The transcriptional regulators RamA and RamB are involved in the regulation of glycogen synthesis in \textit{Corynebacterium glutamicum}

Gerd M. Seibold,1,2 Christian T. Hagmann,1 Melanie Schietzel,1 Denise Emer,1 Marc Auchter,1 Joy Schreiner1 and Bernhard J. Eikmanns1

1Institute of Microbiology and Biotechnology, University of Ulm, D-89069 Ulm, Germany
2Institute of Biochemistry, University of Cologne, D-50674 Cologne, Germany

When grown in glucose-, fructose- or sucrose-containing medium, the amino acid producer \textit{Corynebacterium glutamicum} transiently accumulates large amounts of glycogen (up to 10\% of its dry weight), whereas only a marginal amount of glycogen is formed during growth with acetate. This carbon-source-dependent regulation is at least partially due to transcriptional control of \textit{glgC}, encoding ADP-glucose pyrophosphorylase, the first enzyme of glycogen synthesis from glucose-1-phosphate. Here, we have analysed a possible regulatory role for the transcriptional regulators RamA and RamB on glycogen content of the cells and on control of expression of \textit{glgC} and of \textit{glgA}, which encodes the second enzyme of glycogen synthesis, glycogen synthase. Determination of the glycogen content of RamA- and RamB-deficient \textit{C. glutamicum} indicated that RamA and RamB influence glycogen synthesis positively and negatively, respectively. In accordance with the identification of putative RamA and RamB binding sites upstream of \textit{glgC} and \textit{glgA}, both regulators were found to bind specifically to the \textit{glgC–glgA} intergenic promoter region. Promoter activity assays in wild-type and RamA- and RamB-deficient strains of \textit{C. glutamicum} revealed that (i) RamA is a positive regulator of \textit{glgC} and \textit{glgA}, (ii) RamB is a negative regulator of \textit{glgA} and (iii) neither RamA nor RamB alone is responsible for the carbon-source-dependent regulation of glycogen synthesis in \textit{C. glutamicum}.

INTRODUCTION

The Gram-positive bacterium \textit{Corynebacterium glutamicum} is used for the industrial production of amino acids (Leuchtenberger \textit{et al.}, 2005). As in other bacteria, \textit{C. glutamicum} synthesizes glycogen by the consecutive action of ADP-glucose pyrophosphorylase, glycogen synthase and glycogen branching enzyme, which are encoded by \textit{glgC}, \textit{glgA} and \textit{glgB}, respectively (Tzvetkov \textit{et al.}, 2003; Seibold \textit{et al.}, 2007). In many bacteria, the formation of ADP-glucose from glucose-1-phosphate and ATP by ADP-glucose pyrophosphorylase is regarded as the key regulatory step of glycogen synthesis (Ballicora \textit{et al.}, 2003). In \textit{Escherichia coli}, the regulation of this reaction takes place at several levels: transcription of \textit{glgC} and \textit{glgA} is activated by both the CRP/cAMP-dependent catabolite repression system and the stringent response (Romeo & Preiss, 1989; Romeo \textit{et al.}, 1990). Translation and stability of the \textit{glgC–glgA} mRNA are regulated by the carbon storage regulation system (Baker \textit{et al.}, 2002; Weibacher \textit{et al.}, 2003) and, at the post-translational level, bacterial ADP-glucose pyrophosphorylases are generally allosterically controlled by various effector molecules (reviewed by Ballicora \textit{et al.}, 2003, 2007; Preiss \textit{et al.}, 1966).

We recently reported that during cultivation of \textit{C. glutamicum} in medium containing carbohydrates (e.g. glucose, fructose or sucrose) the intracellular glycogen content reaches up to 10\% of the cell’s dry weight (dw), whereas no glycogen is accumulated when the cells are cultivated with gluconeogenic substrates such as acetate (Seibold \textit{et al.}, 2007). Moreover, we showed that the first step of glycogen synthesis in \textit{C. glutamicum} is regulated by controlling the activity of ADP-glucose pyrophosphorylase and by transcriptional regulation of \textit{glgC} dependent on the carbon source (Seibold \textit{et al.}, 2007). However, the proteins involved in transcriptional control of \textit{glgC} have not been identified so far.

