The \textit{glgB}-encoded glycogen branching enzyme is essential for glycogen accumulation in \textit{Corynebacterium glutamicum}

Gerd M. Seibold,1 Katrin J. Breitinger,2 Raoul Kempkes,1 Leonard Both,1† Matthias Krämer,1 Stefan Dempf2 and Bernhard J. Eikmanns2

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

\textit{Corynebacterium glutamicum} transiently accumulates glycogen as carbon capacitor during the early exponential growth phase in media containing carbohydrates. In some bacteria glycogen is synthesized by the consecutive action of ADP-glucose pyrophosphorylase (GlgC), glycogen synthase (GlgA) and glycogen branching enzyme (GlgB). GlgC and GlgA of \textit{C. glutamicum} have been shown to be necessary for glycogen accumulation in this organism. However, although cg1381 has been annotated as the putative \textit{C. glutamicum} \textit{glgB} gene, cg1381 and its gene product have not been characterized and their role in transient glycogen accumulation has not yet been investigated. We show here that the cg1381 gene product of \textit{C. glutamicum} catalyses the formation of \(\alpha\)-1,6-glycosidic bonds in polysaccharides and thus represents a glycogen branching enzyme. RT-PCR experiments revealed \textit{glgB} to be co-transcribed with \textit{glgE}, probably encoding a maltosyltransferase. Promoter activity assays with the \textit{glgE} promoter region revealed carbon-source-dependent expression of the \textit{glgEB} operon. Characterization of the growth and glycogen content of \textit{glgB}-deficient and \textit{glgB}-overexpressing strains showed that the glycogen branching enzyme GlgB is essential for glycogen formation in \textit{C. glutamicum}. Taken together these results suggest that an interplay of the enzymes GlgC, GlgA and GlgB is not essential for growth, but is required for synthesis of the transient carbon capacitor glycogen in \textit{C. glutamicum}.

INTRODUCTION

The polysaccharide glycogen consists of linear chains of \(\alpha\)-1,4-glycosidically linked glucose molecules with \(\alpha\)-1,6 branches. In bacteria such as \textit{Escherichia coli}, glycogen is synthesized from glucose 1-phosphate by ADP-glucose pyrophosphorylase (GlgC), glycogen synthase (GlgA) and glycogen branching enzyme (GlgB). GlgC catalyses the formation of ADP-glucose from glucose 1-phosphate (Ballicora \textit{et al.}, 2003), and in the following step ADP-glucose is utilized by GlgA for the synthesis of linear chains of \(\alpha\)-1,4-linked glucose molecules (Sheng \textit{et al.}, 2009). Additionally, GlgA also catalyses side-chain elongations. The branching points are introduced by the action of GlgB, catalysing the formation of \(\alpha\)-1,6 branches by cleaving \(\alpha\)-1,4-glycosidic linkages and transferring the non-reducing terminal ends of the resulting fragments to the C6 hydroxyl position of internal glucose molecules (Boyer “et al., 1977). In \textit{Mycobacterium tuberculosis} and \textit{Mycobacterium smegmatis} a novel, additional pathway for glycogen synthesis was recently discovered, which also requires the function of GlgB: maltose 1-phosphate formed by the maltose kinase Pep2 is transferred by the maltosyltransferase GlgE to preferentially branched \(\alpha\)-glucans, forming a glycogen-like polymer (Elbein \textit{et al.}, 2010; Kalscheuer \textit{et al.}, 2010). However, accumulation of the intermediate maltose 1-phosphate has toxic effects, and therefore deletion of the genes for both GlgE and GlgB is detrimental for growth of these bacteria (Kalscheuer \textit{et al.}, 2010). The non-pathogenic, Gram-positive bacterium \textit{Corynebacterium glutamicum} belongs phylogenetically to the same family as \textit{M. tuberculosis} and \textit{M. smegmatis} (Liebl, 2005) and is particularly known for its employment in the industrial production of amino acids (Wendisch \textit{et al.}, 2006). When cultivated in media containing sugars, it transiently accumulates large amounts of glycogen (Seibold \textit{et al.}, 2007). Furthermore, glycogen accumulation in \textit{C. glutamicum} occurs in response to phosphate limitation (Woo \textit{et al.}, 2010). Analyses of the genome sequence of \textit{C. glutamicum} showed the presence of genes for both glycogen synthesis...
pathways (Chandra et al., 2011; Kalinowski et al., 2003). However, GlgC and GlgA are indispensable for glycogen synthesis in C. glutamicum during cultivation with glucose (Seibold et al., 2007; Tzvetkov et al., 2003). As inactivation of either glgA or glgC led to only marginal alterations of growth characteristics of C. glutamicum, the lack of the GlgAC pathway might be partially compensated for by the GlgE pathway, which has not been characterized in this organism. Inactivation of the glgB gene, which encodes the common enzyme of both glycogen synthesis pathways, might therefore be a suitable strategy to identify the general role of glycogen metabolism in C. glutamicum. Nevertheless, a glycogen branching enzyme has also not been identified or characterized in C. glutamicum, although the genome of this organism contains an ORF, i.e. cg1381, annotated as the putative glgB gene (Kalinowski et al., 2003). We show here that this gene in fact encodes a protein of C. glutamicum possessing glycogen branching activity. We analyse the transcriptional organization of glgB and study its expression. Furthermore, we investigate the role of this enzyme for growth of and for glycogen accumulation in C. glutamicum.

