Expression of *Corynebacterium glutamicum* glycolytic genes varies with carbon source and growth phase

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A basic pattern of gene expression and of relative expression levels during different growth phases was obtained for *Corynebacterium glutamicum* R grown on different carbon sources. The *gapA-pgk-tpi-ppc* gene cluster was transcribed as a mono- or polycistronic mRNA, depending on the growth phase. The 1.4 kb (*gapA*) and 2.3 kb (*pgk-tip*) mRNAs were expressed in the early through late exponential phases, whereas the 3.7 kb (*gapA-pgk-tip*) and 5.4 kb (*pgk-tip-ppc*) mRNAs were only detected in the mid-exponential phase. All other glycolytic genes except *pps*, *glk* and *pgi* were transcribed as monocistronic mRNAs under all tested conditions. Identification and alignment of the promoter regions of the transcriptional start sites of glycolytic genes revealed strong similarities to the \( \alpha^+ \) consensus promoter sequences of Gram-positive bacteria. All genes involved in glycolysis were coordinately expressed in medium containing glucose. Growth in the presence of glucose gave rise to abundant expression of most glycolytic genes, with the level of *gapA* transcript being the highest. Glucose depletion led to a rapid repression of most glycolytic genes and a corresponding two- to fivefold increased expression of the gluconeogenic genes *pps*, *pck* and *malE*, which are induced by pyruvate, lactate, acetate and/or other organic acids.

**INTRODUCTION**

*Corynebacterium glutamicum*, a nonpathogenic, Gram-positive soil bacterium, is widely used for industrial production of numerous metabolites including amino acids and organic acids (Kinoshita et al., 1957; Liebl, 2005). In addition, it has gained increasing interest as a suitable model organism for the *Corynebacterineae*, a suborder of the Actinomycetes which also includes the medically important pathogenic genera *Corynebacterium* and *Mycobacterium* (Dover et al., 2004; Funke et al., 1997). *C. glutamicum* is able to grow on a variety of carbohydrates and organic acids as sole carbon and energy sources (Kinoshita & Tanaka, 1972; Liebl, 1991). Due to the commercial importance of the amino acids produced by *C. glutamicum*, the catabolic and anabolic pathways leading to these amino acids have been studied in detail (Chassagnole et al., 2003; Dominguez et al., 1998; Eikmanns, 2005; Sahm et al., 1995; Wittmann & De Graaf, 2005; Yokota & Lindley, 2005). Among these pathways, glycolysis not only generates precursors for anabolism but also improves efficiency by substrate-level phosphorylation. The control of activity of glycolytic enzymes has been the subject of intensive studies, and phenotypes of mutants affecting glycolytic genes have been analysed (Eikmanns, 1992; Gourdon et al., 2000; Gubler et al., 1994; Inui et al., 2004; Netzer et al., 2004; Onumasaba et al., 2004; Park et al., 2000; Peters-Wendisch et al., 1998; Riedel et al., 2001; Schreiner et al., 2005). However, limited work has been devoted to the regulation of the *C. glutamicum* glycolytic pathway at the level of gene expression.

Although several studies of genome-wide expression analysis of *C. glutamicum* using DNA microarrays were recently reported (Barreiro et al., 2004; Huser et al., 2003, 2005; Muffler et al., 2002), the only individual glycolytic transcripts of *C. glutamicum* that have been characterized are the *gapA* cluster and *pyc* gene (Peters-Wendisch et al., 1998; Schwinde et al., 1993). More than a decade ago, Schwinde et al. (1993) identified the genetic organization of the *C. glutamicum* *gapA* cluster corresponding to *gapA*.
Expression of C. glutamicum glycolytic genes

gapA-pgk-tpi, pgk-tpi, and pgk-tpi-ppc. They also determined the transcriptional initiation sites, which were located in front of gap and pgk. Recently, analysis of biochemical properties and physiological roles of gapA and gapB of C. glutamicum encoding glyceraldehyde-3-phosphate dehydrogenases (GAPDHs) demonstrated that the enzyme encoded by gapA is active in glycolysis, whereas the gapB product acts in gluconeogenesis (Oumumasa et al., 2004). Transcriptional analyses revealed that aceE (Schreiner et al., 2005), lpd (Schwinde et al., 2001) and pyc (Peters-Wendisch et al., 1998), encoding C. glutamicum pyruvate dehydrogenase, lipoamide dehydrogenase and pyruvate carboxylase respectively, are monocistronic (2.9, 1.6 and 3.5 kb mRNAs respectively). Their respective transcripts are initiated at A, G and A residues located 121, 0 and 55 bp upstream of the respective translational starts. Riedel et al. (2001) reported that the pck gene, encoding phosphoenolpyruvate carboxykinase, was monocistronic (2.0 kb) and that its transcription in lactate-grown cells of C. glutamicum was higher than in glucose-grown cells.