The two transcriptional regulators RamA and RamB have been proposed to be master regulators for the adjustment of the central metabolism towards the utilization of gluconeogenic substrates for \textit{C. glutamicum} (reviewed by Arndt & Eikmanns, 2008). In the presence of acetate, RamA activates the expression of genes encoding proteins required for the utilization of acetate, such as the genes for...
isocitrate lyase, malate synthase, phosphotransacetylase and acetate kinase (aceA, aceB and pta–ack, respectively) (Cramer et al., 2006). Recently, RamA has also been shown to activate the adhA and ald genes, encoding alcohol and acetaldehyde dehydrogenases (Arndt & Eikmanns, 2007; Auchter et al., 2009), the monocarboxylic acid transporter gene mctC (Jolkver et al., 2008) and to activate transcription of the aceA gene (Cramer & Eikmanns, 2007) and to activate transcription of the rpf2 genes, which encode the S-layer protein PS2 and a resuscitation factor, respectively (Hansmeier et al., 2006; Jungwirth et al., 2008). Furthermore, RamA has been shown to negatively regulate the expression of its own gene (Cramer & Eikmanns, 2007) and to activate transcription of the ramB gene (Cramer et al., 2007). RamB is a negative regulator of the aceA, aceB, pta–ack, adhA, ald and rpf2 genes (Gerstmeir et al., 2004; Arndt & Eikmanns, 2007; Auchter et al., 2009; Jungwirth et al., 2008). Additionally, RamB is subject to negative autoregulation (Cramer et al., 2007) and acts as an activator of the pyruvate dehydrogenase complex subunit E1p gene aceE (Blombach et al., 2009).

Here, we present data showing the direct involvement of RamA in transcriptional activation of the ADP–glucose pyrophosphorylase and glycogen synthase genes glgC and glgA, and thus in glycogen synthesis of C. glutamicum. Furthermore, we show that RamB is also involved in expression control of glgA and thus adjusts the glycogen synthesis rate in C. glutamicum.

METHODS

Bacterial strains, plasmids, oligonucleotides and growth conditions. Bacterial strains and plasmids used in this study are listed in Table 1, and primers are listed in Table 2. E. coli and all C. glutamicum pre-cultures were grown aerobically in TY complex medium (Sambrook et al., 2001) at 37°C and 30°C, respectively, as 60 ml cultures in 500 ml baffled Erlenmeyer flasks on a rotary shaker at 120 r.p.m. For the main C. glutamicum cultures, cells of an overnight pre-culture were washed twice with 0.9% (w/v) NaCl and then inoculated into CGC minimal medium (Eikmanns et al., 1991) containing glucose and/or acetate at concentrations indicated in Results and Discussion. In minimal medium, C. glutamicum was grown aerobically at 30°C as 50 ml cultures in 500 ml baffled Erlenmeyer flasks on a rotary shaker at 120 r.p.m. The growth of E. coli and C. glutamicum was followed by measuring the OD at 600 nm.

DNA preparation, transformation and manipulation. Standard procedures were employed for plasmid isolation and for molecular cloning and transformation of E. coli DH5α, as well as for electrophoresis (Sambrook et al., 2001). C. glutamicum chromosomal DNA was isolated according to the method described by Eikmanns et al. (1994). Transformation of C. glutamicum was performed by electroporation using the methods described by Tauch et al. (2002); the recombinant strains were selected on Luria-Bertani–BHIS agar plates containing kanamycin (25 μg ml⁻¹). E. coli was electroporated according to the method described by Dower et al. (1988). All restriction enzymes, T4-DNA ligase, shrimp alkaline phosphatase and Taq DNA polymerase were obtained from MBI Fermentas and used according to the manufacturer’s instructions.

Cloning the glgA promoter. The promoter probe vector pET2 was used to construct a transcriptional fusion of the glgA promoter to the promoterless cat gene. The glgA promoter fragment was amplified from chromosomal DNA from C. glutamicum wild-type (WT) by PCR with the primers PR-glgAC-for and PRAC2rev. The 434 bp PCR product, covering the region from 308 bp upstream to 125 bp downstream of the annotated translational start codon, was digested with SalI and BamHI and ligated into the multiple cloning site in front of the cat gene in pET2, resulting in plasmid pET-PA. Successful cloning was verified by sequencing.

