The transcriptional regulators RamA and RamB are involved in the regulation of glycogen synthesis in *Corynebacterium glutamicum*

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When grown in glucose-, fructose- or sucrose-containing medium, the amino acid producer *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 *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 *glgC* and of *glgA*, which encodes the second enzyme of glycogen synthesis, glycogen synthase.

Determination of the glycogen content of RamA- and RamB-deficient *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 *glgC* and *glgA*, both regulators were found to bind specifically to the *glgC*–*glgA* intergenic promoter region. Promoter activity assays in wild-type and RamA- and RamB-deficient strains of *C. glutamicum* revealed that (i) RamA is a positive regulator of *glgC* and *glgA*, (ii) RamB is a negative regulator of *glgA* and (iii) neither RamA nor RamB alone is responsible for the carbon-source-dependent regulation of glycogen synthesis in *C. glutamicum*.

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INTRODUCTION

The Gram-positive bacterium *Corynebacterium glutamicum* is used for the industrial production of amino acids (Leuchtenberger *et al.*, 2005). As in other bacteria, *C. glutamicum* synthesizes glycogen by the consecutive action of ADP-glucose pyrophosphorylase, glycogen synthase and glycogen branching enzyme, which are encoded by *glgC*, *glgA* and *glgB*, respectively (Tzvetkov *et al.*, 2003; Seibold *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 *et al.*, 2003). In *Escherichia coli*, the regulation of this reaction takes place at several levels: transcription of *glgC* and *glgA* is activated by both the CRP/cAMP-dependent catabolite repression system and the stringent response (Romeo & Preiss, 1989; Romeo *et al.*, 1990). Translation and stability of the *glgC*–*glgA* mRNA are regulated by the carbon storage regulation system (Baker *et al.*, 2002; Weibacher *et al.*, 2003) and, at the post-translational level, bacterial ADP-glucose pyro-

phosphorylases are generally allosterically controlled by various effector molecules (reviewed by Ballicora *et al.*, 2003, 2007; Preiss *et al.*, 1966).

We recently reported that during cultivation of *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 *et al.*, 2007). Moreover, we showed that the first step of glycogen synthesis in *C. glutamicum* is regulated by controlling the activity of ADP-glucose pyrophosphorylase and by transcriptional regulation of *glgC* dependent on the carbon source (Seibold *et al.*, 2007). However, the proteins involved in transcriptional control of *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 *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

Abbreviations: CAT, chloramphenicol acetyltransferase; dw, dry weight; EMSA, electrophoretic mobility shift assay; WT, wild-type.

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.*, 2009), some further genes encoding enzymes of the central metabolism (Emer *et al.*, 2009; Toyoda *et al.*, 2009; Bussmann *et al.*, 2009) and the *cspB* and *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* DH5 α , 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 μ g 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-*glgA*-for and PRAC2rev. The 434 bp PCR product, covering the region from 308 bp upstream to 125 bp downstream of the annotated *glgA* translational start codon, was digested with *Sall* and *Bam*HI 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

Strain or plasmid	Relevant characteristics	References/Source
<i>E. coli</i> strain		
DH5 α	F ⁻ ϕ 80 <i>lacZ</i> AM15 Δ (<i>lacZYA-argF</i>) U169 <i>endA1 recA1 hsdR17</i> (r _k ⁻ , m _k ⁺) <i>supE44 thi-1 gyrA96 relA1 phoA</i>	Hanahan (1983)
BL21 (DE3)	<i>ompT</i> , <i>hsdSB</i> (r _B ⁻ m _B ⁻), <i>gal</i> , <i>dcm</i> (DE3)	Studier & Moffatt (1986)
<i>C. glutamicum</i> strain		
WT	Wild-type strain ATCC 13032	American Type Culture Collection
RG1	<i>C. glutamicum</i> WT with truncated <i>ramB</i> gene, shortened by 775 bp	Gerstmeir <i>et al.</i> (2004)
RG2	<i>C. glutamicum</i> WT with truncated <i>ramA</i> gene, shortened by 364 bp	Cramer <i>et al.</i> (2006)
Plasmid		
pET2	Promoter probe vector carrying the promoterless <i>cat</i> gene, Kan ^R	Vasicova <i>et al.</i> (1998)
pET2-PC	pET2 containing the <i>glgC</i> promoter fragment	Seibold <i>et al.</i> (2007)
pET2-PA	pET2 containing the <i>glgA</i> promoter fragment	This work
pET28-RamA $\times$ 6His	pET28a containing the <i>ramA</i> gene	Cramer <i>et al.</i> (2006)
pET29-RamB $\times$ 6His	pET28a containing the <i>ramB</i> gene	Gerstmeir <i>et al.</i> (2004)
pDrive	Kan ^R , Amp ^R , <i>lacZ</i> α , <i>ori</i> f1, <i>ori</i> -pUC	Qiagen
pDrive-RACE-PRglgC	pDrive derivative containing the PCR-amplified <i>glgC</i> fragment from the RACE assay	This work
pDrive-RACE-PRglgA	pDrive derivative containing the PCR-amplified <i>glgA</i> fragment from the RACE assay	This work