Therefore, important fundamental questions still remain to be answered at the transcriptional level. For example, what is the transcriptional pattern, and how are the genes regulated? In particular, is the expression of the different glycolytic enzymes coordinately regulated by a shared mechanism? Transcriptional analysis of Bacillus subtilis revealed that glycolytic genes encoding enzymes that perform irreversible steps are induced by glucose and other sugars while those enzymes that are required for both glycolysis and gluconeogenesis are synthesized in both the presence and absence of sugars (Ludwig et al., 2001). Studies of genomic expression of seven glycolytic enzymes in Saccharomyces cerevisiae indicated that constitutive synthesis of glycolytic enzymes occurred when cells were grown in the presence of glucose (Hauf et al., 2000).

Glucose controls utilization of alternative carbon sources by regulating gene expression in response to glucose depletion (de Crombrugge et al., 1984; Kolb et al., 1993). Carbon catabolite repression (CCR) is an environment-sensing mechanism used by bacteria for establishing priorities in carbon metabolism. The transcriptional regulator GlxR from C. glutamicum, which is involved in modulating expression of acetate regulation (aceB) and gluconate catabolism (gnt), shares 27% identity in amino acid sequence and the presence of cAMP-binding domain with cAMP receptor protein (CRP) from Escherichia coli (Kim et al., 2004; Letek et al., 2006). Kim et al. (2004) showed the heterologous complementation of E. coli CRP mutants by GlxR protein and suggested a possible interaction of GlxR with other promoters. However, the CCR mechanism in B. subtilis is different and is not based on the presence of a typical CRP able to bind or respond to cAMP (Lorca et al., 2005; Saier et al., 1995). In contrast to E. coli or B. subtilis, no direct evidence was found of a CCR system in C. glutamicum (Bruckner & Tittgemeyer, 2002; Gerstmeir et al., 2003; Stulke & Hillen, 2000). The regulation of C. glutamicum metabolism in the presence of various carbon sources is clearly different from that of E. coli or B. subtilis (Gerstmeir et al., 2003; Hayashi et al., 2002; Muffler et al., 2002). However, the mechanisms of true induction/repression in the glycolysis pathway have not been studied in depth.

The objective of this study was to perform a detailed transcriptional analysis of genes encoding glycolytic enzymes by characterizing the lengths and start sites of the transcripts. The study also focused on the effects of culture conditions (carbon source and growth phase) on mRNA levels of different sets of genes. One such set includes genes encoding glycolytic enzymes.

METHODS

Growth conditions and analytical methods. C. glutamicum R (Kotra et al., 2001) was used as the source of genomic DNA and total RNA. The bacteria were precultured at 33 °C for 8–12 h in a 500 ml flask containing 100 ml nutrient-rich medium composed of (per litre): 2 g yeast extract, 7 g Casamino acids, 2 g (NH₄)₂CO₃, 7 g (NH₄)₂SO₄, 0.5 g KH₂PO₄, 0.5 g K₂HPO₄, 0.5 g MgSO₄.7H₂O, 6 mg FeSO₄.7H₂O, 4.2 mg MnSO₄.H₂O, 0.2 mg biotin, and 0.2 mg thiamine, with 4% (w/v) glucose. Cell growth was monitored by measuring the OD₆₁₀ using a spectrophotometer (DU/640, Beckman Coulter). Samples of a mid-exponential-phase culture were harvested by centrifugation (8000 g, 4 °C, 10 min). The cell pellet was subsequently washed twice with mineral salts medium, which had the same composition as the nutrient-rich medium except for the absence of yeast extract and Casamino acids. The washed cells were resuspended at a final cell concentration corresponding to an OD₆₁₀ of 0.1 in 500 ml mineral salts medium in a 1 l jar fermenter (BMJ101P, Biotec). The sole carbon and energy source was provided by glucose, acetate, pyruvate, lactate, citrate, succinate or malate (200 mM each). The pH was monitored using a pH controller (DT-1023, Biotec) and maintained at 7.6 by supplementing the medium with 5 M NH₃ and 1 M HCl. Cultivation was performed at 33 °C with constant agitation (1000 r.p.m.) and on (1 vol vol⁻¹ min⁻¹). Cell growth was also continuously monitored by measuring the OD₆₁₀. Cultured samples were centrifuged (10 000 g, 4 °C, 3 min), and the supernatants were analysed for sugars and organic acids. The concentrations of glucose were measured by an enzyme electrode glucose sensor (BF-4, Oji Scientific Instruments). Organic acids were quantified by high-performance liquid chromatograph (8020, Tosoh) equipped with an electric conductivity detector.