RNA techniques. RNA from exponentially growing cultures of C. glutamicum was prepared using the RNeasy kit (Qiagen) as described

### Table 1. Strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristics</th>
<th>References/Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>E. coli strain</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DH5α</td>
<td>F⁻ Δ80lacZΔM15 Δ(lacZΔM15::argF) U169 endA1 recA1 hsdR17 (rK-, mB+) supE44 thi-1 gyrA96 relA1 phoA</td>
<td>Hanahan (1983)</td>
</tr>
<tr>
<td>BL21 (DE3)</td>
<td>ompT, hsdR30 (rK-, mB⁻), gal, dcm (DE3)</td>
<td>Studier &amp; Moffatt (1986)</td>
</tr>
<tr>
<td><strong>C. glutamicum strain</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>Wild-type strain ATCC 13032</td>
<td>American Type Culture Collection</td>
</tr>
<tr>
<td>RG1</td>
<td>C. glutamicum WT with truncated ramB gene, shortened by 775 bp</td>
<td>Gerstmeir et al. (2004)</td>
</tr>
<tr>
<td>RG2</td>
<td>C. glutamicum WT with truncated ramA gene, shortened by 364 bp</td>
<td>Cramer et al. (2006)</td>
</tr>
<tr>
<td><strong>Plasmid</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pET2</td>
<td>Promoter probe vector carrying the promoterless cat gene, KanR</td>
<td>Vasicova et al. (1998)</td>
</tr>
<tr>
<td>pET2-PC</td>
<td>pET2 containing the glgC promoter fragment</td>
<td>Seibold et al. (2007)</td>
</tr>
<tr>
<td>pET2-PA</td>
<td>pET2 containing the glgA promoter fragment</td>
<td>This work</td>
</tr>
<tr>
<td>pET28-RamA × 6His</td>
<td>pET28a containing the ramA gene</td>
<td>Cramer et al. (2006)</td>
</tr>
<tr>
<td>pET29-RamB × 6His</td>
<td>pET28a containing the ramB gene</td>
<td>Gerstmeir et al. (2004)</td>
</tr>
<tr>
<td>pDrive</td>
<td>KanR, AmpR, lacZΔm, oriT, ori-pUC</td>
<td>Qiagen</td>
</tr>
<tr>
<td>pDrive-RACE-PRglgC</td>
<td>pDrive derivative containing the PCR-amplified glgC fragment from the RACE assay</td>
<td>This work</td>
</tr>
<tr>
<td>pDrive-RACE-PRglgA</td>
<td>pDrive derivative containing the PCR-amplified glgA fragment from the RACE assay</td>
<td>This work</td>
</tr>
</tbody>
</table>
**RESULTS AND DISCUSSION**

**Influence of inactivating ramA or ramB on the glycogen content of C. glutamicum**

To study the possible effects of RamA and RamB on glycogen metabolism in *C. glutamicum*, the WT and the *ramA* and *ramB* deletion mutants *C. glutamicum* RG2 and *C. glutamicum* RG1, respectively, were cultured in minimal medium containing either glucose, glucose plus acetate, or acetate as the carbon source, and the glycogen content was measured at different time points. As expected from earlier experiments, when *C. glutamicum* WT was cultivated with glucose as the carbon source during early exponential phase, glycogen accumulated, and decreased in the course of cultivation (Fig. 1a; Seibold *et al.*, 2007). Less glycogen was present in the course of cultivation of *C. glutamicum* WT with glucose plus acetate (Fig. 1b) and only minor amounts of glycogen were found when cultivated with acetate as the sole carbon source (Fig. 1c). As shown before, the RamA-deficient *C. glutamicum* RG2 did not grow on acetate as the sole carbon source (Cramer *et al.*, 2006).