**METHODS**

**Bacterial strains, media and culture conditions.** Bacteria and plasmids used in this study are listed in Table 1. E. coli and all precultures of C. glutamicum were grown aerobically in TY complex medium (Sambrook & Russell, 2001) at 37 and 30 °C, respectively, as 50 ml cultures in 500 ml baffled Erlemeyer flasks on a rotary shaker at 120 r.p.m. For the main cultures of C. glutamicum, 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 1% (w/v) glucose. When appropriate, kanamycin (25 µg ml⁻¹), spectinomycin (100 µg ml⁻¹) and IPTG (250 µM) were added to the media. Growth of E. coli and of C. glutamicum was followed by measuring the optical density at 600 nm (OD₆₀₀). For enzyme analysis of intracellular polysaccharides, 5 ml samples of respective cultures were harvested, cell extracts were prepared and glycogen content was determined with amyloglucosidase as previously described (Seibold et al., 2007).

**DNA preparation, transformation and DNA manipulations.** Standard procedures were employed for plasmid isolation, for cloning and transformation of E. coli DH5α, and for electrophoresis (Sambrook & Russell, 2001). C. glutamicum chromosomal DNA was isolated according to Eikmanns et al. (1994). Transformation of C. glutamicum was performed by electroporation using the methods of Tauch et al. (2002). PCR experiments were performed in a FlexCycler (AnalytikJena). The oligonucleotides listed in Supplementary Table S1 (available with the online version of this paper) were obtained from Eurofins MWG Operon. Cycling times and temperatures were chosen according to fragment length and primer constitution. PCR products were separated on agarose gels and purified using the Nucleospin extract II kit (Macherey-Nagel).

**Inactivation and homologous overexpression of glgB.** Inactivation of the chromosomal glgB gene in C. glutamicum was performed as described previously for the inactivation of the ald gene (Auchter et al., 2009), using plasmid pDrive-IMglgB. This plasmid was constructed by PCR amplification of a DNA fragment covering nucleotides 509–1560 of the annotated glgB gene, using primers glgB-IM-for and glgB-IM-rev. The 1068 bp PCR product was cloned into the TA-cloning vector pDrive according to the manufacturer’s instructions and the resulting vector pDrive-IMglgB was transformed into E. coli. After isolation of the recombinant plasmid, it was electroporated into C. glutamicum WT. Integration of pDrive-IMglgB at the genomic glgB locus in C. glutamicum and thus inactivation of the glgB gene was confirmed by Southern blot hybridization, using a glgB-specific fragment, amplified from chromosomal DNA of C. glutamicum WT using primers glgB-IM-for and glgB-IM-rev. Labelling with digoxigenin-dUTP, hybridization, washing and detection were conducted using the non-radioactive DNA labelling