Nucleic acid isolation. Chromosomal DNA was isolated using a genomic DNA purification kit (Promega) according to the manufacturer’s instructions. Total RNA was extracted from broth cultures using a Qiagen RNeasy kit with the following additional steps. Cultures were added to 2 vols RNAlater solution (Ambion) and centrifuged at 15 000 g for 10 min at 4 °C. The aliquots of cell material were incubated with lysozyme buffer (5 µg ml⁻¹) for 10 min, and immediately resuspended in RLT buffer containing β-mercaptoethanol (Qiagen RNeasy kit). The suspensions were subsequently disrupted using 0.1 mm Zirconia/Silica beads (BioSpec Products) via three 45 s cycles at a speed of 6.5 m s⁻¹ in a Q-BioGene FastPrep FP120 Instrument (Q-BioGene). After removal of the cell debris, RNA was isolated using the Qiagen RNeasy kit following the manufacturer’s instructions. Where necessary, a second DNase digestion was performed with RNase-free DNase (Promega) to completely remove the chromosomal DNA. RNA samples were stored at −80 °C until used.
6-His-tag was removed by treatment with Enterokinase Max 20 mM Tris (pH 7.0) and 1 mM dithiothreitol. Where indicated, the target gene, and was transformed into the template.

**RT-PCR analysis.** RT reactions were performed on total RNA using a commercially available reverse transcription protocol (Promega) with slight modifications to the manufacturer’s recommended protocol. A final volume of 20 µl containing 5 mM MgCl₂, 1 x RT buffer (10 mM Tris/HCl (pH 9.0), 50 mM KCl, 0.1 % (v/v) Triton X-100), 1 mM (each) deoxynucleoside triphosphates, 1 U recombinant RNasin RNase inhibitor, 15 U avian myeloblastosis virus (AMV) reverse transcriptase, 0.25 µM oligonucleotide primer and 10 µM cAMP. The mRNA was quantified with an ABI Prism 7000 RT-PCR system (Applied Biosystems). The sequence analyses including mapping, alignment, promoter searches, annotation and illustration, and all database searches were performed using the Vector NTI software, version 9.1 (Invitrogen) and GENETYX-PC version 7.0 (GENETYX).

**RESULTS**

**Transcription of glycolytic genes.**

In Northern blot analyses, membranes containing RNA were hybridized with intragenic probes derived from the glycolytic genes of *C. glutamicum* (Table 1). RLM-RACE analysis was designed to amplify cDNA only from full-length mRNA, to enable the recovery of the 5’ and 3’ end of the mRNA transcripts. These experiments demonstrated the size of the transcripts and the relative location of the genes. The promoters of *pgs*, *gpm*, *pfk*, *fbα*, *gapB*, *pgm*, *eno*, *aceE*, *lpd*, *pyc*, *pck* and *malE* genes were transcribed as monocistronic transcriptional units with transcripts (protein-coding sequences) of 2.4 (2043), 1.7 (1665), 1.3 (1032), 1.2 (1035), 1.7 (1392), 0.8 (747), 1.4 (1278), 2.9 (2766), 1.5 (1410), 3.6 (3420), 2.0 (1830) and 1.3 kb (1176 bp) respectively (data not shown). The promoters of *pgs*, *gpm*, *pfk*, *fbα*, *gapB*, *pgm*, *eno*, *aceE*, *lpd*, *pyc*, *pck* and *malE* were located 255, 26, 0, 148, 132, 45, 70, 118, 0, 56, 37 and 70 bp directly upstream of the respective start codon (Fig. 1). RLM-RACE mapping analysis of *glk*, *pgi*, *pyk* and *pps* revealed that putative promoter and independent terminator structures were not found upstream and downstream of the respective genes (data not shown). A BLAST search of genomic databases of *C. glutamicum* (Ikeda & Nakagawa, 2003; Kalinowski et al., 2003) indicated that *glk*, *pgi*, *pyk* and *pps* were not clustered with genes encoding other enzymes of the glycolytic pathway in their respective adjacent regions. All glycolytic genes, except *glk*, *pgi*, *pyk* and *pps* along with the gapA-pgk-tpi-ppc cluster, were transcribed as monocistronic mRNAs.
Table 1. Oligonucleotide primers used in this study

