Effect of carbon source availability and growth phase on expression of *Corynebacterium glutamicum* genes involved in the tricarboxylic acid cycle and glyoxylate bypass

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The effect of different carbon sources on the expression of tricarboxylic acid (TCA) cycle genes, along with glyoxylate bypass genes, in *Corynebacterium glutamicum* was determined. All TCA cycle genes were coordinately expressed in medium containing acetate. Growth in the presence of acetate gave rise to abundant expression of most TCA cycle genes, with the level of *gltA* transcript being the highest. However, when the cells entered the stationary phase triggered by acetate exhaustion, all genes were repressed, except *sucCD* and *mdhB*, which were slightly induced. Acetate withdrawal from the growth medium during the exponential phase also led to rapid repression of most TCA cycle genes and a corresponding twofold increase in the expression of *sucCD*, which were strongly induced by citrate and succinate. In addition, glucose depletion during the stationary phase led to a corresponding 8–20-fold induction of the *sucCD*, *aceA* and *aceB* genes. Addition of glucose to acetate medium resulted in about 10-fold induction of *sucCD*. The strong dependence of TCA cycle *sucCD* and glyoxylate bypass *aceA* and *aceB* expression on carbon source availability was confirmed and the regulatory system will be studied precisely.

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INTRODUCTION

Corynebacterium glutamicum, a non-pathogenic, facultative anaerobic Gram-positive soil bacterium, is widely used in the industrial production of numerous metabolites, including amino acids and organic acids (Kinoshita *et al.*, 1957; Liebl, 2005; Nishimura *et al.*, 2007). In contrast to closely related, medically important pathogenic species, such as *Corynebacterium diphtheriae* and *Mycobacterium tuberculosis*, *C. glutamicum* is generally recognized as a non-hazardous organism (Dover *et al.*, 2004; Funke *et al.*, 1997). Furthermore, this organism has gained increasing interest as a suitable model organism for high-G+C-content Gram-positive bacteria in general and for *Corynebacterineae*, a suborder of the Actinomycetes, in particular.

One of the central metabolic pathways in *C. glutamicum* and in other aerobic bacteria is the tricarboxylic acid (TCA) cycle, which is responsible for the complete

oxidation of acetyl-CoA derived from various substrates and for the provision of precursors for amino acid biosynthesis. TCA cycle intermediates are commonly used by other metabolic reactions in a wide variety of cell types. Due to the commercial importance of the amino acids and organic acids produced by *C. glutamicum*, the control of TCA cycle enzyme activities has been the subject of intensive studies, and the phenotypes of mutants affecting TCA cycle genes have been analysed (Eikmanns *et al.*, 1994, 1995; Molenaar *et al.*, 1998, 2000; Usuda *et al.*, 1996; Wittmann & De Graaf, 2005). However, limited work has been devoted to the regulation of the expression of *C. glutamicum* TCA cycle genes in response to different growth phases and carbon sources. Recently we have reported the transcription of *C. glutamicum* genes involved in the TCA cycle and glyoxylate bypass (Han *et al.*, 2008). In fact, aspects of the regulation of only three TCA cycle enzymes in *C. glutamicum*, citrate synthase (encoded by *gltA*), aconitase (encoded by *acn*), and isocitrate dehydrogenase (encoded by *icd*), have been described (Eikmanns *et al.*, 1994, 1995; Krug *et al.*, 2005).

C. glutamicum can use a variety of carbohydrates and organic acids as carbon and energy sources (Kinoshita & Tanaka, 1972;

Abbreviations: CBS, CRP-binding site; CCR, carbon catabolite repression; CRP, cAMP receptor protein; DO, dissolved oxygen; EMSA, electrophoretic mobility shift assay; ORP, oxidation-reduction potentials; qRT-PCR, quantitative real-time reverse transcription PCR; RLM-RACE, RNA ligase-mediated rapid amplification of cDNA ends; TCA cycle, tricarboxylic acid cycle.