Table 2. Oligonucleotides used in this study

Oligonucleotide	Sequence (5'–3')*	Purpose
PR-glgAC-for	CGGGATCCTGCACCCATGCAGTGAAC	pET2-PA, intAC
PRAC2rev	ACGCGTCGACTCCGGAGTTACCA	pET2-PA, intAC
RACE-glgC-SP1	CAGTACCGCGATCTTGAG	5'-RACE, cDNA synthesis
RACE-glgC-SP2	CTGTAAGTTCCGCCGAATGG	5'-RACE, nested primer
RACE-glgA-SP1	GTCTTAATCGCAGGGTTC	5'-RACE, cDNA synthesis
RACE-glgA-SP2	ACCCATGCAGTGAACATC	5'-RACE, nested primer
Oligonucleotide dT anchor primer	GACCACGCGTATCGATGTCGACTTTTTTTT TTTTTTTTTV	5'-RACE, amplification of dA-tailed cDNA
ramBp3b_forw	ACGCGTCGACGATGTGGCCCCGACCACGCCG	ramBp3b
ramBp3c_forw	ACGCGTCGACCTCAGTGCCAAGAGTGTTA	ramBp3c
ramBp_rev	ACTGAGGTGTTGCAAACCTGTTGATTTTCGCT	ramBp3b, ramBp3c

*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-glgC-SP1. The subsequent PCRs were performed using the primer pair RACE-glgC-SP2/oligonucleotide dT anchor primer (the latter is included in the kit). To identify the *glgA* start site, primers RACE-glgA-SP1 and RACE-glgA-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 bicinchoninic 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-glgAC-for and PRAC2rev. The 211 bp fragment *ramBp3b* generated with the primers *ramBp3b_forw* and *ramBp_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* served as negative control for RamB binding and was generated by PCR using the primers *ramBp3c_forw* and *ramBp_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(I-C)] for 20 min at room temperature. The mixture was separated on a 2 % (w/v) agarose gel in 1 × 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 MBGD platform (Uchiyama, 2007) and sequence analysis was done using CoryneRegNet (Baumbach *et al.*, 2006, 2009).

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).

Fig. 1. Glycogen content (mean $\pm$ SD) of *C. glutamicum* WT (black bars), *C. glutamicum* RG2 (light grey) and *C. glutamicum* RG1 (dark grey) at selected time points during cultivation with 1 % (w/v) glucose (a), 0.5 % (w/v) glucose plus 0.5 % (w/v) potassium acetate (b) or 1 % (w/v) potassium acetate (c). The pre-cultures were grown in TY complex medium, the glycogen content at 0 h was <2 mg (g dw)⁻¹ in all three strains. Three to five independent cultures were performed with two determinations per experiment.

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*, *lrp*, *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.

Fig. 2. Genomic locus of the intergenic region of the *C. glutamicum glgA* and *glgC* genes. Coding regions are shown in bold. Putative RamA binding sites are boxed, the putative RamB binding site is highlighted by a grey box, and the *glgA* (TSa) and *glgC* (TSc) transcriptional start sites are indicated by asterisks. The -10 regions are indicated above the sequence.

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 (RamA^{HIS}) 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 RamA^{HIS} 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 pro-

motor 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 RamA^{HIS} with the intAC fragment, the reduced glycogen content and the reduced *glgC* and *glgA* promoter activities in the *ramA* deletion strain *C.*

Fig. 3. Representative EMSA using hexahistidyl-tagged RamA protein (0, 0.15, 0.25, 0.35, 0.5, 0.65 and 0.75 µg), with the 0.46 kb intAC fragment as a probe (10 ng) and the 0.21 kb *ramBp3b* fragment (5 ng) as a negative control.

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

All values are mean $\pm$ SD of three to five independent experiments and two determinations per experiment.

Strain	Specific CAT activity [U (mg protein) ⁻¹]		
	Glucose	Glucose + acetate	Acetate
<i>C. glutamicum</i> WT (pET2-PC)	0.15 $\pm$ 0.01	0.10 $\pm$ 0.01	0.07 $\pm$ 0.01
<i>C. glutamicum</i> RG2 (pET2-PC)	0.10 $\pm$ 0.01	0.05 $\pm$ 0.00	No growth*
<i>C. glutamicum</i> RG1 (pET2-PC)	0.14 $\pm$ 0.01	0.11 $\pm$ 0.02	0.09 $\pm$ 0.01
<i>C. glutamicum</i> WT (pET2-PA)	0.10 $\pm$ 0.01	0.09 $\pm$ 0.01	0.07 $\pm$ 0.00
<i>C. glutamicum</i> RG2 (pET2-PA)	0.04 $\pm$ 0.00	0.05 $\pm$ 0.00	No growth*
<i>C. glutamicum</i> RG1 (pET2-PA)	0.15 $\pm$ 0.00	0.13 $\pm$ 0.01	0.10 $\pm$ 0.01

*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 μ g purified hexahistidyl-tagged RamB fusion protein (RamB^{HIS}) was used in the assay. However, an increase in RamB^{HIS} to up to 1 μ g 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 μ g), 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 glycogen 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 glycogen 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.

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