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

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristics</th>
<th>Reference/source</th>
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<tr>
<td><strong>E. coli strains</strong></td>
<td></td>
<td></td>
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<tr>
<td>DH5α</td>
<td>F⁻ ϕ80lacZΔM15 Δ(lacZYA-argF)U169 phoA supE44 hsdR17 recA1 endA1 gyrA96 thi-1 relA1</td>
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</tr>
<tr>
<td>BL21(DE3)</td>
<td>ompT hsdSB (rB mB) gal dcm (DE3)</td>
<td>Studier &amp; Moffatt (1986)</td>
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<tr>
<td><strong>C. glutamicum strains</strong></td>
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<tr>
<td>WT</td>
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<td>American Type Culture Collection</td>
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<td>IMglgB</td>
<td>C. glutamicum WT with integration of plasmid pDrive in glgB</td>
<td>This work</td>
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<td><strong>Plasmids</strong></td>
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<td>pET16b</td>
<td>AmpRΔ, pBR322 oriV&lt;sub&gt;E&lt;/sub&gt; coli P&lt;sub&gt;r7&lt;/sub&gt;, lacI, overproduction of proteins with an N-terminal decahistidine tag in E. coli</td>
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<td>pET16b containing the glgB gene</td>
<td>This work</td>
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<tr>
<td>pDrive</td>
<td>KmRΔ, AmpRΔ, lacZΔs, oriF1, ori-pUC, PCR cloning vector Qiagen, Hilden, Germany</td>
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<td>pDrive-IMglgB</td>
<td>pDrive derivative containing an internal 1068 bp fragment of glgB Vasicová et al. (1998)</td>
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<td>pET2</td>
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<td>pET2 containing the glgEB promoter fragment Stabler et al. (2011)</td>
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<td>pEKEx5</td>
<td>SpecRΔ, pUK18 oriV&lt;sub&gt;E&lt;/sub&gt; coli pBL1 ori&lt;sub&gt;C&lt;/sub&gt; glutamicum P&lt;sub&gt;lac&lt;/sub&gt;, lacIΔ, production of proteins with N-terminal hexahistidine tag in C. glutamicum</td>
<td>This work</td>
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<td>pEKEx5-glglgB</td>
<td>pEKEx5 containing the glgB gene</td>
<td>This work</td>
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and detection kit and the instructions from Roche Diagnostics. The labelled probe was hybridized to StuI-restricted and size-fractionated chromosomal DNA from C. glutamicum WT and the integration mutant IMglgB. The hybridization resulted in one signal of about 5.6 kb from C. glutamicum WT and one signal of about 10.5 kb from C. glutamicum IMglgB. These sizes were expected for the WT strain and the glgB integration mutant, respectively.

For homologous overexpression of glgB, the gene was amplified from genomic DNA of C. glutamicum with primers pEKEx5- and glgB-rev. The 2212 bp PCR product was cloned using the primer-generated BamH I restriction sites into the expression vector pEKEx5. The constructed vector pEKEx5-glgB allows the IPTG-inducible expression of glgB in C. glutamicum.

Cloning of the glgEB promoter fragment. The glgEB promoter fragment was amplified from chromosomal DNA of C. glutamicum with primers PRglgEB-for and PRglgEB-rev. The PCR product was digested with SalI and BamHI, and the resulting fragment of 344 bp was ligated into the SalI/BamHI-digested plasmid pET2 and transformed into E. coli DH5α. The resulting plasmid pET2-PRglgEB was isolated and introduced in C. glutamicum.

Overproduction and purification of GlgB-HIS. Vector pET16b was used for the synthesis of the N-terminal histidyl-(His)-tagged GlgB fusion protein (GlgB-His). The glgB gene was amplified from chromosomal DNA of C. glutamicum WT by PCR with primers glgB-HIS-for and glgB-HIS-rev. The PCR product was cloned using primer-added NdeI and BamHI restriction sites in plasmid pET16b. The resulting plasmid pET16b-glgB was transformed into E. coli BL21(DE3). The nucleotide sequence of the glgB fragment in plasmid pET16b-glgb was verified by sequence analysis (GATC Biotech).