Liebl, 1991). As carbon sources, glucose and acetate have been shown to serve as substrates for amino acid production by *C. glutamicum*. The genes required for the utilization of a specific carbon source are, in most cases, expressed only if that carbon source is present in the medium and if preferred carbon sources, such as acetate and glucose, are absent from the growth medium. These two processes in *C. glutamicum* are referred to as substrate induction and carbon catabolite repression (CCR), respectively (Letek *et al.*, 2006). CCR is an environment-sensing mechanism used by bacteria to establish priorities in carbon metabolism. Transcriptional regulation is modulated by the level of cAMP and cAMP receptor protein (Crp), a global transcriptional regulator. It has been reported that the activities of *Bacillus subtilis* TCA cycle enzymes, particularly citrate synthase and aconitase, are severely depressed when glucose is supplied (Cox & Hanson, 1968; Hanson & Cox, 1967; Ohne, 1974) and that the dyad symmetry sequence located in the aconitase gene promoter region is the target for CCR (Fouet *et al.*, 1990; Fouet & Sonenshein, 1990; Jin & Sonenshein, 1994; Rosenkrantz *et al.*, 1985). Expression of *Escherichia coli* aconitase genes is also subject to CCR mediated by Crp (Cunningham *et al.*, 1997; Gruer & Guest, 1994; Gruer *et al.*, 1997). The transcriptional regulator GlxR contains a domain with similarity to the cAMP-binding motifs of Crp from *E. coli* and, in fact, cAMP is essential for binding of GlxR to the *aceA/aceB* intergenic region in *C. glutamicum* (Kim *et al.*, 2004; Letek *et al.*, 2006). The regulation of *C. glutamicum* metabolism in the presence of various carbon sources clearly differs from that of *E. coli* or *B. subtilis* (Gerstmeir *et al.*, 2003; Hayashi *et al.*, 2002; Letek *et al.*, 2006; Muffler *et al.*, 2002). No direct evidence has been found of a CCR system in *C. glutamicum* (Bruckner & Titgemeyer, 2002; Gerstmeir *et al.*, 2003; Han *et al.*, 2007), although a CCR-like phenomenon has been reported from cells grown on medium containing glucose with either glutamate (Gerstmeir *et al.*, 2003; Kramer *et al.*, 1990; Kronmeyer *et al.*, 1995) or ethanol (Arndt & Eikmanns, 2007; Kotrbova-Kozak *et al.*, 2007). However, mechanisms of true induction and repression in the TCA cycle have not been studied in depth.

To add to our previous findings on transcription of TCA cycle genes (Han *et al.*, 2008) and to define the regulatory networks responsive to the TCA cycle, we performed a detailed transcriptional analysis of TCA cycle genes in various growth phases and with various carbon sources to assess their effects on mRNA levels of different sets of genes. We uncovered a unique pattern of gene expression that alternates between elevated and diminished levels of contiguous pairs of TCA cycle genes in response to culture conditions. The data indicate that the TCA cycle is regulated by the availability of carbon sources such as acetate and glucose, and that CCR protein might be involved in this global regulation.

METHODS

Growth conditions and analytical methods. *C. glutamicum* R (Kinoshita *et al.*, 1957; Liebl, 2005; Yukawa *et al.*, 2007) was used as

the source of genomic DNA and total RNA. The organism was cultured at 33 °C with constant agitation (1000 r.p.m.) and aeration (1 volume per volume min⁻¹) in 500 ml mineral salts medium described previously (Han *et al.*, 2007) in a 1-litre jar fermenter (BMJ01PI, Biot). The sole carbon and energy source was provided by glucose, acetate, pyruvate, lactate, citrate, succinate or malate (200 mM each). Cells were subcultured in medium containing the same carbon source before they were added to fresh medium as inoculants. The pH was monitored using a pH controller (DT-1023, Biot) and maintained at 7.6 by supplementing the medium with 5 M NH₃ and 1 M HCl. Cell growth was monitored continuously by measuring OD₆₁₀. Cultured samples were centrifuged (10 000 g, 4 °C, 3 min), and the supernatants were analysed for sugars and organic acids. Organic acids were quantified by high-performance liquid chromatography (8020 chromatograph, Tosoh Corporation) equipped with a UV detector and conductivity meter, and a TSKgel OApak-A column (Tosoh Corporation) operating at 40 °C with a 0.75 mM H₂SO₄ mobile phase at a flow rate of 1.0 ml min⁻¹. The concentrations of glucose were measured by an enzyme electrode glucose sensor (BF-4, Oji Scientific Instruments).

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 (Qiagen) as previously described (Han *et al.*, 2007).

Quantitative real-time reverse transcription PCR (qRT-PCR). mRNA was quantified with an ABI Prism 7000 RT-PCR system (Applied Biosystems). A 50 ng total RNA sample was used per RT-PCR with each primer pair using a QuantiTect SYBR Green RT-PCR kit (Qiagen) according to the manufacturer's instructions. Negative controls with no reverse transcriptase were included with each RNA sample to rule out DNA contamination. DNA templates without reverse transcriptase were included as positive controls. Amplicons were run on a 2% (w/v) agarose gel to verify that only a single band was produced. The target gene transcripts were normalized to the reference gene transcript (16S rRNA) from the same RNA sample. Each gene was analysed using RNA isolated from three independent samples. The comparative threshold cycle (C_t) method (Applied Biosystems) was used to quantify relative expression, with a C_t being defined as the cycle at which the reporter fluorescence is distinguishable from the background in the extension phase of the PCR. The C_t values were computed as described previously (Bustin, 2002; Winer *et al.*, 1999). The relative change in transcription rate was determined as 2^{-[ΔΔC_t]}, with ΔΔC_t equal to the difference of the measured crossing points for the test and the control condition.

