The *glgX* gene product of *Corynebacterium glutamicum* is required for glycogen degradation and for fast adaptation to hyperosmotic stress

Gerd M. Seibold and Bernhard J. Eikmanns

Institute of Microbiology and Biotechnology, University of Ulm, D-89069 Ulm, Germany

Correspondence

Bernhard J. Eikmanns

bernhard.eikmanns@uni-ulm.de

Corynebacterium glutamicum cells growing in medium containing sugars accumulate glycogen in the early exponential-growth phase, and start to degrade this polymer at entry into the stationary phase. In a first attempt to investigate glycogen degradation, the *C. glutamicum glgX* gene, which encodes a protein with 46% identity to the isoamylase-type debranching enzyme of *Escherichia coli*, was analysed, expressed and inactivated. The purified *C. glutamicum* gene product showed debranching activity towards glycogen, amylopectin and starch. Chromosomal inactivation of *glgX* in *C. glutamicum* wild-type led to slower growth and to a higher intracellular glycogen pool throughout growth, when compared to those in the parental strain. This result suggests that glycogen synthesis and degradation occur simultaneously in *C. glutamicum*. When exposed to hyperosmotic shock, *C. glutamicum* rapidly degrades glycogen, and at the same time, synthesizes the osmoprotectant trehalose. The *glgX* mutant, however, synthesized only minor amounts of trehalose throughout cultivation, and its growth ceased after hyperosmotic shock. Taken together, the results indicate that the *glgX* gene product is essential for glycogen degradation in *C. glutamicum*, that glycogen is constantly recycled in *C. glutamicum*, and that it serves as a carbon store for trehalose synthesis via the TreYZ pathway after hyperosmotic shock.

Received 13 December 2006

Revised 25 January 2007

Accepted 26 February 2007

INTRODUCTION

Corynebacterium glutamicum is an aerobic, Gram-positive soil bacterium well known for the industrial production of amino acids such as L-glutamate and L-lysine (Eggeling & Bott, 2005). Moreover, this organism has gained interest as a model organism for the group of mycolic acid-containing actinomycetes, which includes several medically important species of the genus *Mycobacterium* (Stackebrandt *et al.*, 1997). *C. glutamicum* grows on a variety of substrates, including sugars, organic acids and alcohols (Liebl, 2005). During cultivation of *C. glutamicum* in media containing sugars, we have recently observed synthesis and degradation of intracellular glycogen (Seibold *et al.*, 2007). Glycogen consists of α -1,4- α -1,6-linked glucose residues (Manners, 1991), and serves as a major reserve polysaccharide in eukaryotes and in a variety of bacteria (Ball & Morell, 2003; Iglesias & Preiss, 1992). Although there have been some investigations of enzymes or genes involved in glycogen synthesis and degradation in other bacteria (Buschiazzi *et al.*, 2004; Horcajada *et al.*, 2006; Yeo & Chater, 2005), most studies have been done with *Escherichia coli* (Ballicora *et al.*, 2003; Preiss, 1996). In this organism, glycogen is synthesized in a two-step process. First, a linear α -1,4-glycosylglucan is formed from glucose

1-phosphate subunits by ADP-glucose pyrophosphorylase and glycogen synthase (Ballicora *et al.*, 2003; Preiss, 1984). The second step, i.e. the introduction of α -1,6-glycosidic linkages in the polymer, is catalysed by the glycogen-branching enzyme (Abad *et al.*, 2002). The genes encoding the three glycogen-synthesizing enzymes are located in a cluster together with genes encoding enzymes involved in degradation of glycogen (Romeo *et al.*, 1988). Glycogen degradation in *E. coli* is accomplished by the interplay of four enzymes (Fig. 1). Glycogen phosphorylase forms glucose 1-phosphate and α -1,6-branched dextrins (Alonso-Casajús *et al.*, 2006). These phosphorylase-limit dextrins serve as substrates for the glycogen-debranching enzyme (Dauvillée *et al.*, 2005). After debranching, the resulting linear maltodextrins serve as substrates for maltodextrin phosphorylase, which forms glucose 1-phosphate, and for maltodextrin glucosidase, which forms glucose (Dippel *et al.*, 2005). None of these enzymes has been experimentally identified in *C. glutamicum*, although two genes in the genome (*cg1479* and *cg2289*) have been annotated as glycogen phosphorylase genes, and a further gene (*cg2310*) has been annotated as *glgX* and putatively encodes a glycogen-debranching enzyme (Kalinowski *et al.*, 2003). In contrast to *E. coli*, *C. glutamicum* degrades glycogen formed in the early exponential growth phase in the late-exponential-growth phase, i.e. before complete consumption of the sugar substrate (Seibold *et al.*, 2007). Therefore,

Abbreviations: dw, dry weight; His-tag, hexahistidyl tag; WT, wild-type.

Fig. 1. Schematic diagram of glycogen degradation in *E. coli*. Abbreviations: GlgP, glycogen phosphorylase; GlgX, debranching enzyme; Glucose-1-P, glucose 1-phosphate; MalP, maltodextrin phosphorylase; MalZ, maltodextrin glucosidase.

it seems unlikely that glycogen in this organism serves as a long-term carbon storage compound, and in fact, glycogen metabolism in *C. glutamicum* has previously been suggested to be important for trehalose synthesis (Padilla *et al.*, 2004; Seibold *et al.*, 2007; Tzvetkov *et al.*, 2003; Wolf *et al.*, 2003). It has been shown that linear maltodextrins (formed as intermediates during glycogen synthesis and degradation) serve as substrates for the two-step trehalose biosynthetic pathway via maltooligosyltrehalose synthase (TreY) and maltooligosyltrehalose trehalohydrolase (TreZ) (Carpinelli *et al.*, 2006; De Smet *et al.*, 2000). Since trehalose in *C. glutamicum* acts as a cell-wall component (Tropis *et al.*, 2005) and as a compatible solute under hyperosmotic and nitrogen-limitation conditions (Wolf *et al.*, 2003), glycogen degradation and its control might be important for the cell-wall composition and for the response to osmotic stress in this organism.