**Table 2. Oligonucleotides used in this study**

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Sequence (5′–3′)*</th>
<th>Purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>PR-glglA–for</td>
<td>CGGATCTCCTGACCCCATGCACTGAAC</td>
<td>pET2-PA, intAC</td>
</tr>
<tr>
<td>PRAC2rev</td>
<td>AGCGGTCTGACCTCCGAGGTCAAC</td>
<td>pET2-PA, intAC</td>
</tr>
<tr>
<td>RACE-glglC–SP1</td>
<td>CAGTACGCGGATCTTGGAG</td>
<td>5′-RACE, cDNA synthesis</td>
</tr>
<tr>
<td>RACE-glglC–SP2</td>
<td>CTTGAGATGCGGCAATTGG</td>
<td>5′-RACE, nested primer</td>
</tr>
<tr>
<td>RACE-glglA–SP1</td>
<td>GTCTTAATCGCAGGAGGTTT</td>
<td>5′-RACE, cDNA synthesis</td>
</tr>
<tr>
<td>RACE-glglA–SP2</td>
<td>ACCCATGCACTGAAACATC</td>
<td>5′-RACE, nested primer</td>
</tr>
<tr>
<td>Oligonucleotide</td>
<td>dT-anchor primer</td>
<td>5′-RACE, amplification of dA-tailed cDNA</td>
</tr>
<tr>
<td>ramBp3b_forw</td>
<td>ACGCGTTCGAGGATGTGGCCCAGGAAATGC</td>
<td>ramBp3b</td>
</tr>
<tr>
<td>ramBp3c_forw</td>
<td>ACGGCTGACCTGACGGAAGAGTTGTTA</td>
<td>ramBp3c</td>
</tr>
<tr>
<td>ramBp_rev</td>
<td>ACTGAGGTGTTGCAAACTTGTTGATTTTCGCT</td>
<td>ramBp3b, ramBp3c</td>
</tr>
</tbody>
</table>

* Restriction sites are underlined; V represents an A, C or G.

previously (Seibold *et al.*, 2007). To identify the transcriptional start site of *glgC* with the 5′/3′-RACE kit from Roche Diagnostics, 3 μg total RNA was used as a template for the specific *glgC* cDNA synthesis using primer RACE-glglC–SP1. The subsequent PCRs were performed using the primer pair RACE-glglC–SP2/oligonucleotide dT-anchor primer (the latter is included in the kit). To identify the *glgA* start site, primers RACE-glglA–SP1 and RACE-glglA–SP2 were used. The purified PCR products were ligated into plasmid pDrive (Qiagen), resulting in recombinant plasmids pDrive-RACE-PRglgC and pDrive-RACE-PRglgA. These plasmids were sequenced and the transcriptional start sites were deduced from the sequences obtained.

**Enzyme assays.** To determine chloramphenicol acetyltransferase (CAT) activities in cell extracts, *C. glutamicum* cells were grown in minimal medium to the exponential growth phase, washed twice in 20 ml 20 mM Tris/HCl buffer, pH 7.8, and resuspended in 1 ml of the same buffer containing 10 mM MgCl₂ and 1 mM EDTA. The cell suspension was added to 2 ml screw cap vials together with 250 mg glass beads (150–212 μm; Sigma-Aldrich) and mechanically disrupted with a RiboLyser (setting 6.5; Hybaid) six times for 25 s at 4 °C with intermittent cooling on ice for 2 min. After disruption, glass beads and cellular debris were removed by two consecutive centrifugation steps (13 000 × g; 4 °C, 10 min and 45 000 × g; 4 °C, 60 min) and the supernatant was used to determine the specific CAT activity (Gerstmeir *et al.*, 2004). The bicinonicin acid protein assay kit (Pierce) was used to determine protein concentrations according to the manufacturer’s instructions, with BSA as the standard.

**Protein purification and electrophoretic mobility shift assays (EMSAs).** RamA and RamB were synthesized as hexahistidyl-tagged fusion proteins and purified by Ni²⁺-affinity chromatography as described previously (Gerstmeir *et al.*, 2004; Cramer *et al.*, 2006). Binding of purified RamA and RamB was tested by EMSAs using DNA fragments generated by PCR and purified using the Nucleospin Extract kit (Macherey-Nagel). The fragment intAC, carrying the *glgC* intergenic region, was amplified using primers PR-glgAC–for and PR-glgAC–rev. These plasmids were sequenced and the transcriptional start site of *glgC* with the 5′/3′-RACE kit from Roche Diagnostics, 3 μg total RNA was used as a template for the specific *glgC* cDNA synthesis using primer RACE-glglC–SP1. The subsequent PCRs were performed using the primer pair RACE-glglC–SP2/oligonucleotide dT-anchor primer (the latter is included in the kit). To identify the *glgA* start site, primers RACE-glglA–SP1 and RACE-glglA–SP2 were used. The purified PCR products were ligated into plasmid pDrive (Qiagen), resulting in recombinant plasmids pDrive-RACE-PRglgC and pDrive-RACE-PRglgA. These plasmids were sequenced and the transcriptional start sites were deduced from the sequences obtained.