Purification of GlxR. Primers glxR-TF (5'-caccGTGGAAGGTG-TACAGGAGAT) and glxR-TR (5'-TCGAGCGCGACGTGCCAAAT) were used to amplify the *glxR* gene from *C. glutamicum*. The amplified *glxR* gene was cloned into pET100/D-TOPO, which allows translational fusion between a C-terminal 6-His tag and the target gene, and then transformed into *E. coli* BL21 Star for overexpression (Invitrogen). The enzyme was purified using a nickel-nitrilotriacetic acid column (Invitrogen) and dialysed against 20 mM Tris (pH 7.0) and 1 mM dithiothreitol. Where indicated, the 6-His tag was removed by treatment with EnterokinaseMax (Invitrogen) after purification. Samples taken at different stages of the purification process and the purified protein were analysed by SDS-10% PAGE as described by Laemmli (1970). Samples mixed with Tris/glycine SDS sample buffer (Invitrogen) (1:1, v/v) were denatured at 100 °C for 5 min and applied to a 10% polyacrylamide gel using a mini-Protein II system (Bio-Rad). Gels were stained with colloidal Coomassie Blue stain (Genomic Solutions) to visualize proteins.

Electrophoretic mobility shift assay (EMSA). To test the binding of GlxR to putative target promoters, purified GlxR protein (50–500 ng) was mixed with DNA fragments (mostly 200 bp; final concentration, 10–100 ng) in a total volume of 10 μ l. The binding buffer (pH 7.4) contained 150 mM KCl, 0.1 mM dithiothreitol, 0.1 mM EDTA, 10 mM Tris and 0.2 mM cAMP. The reaction mixture was incubated at room temperature for 30 min and then loaded onto a 5% native polyacrylamide gel. Electrophoresis was performed at room temperature and 200 V using 0.5 $\times$ TBE buffer (44.5 mM Tris base, 44.5 mM boric acid and 1 mM EDTA) supplemented with 0.2 mM cAMP. The gels were subsequently stained using the EMSA kit (Invitrogen) as described by the manufacturer and photographed. All PCR products used in EMSA were purified with the PCR purification kit (Qiagen) and eluted in distilled water.

RESULTS

Effect of growth phase on expression of genes involved in the TCA cycle and glyoxylate bypass

To verify whether the TCA cycle genes are coordinately regulated, changes in the expression levels of several TCA cycle genes were monitored during cultivation of *C. glutamicum* with either acetate (Fig. 1) or glucose (Fig. 2) as the sole carbon and energy source. RNA was prepared from the cultures at different growth stages (Figs 1N and 2N). The RNA was subjected to qRT-PCR analyses using specific primer sets for the TCA cycle genes (Table 1). To

ensure that the resulting PCR products were amplified from cDNA, instead of from contaminating chromosomal DNA, reverse transcriptase or total RNA was omitted in control experiments. In these controls, no signal was detected (data not shown).

The pattern of induction by acetate at different growth phases was very similar among all TCA cycle gene transcripts (Fig. 1). Although the errors in expression level were wide, the levels of initial (0 h) transcription of *gltA* and *sucCD* were considerably higher compared with those of the other TCA genes. In the early stages of growth (1 h), most TCA cycle gene transcripts were induced in the presence of acetate; the levels of *gltA* and *aceA* mRNA were highest in TCA cycle and glyoxylate bypass, respectively. As the culture entered the mid-exponential growth phase at 2–3 h, the transcription of most TCA cycle genes increased simultaneously and reached maximum levels. After this, the transcription level decreased as the cells adapted to the late exponential phase. Generally, the degree of expression at each growth phase, especially stationary phase, appeared to correlate with the acetate consumption rate (Fig. 1N and O). Unlike that of other genes, the expression levels of *sucCD* and *mdhB* mRNA were maximal when the cells had completely consumed the acetate in the medium at stationary phase and then gradually decreased as the cells reached the late stationary phase (Fig. 1F and J). There was a 12-fold increase in *sucCD* transcripts associated with

Fig. 1. Relative levels of transcripts of TCA cycle genes at different growth phases in acetate culture. Transcription levels refer to fold changes. Transcriptional expression (A–M) was determined by quantitative RT-PCR with RNA (25 ng) isolated from *C. glutamicum* cultivated on mineral salts medium containing 200 mM acetate as the sole carbon and energy source. Primers specific for the TCA cycle genes were used to amplify fragments by PCR (see Table 1). (N) *C. glutamicum* growth curve (OD_{610}). (O) Concentration of acetate (mM). The pH was maintained at 7.6. The error bars represent the standard deviation of three independent experiments. The vertical dashed lines in panels F and J indicate an incubation time of 7 h; data to the right of these lines are plotted on the scales to the right. In this and subsequent figures, the data plotted are means $\pm$ SD ($n=3$).

Fig. 2. Relative levels of transcripts of TCA cycle genes at different growth phases in glucose culture. The primers, methods, and other details correspond to those described in Fig. 1. (O) Concentration (mM) of glucose (●), acetate* (■), pyruvate* (◆), lactate* (▲, 10×) and malate* (×) (* indicates use of y-axis scale on right of graph).

simultaneous depletion of acetate in the stationary phase (9 h) relative to the values observed in the mid-exponential phase (3 h) (Fig. 1F). In addition, there was a sevenfold increase in *mdhB* mRNA in the stationary phase (9 h) compared with the value measured in the exponential phase (2 h) (Fig. 1J). These observations indicate that most TCA cycle genes of *C. glutamicum* are constitutively expressed but are induced to express at higher levels in the presence of acetate after a rather short lag period. Reduced expression was observed at later growth stages. The close correlation between the transcription time-courses of TCA cycle genes suggests that these genes are expressed simultaneously to enable acetate utilization.