We show here that the *glgX* gene (*cg2310*) of *C. glutamicum* in fact encodes a functional glycogen-debranching enzyme. Furthermore, we investigated the role of this enzyme for growth of, and for the intracellular glycogen concentration in *C. glutamicum*. Due to the supposed role of glycogen degradation in trehalose formation, we further examined the role of the glycogen-debranching enzyme for growth after a shift to hyperosmotic conditions.

METHODS

Bacterial strains, plasmids and culture conditions. The bacteria used in this study were *E. coli* DH5 α (Hanahan, 1983), *E. coli* ER2566 (New England Biolabs) and *C. glutamicum* wild-type (WT) (strain ATCC 13032; American Type Culture Collection). Vector pET28a (Novagen) was used for the generation of the His-tagged GlgX. *E. coli*

and all pre-cultures of *C. glutamicum* were grown aerobically in TY complex medium (Sambrook & Russel, 2001) at 37 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 cultures of *C. glutamicum*, cells of an overnight pre-culture were washed with 0.9 % (w/v) NaCl, and then inoculated into CgXII minimal medium (Eikmanns *et al.*, 1991) containing glucose at the concentrations indicated in Results. *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., or as 250 ml cultures in a parallel fermentation system (Fedbatch-pro system; DASGIP) as described previously (Seibold *et al.*, 2007). In this system the pH was maintained at 7.0, and dissolved oxygen was adjusted to 30 % saturation. Growth of *E. coli* and *C. glutamicum* was followed by measuring OD₆₀₀.

DNA isolation, transfer and manipulations. Standard procedures were employed for plasmid isolation, cloning and transformation of *E. coli* DH5 α , as well as for electrophoresis (Sambrook & Russel, 2001). Isolation of *C. glutamicum* chromosomal DNA, transformation of *C. glutamicum*, and selection of the transformants were performed as described by Eikmanns *et al.* (1994).

All enzymes used were obtained from MBI-Fermentas and used according to the instructions of the manufacturer. PCR experiments were performed in a Biometra personal cyclor (Biotron). dNTPs were obtained from MBI-Fermentas, and oligonucleotide primers from MWG-Biotech. Cycling times and temperatures were chosen according to fragment length and primer constitution. PCR products were purified using the Nucleospin Extract II kit (Macherey–Nagel).

Construction of *C. glutamicum* WT-ImX. Inactivation of the chromosomal *glgX* gene in *C. glutamicum* was performed as described previously for the inactivation of the *glgC* gene (Seibold *et al.*, 2007), using pK19*mobsacB*-Ins*glgX*. This plasmid was constructed by PCR amplification of a DNA fragment covering nt 439–1604 of the annotated *glgX* gene, including the *EcoRI* restriction sites at positions 459 and 1594, using primers IM*glgX*For (5'-GCAGATAAC-AACGAGACTC-3') and IM*glgX*Rev (5'-GAGGAACCACTTAGTC-GGG-3'). After digestion of the PCR product with *EcoRI*, the fragments were separated on an agarose gel, and an 1136 bp internal fragment of *glgX* was eluted from the gel using the Nucleospin Extract II kit. This fragment was ligated into the *EcoRI*-restricted pK19*mobsacB*, and the resulting vector pK19*mobsacB*-Ins*glgX* was transformed into *E. coli*. After isolation of the recombinant plasmid, it was electroporated into *C. glutamicum* WT. Integration of pK19*mobsacB*-Ins*glgX* at the genomic *glgX* locus in *C. glutamicum* was confirmed by Southern blot hybridization, using a *glgX*-specific 535 bp fragment (covering nt 739–1274 of the *glgX* gene), amplified from chromosomal DNA of *C. glutamicum* WT using primers GlgXInsFor (5'-ACGCTGCCAACAAGAACC-3') and GlgXInsRev (5'-ACCACGCCAGAAATCACG-3'). Labelling, hybridization, washing and detection were conducted using the non-radioactive DNA labelling and detection kit, according to the instructions from Roche Diagnostics. The labelled probe was hybridized to *Bam*HI-restricted and size-fractionated chromosomal DNA from *C. glutamicum* WT and the integration mutant WT-ImX. The hybridization resulted in one signal of ~5 kb from *C. glutamicum* WT and two signals of ~4.2 and ~7.8 kb from *C. glutamicum* WT-ImX. These sizes were expected for the WT strain and the *glgX* integration mutant, respectively.

Construction of *E. coli* ER2566 (pET28a-*glgX*His). Vector pET28a was used for the synthesis of the hexahistidyl (His)-tagged GlgX fusion protein (GlgX-His). The *glgX* gene was amplified from chromosomal DNA of *C. glutamicum* WT by PCR with primers GlgXHisFor (5'-CATGCCATGGCATCAACGATTGAGCACTCGT-3', *NcoI* site underlined) and GlgXHisRev (5'-CGCCTCGAGCT-TCTCTTCTTCGTCGCTTC-3', *XhoI* site underlined). The PCR

product was restricted with *Nco*I and *Xho*I, ligated into the *Nco*I/*Xho*I-restricted plasmid pET28a, and transformed into *E. coli* ER2566. The nucleotide sequence of the *glgX* fragment in pET28a-*glgX*[His] was verified by sequence analysis (MWG Biotech).

Protein analysis. Protein concentrations were determined using the bicinchoninic acid protein assay reagent kit (Pierce), with BSA as the standard. SDS-PAGE was performed according to Laemmli (1970). Western blot experiments for detection of the His-tagged GlgX protein were performed using His-tag mAbs and LumiBlot reagents from Novagen.

Purification of the GlgX-His protein. The crude extract for GlgX-His purification was obtained as described previously for purification of RamA (Cramer *et al.*, 2006). The crude extract was applied to a HisTrap FF crude column (GE Healthcare) equilibrated with binding buffer (20 mM sodium phosphate, 0.5 M NaCl, 30 mM imidazole, pH 7.5). The stepwise elution of proteins with imidazole was performed and monitored with an Äkta Purifier system (GE Healthcare). The first washing buffer to elute non-specifically bound proteins contained 75 mM imidazole, 0.5 M NaCl, and 20 mM sodium phosphate, pH 7.5. GlgX-His was eluted with buffer containing 250 mM imidazole. For further purification and removal of imidazole, the fraction was applied to a HiLoad 16/60 Superdex 200 prep grade gel-filtration column (GE Healthcare) equilibrated with 40 mM acetate buffer, pH 5.2. Elution was performed with 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 GlgX-His was compared to that of standard proteins (HMW Calibration kit, GE Healthcare).