**Enzyme assays.** To determine chloramphenicol acetyltransferase (CAT) activities in cell extracts, *C. glutamicum* cells were grown in minimal medium to the exponential growth phase, washed twice in 20 ml 20 mM Tris/HCl buffer, pH 7.8, and resuspended in 1 ml of the same buffer containing 10 mM MgCl₂ and 1 mM EDTA. The cell suspension was added to 2 ml screw cap vials together with 250 mg glass beads (150–212 μm; Sigma-Aldrich) and mechanically disrupted with a RiboLyser (setting 6.5; Hybaid) six times for 25 s at 4 °C with intermittent cooling on ice for 2 min. After disruption, glass beads and cellular debris were removed by two consecutive centrifugation steps (13 000 × g; 4 °C, 10 min and 45 000 × g; 4 °C, 60 min) and the supernatant was used to determine the specific CAT activity (Gerstmeir *et al.*, 2004). The bicinonicin acid protein assay kit (Pierce) was used to determine protein concentrations according to the manufacturer’s instructions, with BSA as the standard.

**Protein purification and electrophoretic mobility shift assays (EMSAs).** RamA and RamB were synthesized as hexahistidyl-tagged fusion proteins and purified by Ni²⁺-affinity chromatography as described previously (Gerstmeir *et al.*, 2004; Cramer *et al.*, 2006). Binding of purified RamA and RamB was tested by EMSAs using DNA fragments generated by PCR and purified using the Nucleospin Extract kit (Macherey-Nagel). The fragment intAC, carrying the *glgA–glgC* intergenic region, was amplified using primers PR-glglAC–for and PRAC2rev. The 211 bp fragment ramBp3b_forw generated with the primers ramBp3b_forw and ramBp3c_rev was used as a negative control for RamA binding and as a positive control for RamB binding (Cramer *et al.*, 2007). The 135 bp fragment ramBp3c_rev served as a negative control for RamB binding and was generated by PCR using the primers ramBp3c_forw and ramBp3c_rev (Cramer *et al.*, 2007). In the binding assays, 5–30 ng of the fragments was incubated with various amounts of RamA or RamB (0–1 μg; corresponds to a molar excess of protein over DNA of 0–250-fold in the case of RamA and of 0–180-fold in the case of RamB) in 20 μl 10 mM Tris/HCl reaction buffer, pH 7.6, containing 50 mM NaCl, 1 mM DTT, 1 mM EDTA, 10% (w/v) glycerol and 1 μg Poly[d(C–C)] for 20 min at room temperature. The mixture was separated on a 2% (w/v) agarose gel in 1 x TAE buffer (40 mM Tris/HCl, pH 7.5, 20 mM acetate, 1 mM EDTA) at 70 V and 80 mA and stained with ethidium bromide.

**Analysis of intracellular carbohydrates.** For enzymic analysis of intracellular polysaccharides in *C. glutamicum*, 5 ml samples of cultures were harvested, cell extracts were prepared and glycogen content was determined with amyloglucosidase (Roche Diagnostics) as described previously (Seibold *et al.*, 2007).

**Computational analysis.** Comparative genome analysis was performed using the MGB platform (Uchiyama, 2007) and sequence analysis was done using CoryneRegNet (Baumbach *et al.*, 2006, 2009).
However, when cultivated with glucose or with glucose plus acetate as carbon sources, the glycogen content in the RamA-deficient *C. glutamicum* RG2 was significantly lower than in *C. glutamicum* WT (Fig. 1a and b). These results indicate that RamA positively influences glycogen synthesis, possibly by transcriptional activation of *glgC*, which has been shown to be expressed in a carbon-source-dependent manner (Seibold et al., 2007). However, although the glycogen content of *C. glutamicum* RG2 was lower than in the WT strain, it differed during cultivation on glucose with or without acetate (Fig. 1). This result indicates that RamA is not responsible for the carbon-source-dependent differences in glycogen content. The pattern observed for glycogen accumulation in the RamB-deficient mutant *C. glutamicum* RG1 is different from that of *C. glutamicum* WT, i.e. most glycogen was found in the late exponential growth phase (8 h after inoculation), independent of the carbon source used for cultivation (Fig. 1). Furthermore, the *ramB* mutant strain also accumulated significant amounts of glycogen when cultivated with acetate as the sole carbon source (Fig. 1c). These results indicate that in the presence of acetate in the growth medium, RamB might act as a repressor of genes encoding enzymes required for glycogen synthesis and is possibly responsible for the carbon-source-dependent regulation of glycogen content in *C. glutamicum*.