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence (5'-3')</th>
</tr>
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<tr>
<td>ptsG-F</td>
<td>GTCCAAACTGACGAGGACAT</td>
</tr>
<tr>
<td>ptsG-5'-In</td>
<td>ATTCCTTACTGAGGAAACGAGA</td>
</tr>
<tr>
<td>ptsG-5'-Out</td>
<td>ATTCCTTACTGAGGAAACGAGA</td>
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<tr>
<td>ptsG-UF</td>
<td>CCGTTTTGCTTTTAAAATAAAAA</td>
</tr>
<tr>
<td>gfp-F</td>
<td>CCAAAAAACGAGGAGTACCTTTT</td>
</tr>
<tr>
<td>gapA-F</td>
<td>GCCAACCTGACGAGGACAT</td>
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<tr>
<td>gapA-5'-In</td>
<td>CCGTGAGGAGGAGTACCTTTT</td>
</tr>
<tr>
<td>gapA-5'-Out</td>
<td>CCGTGAGGAGGAGTACCTTTT</td>
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<tr>
<td>gapA-UF</td>
<td>ACAATTTGCTTTTAAAATAAAAA</td>
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<tr>
<td>gsk-F</td>
<td>CCACAAAACGAGGAGTACCTTTT</td>
</tr>
<tr>
<td>gapB-5'-In</td>
<td>GCCAACCTGACGAGGACAT</td>
</tr>
<tr>
<td>gapB-5'-Out</td>
<td>GCCAACCTGACGAGGACAT</td>
</tr>
<tr>
<td>gapB-UF</td>
<td>GCCAACCTGACGAGGACAT</td>
</tr>
<tr>
<td>gapC-F</td>
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</tr>
<tr>
<td>gapC-5'-In</td>
<td>CCGTTTTGCTTTTAAAATAAAAA</td>
</tr>
<tr>
<td>gapC-5'-Out</td>
<td>CCGTTTTGCTTTTAAAATAAAAA</td>
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<td>gapC-UF</td>
<td>ACAATTTGCTTTTAAAATAAAAA</td>
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<td>pgd-F</td>
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</tr>
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<td>pgm-UF</td>
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</tr>
<tr>
<td>eno-F</td>
<td>GTGGTTTACGAGGAGTACCTTTT</td>
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Results of promoter analyses of all genes involved in glycolysis are summarized in Fig. 1. When the DNA sequences in the promoter regions were scanned for promoter-like −10 and −35 elements, including was used for EMSA.

Expression of C. glutamicum glycolytic genes

Results of promoter analyses of all genes involved in glycolysis are summarized in Fig. 1. When the DNA sequences in the promoter regions were scanned for promoter-like −10 and −35 elements by Vector NTI and GENETYX software, candidate sequences were easily identified. A −10 sequence identical to the consensus −10 (TATAtt) for the primary σ70-type promoters in B. subtilis (σ70) and E. coli (σ70) (Chang et al., 1992; Harley & Reynolds, 1987) is shown. In looking for a pattern, the
The presence of the mono-, bi- and tri-cistronic transcripts suggested the existence of a promoter upstream of gapA and pgk. In agreement with the previous report (Schwinde et al., 1993), the gapA transcriptional start point was

Fig. 1. Determination and alignment of putative C. glutamicum promoters located 10 and 35 bases upstream of the transcription start point (TSP). TSPs were determined by sequencing of 5'–RLM-RACE PCR products, and are indicated by bold letters. The nucleotide numbering begins from the first codon shown on the right. The consensus sequence for the −10 and −35 promoter sequences derived from this alignment is given at the bottom. It consists of nucleotides that are present in any given position in more than 50% of the sequences. Promoter sequence nucleotides that match those of the consensus sequence are shown in black boxes. Asterisks (*) indicate previously mapped promoters: gapA and pgk (Schwinde et al., 1993), aceE (Schreiner et al., 2005), lpd (Schwinde et al., 2001), and pyc (Peters-Wendisch et al., 1998). Their TSPs (except for pgk) are in agreement with the findings of this study.

Transcription of the gapA cluster at different growth phases

The gapA, pgk, tpi and ppc genes form a gene cluster (Fig. 2A). In order to determine gapA cluster transcriptional organization at different growth phases, RNA was isolated from glucose cultures at different growth phases (Fig. 2D). Transcription of operons such as gapA-pgk-tpi and pgk-tpi-ppc was the subject of a previous study (Schwinde et al., 1993). As observed previously, Northern analysis using the gapA probe revealed two mRNAs, of 1.4 kb and 3.7 kb (Fig. 2B-1). Based on transcript sizes and Northern analyses using other probes such as pgk or tpi, the 2.3 kb and 3.7 kb mRNAs were demonstrated to correspond to bicistronic pgk-tpi and tricistronic gapA-pgk-tpi transcripts, respectively, that are terminated at the terminator between tpi and ppc (Fig. 2B-2). However, the larger transcript (3.7 kb) was detectable only as a very faint band in all growth phases except the mid-exponential phase (Fig. 2B). In addition, the 5.4 kb mRNA represents a pgk-tpi-ppc transcript (Fig. 2B-2) of the operon terminating at the terminator structure downstream of ppc. The levels of 16S and 23S rRNA (1.5 and 2.9 kb respectively, Fig. 2B-3) were constant throughout different growth phases, indicating that equal amounts of total RNA were loaded into each well of the gel.

Quantitative RT-PCR analysis showed that the level of expression of the intergenic region between the gapA and pgk genes (Fig. 2C-ii) was significantly decreased compared to gapA or pgk (Fig. 2C-i, C-iii). Transcripts of the intergenic region between the tpi and ppc genes (Fig. 2C-vi) were also at least 10-fold lower than those of the intragenic regions on either tpi or ppc (Fig. 2C-v, C-vii). However, expression levels of an intergenic region between the pgk and tpi genes (Fig. 2C-iv), in which neither a promoter nor a terminator was found, were higher than those of pgk (Fig. 2C-iii) and not significantly different from those of tpi (Fig. 2C-v).