The crude extract for GlgB-His purification was obtained as described previously for purification of RamaA (Cramer et al., 2006). The crude extract was applied to a HiTrap FF crude column (GE Healthcare) equilibrated with binding buffer (20 mM sodium phosphate, 0.5 M sodium chloride, 30 mM imidazole, pH 7.5). Unspecifically bound proteins were eluted at a concentration of 50 mM imidazole. The Histagged GlgB-His was eluted with a buffer containing 250 mM imidazole. For further purification and removal of imidazole the fraction was applied to a Superdex 200 10/300 GL gel filtration column (GE Healthcare) equilibrated with GF buffer (20 mM sodium phosphate, 50 mM sodium chloride, pH 7.4). Elution was performed at a constant flow (1 ml min⁻¹). The fractions were collected and screened by Western blot analysis. For molecular mass determination, the retention volume of GlgB-His was compared with those of standard proteins (HMW calibration kit; GE Healthcare).

Enzyme assays. Branching enzyme activity was analysed using the iodine assay and the bicinchoninic acid (BCA) assay essentially as described by Utsumi et al. (2009). In detail, the initial reaction mixtures contained 10 mM potassium phosphate (pH 7.4), 5 mM sodium chloride, 2 mg amylase (Calbiochem) or 10 mg amylpectin (Calbiochem), and 0.5–2 mg enzyme preparations in a total volume of 1 ml. The reaction mixture was incubated at 30 °C. At different time intervals aliquots were taken and incubated at 100 °C for 10 min to stop the reaction. For the iodine assay 100 µl iodine reagent [0.01 % (w/v) I₂, 0.1 % (w/v) KI] was added to 50 µl aliquots from the reaction mixture, and the changes of absorbance at 660 and 560 nm were analysed spectrophotometrically in 96-well microtitre plates using an Infinite M200 plate reader (Tecan).

For the BCA assay 100 µl 0.1 M sodium acetate buffer (pH 4.8) containing 0.5 U isomaltase from Pseudomonas sp. (Sigma) was added to 100 µl of the reaction mixture and incubated at 30 °C for 12 h. To stop the debranching, 200 µl 0.1 M sodium hydroxide was added. Aliquots of this reaction were mixed with 225 µl BCA working reagent [freshly prepared from Solution A (91.1 mg disodium 2,2′-bicinchoninitate, 3.2 g sodium carbonate monohydrate, 1.2 g sodium bicarbonate in a total volume of 50 ml) and Solution B (62 mg copper sulfate pentahydrate, 63 mg serine in a total volume of 50 ml)] and diluted with water to a total volume of 450 µl. The mixture was then incubated at 80 °C for 10 min in a water bath, and cooled to room temperature. The absorbance of the mixture at 560 nm was analysed spectrophotometrically. Glucose solutions (0–25 µM) were used for the calibration.

To determine chloramphenicol acetyltransferase (CAT) activity, C. glutamicum cells were harvested, washed twice in 0.1 M Tris/HCl, pH 7.8, and resuspended in the same buffer containing 10 mM MgCl₂ and 1 mM EDTA. The specific CAT activity was determined as described by Schreiner et al. (2005).

Protein analysis. Protein concentrations were determined using the Roti-Nanoquanti kit (Roti) with BSA as the standard. SDS-PAGE was performed according to Laemmli (1970). Standard loading buffer (4 x) contained 8 % (w/v) SDS, 20 % (v/v) glycerol, 10 mM EDTA, 100 mM Tris/HCl, pH 6.8, and 1 mg bromophenol blue ml⁻¹; for analysis of the effects of reducing agents, 2 % (v/v) β-mercaptoethanol was added to the loading buffer. Western blot experiments for detection of the His-tagged GlgB protein were performed as previously described (Sebold & Eikmanns, 2007).