### Identifying the transcriptional start sites in the *glgA–glgC* intergenic region and the putative binding sites for RamA and RamB

The genes encoding the enzymes that catalyse the first two steps of glycogen synthesis, *glgC* and *glgA*, are adjacent on the genome sequence of *C. glutamicum*, separated by 153 bp and are divergently transcribed (Kalinowski et al., 2003; Seibold et al., 2007). Both genes are transcribed monocistronically (Seibold et al., 2007; data not shown). To determine the transcriptional start sites of both genes, we performed 5′-RACE with total RNA of both glucose- and acetate-grown *C. glutamicum* WT. After cDNA synthesis of the 5′-end transcripts of *glgA* and *glgC* with specific primers for each gene, the amplified cDNAs were subcloned in pDrive and the resulting plasmids were sequenced. In three independent experiments with RNA from cells cultivated with glucose as well as with acetate, the transcriptional start site of *glgC* (*Tsc*) was found to be identical to the translational start site (see Fig. 2). Upstream of *Tsc*, we found the motif TATGGT, which is identical in four of six bases to the −10 consensus motif (TAC/TAAT) described for corynebacteria (Pátek et al., 2003) and which is identical to the −10 motif described for the *ald* gene (Auchter et al., 2009). The transcriptional start site of *glgA* (*Tsa*) was identified by three independent 5′-RACE experiments and found to be the A residue of the annotated ATG start codon (see Fig. 2). A possible −10 motif (GCTAAT) would be the GCTAAT sequence, which is also conserved in four of six bases compared to the −10 consensus motif (Pátek et al., 2003). Overlap of transcriptional and translational start points was observed quite frequently in *C. glutamicum* (brnF, *lpdA*, *ilvA*, *leuA*, *betP* and several other genes) (Pátek et al., 2003); however, the interaction of the respective mRNAs with the 16S rRNA has not been investigated in *C. glutamicum*.

The known binding motifs for both transcriptional regulators RamA and RamB have recently been listed (reviewed by Arndt & Eikmanns, 2008) and the derived consensus sequences were used to screen the *glgA–glgC* intergenic region for such motifs. As shown in Fig. 2, three putative RamA binding sites (TGGGGGC, AGGGGC and ACCCCA) are located in the intergenic region, centred 52, 65 and 81 bp upstream of the *glgA* transcriptional start site and 102, 89 and 73 bp upstream of the *glgC* transcriptional start site, respectively. A putative RamB binding site (TAATCTTTGAAAT), consistent in 9 of 13 bases with the AA/GAACTTTGCAAA consensus RamB-binding motif (Gerstmeir et al., 2004), is located next to the *glgA* transcriptional start site and overlaps the putative −10 region of *glgA* (see Fig. 2). Further analysis of the *glgA–glgC* intergenic region for putative binding sites of other known corynebacterial transcriptional regulators (using the CoryneRegNet database; Baumbach et al., 2006, 2009) revealed no binding site of high significance apart from those described for RamA and RamB.
RamA is a positive regulator of glgA and glgC transcription

EMSAs were performed to confirm the binding of RamA to the glgA–glgC intergenic region postulated above. For this purpose, different amounts of purified hexahistidyl-tagged RamA fusion protein (RamAHIS) were incubated with the intAC DNA fragment, which comprises the intergenic glgC–glgA region. As shown in Fig. 3, the intAC probe was already slightly retarded by incubation with 0.15 μg RamA; an increase in RamAHIS concentration to 0.65 μg caused a complete shift. It is evident that two DNA–RamA complexes were formed, possibly reflecting the binding to two binding sites on the DNA fragment used. No retardation was observed with the control fragment ramBp3b, which possesses no RamA binding site (Cramer et al., 2007) and which was added as a negative control to the reaction mixtures (Fig. 3). From these results, we conclude that RamA specifically interacts with the glgA–glgC intergenic region.