Glycogen degradation/debranching activity of purified GlgX-His enzyme. For analysis of the degradation activity of the purified GlgX-His on glycogen, amylopectin and soluble starch, 7.5 µg protein was added to a solution of the respective polysaccharide (10 mg ml⁻¹ in 40 mM acetate buffer, pH 5.3). After incubation at 30 °C, the GlgX-His enzyme was inactivated by incubation at 95 °C for 10 min. The amount of sugars released after treatment with GlgX-His compared to untreated polysaccharide was determined essentially as described by Miller (1959). Dinitrosalicylic acid solution (0.4 ml, containing 1%, w/v, dinitrosalicylic acid, 20%, w/v, NaOH, and 30%, w/v, potassium sodium tartrate) was added to 0.4 ml sample. Subsequently, the samples were boiled for 5 min and centrifuged for 5 min at 13 000 g to precipitate insoluble particles. The supernatant (200 µl) was diluted with 800 µl water and *A*₅₄₆ was recorded. The amount of reducing sugars formed was determined by comparison with a maltose standard.

The debranching activity of GlgX-His with glycogen, amylopectin and soluble starch as substrates was tested qualitatively by following changes in the spectrum of the iodine-carbohydrate complex in a spectrophotometer between 400 and 600 nm (Jeanningros *et al.*, 1976). The respective carbohydrate (5 mg ml⁻¹) was dissolved in 40 mM acetate buffer, pH 5.3. After addition of 5 µg purified enzyme, the reaction mixture was incubated at 30 °C. At time points 0, 1, 2, 4 and 24 h after the start of incubation, samples of 50 µl were taken and heat-inactivated (10 min, 95 °C). Iodine reagent (2.5 ml; 0.02%, w/v, iodine and 0.2%, w/v, potassium iodine in 0.05 M HCl) was added and the absorbance spectrum of the complex formed was determined.

Debranching activity of the GlgX-His protein was also determined in combination with α - and β -amylases. Aliquots of the GlgX-His-treated and untreated carbohydrate solutions (see above) were incubated with α -amylase (5 U ml⁻¹, 2 h, 30 °C, in 0.1 M phosphate buffer, pH 6.9) or with β -amylase (5 U ml⁻¹, 2 h, 30 °C, in 0.1 M acetate buffer, pH 4.8). Afterwards, the release of reducing sugars from the substrate was measured with dinitrosalicylic acid solution, as described above.

Quantification of glucose. For analysis of the glucose concentration in the culture broth, 1 ml culture was withdrawn and centrifuged (13 000 g, 10 min, 4 °C), and the supernatant was used for the determination. Glucose was determined enzymically using hexokinase/glucose-6-phosphate dehydrogenase (Roche Diagnostics) and spectrophotometric quantification (at 340 nm) of the NADPH formed.

Analysis of intracellular glycogen and trehalose. For enzymic analysis of intracellular glycogen and trehalose, 5 ml samples of respective cultures were harvested, and the cell extracts were prepared exactly as described previously (Seibold *et al.*, 2007). Glycogen was determined with amyloglycosidase, as described previously (Seibold *et al.*, 2007). For trehalose determination, each sample was divided into two 100 µl aliquots (reactions A and B), and 1.5 µl trehalase (10 U ml⁻¹; Sigma) was added to reaction A, while reaction B remained as a reference. Both reaction A and reaction B were incubated with shaking for 2 h at 37 °C. The concentration of the liberated glucose was then measured in both control (–trehalase) and experimental (+trehalase) tubes, as described above. The calculations of the intracellular trehalose content in relation to the cell dry weight (dw) were performed as described previously for the glycogen content (Seibold *et al.*, 2007).

TLC was used to distinguish between intracellular glycogen, trehalose and other carbohydrates, which might serve as substrates for amyloglycosidase or trehalase. Sample preparation, application, separation and visualization of the carbohydrates on the TLC plate were performed as described previously (Seibold *et al.*, 2007). Carbohydrates were identified by comparison with the migration of known standards, i.e. glucose, trehalose, maltose, maltotriose, maltoheptaose and glycogen (obtained from Sigma).

Computational analysis. Database searches and alignments were carried out by using BLAST (Altschul *et al.*, 1990) and CLUSTALW (Thompson *et al.*, 1994). The TrEMBL accession numbers for (putative) GlgX protein sequences and the corresponding genes annotated as *glgX* are: *C. glutamicum*, Q8NNT0, cg2310; *Corynebacterium diphtheriae*, Q6NGF3, DIP1572; *Corynebacterium efficiens*, Q8FNY3, CE2010; *Corynebacterium jeikeium*, Q4JW65, jk0780, *Mycobacterium bovis*, P0A4Y5, Mb1591c; *Mycobacterium tuberculosis*, P0A4Y4, MT1615; *E. coli*, P15067, b3431.

RESULTS

Nucleotide sequence of the *C. glutamicum glgX* gene and analysis of the deduced GlgX amino acid sequence

The *C. glutamicum* cg2310 gene has been sequenced in the course of the determination of the genome sequence, and annotated as *glgX* due to similarities of the deduced polypeptide with the debranching enzyme of *E. coli* (Kalinowski *et al.*, 2003). The *C. glutamicum glgX* gene has a length of 2511 bp, and the predicted gene product consists of 836 aa with a molecular mass of 94 334 Da. The best homology scores with probable debranching enzymes were observed with enzymes deduced from other *Corynebacterineae*, such as *C. efficiens* (86% identity), *C. diphtheriae* (80%), *C. jeikeium* (72%), *M. tuberculosis* (68%) and *M. bovis* (68%), while the best score with a functionally proven enzyme was that with the *E. coli* debranching enzyme GlgX (46% identity). The *glgX* gene

of *C. glutamicum* is preceded by an ORF (*cg2309*) which probably encodes a TetR-like regulator, and is followed by *cg2311*, which putatively encodes an S-adenosylmethionine (SAM)-dependent methyltransferase (Kalinowski *et al.*, 2003). A similar genomic organization can be observed in *C. efficiens* (Tauch *et al.*, 2005) and *C. diphtheriae* (Cerdeno-Tarraga *et al.*, 2003), whereas in *C. jeikeium*, the deduced *glgX* gene seems to form an operon with *treY* (Nishio *et al.*, 2003). This is also the case in *M. tuberculosis* and *M. bovis*, in which the annotated *glgX* gene is directly followed by the genes encoding TreY and TreZ, which may form *glgX-treY-treZ* operons (Fleischmann *et al.*, 2002; Garnier *et al.*, 2003). In *C. glutamicum*, *glgX* is located ~10 kb apart from *treY*, and the *treY* and *treZ* genes are separated by ~8 kb.