To determine whether glucose activates TCA cycle gene transcription, RNA was isolated from *C. glutamicum* at different growth phases in the presence of glucose. The levels of most TCA cycle gene transcripts increased simultaneously from the mid-exponential phase to the early stationary phase, and decreased during the mid-stationary phase (Fig. 2). The pattern of glucose induction was similar among most TCA cycle genes. However, the mRNA levels of *sucCD*, *mdh*, *mdhB*, *aceA* and *aceB* increased simultaneously when the cells had completely consumed the glucose in the medium during the stationary phase (Fig. 2). In particular, the highest expression of the *sucCD* operon, which encodes succinyl-CoA synthetase, was observed when the cells were starved, regardless of whether the cells were grown on acetate or glucose (Figs 1F and 2F). In addition, glucose depletion most strongly induced *aceA* and *aceB* (Fig. 2L and M). The levels of *aceA* and *aceB* transcripts in acetate-grown cells were up to

30-fold higher than those in glucose-grown cells (Figs 1L, and M, 2L and 2M). These data also indicated that either acetate (approx. 30 mM) or lactate (approx. 400 mM) accumulated in the medium at 16 h induced these genes, when the glucose has been completely consumed (Fig. 2O). However, these accumulated carbon sources may not affect the transcripts of genes strongly when glucose remains abundant (Gerstmeir *et al.*, 2003). Interestingly, glucose induced high levels of expression of TCA cycle genes during the early stationary phase, whereas the transcript levels were highest at the mid-exponential phase in acetate medium (Figs 1 and 2).

Effect of carbon depletion on expression of TCA cycle genes

To determine whether carbon source withdrawal induces or represses TCA cycle gene expression, transcript levels were determined in *C. glutamicum* before and after starvation of cells grown on acetate (Fig. 3I) or glucose (Fig. 3II) as the sole carbon and energy source. First of all, a carbon-starvation condition was created when exponential-phase cells from medium containing either acetate or glucose were washed with the same medium lacking any carbon source. Expression of most TCA cycle genes, except *sucCD*, *mdh* and *mdhB*, which were clearly observed in acetate-fed cells, dramatically decreased when the cells were starved for 2 h (Fig. 3I). In agreement with the previous section, the genes that were most positively regulated by acetate depletion were *sucCD* and *mdhB* (Figs 3I F and J). Acetate starvation for about 1.5 h provoked an