The strongest signal was clearly recognized in the −10 region, providing the consensus sequence TATAAT. Some bases upstream and downstream of the −10 hexamer (underlined) are conserved to GnTATaaTTGGGG. Obvious similarity was observed in regions between −10 and −35 with the consensus sequence TTTnTnT. This consensus sequence was not found in promoters of non-glycolytic genes described previously (Patek et al., 2003). The promoter regions of gapA and gapB genes, encoding GAPDH, were very similar in the −10 hexamer region and in the region between the −10 and transcriptional initiation site. According to the base distribution in the −35 region, the hexamer TTGACa was evaluated as the −35 consensus sequence. In addition, a number of hexamer (underlined) are conserved to GnTATaaTTGGGG. Obvious similarity was observed in regions between −10 and −35 with the consensus sequence TTTnTnT. This consensus sequence was not found in promoters of non-glycolytic genes described previously (Patek et al., 2003). The promoter regions of gapA and gapB genes, encoding GAPDH, were very similar in the −10 hexamer region and in the region between the −10 and transcriptional initiation site. According to the base distribution in the −35 region, the hexamer TTGACa was evaluated as the −35 consensus sequence. In addition, a number of consensus sequence for the 35 promoter regions derived from this alignment is given at the bottom. It consists of nucleotides that are present in any given position in more than 50% of the sequences. Promoter sequence nucleotides that match those of the consensus sequence are shown in black boxes. Asterisks (*) indicate previously mapped promoters: gapA and pgk (Schwinde et al., 1993), aceE (Schreiner et al., 2005), lpd (Schwinde et al., 2001), and pyc (Peters-Wendisch et al., 1998). Their TSPs (except for pgk) are in agreement with the findings of this study.

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identified at a single cytosine located 184 bases upstream of the ATG start codon (Fig. 1). On the other hand, the transcriptional start of pgk was detected at a guanine residue 95 bp upstream of the ATG start site (Fig. 1), indicating that pgk could be transcribed independently of gapA. However, this promoter region was different from that identified in previous experiments (Schwinde et al., 1993), in which the main signal indicated that it was 58 bp upstream of the ATG start codon. In the pgk primer extension analysis by Schwinde et al. (1993), a main signal (−58) and several week signals were detected. The position at 95 bp identified in this study, however, corresponded to neither a main signal nor a weak one. No promoter was observed upstream of tpi or ppc (Figs 1 and 2). Comparison of the DNA sequences of all glycolytic genes between C. glutamicum R and those of C. glutamicum ATCC 13032 (Ikeda & Nakagawa, 2003) registered in the National Center for Biotechnology Information (NCBI) nucleotide databases revealed 97% identity both within the coding region and upstream of the coding region (data not shown). Complete (100%) sequence identity of the promoter region between strain R and ATCC 13032 was observed upstream of pgk, but the location of the promoter was different between strains R and ATCC 13032.

**Relative expression levels of all genes involved in glycolysis at different growth phases**

To determine whether the glycolytic genes are coordinately regulated, changes in the expression levels of several glycolytic genes were monitored during the cultivation of
C. glutamicum on glucose as the sole carbon source. RNA was prepared from the culture at different stages of growth (Figs 2D and 3S) and was subjected to qRT-PCR analyses using specific primer sets for the glycolytic genes (Figs 2 and 3; Table 1). To ensure that the resulting PCR products were amplified from cDNA and not contaminating chromosomal DNA, control experiments were performed in which reverse transcriptase or total RNA was omitted. In these controls, no signal was detected (data not shown).

As shown in Figs 2 and 3, the pattern of induction by glucose at different growth phases was very similar among all glycolytic gene transcripts. In the presence of glucose at the early stages of growth (4 h), all glycolytic gene transcripts were induced, with the level of gapA mRNA being the highest. As the culture entered the exponential growth phase at 6 h, transcription of all glycolytic genes increased simultaneously and reached a maximum level in the mid-exponential growth phase (8 h). After this, the transcription levels decreased while the cells adapted to the late exponential phase. Generally, it appeared that the degree of expression at each growth phase correlated approximately with the glycolytic rate (measured as glucose consumption; Fig. 2E).

Unlike other genes, the expression levels of pps, pck and malE mRNA were maximal when the cells had completely consumed the glucose in the medium at stationary phase (16 to 18 h; Fig. 3S) and then gradually decreased as the cells reached the later stage of stationary phase (Fig. 3P–R). There was a 10-fold increase in pps transcripts associated with simultaneous depletion of glucose in the stationary phase relative to the values observed in the mid-exponential phase (Fig. 3K, P). In addition, there were twofold increases of both pck and malE mRNA in the stationary phase compared with the value measured in exponential phase (Fig. 3N, O, Q, R). The transcripts of the gluconeogenic genes gapB, pps, pck and malE were similar to or less abundant in cells grown in the presence of glucose (Figs 3 and 7). The genes expressed irrespective of the presence of glucose in the medium were confirmed to be constitutively expressed at all growth stages tested.