Inactivation of the *glgX* gene and analysis of the mutant

To study the role of the *glgX* gene product (GlgX protein) in growth and glycogen degradation in *C. glutamicum*, the chromosomal *glgX* gene was inactivated by chromosomal insertion of the vector pK19*mobsacB*-*Ins*glgX, resulting in strain *C. glutamicum* WT-ImX. During cultivation in minimal medium containing 1% glucose, growth behaviour and glycogen content of the mutant *C. glutamicum* WT-ImX were significantly different from those of *C. glutamicum* WT (Fig. 2). In comparison to the WT, the growth rate in the exponential-growth phase was reduced (WT, 0.37 h⁻¹; WT-ImX, 0.26 h⁻¹); the final optical density of the mutant strain was significantly lower (14.1

Fig. 2. Growth (lines) and glycogen content (bars) of *C. glutamicum* WT (black circles and bars) and *C. glutamicum* WT-ImX (grey triangles and bars) during cultivation in minimal medium with 1% glucose as a carbon source. Glycogen was determined enzymically as liberated glucose after amyloglycosidase treatment (two determinations per sample). Three independent cultivations were performed, and the SD of the glycogen determinations was <10%.

and 10.5, respectively), and throughout cultivation, *C. glutamicum* WT-ImX showed an increased glycogen content. Moreover, the glycogen content decreased only partially in the course of the stationary phase, leading even 16 h after entry into the stationary phase to a glycogen content of 22.5 mg glucose (g dw)⁻¹ (Fig. 2). In contrast, at that time point, only minor amounts of glycogen were present in *C. glutamicum* WT [6 mg (g dw)⁻¹]. The intracellular carbohydrates were additionally tested at selected time points via TLC analysis. In accordance with the enzymic measurements shown in Fig. 2, the TLC analysis revealed that almost no glycogen was present 24 h after inoculation in *C. glutamicum* WT cells, whereas glycogen was present in the mutant (Fig. 3). Analysis of the samples taken in the course of the exponential-growth phase revealed that *C. glutamicum* WT samples, but not the mutant samples, contained several other smaller carbohydrates in addition to glycogen, with sizes in the range of maltotetraose to maltooctaose (Fig. 3). These carbohydrates obviously are intermediates of glycogen degradation.

Purification of GlgX-His and analysis of its activity

To further analyse the function of the *glgX* gene and its role in glycogen degradation in *C. glutamicum*, we purified and characterized the respective GlgX protein. GlgX was synthesized as a C-terminal His-tagged fusion protein in *E. coli* ER2566 and purified using centrifugation, affinity

Fig. 3. TLC analysis of intracellular carbohydrates of *C. glutamicum* WT and *C. glutamicum* WT-ImX during cultivation in minimal medium with 1% glucose as a carbon source. The samples were taken at 5, 8 and 24 h after inoculation. Lanes S1 and S2 represent the separation of the standard sugars: standard solution S1 contained trehalose (tre), and standard solution S2 contained glucose (glc), maltose (m2), maltotriose (m3), maltoheptaose (m7) and glycogen (glyc).

chromatography and size-exclusion chromatography. SDS-PAGE analysis of the products of each purification step finally revealed a single protein of ~110 kDa, which could also be detected by Western blot analysis with antibodies binding to the C-terminal His-tag (data not shown). Size-exclusion chromatography with the GlgX-His and standard proteins revealed a native molecular mass of 417 000 Da (data not shown). This result allows the assumption that native GlgX-His forms a homotetramer.

To determine the extent of degradation activity of GlgX towards glycogen, soluble starch and amylopectin, they were incubated with purified GlgX-His protein, and the number of reducing ends formed was determined with dinitrosalicylic acid. Incubation of the polysaccharides with purified GlgX-His protein for 30 min led to liberation of 0.11–0.17 milligram maltose equivalents per millilitre compared to that in untreated substrates.

Debranching activity of the purified GlgX-His protein was assayed with glycogen, soluble starch and amylopectin as substrates by following changes in iodine colouration. A change in the absorbance maximum of the iodine-carbohydrate complex (from 471 to 477 nm, with glycogen as a substrate), and an increase in absorption of the complex formed was observed after incubation of purified GlgX-His with each of the three substrates. Although this method does not allow quantitative determination, the result shows that GlgX-His is able to cleave α -1,6-glycosidic linkages.

To further test for debranching activity of GlgX, glycogen, starch and amylopectin were pre-treated with GlgX-His, incubated with α - or β -amylase, and the number of reducing ends formed was determined with dinitrosalicylic acid. Degradation of branched polysaccharides (such as glycogen or starch) by α - and β -amylase is limited by α -1-6-glycosidic linkages, which leads to the formation of α - or β -amylase-limit dextrins (Friedberg & Rhodes, 1986; Ikawa *et al.*, 1998). Pre-treatment of glycogen or starch with a debranching enzyme should reduce the amount of these amylase-limit dextrins formed, and increase the formation of reducing ends by α - and/or β -amylase. Comparing the number of reducing ends formed by the action of α - or β -amylase alone with the number of reducing ends in assays with GlgX-His pre-treatment showed that, in the latter assays, up to an additional 0.42 milligrams maltose equivalents per millilitre was liberated (Fig. 4). No additional reducing ends were liberated by the action of GlgX-His on amylose, a polysaccharide consisting of α -1,4-linked glucose residues only (data not shown). Thus, incubation of the branched polysaccharides glycogen, starch and amylopectin with purified protein GlgX-His led to the formation of additional liberated maltose equivalents, compared to an assay with only α - or β -amylase-treated substrates. These results indicate that due to the action of GlgX-His, the availability of α -1,4-glycosidic-linked glucose residues for α - and for β -amylase is increased. These results and the observations made with