Table 1. Oligonucleotide primers used in this study

Gene/ operon	Protein encoded/references	Primer name*	Sense strand (5'–3')	Antisense strand (5'–3')
<i>gltA</i>	Citrate synthase Eikmanns <i>et al.</i> (1994)	<i>gltA</i> -RT <i>gltA</i> -ES	TGTTTGAAAGGGATATCGTG GGCGAATTGCATGGGGAAAG	AGTCTCAGACAGCATCTTGC ACCAACTGGGATGCGCCAAG
<i>acn</i>	Aconitate hydratase Krug <i>et al.</i> (2005)	<i>acn</i> -RT <i>acn</i> -ES	GCACCCCTTGAAAGTTGGCGAC CTAAAAACCTTCATTAGTGTCG	CGGTGCGAAGAAGGTTCTCT TTTTTAGGGTGAGATTTTCGC
<i>icd</i>	Isocitrate dehydrogenase Eikmanns <i>et al.</i> (1995)	<i>icd</i> -RT <i>icd</i> -ES	AAGCACCGTTGCTCGCGACCTA TCTTATGGGAAACTCCCGAG	CGGCCAGCCAGTGAAATGTC TGGGCTCTTTTGTTTCAGCTA
<i>odhA</i>	2-oxoglutarate dehydrogenase E1 Usuda <i>et al.</i> (1996)	<i>odhA</i> -RT <i>odhA</i> -ES	ATGCTACAACCTGGGGCTTAG CGCCGAACATCATCGAGCCTT	CTCGCCTCTTTTCCTTGCTT GCGTAGGTGCCGTTACACAGA
<i>sucB</i>	2-oxoglutarate dehydrogenase E2 Kalinowski <i>et al.</i> (2003)	<i>sucB</i> -RT <i>sucB</i> -ES	ATGGCGTTCTCCGTAGAGAT CGACCAAATAAAATTGAACAC	GGCTCATCTACCTCAACAGT GTGTCGTTAAACCGAAACT
<i>sucCD</i>	Succinyl-CoA synthetase Kalinowski <i>et al.</i> (2003)	<i>sucCD</i> -RT <i>sucCD</i> -ES	TGTGCCAGTGTTGAAGGGAA TCGACTTCTTGCTTACGACT	ACCTTGACCTGAGCCTTGAC CCCATCAAAGTAAGTGTTC
<i>sdhCAB</i>	Succinate dehydrogenase Kalinowski <i>et al.</i> (2003)	<i>sdhCAB</i> -RT <i>sdhCAB</i> -ES	ATGAGCACTCACTCTGAAAC CATCAGGCTCCCGCGTGGT	TATTTCCACATGTCTTTGGT GGCACCTCCAGTGTCGTGTG
<i>fum</i>	Fumarate hydratase Genda <i>et al.</i> (2006)	<i>fum</i> -RT <i>fum</i> -ES	ATGACCGAGCAGGAATTCCG TACGGAGCGAAGATCAGTTG	GATTCCAGACCACGACCAGA TACGGCTGTCACAAGCTTAC
<i>mdh</i>	Malate dehydrogenase Genda <i>et al.</i> (2003); Molenaar <i>et al.</i> (2000)	<i>mdh</i> -RT <i>mdh</i> -ES	ATGAATTCCCCCGCAGAACGT GTCTAGGTTTTCGGCATCTC	TCAGCTCAACAGGGGTTTCA CCTCTAAGAACCCATGCTAT
<i>mdhB</i>	Malate/L-lactate dehydrogenase Kalinowski <i>et al.</i> (2003)	<i>mdhB</i> -RT <i>mdhB</i> -ES	AAGTCACTGTCAACGCCCAA CCGCGTTGATTATGCTGAT	CACCAAACATATCAGCGACAA TCCCTCATTTTCGATTTAGGT
<i>mgo</i>	Malate: quinine oxidoreductase Molenaar <i>et al.</i> (1998)	<i>mgo</i> -RT <i>mgo</i> -ES	ATGTCAGATTCCCCGAAGAA TCATATGTATAACGATAGGTGCG	AGACGATCTGAGTCCAGCTT ATCTCATAAATGATCGCCAG
<i>aceA</i>	Isocitrate lyase Gerstmeir <i>et al.</i> (2003); Reinscheid <i>et al.</i> (1994b)	<i>aceA</i> -RT <i>aceA</i> -ES	ATGTCAAACGTTGGAAAGCC AAAAGGGGTTTGCGAACTTT	TGTGCTCCTCGATGACGGAA ACTGTCACTGTTTCGACGTG
<i>aceB</i>	Malate synthase Gerstmeir <i>et al.</i> (2003); Reinscheid <i>et al.</i> (1994a)	<i>aceB</i> -RT <i>aceB</i> -ES	TGACTGAACAGGAAGTGTGTC TTGGTGCCTCAATCGGTTTG	AGGGAGTACCGCTTCGGTTA ATCTAGGTGCCGCTCTTCTT
		<i>aceAB</i> -ES	ACTGTCACGTGTTTCGACGTG	ATCTAGGTGCCGCTCTTCTT
16S rRNA	16S rRNA Yukawa <i>et al.</i> (2007)	16S rRNA-1 16S rRNA-2	AGAGTTTGATCCTGGCTCA ACGTTCCCGGGCCTTGTACA	CGGTACCTTGTACGAC ACGTGTTACTACCCGTTTCG

*Primers -RT, used for qRT-PCR; -ES, used for EMSA.

approximately threefold increase in *sucCD* mRNA (Fig. 3I F). Expression of *mdhB* was also stimulated by acetate starvation, increasing 1.5-fold after 2 h (Fig. 3I J). After about 1 h acetate starvation, transcripts of *mdh* were depressed, but later markedly increased until about 2 h of starvation. The levels of *sdhCAB* transcripts increased during the initial 0.5 h of starvation, but decreased during the next 1 h (Fig. 3I G). We next explored whether this induction was specific to acetate withdrawal or was a general consequence of carbon depletion. Accordingly, cells were grown on fermentable glucose and then washed in medium not containing the carbon source. Upon glucose depletion, the expression pattern of TCA cycle genes differed from that upon acetate depletion (Fig. 3). In glucose-starved cells, transcripts of all TCA cycle genes were depressed during the initial 0.5 h of starvation, but increased during the next 1 h (Fig. 3II), with the only variation being in the level of expression. Upon further starvation (2 h), most transcripts decreased (Fig. 3II).

However, up to threefold increases of *mdh*, *mdhB* and *mgo* transcripts were observed after 2 h starvation (Fig. 3II K).