The observations indicate that most of the glycolytic genes of C. glutamicum are expressed constitutively but are induced to express at higher levels in the presence of glucose after a rather short lag period. Reduced expression was observed during later stages of growth. The close correlation between the time courses of transcription of glycolytic genes supports the view that

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**Fig. 3.** Relative levels of transcripts of glycolytic genes unlinked to the gapA cluster at different growth phases in glucose culture. The times on the graphs in A–O represent the times of the growth curve in Fig. 2D. (P–R) Expression of pps, pck and malE genes at stationary phase. (S) Growth curve. (T) Time course of glucose concentration. The primers, methods and details correspond to those described in Fig. 2.
the glycolytic genes are simultaneously expressed to enable glucose utilization.

**Expression of glycolytic genes before and after glucose starvation**

To find out whether carbon source withdrawal induces or represses glycolytic gene expression, transcript levels were determined in *C. glutamicum* before and after starvation of cells grown on either glucose (Fig. 4I) or acetate (Fig. 4II) as sole carbon and energy sources. Firstly, glucose starvation conditions were created when exponential-phase cells from a glucose-containing medium were washed in medium lacking glucose. Expression of most glycolytic genes in a glucose-grown culture, which were clearly observed in unstarved cells, dramatically decreased when the cells were starved for 2 h (Fig. 4I). In agreement with the previous section (Fig. 3P–R), the genes that were most positively regulated by glucose depletion were gluconeogenic genes such as *pps*, *pck* and *malE* (Fig. 4I-K, O, P). Glucose starvation for about 1.5 h provoked a 10-fold increase of *pps* mRNA (Fig. 4I-K). Expression of *pck* was also strongly stimulated by glucose starvation, increasing 3.5-fold after about 1 h (Fig. 4I-O). After about 0.5 h glucose starvation, transcripts of *gapB* and *malE* were depressed, but later markedly increased until about 2 h starvation. In contrast to the other glycolytic genes, the level of *glk* transcripts increased during the initial 0.5 h of starvation, but decreased during next 1 h (Fig. 4I-B). Upon further starvation (2 h), the transcripts increased (Fig. 4I-B).

We next investigated whether this induction was specific to glucose withdrawal or a general consequence of carbon depletion. Accordingly, cells were grown on acetate and then washed in the absence of the carbon source. The expression pattern of glycolytic genes upon acetate depletion was similar to that upon glucose depletion, the only variation being in the level of expression (Fig. 4). In acetate-starved cells, transcripts of most glycolytic genes were depressed (Fig. 4II). However, a threefold increase of *pps* transcript was observed after 0.5 h starvation (Fig. 4II-K). Furthermore, acetate depletion was associated with an increase in *pyc* and *malE*, but not *pck* expression, after about 0.5 h (Fig. 4II-N, P, O). After 2 h starvation, *glk* and *gapB* transcripts regained their pre-starvation levels (Figs 4II-B, G).

**Glycolytic gene expression response to glucose addition to acetate culture**

To determine whether glucose as a preferred carbon and energy source acts by repressing transcription of glycolytic genes involved in catabolism of subordinate substrates such as acetate, we performed qRT-PCR analysis with specific primer sets. *C. glutamicum* was grown with acetate (200 mM) until the mid-exponential phase of growth.

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**Fig. 4.** Levels of transcripts of glycolytic genes analysed by qRT-PCR before and after carbon starvation at exponential phase. Cells grown in mineral salts medium containing 200 mM glucose (I) or acetate (II) as a carbon and energy source were harvested at exponential phase (OD610 10) by centrifugation at 6000 g for 5 min and washed twice with non-carbon mineral salts medium. Cells were resuspended in non-carbon mineral salts medium to a final OD610 of 1–2 and incubated for 2 h at 33 °C. The primers, methods and details correspond to those described in Fig. 2.

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and then incubated for an additional 2 h after supplementation with 200 mM glucose. Addition of glucose in the presence of acetate strongly induced the expression of most glycolytic genes (Fig. 5). However, the pck transcripts were repressed after the culture had been incubated for 0.5 h (Fig. 5O). It was separately observed that the most favourable inducer of pck mRNA was acetate rather than glucose (see Fig. 7xix). Glucose addition induced the expression of the pps, pyc and malE genes during the initial 1–1.5 h incubation, and then a clear reduction of expression to basal levels was observed after 2 h (Fig. 5K, N, P). In this study, transcription of only few gluconeogenic genes, such as pck, was shown to be repressed when glucose was present but slightly derepressed upon co-utilization of glucose and acetate. This analysis demonstrated that glucose causes a rapid repression of certain genes of gluconeogenic metabolism of C. glutamicum.