Fig. 4. Debranching activity of purified *C. glutamicum* GlgX-His protein. The release of maltose equivalents was measured with dinitrosalicylic acid from the substrates glycogen, starch and amylopectin. Bars represent additional reducing ends liberated by α -amylase (light grey) or β -amylase (dark grey) after pre-treatment of the respective substrate with GlgX-His, when compared to non-pre-treated substrates. The reducing ends formed by α -amylase without GlgX-His pretreatment correspond to 1.85 mg ml⁻¹ (glycogen), 3.51 mg ml⁻¹ (starch) and 2.77 mg ml⁻¹ (amylopectin) maltose equivalents; those formed by β -amylase correspond to 3.26 mg ml⁻¹ (glycogen), 4.65 mg ml⁻¹ (starch) and 4.33 mg ml⁻¹ (amylopectin) maltose equivalents.

the *glgX* mutant show that GlgX is involved in glycogen degradation, and indicate that it represents the debranching enzyme of *C. glutamicum*.

Role of the debranching enzyme GlgX for growth under hyperosmotic conditions

Previous studies have revealed a link between glycogen and trehalose synthesis in *C. glutamicum*, suggesting a role of glycogen metabolism for fast adaptation to hyperosmotic growth conditions (Padilla *et al.*, 2004; Seibold *et al.*, 2007; Tzvetkov *et al.*, 2003; Wolf *et al.*, 2003). We therefore examined the effects of salt addition in the exponential-growth phase on growth, substrate consumption, glycogen level and intracellular trehalose formation of *C. glutamicum* WT, using minimal medium with 1.5 % glucose as a carbon source. Addition of 750 mM NaCl 5.5 h after inoculation to the culture broth led to a decrease in growth rate from 0.35 to 0.19 h⁻¹, and to a decrease in glucose consumption rate (Fig. 5a). Immediately after the addition of salt, the glycogen level dropped severely from 62 to 4 mg glucose (g dw)⁻¹ (Fig. 5b). In the course of further growth, however, the glycogen content rose again. Interestingly, shortly after hyperosmotic shock, the intracellular trehalose concentration was significantly higher when compared to that in the cells not treated with NaCl (Fig. 5c). This result suggests that, as a consequence of hyperosmotic shock, glycogen is degraded, and trehalose is formed from the degradation products of glycogen. To investigate this

Fig. 5. Effects of hyperosmotic shock by addition of 750 mM NaCl on growth (circles), glucose consumption (triangles), glycogen content (squares) and trehalose content (diamonds) of *C. glutamicum* WT (a–c) and *C. glutamicum* WT-ImX (d–f) during cultivation in minimal medium with 1.5% glucose as a carbon source. Solid symbols, the respective strain without salt addition; open symbols, with salt addition, at the time point indicated by the arrows.

hypothesis and to study the function of GlgX in response to hyperosmotic conditions, we performed the same experiments with mutant *C. glutamicum* WT-ImX, which is unable to degrade glycogen. Addition of 750 mM NaCl at the same optical density as in the experiment with *C. glutamicum* WT immediately halted growth and glucose consumption (Fig. 5d). As expected from the results described above, more glycogen was present throughout the growth of cells of the mutant strain WT-ImX, compared to *C. glutamicum* WT (Fig. 5b, e). Moreover, the addition of salt to the medium did not lead to a decrease in glycogen content of the mutant strain WT-ImX (Fig. 5e). These results indicate that the GlgX protein is essential for glycogen degradation after hyperosmotic shock. The intracellular trehalose concentration of the *glgX* mutant strain (Fig. 5f) throughout cultivation was lower than the concentration observed in the WT strain. Moreover, the addition of NaCl to the medium did not lead to an increase in the trehalose content (Fig. 5f).

The intracellular glycogen and trehalose content after hyperosmotic shock in *C. glutamicum* WT and in the mutant strains was also investigated in a separate experiment by TLC analysis. For this purpose, culture samples were taken immediately (30 s) before (at 6 h for the WT and 10 h for the *glgX* mutant strain) and 30 min after the addition of 750 mM NaCl (6.5 h for the WT and 10.5 h for the mutant). Further samples from the WT and WT-ImX strain were taken at the late-stationary phase, i.e. 24 h after inoculation. As shown in Fig. 6, glycogen and smaller carbohydrates were present in cell extracts of *C. glutamicum* WT at the exponential-growth phase (6 and 6.5 h after inoculation). In the 24 h sample, no glycogen was detected. After the addition of salt to the medium at 6.5 h, an additional spot with retention behaviour corresponding to that of trehalose occurred, and was still detectable in the sample taken at the stationary phase (24 h after inoculation, Fig. 6). As in the TLC analysis of cell extracts from the mutant strain WT-ImX grown without

Fig. 6. TLC analysis of intracellular carbohydrates of *C. glutamicum* WT and *C. glutamicum* WT-ImX during cultivation in minimal medium with 1.5% glucose as a carbon source, before and after hyperosmotic shock. NaCl was added to *C. glutamicum* WT 6 h after inoculation and to *C. glutamicum* WT-ImX 10 h after inoculation. The numbers given above WT and WT-ImX indicate the time points (hours after inoculation) of sample collection. Lanes S1 and S2 represent separation of the standard sugars: standard solution S1 contained trehalose (tre), and standard solution S2 contained glucose (glc), maltose (m2), maltotriose (m3), maltotetraose (m7) and glycogen (glyc).

addition of sodium chloride (Fig. 3), all samples of the mutant taken before (10 h after inoculation) and after salt addition (at 10.5 h) contained glycogen, but no trehalose or other carbohydrate (Fig. 6). These results indicate that glycogen degradation is necessary for fast synthesis of trehalose as a compatible solute, and for survival after hyperosmotic shock.