Effect of glucose supplementation of acetate cultures on expression of TCA cycle genes

To determine whether glucose acts by repressing transcription of TCA cycle genes involved in the catabolism of acetate, we performed qRT-PCR analysis with specific primer sets. *C. glutamicum* was grown with acetate (200 mM) until the early exponential phase and then incubated for an additional 2 h after supplementation with 200 mM glucose. Addition of glucose in the presence of acetate simultaneously induced the expression of 8 out of 13 genes: *gltA*, *acn*, *odhA*, *sucCD*, *sdhCAB*, *mgo*, *aceA* and *aceB* (Fig. 4). However, most transcripts, except *sucCD*, were repressed after the culture had been incubated for 1 h. These transcripts were slightly induced again during the next 1.5 h incubation, but were almost completely

Fig. 3. Level of transcripts of TCA cycle genes analysed by qRT-PCR before and after carbon starvation at exponential phase. Cells grown in mineral salts medium containing 200 mM acetate (I) or glucose (II) as a carbon and energy source were harvested at exponential phase (OD_{610} 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 OD_{610} of 1–2 and incubated for 2 h at 33 °C. The primers, methods, and other details correspond to those described in Fig. 1. In panel L, the left scale refers to *aceA* and the right scale to *aceB*.

repressed upon further incubation (2 h) (Fig. 4). The expression of the *sucCD* operon was most strongly regulated by glucose supplementation (Fig. 4F). Glucose addition provoked about a 10-fold increase in *sucCD* transcripts during the 1–1.5 h incubation, and the expression was then clearly reduced to basal level after 2 h incubation. In contrast to other genes, the levels of *mdh* and *mdhB* transcripts increased during the 2 h incubation (Figs 4I and J). Glucose addition was associated with decreased *icd*, *sucB* and *fum* expression, after about 0.5–1 h (Figs 4C, E and H). After 1.5 h incubation, *sucB* transcripts regained pre-incubation levels (Fig. 4E), while *icd* and *fum* transcripts were slightly induced (Fig. 4C and H). However, levels of all of these transcripts were depressed after further incubation (2 h) (Fig. 4). In this study, transcription of most TCA cycle genes was induced when glucose was present with acetate for 0.5–1.5 h, but was completely repressed after further incubation with co-utilization of glucose and acetate. This analysis demonstrates that glucose represses most genes of TCA cycle metabolism in *C. glutamicum*.

Analysis of GlxR binding to the promoter region of TCA cycle genes

To identify GlxR's involvement in controlling the expression of TCA cycle genes, we assessed the ability of purified GlxR to bind to DNA fragments carrying the promoter region of the TCA cycle genes by EMSA. We searched the nucleotide sequence of *C. glutamicum* for DNA elements with high levels of identity to the GlxR-binding motifs in the promoter/operator regions of TCA cycle genes. A computational search (Vector NTI, Invitrogen) allowing the highest match (up to 3 mismatches out of 10) for each gene with the consensus Crp-binding site (CBS; 5'-TGTGA-N₆-ACACT-3') revealed 13 sites in or around the promoter regions of TCA cycle genes (Fig. 5A and C). Out of the 13 sites, 6 exhibited high similarity (up to two mismatches) with the CBS consensus motif in the upstream regions of *gltA*, *acn*, *sucCD*, *sdhCAB*, *aceA* and *aceB* (Fig. 5A), with *sucC* having the highest homology with the consensus motif (no mismatch). The nucleotides of the GlxR-binding motifs in the *aceB* promoter region have

Fig. 4. Time-course of transcription during growth of *C. glutamicum* on acetate medium with subsequent glucose supplementation, as determined by qRT-PCR. Total RNA (50 ng) was isolated from cells grown on 200 mM acetate medium supplemented (0 h) with 200 mM glucose. The primers, methods and other details correspond to those described in Fig. 1. In panel L, the left scale refers to *aceA* and the right scale to *aceB*.

been described previously (Kim *et al.*, 2004; Letek *et al.*, 2006), with two mismatches (Fig. 5A).

His-tagged GlxR protein was purified from *E. coli* and analysed by SDS-PAGE (Fig. 5B); its molecular mass coincides with the expected size (25 kDa) deduced from the gene sequence. Treating His-tagged GlxR with EnterokinaseMax (Invitrogen) to remove the His tag had no effect on binding (data not shown). In agreement with previous reports (Kim *et al.*, 2004; Letek *et al.*, 2006), the presence of retarded bands was directly dependent on the presence of cAMP in the reaction mixture. No retarded bands were observed in the absence of cAMP or His-tagged GlxR (data not shown). Different amounts of purified GlxR protein (50–500 ng) and DNA fragments (10–100 ng) were used to optimize the binding reaction (data not shown).

As indicated in the previous sections, expression of *sucCD* was subject to tight carbon regulation. Hence, we first tested whether GlxR recognizes and binds to the promoter region of the *sucCD* operon by performing mobility shift assays with a 200 bp DNA fragment containing the *sucC* promoter region (Fig. 5D). When cAMP was added to the

binding mixture, a clear band shift was observed for *sucC* as it was with the positive control *aceB* (Fig. 5D). In addition, DNA fragments containing the sequences upstream of *gltA*, *acn*, *sdhCAB*, *aceA* and *aceB* (Fig. 5C) appear to bind with GlxR (Fig. 5D). When the DNA fragment of the sequence upstream of *fum* was used, clear retardation was observed (Fig. 5D), although all possible binding sites upstream of *fum* exhibited relatively low similarity (three mismatches) with the consensus motif.