**GlxR binding analysis to the promoter region of glycolytic genes**

To examine GlxR’s involvement in controlling the expression of glycolytic genes, we determined the ability of purified GlxR to bind to DNA fragments carrying the promoter region of the glycolytic genes in EMSAs. We searched the nucleotide sequence of C. glutamicum for DNA elements with high levels of identity to the GlxR binding motifs found in the promoter/operator region of glycolytic genes. Computational searches (Vector NTI, Invitrogen) revealed the highest match (59.4 % identity) with the consensus CRP-binding site (CBS: 5’TGTGA-N₆-ACACT-3’) in the upstream regions of fba (gGTcA-N₆-ACACT), gapA (TGTGA-N₆-tCACa) and pck (TGaGA-N₆-ACACa). The nucleotide-level identity of GlxR binding motifs in the aceB promoter region previously described (gGTGA-N₆-tCACACT) (Kim et al., 2004; Letek et al., 2006) was 59.4 %.

As indicated in the previous section (Fig. 5O), expression of pck was found to be subject to glucose repression. Hence we first tested whether GlxR recognizes and binds to the promoter region of the pck gene by doing EMSAs with a 200 bp DNA fragment containing the pck promoter region (Fig. 6, lanes 27 and 28). When cAMP was added to the binding mixture, a clear band shift was observed for pck (Fig. 6, lane 28) as with the positive control aceB (Fig. 6, lane 32). In addition, the DNA fragment containing sequences far upstream of gapA (288–488 bases upstream of the ATG start codon) appeared to bind to GlxR (Fig. 6, lane 10). Interaction of GlxR with the promoter regions of other glycolytic genes, including fba, was not found (Fig. 6).

**Induction of glycolytic genes in response to different carbon sources**

To complete our understanding of the regulation of expression by glycolytic and gluconeogenic carbon sources, we measured the amount of glycolytic gene transcripts in C. glutamicum cells grown in medium containing different carbon substrates as a sole carbon and energy source. The levels of the glycolytic gene transcripts varied considerably with the nature of the carbon source (Fig. 7i–xx). In the presence of glucose, most glycolytic genes (ptsG, gpm, pfk, fba, tpi, gapA, pgk, pgm, pyk, aceE and ppc) were induced to their highest levels (Fig. 7). Gluconeogenic carbon sources such as pyruvate and lactate along with acetate were observed to stimulate the expression of glk, pgi, gapB, pyc, pck and malE, which might be involved in gluconeogenic metabolism (Fig. 7). Succinate was a relatively good inducer for the glycolytic genes pgi, gapB, pps, aceE, pyc...
and malE (Fig. 7). Relatively lower levels of transcripts of all tested genes, except eno and pps, were observed in citrate- or malate-grown cultures. Expression of gapA (Fig. 7viii) was about threefold lower and gapB expression (Fig. 7ix) about twofold higher for gluconeogenic carbon sources than for glucose cultures. These results indicate that glucose generally induced glycolytic enzymes, but acetate, pyruvate and lactate did induce a number of gluconeogenic genes such as gapB, pps, pyc and pck.

**DISCUSSION**

To investigate the expression pattern of glycolytic genes, mRNA was isolated from glucose-grown cells taken at various time points. These studies revealed that these genes were coordinately and specifically upregulated by the presence of glucose in the growth medium, and in all cases, qRT-PCR, RLM-RACE and Northern blot experiments revealed that this regulation was due to transcriptional
control of the respective genes. These results suggest that a common mechanism may exist at the transcriptional level for regulation of glycolytic genes by glucose. An interesting influence of growth phase on the transcriptional organization of the gapA operon was found. The transcription of the gapA-pgk-tpi cluster involved mono-, di- and tricistronic mRNAs (Schwinde et al., 1993). This study indicated that highly regulated synthesis of gapA was found during most of the exponential growth phase, with the level being the highest at the mid-exponential phase (Fig. 2). However, expression of the downstream gapA gene was only significantly regulated during the mid-exponential phase. The present observations indicated that most of the glycolytic genes of C. glutamicum were constitutively expressed in the presence or absence of glucose. Although being a general inducing compound for glycolytic genes, glucose strongly induced expression of most of the glycolytic genes such as gapA (Figs 2, 3 and 7). On the other hand, gapB was induced during growth of C. glutamicum on pyruvate, lactate or acetate (Fig. 7). These results indicated that gapA and gapB in C. glutamicum are inversely regulated, which supported previous reports for B. subtilis (Fillinger et al., 2000; Yoshida et al., 2001). Recently, the involvement of the gapB product of C. glutamicum in gluconeogenesis was confirmed by a gene inactivation study and validation of expression data at the level of enzyme activity (Oumasaba et al., 2004). It is apparent that C. glutamicum needs to perform gluconeogenesis rather than glycolysis in the absence of glucose. In general, the expression profiles obtained from C. glutamicum grown on different carbon sources agree with the direction of intracellular carbon fluxes. In glucose cultures, the phosphotransferase system for glucose uptake and glycolytic genes were highly upregulated compared with those in gluconeogenic cultures. We observed a strong correlation between the apparent glycolytic rate and the degree of induction of glycolytic gene expression.