DISCUSSION

In contrast to glycogen synthesis, glycogen degradation has not been investigated so far in *C. glutamicum*. We showed here that inactivation of the chromosomal *glgX* gene in *C. glutamicum* led throughout growth to a higher intracellular glycogen content when compared to that in *C. glutamicum* WT, and only partial degradation of glycogen (possibly due to activity of glycogen phosphorylase; see Fig. 1) at the end of the exponential-growth phase was observed with the mutant. Furthermore, we showed that the purified *glgX* gene product was able to cleave α -1,6-glycosidic linkages in glycogen, starch and amylopectin. These results indicate that the *C. glutamicum glgX* gene product is in fact involved in glycogen degradation, and that *glgX* encodes a glycogen-debranching enzyme.

It is obvious that glycogen degradation in *C. glutamicum* WT takes place in the late-exponential-growth phase. However, at the beginning of the exponential-growth phase, the glycogen content of the *glgX* mutant strain was much higher than that of *C. glutamicum* WT (Fig. 2), indicating that the debranching reaction has already taken place at this growth phase, i.e. when glycogen is still being synthesized. Parallel synthesis and degradation of glycogen have also been observed in *E. coli*. In this organism, the key enzymes for glycogen degradation, glycogen phosphorylase and debranching enzyme, are present and active throughout growth, even when there is net glycogen accumulation (Dauvillée *et al.*, 2005). In agreement with these observations, the genes encoding the *E. coli* proteins involved in glycogen degradation and synthesis (*glgC*, *glgA*, *glgB*, *glgP* and *glgX*) are clustered and arranged in operons (Romeo *et al.*, 1988). However, in *C. glutamicum*, the *glg* genes or their putative homologues are obviously scattered within the genome (Seibold *et al.*, 2007).

Polysaccharide preparations of *C. glutamicum* WT-ImX lacked maltodextrins (Fig. 3). This result was rather surprising, since during glycogen synthesis, the glycogen synthase should form linear glucans, which subsequently obtain α -1,6-glycosidic-linked residues by the action of the branching enzyme GlgB. We have recently observed that GlgB of *C. glutamicum* acts very efficiently on maltodextrins, and is involved in the fast formation of large polysaccharides (unpublished data). If glycogen branching takes place simultaneously with glycogen debranching, an equilibrium between linear and α -1,6-glycosidic-linked maltodextrins (thereby forming a glycogen-like structure) is established. The lack of debranching, caused by disruption of the *glgX* gene, would then shift the equilibrium in the direction of branched maltodextrins. This seems to be the case in *C. glutamicum* WT-ImX, in which only a large glycogen-like polysaccharide is detectable, and no more maltodextrins are visible in carbohydrate preparations of cell extracts. We therefore believe that the maltodextrins observed in the WT are an additional indicator for degradation processes taking place in parallel to synthesis. This hypothesis is in agreement with a possible role of glycogen as a carbon capacitor, as has previously been suggested for *Mycobacterium smegmatis* (Belanger & Hatfull, 1999). According to this model, the flow of carbon into glycolysis is modulated by the glycogen pool to prevent wasteful expenditure of resources.

For survival of the bacterial cell, it is necessary to react fast to sudden changes in osmolality (Wood, 1999). *C. glutamicum* responds to a sudden osmotic upshift by the uptake of betaine, ectoine or proline (Morbach & Krämer, 2003, 2005). When these compounds are not available, *C. glutamicum* synthesizes compatible solutes such as proline and trehalose (Morbach & Krämer, 2003, 2005). For trehalose synthesis, *C. glutamicum* possesses the OtsAB and the TreYZ pathway (Tzvetkov *et al.*, 2003; Wolf *et al.*, 2003). Absence of intracellular trehalose has been observed when both the OtsAB and the TreYZ pathways are

inactivated in *C. glutamicum* (Tzvetkov *et al.*, 2003; Wolf *et al.*, 2003). The same phenotype has been observed when glycogen synthesis is abolished by disruption of the glycogen synthase gene *glgA* in *C. glutamicum* Δ otsAB (Tzvetkov *et al.*, 2003). Moreover, overexpression of the *treYZ* genes in *C. glutamicum* causes a decrease in the intracellular glycogen content (Carpinelli *et al.*, 2006). All these findings indicate a link between trehalose formation through the TreYZ pathway and glycogen synthesis in the cells. We showed here that a shift to hyperosmotic growth conditions during the exponential-growth phase of *C. glutamicum* WT caused an immediate decrease in the intracellular glycogen content. In parallel to the decrease in glycogen content, an increase in the intracellular trehalose concentration took place. In contrast, the mutant strain *C. glutamicum* WT-ImX reacted with a complete cessation of growth on exposure to hyperosmotic conditions. Neither enzymically nor by TLC analysis could the formation of trehalose be observed in cell extracts of *C. glutamicum* WT-ImX. This corresponds to the same phenotype as *C. glutamicum* Δ treY after an upshift of osmolality (Wolf *et al.*, 2003). Since the *glgX* mutant contains glycogen, in principle, it should synthesize and contain all the intermediates of glycogen synthesis, including the linear chains of α -1,4-glycosidic-linked glucose residues, the products of glycogen synthase (Tzvetkov *et al.*, 2003), and the substrate for TreZ (Carpinelli *et al.*, 2006). The fact that the mutant contains no trehalose thus indicates that mainly the maltodextrins derived from the GlgX activity in the course of glycogen degradation are used for trehalose synthesis via the TreYZ pathway, and not the maltodextrins derived from *de novo* synthesis through the action of ADP-glucose pyrophosphorylase and glycogen synthase. In other words, the GlgX protein is essential for the accumulation of free maltodextrins, which in turn, are required for trehalose synthesis.

