +, mutated fragment shifted with higher affinity than the wild-type (WT) fragment; +, mutated fragment shifted like the WT fragment; o, mutated fragment hardly shifted; −, mutated fragment not shifted at all. Bases indicated with white characters shaded in black are important for IolR binding.

FIG 8 (A) Growth of C. glutamicum wild-type (WT) (black squares) and ΔiolR (white squares) strains in minimal medium with 1% (wt/vol) acetate. (B) Growth of WT (pEK0) (black squares), WT (pEK0_pckB) (black triangles), ΔiolR (pEK0) (white squares), and ΔiolR (pEK0_pckB) (white triangles) strains in minimal medium with 1% acetate. (C) Growth of WT (black squares) and ΔiolR (white squares) strains in minimal medium with 2% (wt/vol) myo-inositol. (D) Growth of WT (pEK0) (black squares), WT (pEK0_pckB) (black triangles), ΔiolR (pEK0) (white squares), and ΔiolR (pEK0_pckB) (white triangles) strains in minimal medium with 2% myo-inositol. The strains were cultivated in microtiter plates using the Biolector system. Mean values and standard deviations of the results of the independent experiments performed with two replicates each are shown; the backscatter was converted to OD₆₀₀ values as described in Materials and Methods.
growth rate of 0.34 ± 0.02 h⁻¹ compared to strain ΔiolR (pEK0) (0.42 ± 0.01 h⁻¹) (Fig. 8D). Similar effects were not observed when these strains were cultivated on glucose (data not shown), indicating that high PEP carboxykinase activity negatively impacts myo-inositol utilization.

DISCUSSION

The anaplerotic and gluconeogenic reactions at the PEP-pyruvate-oxaloacetate node are of importance for the production of TCA cycle-derived amino acids and organic acids. In addition to the C3-carboxylating enzymes PEP carboxylase and pyruvate carboxylase, C. glutamicum possesses the C4-decarboxylating enzymes PEP carboxykinase, oxaloacetate decarboxylase, and malic enzyme, and all of them are present with significant specific activities in extracts of cells grown on glucose (20, 63). ¹³C-based metabolic flux analysis repeatedly revealed that a high C4-decarboxylating flux exists in vivo in C. glutamicum which varies significantly under different growth and/or production conditions and is supposed to be regulated by mechanisms at the enzyme activity and gene expression levels (20). Quantification of the individual in vivo fluxes at the PEP-pyruvate-oxaloacetate node (64) indicated PEP carboxykinase to be the responsible enzyme by which two-thirds of anaplerotically synthesized oxaloacetate are recycled to PEP, resulting in a futile cycle under glycolytic conditions. The advantage of this futile cycling and the physiological function of PEP carboxykinase under glycolytic conditions are far from being understood and require a better understanding of the regulation of the genes and enzymes at the PEP-pyruvate-oxaloacetate node under different metabolic conditions. Here, we focused on transcriptional regulation of the pck gene under glycolytic and gluconeogenic conditions. We report that in addition to GlxR and RamA, the GntR-type regulators GntR1, GntR2, and IolR bind to the pck promoter and are capable of modulating expression of the pck gene. Additionally, we uncovered the role of IolR in regulation of genes involved in myo-inositol catabolism (Fig. 9).

![Diagram of complex regulation of the pck gene](http://jb.asm.org/)

Although direct interaction of GlxR and the pck promoter has been shown (40, 41), the GlxR protein was not enriched with the pck promoter probe in our DNA affinity chromatography experiments. The GlxR protein was initially characterized as a transcriptional regulator that represses the aceB gene of the glyoxylate bypass (65). Additional studies showed that GlxR is a global regulator with dual functionality that is predicted to directly regulate about 14% of the annotated C. glutamicum genes (66–69).

Binding of GlxR to several of its target promoters was shown to be strictly dependent on the presence of cAMP in vitro (40, 41, 65, 68, 70–73). The intracellular concentration of cAMP in C. glutamicum seems to vary with the carbon source and the growth phase of the culture, as it is, for instance, high when cells are grown on glucose and low when cells are grown on acetate medium (65, 74). These differences in cAMP levels during growth on glucose and acetate together with the presence of a GlxR binding site in the pck promoter suggest that GlxR is involved in carbon source-dependent regulation of pck. The GlxR binding site is located from position -62 to position -47 relative to the TSS of pck (40, 41), and, according to this position, activation of pck by GlxR has been predicted (41).