To test for transcriptional regulation of glgC and glgA by RamA in vivo, transcriptional fusions between the promoter regions of both genes and the promoterless CAT gene in the promoter probe vector pET2 (i.e. plasmids pET-PC and pET-PA) were transformed into C. glutamicum WT and the ramA deletion mutant C. glutamicum RG2. CAT activities were determined in the plasmid-carrying strains during early exponential growth in minimal medium with 1 % (w/v) glucose, 0.5 % (w/v) glucose plus 0.5 % (w/v) potassium acetate or 1 % (w/v) potassium acetate. As already indicated by previous CAT reporter studies with glgC (Seibold et al., 2007), the glgC promoter activity varied in a carbon-source-dependent manner in C. glutamicum WT. The activity was highest in cells cultivated with glucose and was lower when acetate was the only, or an additional, carbon source (Table 3). The glgA promoter activities varied in a similar way in C. glutamicum WT cells (Table 3). Independent of the carbon source (glucose or acetate, respectively), the promoter activities for both glgC and glgA were 30–70 % lower in the ramA-deficient mutant C. glutamicum RG2. Due to the direct interaction of RamAHIS with the intAC fragment, the reduced glycogen content and the reduced glgC and glgA promoter activities in the ramA deletion strain C.
Regulation of glycogen synthesis in *C. glutamicum*

### Table 3. Specific CAT activities of *C. glutamicum* WT, RG2 and RG1 cells carrying the glgC or glgA promoter fragment in plasmid pET2 and cultivated in minimal medium containing 1% (w/v) glucose, 1% (w/v) potassium acetate, or 0.5% (w/v) glucose plus 0.5% (w/v) potassium acetate as carbon source

<table>
<thead>
<tr>
<th>Strain</th>
<th>Specific CAT activity [U (mg protein)]^{-1}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Glucose</td>
</tr>
<tr>
<td><em>C. glutamicum</em> WT (pET2-PC)</td>
<td>0.15 ± 0.01</td>
</tr>
<tr>
<td><em>C. glutamicum</em> RG2 (pET2-PC)</td>
<td>0.10 ± 0.01</td>
</tr>
<tr>
<td><em>C. glutamicum</em> RG1 (pET2-PC)</td>
<td>0.14 ± 0.01</td>
</tr>
<tr>
<td><em>C. glutamicum</em> WT (pET2-PA)</td>
<td>0.10 ± 0.01</td>
</tr>
<tr>
<td><em>C. glutamicum</em> RG2 (pET2-PA)</td>
<td>0.04 ± 0.00</td>
</tr>
<tr>
<td><em>C. glutamicum</em> RG1 (pET2-PA)</td>
<td>0.15 ± 0.00</td>
</tr>
</tbody>
</table>

*The RamA-deficient *C. glutamicum* RG2 and plasmid-carrying derivatives did not grow on acetate as the sole carbon source (Cramer et al., 2006).

*glutamicum* RG2, we conclude that RamA is a transcriptional activator of both glgA and glgC and thus is a positive regulator of glycogen synthesis in *C. glutamicum*.

### RamB is a negative transcriptional regulator of glgA

The interaction of RamB with the glgA–glgC intergenic region was also investigated with EMSAs. As shown in Fig. 4, a slight retardation of the intAC probe was observed when 0.25 µg purified hexahistidyl-tagged RamB fusion protein (RamB\(^{HIS}\)) was used in the assay. However, an increase in RamB\(^{HIS}\) to up to 1 µg in the EMSA did not cause a complete shift of the probe, indicating a rather weak binding of RamB\(^{HIS}\) to the DNA fragment. From these data, we conclude that, in spite of four mismatches in the 13 bp RamB consensus sequence, the postulated RamB binding motif between the −10 region and the transcriptional start site is sufficient to bind RamB.