Wolf *et al.* (2003) have found that inactivation of the TreYZ pathway leads to a reduction in the intracellular trehalose content; however, in contrast to our *glgX* mutant, the growth of the Δ treY mutant after osmotic up-shift is not significantly impaired. Those authors explain this result by the dominant role of proline as a compatible solute under their experimental conditions. In view of this hypothesis, the growth impairment of *C. glutamicum* WT-ImX on exposure to hyperosmotic conditions is surprising, since proline synthesis should not be affected by *glgX* inactivation. In fact, the phenotype of *C. glutamicum* WT-ImX resembles that of the mutant deficient in both the TreYZ and OtsAB pathways (i.e. cytoplasmic trehalose and complete cessation of growth after hyperosmotic shock), and therefore we conclude that *glgX* inactivation might also have an influence on the activity of the OtsAB pathway. In view of this hypothesis, it is interesting to note that in *E. coli*, immediately after hypersomotic shock, the uptake of glucose is drastically inhibited (Roth *et al.*, 1985), which leads to a shortage of metabolites such as glucose 1-phosphate, ADP-glucose and UDP-glucose. By analogy,

metabolites derived from glucose might also become limiting in *C. glutamicum* after hyperosmotic shock. The pathways for trehalose synthesis require such metabolites either directly (OtsAB pathway, glucose 1-phosphate and UDP-glucose) or indirectly (*de novo* trehalose formation from glycogen or intermediates of glycogen synthesis). The formation of trehalose from maltodextrins derived from the degradation of intracellular glycogen by GlgX provides *C. glutamicum* with a mechanism for fast adaptation to hyperosmotic conditions, without uptake of carbohydrates.

ACKNOWLEDEMENTS

The authors thank P. Dangel for excellent technical assistance. The support of Degussa AG, Halle-Künsebeck, is gratefully acknowledged.

REFERENCES

- Abad, M. C., Binderup, K., Rios-Steiner, J., Arni, R. K., Preiss, J. & Geiger, J. H. (2002). The X-ray crystallographic structure of *Escherichia coli* branching enzyme. *J Biol Chem* **277**, 42164–42170.
- Alonso-Casajús, N., Dauvillée, D., Viale, A. M., Munoz, F. J., Baroja-Fernandez, E., Moran-Zorzano, M. T., Eydallin, G., Ball, S. & Pozueto-Romero, J. (2006). Glycogen phosphorylase, the product of the *glgP* gene, catalyzes glycogen breakdown by removing glucose units from the nonreducing ends in *Escherichia coli*. *J Bacteriol* **188**, 5266–5272.
- Altschul, S. F., Gish, W., Miller, W., Myers, E. W. & Lipman, D. J. (1990). Basic local alignment search tool. *J Mol Biol* **215**, 403–410.
- Ball, S. G. & Morell, M. K. (2003). From bacterial glycogen to starch: understanding the biogenesis of the plant starch granule. *Annu Rev Plant Biol* **54**, 207–233.
- Ballicora, M. A., Iglesias, A. A. & Preiss, J. (2003). ADP-glucose pyrophosphorylase, a regulatory enzyme for bacterial glycogen synthesis. *Microbiol Mol Biol Rev* **67**, 213–225.
- Belanger, A. E. & Hatfull, G. F. (1999). Exponential-phase glycogen recycling is essential for growth of *Mycobacterium smegmatis*. *J Bacteriol* **181**, 6670–6678.
- Buschiazio, A., Ugalde, J. E., Guerin, M. E., Shepard, W., Ugalde, R. A. & Alzari, P. M. (2004). Crystal structure of glycogen synthase: homologous enzymes catalyse glycogen synthesis and degradation. *EMBO J* **23**, 3196–3205.
- Carpinelli, J., Krämer, R. & Agosin, E. (2006). Metabolic engineering of *Corynebacterium glutamicum* for trehalose overproduction: role of the TreYZ trehalose biosynthetic pathway. *Appl Environ Microbiol* **72**, 1949–1955.
- Cerdeno-Tarraga, A. M., Efstratiou, A., Dover, L. G., Holden, M. T. G., Pallen, M., Bentley, S. D., Besra, G. S., Churcher, C., James, K. D. & other authors (2003). The complete genome sequence and analysis of *Corynebacterium diphtheriae* NCTC13129. *Nucleic Acids Res* **31**, 6516–6523.
- Cramer, A., Gerstmeir, R., Schaffer, S., Bott, M. & Eikmanns, B. J. (2006). Identification of RamA, a novel LuxT-type transcriptional regulator of genes involved in acetate metabolism of *Corynebacterium glutamicum*. *J Bacteriol* **188**, 2554–2567.
- Dauvillée, D., Kinderf, I. S., Zhongyi, L., Kosar-Hashemi, B., Samuel, M. S., Rampling, L., Ball, S. & Morell, M. K. (2005). Role of the *Escherichia coli glgX* gene in glycogen metabolism. *J Bacteriol* **187**, 1465–1473.