Another interesting observation was that glucose starvation and/or carbon source utilization appeared to have a major effect on transcriptional control of the genes of the PEP-pyruvate-oxaloacetate node (Pck converts oxaloacetate to PEP, and MalE plus Pps convert malate to PEP), with gluconeogenic carbon sources such as acetate, pyruvate and lactate inducing maximum expression levels. Expression of pps, pck and malE was two- to fivefold higher when glucose was depleted in the medium (Figs 3 and 4). The most striking difference in regulation of pck between glucose-grown and gluconeogenic carbon source-grown C. glutamicum cultures was manifested in the threefold elevated transcription in cells grown on pyruvate and the twofold higher transcription in cells grown on lactate or acetate when compared with cells grown on glucose (Fig. 7). The gluconeogenic function of Pck in C. glutamicum was shown by inactivation experiments (Riedel et al., 2001). In contrast to the wild-type strain, a defined Pck-deficient mutant was unable to grow on substrates that required gluconeogenesis. As a consequence of carbon-source-dependent regulation of malE in C. glutamicum (Figs 3, 4 and 7), malic enzyme may not contribute to gluconeogenesis during growth on carbon sources such as acetate or citrate. During growth of C. glutamicum on pyruvate or lactate, malic enzyme activity was higher than during growth on acetate, and under these conditions malic enzyme has been proposed to have a role in the generation of NADPH on substrates known to have a low flux through the pentose pathway (Gourdon et al., 2000; Netzer et al., 2004). The transcriptional control of pyruvate carboxylase by carbon source was verified by qRT-PCR data, with pyruvate being the best carbon source for achieving maximum expression levels (Fig. 7). Such an effect has been previously observed in C. glutamicum (Koffas et al., 2002; Peters-Wendisch et al., 1998) and other organisms such as S. cerevisiae (Brewster et al., 1994). Our results indicate that certain carbon sources preferentially induce high levels of expression of one gene or a set of genes. These results give a general picture of the potential of glycolytic and/or gluconeogenic gene expression control in response to carbon source and/or growth phase. Furthermore, these results support the hypotheses that there is coordinated expression of some glycolysis and gluconeogenesis genes, that a glucose starvation type of mechanism regulates gluconeogenic gene expression in glucose-deficient cells, and that the presence of accumulated gluconeogenic carbon sources has an effect on carbon utilization by the cells.

The glxR gene was identified as a putative crp (catabolite repression protein) gene in C. glutamicum using the crp homologues of Mycobacterium and Streptomyces (Kim et al., 2004; Letek et al., 2006). EMSA using purified GlxR from C. glutamicum with DNA fragments of gapA or pck showed a specific DNA–GlxR interaction (Fig. 6). However, the physical positions of a CBS upstream of pck and gapA could be a critical determining factor for the modulation of gene expression, as with catabolite responsive element sequences (Miwa & Fujita, 2001). In the case of pck, the hypothetical CBS is located close to the −35 box, and therefore its inherent promoter activity could be lower and could strengthen the repressor effect of the carbon sources (Figs 3Q, 4I-O, 5O and 7ix). The contrary may be true for gapA, where the CBS is located far upstream of the −35 box. Therefore, based on our results for the regulation of the pck genes on different carbon sources and the interaction of GlxR with the promoter region of pck, we can conclude that pck, encoding PEP carboxykinase and therefore in principle capable of catalysing the first step in gluconeogenesis in C. glutamicum, is subject to carbon catabolite regulation mediated by cAMP and GlxR.

The first detailed compilation of promoters of the entire set of genes involved in glycolysis is reported in this paper. Alignment of these promoters revealed that they were similar to the consensus B. subtilis (e3) promoter (Chang et al., 1992). The close similarity of the promoter to the consensus eA sequence among glycolytic genes suggests that these promoters, if not subjected to any regulatory constraints, would act as strong promoters in vivo (Hawley & McClure, 1983). Promoter recognition specificity is
conferred on RNA polymerase by the sigma factor. The sigma factor gene sigA was cloned and the encoded protein exhibited significant homology to the primary sigma factor of related Gram-positive micro-organisms (Haldenwang, 1995; Predich et al., 1995; Sauer et al., 1994). SigA, the principal sigma factor of corynebacteria, was predominantly expressed during the early exponential phase. The mechanism of control of gene transcription in glycolytic genes appeared to be similar, where the sigma factor showed the same promoter recognition properties. More work is needed to identify the factors determining promoter recognition by the RNA polymerase from C. glutamicum. We hope to gain insight into the properties of C. glutamicum promoter regions in order to genetically engineer and to control expression of these genes. Overall, this research should lead to an improved understanding of how glycolytic and gluconeogenic genes are regulated in C. glutamicum.

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