To analyse the effects of RamB on the glgA and glgC promoter activities in *vivo*, the promoter probe vectors pET-PC and pET-PA were transformed into the RamB-deficient mutant *C. glutamicum* RG1 and CAT activities were determined. The glgC and glgA promoter activities in the ramB deletion mutant varied depending on the carbon source in the same way as in *C. glutamicum* WT cells (Table 3). As no differences in transcription activity were observed for glgC in *C. glutamicum* WT and *C. glutamicum* RG1 cells (Table 3), we conclude that RamB is not involved in the transcriptional regulation of glgC. However, on all media tested, the transcription activity of glgA was about 50% higher in the RamB-deficient mutant *C. glutamicum* RG1 than in *C. glutamicum* WT. These data, in combination with the localization of the RamB binding site near the glgA translational/transcriptional start site (see Fig. 2) and the results of the EMSA experiments (Fig. 4), indicate that RamB acts as a repressor of glgA transcription.

### Conclusion

Taken together, our data show that RamA and RamB are involved in the transcriptional regulation of glgC and glgA and thus in control of glycogen synthesis in *C. glutamicum*. Whereas RamA positively controls both glgC and glgA, RamB negatively controls only the latter. The gene arrangement of glgC and glgA as adjoining but divergently transcribed genes in *C. glutamicum* allows differential expression of both genes and is similar to the gene arrangements present in other glycogen-synthesizing *Corynebacterineae*, such as *Mycobacterium tuberculosis*, *Mycobacterium smegmatis*, *Mycobacterium phlei* and *Rhodococcus jostii* (Sambou et al., 2008; Elbein & Mitchell, 1973; Antoine & Tepper, 1969; Hernández et al., 2008). In contrast, the two genes are organized as operon-like structures in other glycogen-synthesizing bacteria, e.g.

---

**Fig. 4.** Representative EMSA using hexahistidyl-tagged RamB protein (0, 0.25, 0.5 and 1 µg), with the 0.46 kb intAC fragment (10 ng) as a probe and fragments ramBp3b and ramBp3c (10 ng each) as positive and negative controls, respectively. The fragments used for the binding assays are indicated below the different sections of the gel.
E. coli, Bacillus subtilis, Agrobacterium tumefaciens, Vibrio cholerae and Rhodobacter sphaeroides (Romeo et al., 1988; Kiel et al., 1994; Ugalde et al., 1998; Bourassa & Camilli, 2009; Igarashi & Meyer, 2000), and thus, they are co-ordinately controlled at the transcriptional level in these bacteria. In view of the finding that glycolgen synthesis in most bacteria, and probably also in C. glutamicum, is regulated mainly by controlling the activity of ADP-glucose pyrophosphorylase (Ballicora et al., 2003; Seibold et al., 2007), the relatively weak differential expression control of glgA and glgC by RamA and RamB possibly serves as a fine-tuning of glycolgen metabolism. However, in contrast with RamA- and RamB-mediated transcription control of aceA, aceB, pta–ack, adhA, acn and gapA (Arndt & Eikmanns, 2008; Emer et al., 2009; Toyoda et al., 2009), both RamA-mediated activation of glgC and glgA and RamB-mediated repression of glgA are not dependent on the carbon sources tested. Thus, the situation is similar to that of RamA-/RamB-mediated expression control of the sdhCAB operon and the ald gene (Auchter et al., 2009; Bussmann et al., 2009); it becomes clear that a further transcriptional regulator(s) should be involved in carbon-source-dependent regulation of glgC and glgA. However, in spite of a DNA affinity approach (data not shown) we were so far not able to identify a further regulator involved in glgC and glgA expression control.

ACKNOWLEDGEMENTS

The authors thank Ute Meyer and Eva Glees for excellent technical assistance. The support of the BMBF (grants 0313704 ‘SysMAP’ and 0313805G ‘GenoMik-Plus’) and of Evonik Degussa GmbH, Halle-Künsebeck, is gratefully acknowledged.

REFERENCES


Hansmeier, N., Albersmeier, A., Tauch, A., Damberg, T., Ros, R., Anselmetti, D., Pühler, A. & Kalinowski, J. (2008). The surface (S)-layer gene cspB of Corynebacterium glutamicum is transcriptionally...
activated by a LuxR-type regulator and located on a 6 kb genomic island absent from the type strain ATCC 13032. Microbiology 152, 923–935.


Edited by: L. Heide