Besides GlxR, RamA also is a master regulator in C. glutamicum involved in adjusting the expression levels of genes related to carbohydrate metabolism, specifically, those related to ethanol and acetate metabolism, sugar uptake, glycolysis, TCA cycle, anaplerosis, and gluconeogenesis (43, 75). The ramA gene is upregulated 2- to 3-fold in the presence of acetate in the growth medium, which results in 2-fold-larger amounts of RamA in C. glutamicum cells grown on acetate instead of glucose (76). Independent of the acetate-dependent upregulation, the ramA gene is also subject to negative autoregulation (76). In accordance with the refined RamA consensus DNA binding motifs A/T/C-GGGG-N and A/T/C-CCCC-N (43), we proposed five potential RamA binding sites within the pck promoter fragment used in this study for DNA affinity chromatography, which are located at positions -1 to -7,
relative to the TSS of RamA-His6 to the pck promoter region (43) was confirmed by EMSAs, which showed interaction of RamA with all of the predicted sites. The 1.9-fold-higher mRNA level of the pck gene (43) and the 2.2-fold-higher PEP carboxykinase activity measured in the RamA-deficient strain in comparison to the WT during growth with glucose clearly indicate that RamA functions as a repressor of pck expression under these conditions. However, in a previous study, we also observed RamA to activate pck in cells grown in minimal medium with glucose plus acetate or in complex medium (43). This dual function of RamA as activator and repressor was also observed for the gap, cg3226, sucC, ptsA, ackA, and ald genes (43) and might be due to interaction or competition with a further (regulatory) protein or an effector(s) specific for the one or the other condition.

C. glutamicum is able to use gluconate as the sole carbon and energy source (68). The two functionally redundant transcriptional regulators GntR1 and GntR2 are involved in coordinating the expression of genes involved in gluconate catabolism and glucose uptake (44). Gluconate and glucono-δ-lactone were shown to interfere with binding of GntR1 and GntR2 to their target promoters, leading to derepression of genes involved in gluconate catabolism (gntP, gntK) and reduced expression of ptsG and ptsS, encoding the glucose- and sucrose-specific enzyme II components of the phosphotransferase system (44). In this study, GntR1 and GntR2 were isolated by DNA affinity chromatography with the pck promoter and binding was confirmed by EMSAs with purified GntR1 and GntR2. As indicated by (i) the position of the binding site far upstream of the pck TSS, (ii) the 2-fold-lower pck transcript levels in a ΔgntR1 ΔgntR2 double mutant grown on glucose (∆gntR1 ΔgntR2 strain/WT strain mRNA ratio = 0.52; P < 0.05 [44]), and (iii) the about-2-fold-reduced PEP carboxykinase activity of the ΔgntR1 ΔgntR2 double mutant, both GntR1 and GntR2 function as activators of pck transcription in the absence of gluconate or glucono-δ-lactone. A reduced expression of pck during growth on gluconate compared to glucose might be related to the differences in the uptake and catabolism of this sugar acid. In contrast to glucose, gluconate is imported by the permease GntP and phosphorylated by ATP rather than by the PEP-dependent phosphotransferase system. Catabolism of 6-phosphogluconate occurs via the pentose phosphate pathway rather than via glycolysis. These differences might lead to a higher PEP availability and a reduced demand for PEP carboxykinase activity.

Like RamA, GntR1, and GntR2, the GntR-type transcriptional regulator IolR was enriched by DNA affinity chromatography performed with the pck promoter region. The binding site of IolR within the pck promoter is located far upstream of the TSS, and the about-3-fold-lower mRNA level and the about-2-fold-lower specific PEP carboxykinase activity in the ΔiolR mutant than in the WT indicate that IolR acts as an activator. These results are entirely different from those of a recent study by Hyeon et al. (77). Those authors described Cg0196 (IolR) as a novel regulator which negatively regulated pck expression when the cells were grown on glucose as the carbon source, and thus they designated the Cg0196 protein PckR. Repression of pck was reported to be mediated by binding of PckR to the 18-bp motif CTAAAGTTTTTAACTAGTT, which overlaps the −35 region of the pck promoter. By DNA microarray analysis, Hyeon et al. solely identified glgA (cg1268), encoding a glycosyl transferase (78), as an additional potential target of PckR besides the pck gene. We also tested the interaction of purified IolR with a DNA probe containing the 18-bp motif; however, in EMSAs we could not observe any binding (Fig. 3, fragment P11003). The genomic location of Cg0196 along with the results of our DNA microarray and DNA binding studies clearly show that the primary function of Cg0196 is the repression of genes involved in myo-inositol metabolism. This view is also supported by the fact that a cg0196 deletion mutant showed growth on myo-inositol superior to that of the wild-type strain (Fig. 8C); therefore, we think that the designation of this protein as IolR is appropriate.