RNA techniques. Isolation of total RNA from C. glutamicum cells was performed using the Nucleospin RNAII kit (Macherey-Nagel) as described by Wolf et al. (2003). For analysis of the glgE–glgB operon structure by RT-PCR, total RNA was prepared and DNA contaminations were removed by treatment with DNase (New England Biolabs) and subsequent purification on Nucleospin columns. Reverse transcription was carried out with the Revert Aid H Minus first-strand cDNA synthesis kit (MBI Fermentas) as recommended by the supplier, using random hexamer for the reverse transcription and the combinations of primers indicated in Results for PCR.

Computational analysis. Database searches were carried out by using the BLAST program (Altschul et al., 1990). The UniProt accession numbers for protein sequences and the corresponding ORFs annotated as GlgB are: C. glutamicum Q8SR64, cg1381; M. tuberculosis Q10625, Rv1326c; M. smegmatis A0114y, MSMEG_4918; and E. coli P07762, b3432. Those annotated as GlgE are: C. glutamicum Q635V8, cg1382; M. tuberculosis P63531, Rv1327c; and M. smegmatis Q9R4P8, MSMEG_4916. Mfold software (Zuker, 2003) was employed for calculation of the ΔG° (free energy under standard conditions) of the glgEB terminator structure.

RESULTS

Analysis of the glgB gene and its transcriptional organization

The C. glutamicum cg1381 gene was sequenced in the course of the determination of the genome sequence, and was designated glgB, encoding glycogen branching enzyme (Kalinowski et al., 2003). It has a length of 2196 bp and the predicted gene product consists of 731 amino acids with a molecular mass of 82.6 kDa. The glgB gene of C. glutamicum is preceded by an ORF (cg1382) annotated as glgE (and putatively encoding maltosyltransferase), and is followed by a region of dyad symmetry (centred 28 bp downstream of the TAG stop codon; ΔG° = −29.1 kcal mol⁻¹, −121.8 kJ mol⁻¹), similar to rho-independent
transcription terminators. The last result indicates transcriptional termination downstream of the glgB gene. An identical genomic organization can also be observed in Mycobacterium and Rhodococcus species, in which glgE also directly precedes glgB (Hernández & Alvarez, 2010; Kalscheuer et al., 2010). The glgE gene products of M. tuberculosis and M. smegmatis (59% and 60% identity to GlgE of C. glutamicum, respectively) were recently characterized as maltosyltransferases involved in α-glucan synthesis in these two mycobacteria (Elbein et al., 2010; Kalscheuer et al., 2010).

The close proximity of glgE and glgB suggests that the two form an operon. To test for co-transcription of glgE and glgB, we performed RT-PCRs using a pair of primers (glgEB-RT-F2-for and glgEB-RT-F2-rev) covering the intergenic region (Fig. 1a). As controls, internal fragments of glgE (glgEB-RT-F1-for; glgEB-RT-F1-rev) and glgB (glgEB-RT-F3-for; glgEB-RT-F3-rev) were also amplified. With total RNA as template, no PCR products were observed (Fig. 1b), indicating that the RNA preparation was free from DNA contamination. By application of genomic DNA as well as cDNA with all three primer combinations, amplicons of the expected sizes were obtained (Fig. 1b). These results indicate that glgE and glgB are co-transcribed in C. glutamicum as bicistronic mRNA.

To demonstrate the presence of a glgEB-specific promoter and to investigate transcriptional regulation of this operon, a transcriptional fusion between the putative glgEB promoter region and the promoterless CAT gene was constructed in the promoter-probe vector pET-2. The resulting plasmid, pET-2-PRglgEB, was transformed into C. glutamicum WT. As recent studies have shown that glycogen metabolism is regulated in a carbon-source-dependent manner (Seibold et al., 2007, 2010), CAT activity was determined in C. glutamicum WT(pET-2-PRglgEB) during early exponential growth in minimal medium containing 1% (w/v) glucose or 1% (w/v) acetate. CAT activity in C. glutamicum WT(pET2-PRglgEB) cells during growth in minimal medium containing acetate [0.25 ± 0.04 U (mg protein)⁻¹, mean ± SD of three determinations] was about twofold lower than in cells cultivated with glucose as carbon source [0.49 ± 0.04 U (mg protein)⁻¹]. No CAT activity was present in cells of C. glutamicum WT(pET2) carrying the empty vector. These results confirm the presence of a promoter upstream of the glgE gene and indicate weak transcriptional control of the glgEB operon by the carbon source of the growth medium as mentioned above for the genes glgC and glgA (Seibold et al., 2007, 2010).