Induction of TCA cycle genes in response to various carbon sources

To further our understanding of gene expression regulation by various carbon sources, we measured the amount of TCA cycle gene transcripts in *C. glutamicum* cells grown in medium containing different carbon substrates as the sole carbon and energy source. Cells were subcultured in the medium containing the same carbon source before they were added to fresh medium as inocula (OD_{610} 1.8–2.0). After 1 h incubation, cell growth in the medium was similar with the various carbon sources tested (Fig. 6xiv), although glucose culture showed the highest cell growth. The levels of the TCA cycle gene transcripts varied considerably depending on the nature of the carbon source (Fig. 6i–xiii). Some carbon sources clearly induced stronger expression of certain sets of genes. Acetate caused the expression of all the TCA cycle genes, including *gltA*, *acn*, *fum*, *mdh*, *mqo*, *aceA* and *aceB*, most strongly. In the presence of other non-fermentable carbon sources such as pyruvate or lactate, most TCA cycle genes (*acn*, *icd*, *odhA*, *sucB*, *fum*, *mdh*, *mdhB*, *mqo*, *aceA* and *aceB*) were induced to their highest levels (Fig. 6). With non-fermentable carbon along with acetate, the expression of *aceA* and *aceB* was particularly high (Fig. 6xii and xiii). Significant expression of some TCA cycle genes was observed with glucose, but *acn*, *sucCD*, *sdhCAB*, *aceA* and *aceB* had the lowest expression levels (Fig. 6vi, vii, xii and xiii). TCA intermediates, such as citrate and succinate, stimulated the expression of most of the genes, including *gltA*, *acn*, *icd*, *odhA*, *sucB*, *sucCD*, *sdhCAB*, *fum*, *mdh*, *mdhB* and *mqo*, although less effectively (Fig. 6). The *sucCD* operon of *C. glutamicum* showed a markedly different pattern of expression from that of other TCA cycle genes. On succinate-containing medium, mRNA levels of *sucCD* remained at least twofold higher than on acetate, glucose, pyruvate and lactate medium (Fig. 6vi–vii). Similar to *sucCD*, succinate induction of *sdhCAB* was observed, and citrate allowed the most significant expression of the *sdhCAB* gene (Fig. 6vii). Low expression levels of most TCA cycle genes were detected on medium containing malate (Fig. 6).

DISCUSSION

The TCA cycle is the major catabolic pathway of *C. glutamicum* for generation of carbon backbones and energy. The main objective of this study was to examine

Fig. 5. EMSA with GlxR and TCA cycle genes. (A) Hypothetical presence of CBS consensus motifs in the promoter region. The bold capitalized letters indicate nucleotides conserved with the consensus sequence for catabolite repression shown above. (B) SDS-PAGE of GlxR. Cell extracts of uninduced *E. coli* (lane 1) and induced *E. coli* (lane 2); elution fractions containing His-tagged GlxR protein (lane 3); elution fraction containing GlxR without His-tag (lane 4). Molecular mass markers (M) are indicated. (C) Schematic representation of the promoter region. The locations of CBS (C) consensus motifs are indicated by the black boxes. The transcription start points and the promoters (−10 and −35) had been determined previously (Han *et al.*, 2008) and are indicated by the bent arrows and white boxes, respectively. Locations of the DNA fragments used in EMSA obtained by PCR amplification using specific primers are indicated by double headed arrows. (D) EMSA of purified GlxR protein (about 200 ng) incubated with DNA fragments (about 20 ng) containing the promoter region in the binding buffer including 0.2 mM cAMP; the mixtures were analysed by 5% PAGE. Lanes labelled '−' contain only the DNA fragment in the reaction mixture; lanes labelled '+' contain purified GlxR protein with DNA fragments. Arrows indicate shifted bands.

in detail the transcriptional regulation of the TCA cycle in *C. glutamicum*. To investigate the general and specific regulatory patterns in the expression of TCA cycle genes, mRNA was isolated from acetate- or glucose-grown cells taken at various time points. These studies revealed that the TCA cycle genes were coordinately and specifically regulated by acetate and/or glucose in the growth medium, and that this regulation was due to transcriptional control of the respective genes. For instance, acetate induced the transcription of most TCA cycle genes and the time-course

of gene transcription was approximately the same in all cases. These data clearly show that all genes involved in corynebacterial TCA cycle are coordinately expressed at the transcriptional level in different growth phases by various substrates such as acetate, glucose, pyruvate and lactose. In general, the expression profiles obtained from *C. glutamicum* grown in acetate and glucose agree with the direction of intracellular carbon flux. In acetate cultures, genes involved in the TCA cycle (*gltA*, *acn*, *sucCD*, *sdhCAB* and *fum*) and the glyoxylate bypass (*aceA* and *aceB*) were highly