The iolR gene is located in the opposite direction immediately upstream of a large cluster of genes (see Fig. S1 in the supplemental material) encoding enzymes essential for myo-inositol metabolism (45). Clustering of iol genes is also common in Clostridium perfringens and B. subtilis, and transcription is negatively regulated by the repressor IolR encoded upstream and in the opposite orientation (79–81). Upon deletion of iolR in C. glutamicum, the mRNA level of the iol genes was increased 50- to 150-fold. We identified several IolR binding sites within the promoters of iolR, iolC, and iolT1 which led to the identification of the consensus motif KGWCHTRACA, which perfectly matches the palindromic binding site of HutC of Pseudomonas putida, the first studied member of all HutC-like GntR family regulators (5’-TTGTG.t.AC AA-3’ [periods represent variable distances between the half-sites of the highly conserved bases of the HutC consensus sequence, and lowercase letters represent nucleotides that are conserved in 80% to 90% of the binding sites used for the generation of the motif sequence]) (82). The location of the IolR binding sites in the direct vicinity of the TSS of iolR, iolC, and iolT1 is in accordance with the observed function of IolR as repressor of the iol gene cluster and the iolT1 gene and suggests that the iolR gene is subject to negative autoregulation.

Comparing the genes showing altered expression during growth of the WT on myo-inositol versus glucose (45) with the genes showing different mRNA levels in the ΔiolR mutant during growth on glucose (this study), a striking subset of common genes can be found. Among those with reduced mRNA levels during growth of the WT on myo-inositol and during growth of the ΔiolR mutant on glucose are mez, encoding malic enzyme, sucC, encoding the β-subunit of succinyl-CoA synthetase, and ptsS, encoding the sucrose-specific IIABC component of the phosphotransferase system, indicating significant carbon source-dependent regulation of genes of the central metabolism. Additionally, upon iolR deletion we observed reduced mRNA levels of genes generally linked to gluconeogenesis, i.e., pck, aceA, aceB, and gapX, which did not exhibit significantly different transcript levels in the previous study performed with myo-inositol or glucose as the substrate (45). However, with the exception of the pck promoter, no direct interaction of IolR with the promoters of mez, aceA, aceB, and gapX could be observed. Thus, it is very unlikely that IolR is directly responsible for the reduced expression of these genes and the reduced growth rate observed for the iolR-deficient strain during growth on gluconeogenic carbon sources. Although gntR2 was found to be significantly upregulated in the ΔiolR mutant and would have been a likely candidate for cross-regulation, no specific interaction of IolR and the gntR2 promoter region was observed.

The small set of genes directly regulated by IolR together with the strongly elevated levels of the iol genes in the ΔiolR mutant indicate that IolR primarily plays a role in derepressing genes nec-
essary for myo-inositol degradation and additionally slightly modifies pck expression for fine-tuning the metabolic flux at the PEP-pyruvate-oxaloacetate node during myo-inositol degradation. This view is also supported by the fact that high PEP carboxykinase activity seems to have a negative impact on myo-inositol utilization (Fig. 8). Assuming that myo-inositol is funneled into the central metabolism of C. glutamicum via the intermediates dihydroxyacetone phosphate and acetyl-CoA, as described for B. subtilis (62), a high level PEP carboxykinase activity could lead to a decreased concentration of oxaloacetate, which is required as an acceptor for acetyl-CoA in the citrate synthase reaction.

In conclusion, we have elucidated the role of IolR as a transcriptional repressor of genes involved in myo-inositol catabolism and showed that PEP carboxykinase synthesis in C. glutamicum is controlled by at least four different transcriptional regulators, RamA, GntR1, GntR2, and IolR. This complex regulation might be the consequence of the high flexibility at the PEP-pyruvate-oxaloacetate node in this organism and the need to tightly adjust the gluconeogenic and anaplerotic reactions to a given condition.

REFERENCES