**glgB of C. glutamicum encodes a glycogen branching enzyme**

Database and alignment studies with the protein encoded by C. glutamicum glgB revealed significant similarities to the functionally and structurally well-characterized glycogen branching enzymes of E. coli (Abad et al., 2002; Boyer & Preiss, 1977; Devillers et al., 2003; 45% identity) and M. tuberculosis (Garg et al., 2007; Pal et al., 2010; 57% identity). To analyse the function of the glgB gene product of C. glutamicum, we purified and analysed the respective GlgB protein. GlgB was synthesized as the N-terminal His-tagged fusion protein (GlgB-His) in E. coli BL21(DE3) and purified using ultracentrifugation, affinity chromatography and size-exclusion chromatography. SDS-PAGE analysis of the products of each purification step showed the isolation
of four protein bands of approximately 80, 85, 130 and 200 kDa (Fig. 2), all of which could also be detected by Western blot analysis with antibodies binding to the N-terminal His-tag (data not shown). Size-exclusion chromatography with GlgB-His and standard proteins was utilized to determine the oligomeric status. The GlgB-His protein eluted as a homogeneous peak at a volume of 13.9 ml, corresponding to the molecular mass of approximately 74 kDa when compared with standard proteins (Supplementary Fig. S1). This indicates that native GlgB-His should exist as monomer, as was recently also shown for the glycogen branching enzyme of \textit{M. tuberculosis} (Garg et al., 2007).

Cysteines Cys$_{193}$ and Cys$_{617}$ of GlgB of \textit{M. tuberculosis} form intramolecular disulfide bonds, which results in the presence of an additional band in non-reducing SDS-PAGE (Garg et al., 2007, 2009). Also, the primary amino acid sequence of GlgB-His contains three cysteine residues (Cys$_{22}$, Cys$_{156}$, Cys$_{657}$), which could form disulfide bonds. Therefore, we also analysed GlgB-His by SDS-PAGE in the presence of reducing agents. Treatment of GlgB-His with \textit{\beta}-mercaptoethanol resulted in the formation of a single band of 85 kDa (Fig. 2). Analysis of GlgB-His by gel filtration after short storage (12 h) showed elution of two distinct peaks (at 11.8 and 13.9 ml, respectively) corresponding to the molecular mass of the monomer (74 kDa) and the dimer (174 kDa; data not shown). Taken together, these results show the successful purification of GlgB-His, which in the course of storage probably forms intra- and intermolecular disulfide bonds, leading to dimerization.

Activity of the purified GlgB-His was analysed with both amylose (Fig. 3a) and amyllopectin (Fig. 3b) as substrate by measuring the decrease in absorbance of the iodine–polysaccharide complexes over time as described in Methods. Amylose was utilized as a substrate as it is a glucose polymer consisting primarily of \textalpha{}-1,4-glycosidically linked glucose molecules; amyllopectin was used as it is a glucose polymer composed of \textalpha{}-1,4- and \textalpha{}-1,6-glycosidically linked glucose molecules. As shown in Fig. 3, purified GlgB-His was active towards both substrates and the rate of decrease of absorbance was dependent on the amount of protein used in the assay. Amylose appears to be the better substrate for GlgB-His, as the decrease in absorbance over time is generally larger than with amyllopectin. However, direct comparison of the utilization of the two substrates by GlgB-His was not feasible as absorbance of the amyllopectin–iodine complex at 560 nm is not linear to the absorbance of the amylose–iodine complex at 660 nm.