Fig. 6. Levels of induction of *C. glutamicum* TCA cycle genes by different carbon sources. Total RNA (25 ng) was isolated from *C. glutamicum* grown on mineral salts medium for 30 min (black bars) and 60 min (white bars) after addition of various sole carbon and energy sources: 200 mM of A, acetate; G, glucose; P, pyruvate; L, lactate; C, citrate; S, succinate or M, malate. The OD₆₁₀ is also shown (xiv). The primers, methods and other details correspond to those described in Fig. 1.

upregulated compared with the situation in glucose cultures. The upregulation of these genes is reasonable because these gene products play an important role in acetate metabolism (Przybyla-Zawislak *et al.*, 1999; Wendisch *et al.*, 2000). Similar upregulation of TCA cycle genes in acetate has been reported in *B. subtilis* (Nakano *et al.*, 1998). In contrast, TCA cycle genes are repressed at high glucose concentrations in the presence of acetate. The transcriptional analysis provided an overall view of the expression of almost all TCA cycle genes and was in good agreement with previous reports of enzyme activities, metabolic arrays and DNA microarrays (Bott, 2007; Kromer *et al.*, 2004; Zhao & Lin, 2002). The results presented in this work suggest that a common and specific mechanism may exist at the transcriptional level for regulation of TCA cycle genes by different carbon sources, and that this pathway is coordinately regulated to balance the need for energy production and the generation of precursors for anabolism.

TCA cycle genes are regulated by several of the following regulators: Crp, the catabolite repressor activated by cAMP (Saier & Ramseier, 1996; Zhao & Lin, 2002); Fnr, whose modulon encodes proteins that are involved in cellular adaptation to growth in anoxic environments (Lynch & Lin, 1996; Unden & Schirawski, 1997; Unden *et al.*, 2002); and ArcA/ArcB, a signal transduction system that regulates gene expression in response to redox conditions (Liu & De Wulf, 2004; Lynch & Lin, 1996; Perrenoud & Sauer, 2005). However, expression of genes encoding enzymes of the TCA cycle and glyoxylate bypass in *C. glutamicum* has been shown previously to be subject to at least the following five transcriptional regulators: AcnR, a TetR-type repressor of the *acn* gene (Krug *et al.*, 2005); RipA, a repressor of the *acn* gene under iron limitation (Wennerhold *et al.*, 2005); RamB, a negative regulator of glyoxylate bypass genes (*aceA* and *aceB*) involved in acetate metabolism (Cramer *et al.*, 2006; Gerstmeir *et al.*, 2004); DtxR, a transcriptional regulator of the *sdhCAB* gene in iron metabolism (Brune *et al.*, 2006); and GlxR, the repressor of the glyoxylate

bypass genes (Kim *et al.*, 2004). It is possible that multiple regulators control TCA cycle genes to manifest a complex response to the environment. The glyoxylate bypass genes are transcriptionally regulated by a dual mechanism with GlxR and RamB (Gerstmeir *et al.*, 2004; Kim *et al.*, 2004). Krug *et al.* (2005) reported that the increased aconitase activity of cells grown on acetate is partially because of decreased repression by AcnR and that an additional transcriptional regulator (besides AcnR) or another regulatory mechanism for aconitase might exist. The only regulatory system that has ubiquitous effects on many different genes is catabolite derepression by cAMP receptor protein (Eraso & Gancedo, 1984; Kolb *et al.*, 1993; Saier *et al.*, 1996). This regulator activates many genes in the presence of acetate.

One of the features of transcriptional analysis is its utility in predicting gene functions. Although the first three enzymes (*GltA*, *Acn* and *Icd*) at the entry of the TCA cycle are needed for oxoglutarate generation, the regulation of the first two enzymes (*GltA* and *Acn*) is somewhat different from that of *Icd* (Krug *et al.*, 2005; Nakano *et al.*, 1998). In contrast to the mRNA levels of the *icd* gene, the level of *gltA* transcripts on acetate-containing medium remained about 1.2-fold higher than on glucose medium, whereas the mRNA level of *acn* was at least twofold higher on acetate medium than on glucose medium (Fig. 6). These results suggest that *gltA* and *acn*, but not always *icd*, are closely regulated, which may be due in part to the fact that citrate is an inducer of *acn*. Furthermore, EMSA showed that proteins such as AcnR (Krug *et al.*, 2005) and GlxR (Fig. 5) bind to the dyad symmetry sequences in the *acn* promoter region. In addition, both *gltA* and *acn* have GlxR operator sites, the target sequence for probable catabolite repression, in their regulatory regions that bind to GlxR. Taken together, these results suggest that *gltA* and *acn* regulation might be controlled by the same mechanism. The malate:quinine oxidoreductase gene, *mgo*, is down-regulated by acetate depletion at exponential phase, in contrast to malate dehydrogenase, *mdh*, which is

upregulated. In addition, the two malate dehydrogenase genes, *mdh* and *mdhB*, are non-regulated and upregulated, respectively, in acetate depletion at stationary phase. This result suggests that *mdh* plays a different role from *mdhB*. The expression pattern also suggests that the *mgo* gene may serve other functions. The inconsistencies between regulation of gene expression and presumed function call for further investigations.

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