To test that the glycogen branching enzyme catalysed formation of \textalpha{}-1,6-glycosidic bonds, amylose was first treated with GlgB-His, then the branching reaction was stopped by boiling, the reaction products were treated with isoamylase to specifically cleave \textalpha{}-1,6-glycosidic bonds in the polysaccharides, and finally the amount of liberated reducing ends was quantified using the BCA assay. A specific activity of 0.76 \textmu{}mol min$^{-1}$ (mg protein)$^{-1}$ was measured for purified GlgB-His with amylose as a substrate. As a control, GlgB-His treated with amylose but not with isoamylase was analysed using the BCA assay. No increase of reducing ends by GlgB-His treatment was observed. This result shows that GlgB-His activity causes no formation of additional reducing ends, and therefore the above-noted GlgB-His discoloration of the amylose–iodine complex (Fig. 3a) is not due to pure degradation of amylose by GlgB-His. Taken together, these results indicate that the \textit{glgB}-encoded protein of \textit{C. glutamicum} is indeed a glycogen branching enzyme, as it catalyses the formation of \textalpha{}-1,6-glycosidic bonds.

Role of the glycogen branching enzyme in glycogen accumulation during cultivation with glucose

To study the role of the glycogen branching enzyme in growth and glycogen metabolism in \textit{C. glutamicum}, the chromosomal \textit{glgB} gene was inactivated by chromosomal insertion of the vector pDrive-IMglgB, resulting in strain \textit{C. glutamicum} IMglgB. When this strain was cultivated in minimal medium with 1% (w/v) glucose as sole carbon source, glycogen accumulation was nearly abolished (Fig. 4a), while \textit{C. glutamicum} WT transiently accumulated glycogen.
glycogen during the exponential growth phase, as previously described (Seibold et al., 2007). However, no differences were observed in growth between C. glutamicum WT and C. glutamicum IMgglgB: their specific growth rates (WT 0.36 ± 0.02 h⁻¹; IMglgB 0.35 ± 0.03 h⁻¹; means ±SD of three determinations) and final optical densities (WT 15.7 ± 1.2; IMglgB 15.8 ± 1.9) were nearly identical. To rule out the possibility that the effect on glycogen accumulation in C. glutamicum IMgglgB is due to polar effects on glgE, whose gene product was shown to be involved in α-glucan synthesis in M. tuberculosis (Elbein et al., 2010; Kalscheuer et al., 2010), we did complementation studies with the plasmid pEKE5-glgB. As shown in Fig. 4(b), plasmid pEKE5-glgB was able to restore glycogen accumulation in C. glutamicum IMgglgB during cultivation in minimal medium with glucose, while glycogen accumulation was still diminished in the strain carrying the empty plasmid pEKE5. No differences in growth were observed between C. glutamicum IMglgB(pEKE5-glgB) and C. glutamicum IMgglgB(pEKE5) (specific growth rates of 0.24 ± 0.03 and 0.24 ± 0.02 h⁻¹, respectively), although growth of both strains was significantly slower than that of C. glutamicum IMgglgB. The effect on growth is probably caused by plasmid pEKE5, although the reason for this is not clear. Overexpression of glgB in C. glutamicum WT was achieved also using plasmid pEKE5-glgb. As depicted in Fig. 4(c), C. glutamicum WT(pEKE5-glgB) transiently accumulated significantly more glycogen than the control strain C. glutamicum WT(pEKE5) [83 ± 7 and 71 ± 4 mg glucose (g dw⁻¹) 6 h after inoculation, respectively], whose glycogen content was about the same as that of C. glutamicum WT [67 ± 5 mg glucose (g dw⁻¹)]. The increased glycogen content of C. glutamicum WT(pEKE5-glgB) is not caused by slower growth (specific growth rate 0.23 ± 0.04 h⁻¹), as the control strain C. glutamicum WT(pEKE5) (0.24 ± 0.02 h⁻¹) showed similar growth to C. glutamicum WT.

Taken together, these results imply that the glycogen branching enzyme encoded by glgB is necessary for glycogen accumulation in C. glutamicum.

**DISCUSSION**

Our data show that (i) glgB and glgE are organized as an operon in C. glutamicum, (ii) glgB and glgE are expressed in the exponential growth phase, (iii) glgB encodes a glycogen branching enzyme and (iv) glgB is necessary for glycogen accumulation in C. glutamicum. As shown here, the glgEB operon of C. glutamicum is not part of a large operon or gene cluster for glycogen metabolism. Large operons comprising at least the glgC, glgB, glgA and glgP genes have been identified in E. coli, Bacillus subtilis and Agrobacterium tumefaciens (Kiel et al., 1994; Montero et al., 2011; Ugalde et al., 1998). These glycogen metabolism operons are organized as single transcriptional units, can include further genes such as glgX in the glgxXCAP operon of E. coli or pgm in the glgpBCA-pgm operon of A. tumefaciens, and can additionally possess alternative internal promoters (Montero et al., 2011; Ugalde et al., 1998). In contrast, the C. glutamicum genes encoding the enzymes for glycogen metabolism are generally dispersed over the entire genome sequence (Kalinowski et al., 2003). No physiological advantages or consequence of the scattering of glycogen metabolism genes in the genome have been shown, although this organization facilitates physiological studies on the consequences of the inactivation of single genes, as performed in this study for the glgB gene of C. glutamicum.

In Corynebacteriaceae GlgB is of particular interest as in M. tuberculosis and M. smegmatis it is essential for the alternative glycogen synthesis pathway catalysed by the consecutive action of maltose kinase (Pep2), GlgE and GlgB (Kalscheuer et al., 2010). In C. glutamicum this extra pathway probably operates in addition to the common glycogen synthesis pathway via GlgA and GlgC, as we show here for transcription of glgE. In cell extracts from M. smegmatis, accumulation of the toxic intermediate maltose 1-phosphate, the substrate of GlgE, in a ΔglgB strain occurred in a similar manner as in a ΔglgE strain (Kalscheuer et al., 2010), indicating that GlgB activity limits GlgE activity in M. smegmatis. However, in contrast to the situation in M. tuberculosis and M. smegmatis, disruption of
the glgB gene in *C. glutamicum* IMglgB did not cause growth defects, indicating that the growth-inhibiting intermediate maltose 1-phosphate is not accumulated. As glycogen accumulation was abolished in *C. glutamicum* IMglgB, and plasmid-encoded expression of glgB complemented the phenotype of *C. glutamicum* IMglgB, inactivation of the glgB gene was in fact successful. We therefore conclude that either due to the cultivation conditions applied here maltose 1-phosphate is not formed in *C. glutamicum*, or the GlgE of *C. glutamicum* does not require branched glucans as acceptor molecules. One might think that if the latter is the case similar amounts of glucans/glycogen should be present in *C. glutamicum* IMglgB as in *C. glutamicum* WT, which was not the case. The absence of glycogen in *C. glutamicum* IMglgB could be caused either by abolished glycogen synthesis, as is clearly the case in glgC and glgA inactivation mutants of *C. glutamicum* (Seibold et al., 2007; Tzvetkov et al., 2003), or by accelerated degradation of the linear intermediates of glycogen synthesis. The latter proposal is based on the observation that inactivation of glgX, which encodes the glycogen debranching enzyme, significantly increases the glycogen content of *E. coli* and *C. glutamicum* (Dauvillée et al., 2005; Seibold & Eikmanns, 2007), because accessibility of glucose polymers for degradation by the α-glucan phosphorylases is limited by their degree of branching (Alonso-Casajús et al., 2006).

In conclusion, independent of the metabolic pathways contributing to glycogen synthesis in *C. glutamicum* (GlgA-GlgC or Pep2-GlgE), the glgB-encoded glycogen branching enzyme is necessary for accumulation of glycogen in this organism. Further investigation of both glycogen synthesis pathways and the pathway for glycogen degradation in *C. glutamicum*, their regulation and their interplay seems necessary for a comprehensive understanding of the control of glycogen content in this model organism for Corynebacterianeae.

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