- De Smet, K. A. L., Weston, A., Brown, I. N., Young, D. B. & Robertson, B. D. (2000). Three pathways for trehalose biosynthesis in mycobacteria. *Microbiology* **146**, 199–208.
- Dippel, R., Bergmiller, T., Böhm, A. & Boos, W. (2005). The maltodextrin system of *Escherichia coli*: glycogen-derived endogenous induction and osmoregulation. *J Bacteriol* **187**, 8332–8339.
- Eggeling, L. & Bott, M. (2005). *Handbook of Corynebacterium glutamicum*. Boca Raton, FL: CRC Press.
- Eikmanns, B. J., Metzger, M., Reinscheid, D., Kircher, M. & Sahm, H. (1991). Amplification of three threonine biosynthesis genes in *Corynebacterium glutamicum* and its influence on carbon flux in different strains. *Appl Microbiol Biotechnol* **34**, 617–622.
- Eikmanns, B. J., Thum-Schmitz, N., Eggeling, L., Ludtke, K. U. & Sahm, H. (1994). Nucleotide sequence, expression and transcriptional analysis of the *Corynebacterium glutamicum* *gltA* gene encoding citrate synthase. *Microbiology* **140**, 1817–1828.
- Fleischmann, R. D., Alland, D., Eisen, J. A., Carpenter, L., White, O., Peterson, J., DeBoy, R., Dodson, R., Gwinn, M. & other authors (2002). Whole-genome comparison of *Mycobacterium tuberculosis* clinical and laboratory strains. *J Bacteriol* **184**, 5479–5490.
- Friedberg, F. & Rhodes, C. (1986). Cloning and characterization of the beta-amylase gene from *Bacillus polymyxa*. *J Bacteriol* **165**, 819–824.
- Garnier, T., Eiglmeier, K., Camus, J.-C., Medina, N., Mansoor, H., Pryor, M., Duthoy, S., Grondin, S., Lacroix, C. & other authors (2003). The complete genome of *Mycobacterium bovis*. *Proc Natl Acad Sci U S A* **100**, 7877–7882.
- Hanahan, D. (1983). Studies on transformation of *Escherichia coli* with plasmids. *J Mol Biol* **166**, 557–580.
- Horcajada, C., Guinovart, J. J., Fita, I. & Ferrer, J. C. (2006). Crystal structure of an archaeal glycogen synthase. *J Biol Chem* **281**, 2923–2931.
- Iglesias, A. A. & Preiss, J. (1992). Bacterial glycogen and plant starch biosynthesis. *Biochem Educ* **20**, 196–203.
- Ikawa, K., Araki, H., Tsujino, Y., Hyashi, Y., Igarashi, K., Hatada, Y., Hagihara, H., Ozawa, T., Ozaki, K. & other authors (1998). Hyperexpression of the gene for a *Bacillus* α -amylase in *Bacillus subtilis* cells: enzymatic properties and crystallization of the recombinant enzyme. *Biosci Biotechnol Biochem* **62**, 1720–1725.
- Jeanningros, R., Creuzet-Sigal, N., Frixon, C. & Cattaneo, J. (1976). Purification and properties of a debranching enzyme from *Escherichia coli*. *Biochim Biophys Acta* **438**, 186–199.
- Kalinowski, J., Bathe, B., Bartels, D., Bischoff, M., Bott, M., Burkowski, A., Dusch, N., Eggeling, L., Eikmanns, B. J. & other authors (2003). The complete *Corynebacterium glutamicum* ATCC 13032 genome sequence and its impact on the production of L-aspartate-derived amino acids and vitamins. *J Biotechnol* **104**, 5–25.
- Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.
- Liebl, W. (2005). *Corynebacterium* taxonomy. In *Handbook of Corynebacterium glutamicum*, pp. 9–34. Edited by L. Eggeling & M. Bott. Boca Raton, FL: CRC Press.
- Manners, D. J. (1991). Recent developments in our understanding of glycogen structure. *Carbohydr Polym* **16**, 37–82.
- Miller, G. L. (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Anal Chem* **31**, 426–428.
- Morbach, S. & Krämer, R. (2003). Impact of transport processes in the osmotic response of *Corynebacterium glutamicum*. *J Biotechnol* **104**, 69–75.
- Morbach, S. & Krämer, R. (2005). Osmoregulation. In *Handbook of Corynebacterium glutamicum*, pp. 417–435. Edited by L. Eggeling & M. Bott. Boca Raton, FL: CRC Press.
- Nishio, Y., Nakamura, Y., Kawarabayashi, Y., Usuda, Y., Kimura, E., Sugimoto, S., Matasui, K., Yamagishi, A., Kikuchi, H. & other authors (2003). Comparative genome sequence analysis of the amino acids replacements responsible for the thermostability of *Corynebacterium efficiens*. *Genome Res* **13**, 1572–1579.
- Padilla, L., Morbach, S., Krämer, R. & Agosin, E. (2004). Impact of heterologous expression of *Escherichia coli* UDP-glucose pyrophosphorylase on trehalose and glycogen synthesis in *Corynebacterium glutamicum*. *Appl Environ Microbiol* **70**, 3845–3854.
- Preiss, J. (1984). Bacterial glycogen synthesis and its regulation. *Annu Rev Microbiol* **38**, 419–458.
- Preiss, J. (1996). ADPglucose pyrophosphorylase: basic science and applications in biotechnology. *Biotechnol Annu Rev* **2**, 259–279.
- Romeo, T., Kumar, A. & Preiss, J. (1988). Analysis of the *Escherichia coli* glycogen gene cluster suggests that catabolic enzymes are encoded among biosynthetic genes. *Gene* **70**, 363–376.
- Roth, W. G., Leckie, M. P. & Dietzler, D. N. (1985). Osmotic stress drastically inhibits active transport of carbohydrates by *Escherichia coli*. *Biochem Biophys Res Commun* **126**, 434–441.
- Sambrook, J. & Russell, D. W. (2001). *Molecular Cloning: a Laboratory Manual*, 3rd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Seibold, G. M., Dempf, S., Schreiner, J. & Eikmanns, B. J. (2007). Glycogen formation in *Corynebacterium glutamicum* and role of ADP-glucose pyrophosphorylase. *Microbiology* **153**, 1275–1285.
- Stackebrandt, E., Rainey, F. A. & Ward-Rainey, N. L. (1997). Proposal for a new hierarchic classification system, *Actinobacteria* classis nov. *Int J Syst Bacteriol* **47**, 479–491.
- Tauch, A., Kaiser, O., Hain, T., Goesmann, A., Weisshaar, B., Albersmeier, A., Bekel, T., Bischoff, N., Brune, I. & other authors (2005). Complete genome sequence and analysis of the multiresistant nosocomial pathogen *Corynebacterium jeikeium* K411, a lipid-requiring bacterium of the human skin flora. *J Bacteriol* **187**, 4671–4682.
- Thompson, J. D., Higgins, D. G. & Gibson, T. J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* **22**, 4673–4680.
- Tropis, M., Meniche, X., Wolf, A., Gebhardt, H., Strelkov, S., Chami, M., Schomburg, D., Krämer, R., Morbach, S. & Daffé, M. (2005). The crucial role of trehalose and structurally related oligosaccharides in the biosynthesis and transfer of mycolic acids in corynebacterineae. *J Biol Chem* **280**, 26573–26585.
- Tzvetkov, M., Klopprogge, C., Zelder, O. & Liebl, W. (2003). Genetic dissection of trehalose biosynthesis in *Corynebacterium glutamicum*: inactivation of trehalose production leads to impaired growth and an altered cell wall composition. *Microbiology* **149**, 1659–1673.
- Wolf, A., Krämer, R. & Morbach, S. (2003). Three pathways for trehalose metabolism in *Corynebacterium glutamicum* ATCC 13032 and their significance in response to osmotic stress. *Mol Microbiol* **49**, 1119–1134.
- Wood, J. M. (1999). Osmosensing by bacteria: signals and membrane-based sensors. *Microbiol Mol Biol Rev* **63**, 230–262.
- Yeo, M. & Chater, K. (2005). The interplay of glycogen metabolism and differentiation provides an insight into the developmental biology of *Streptomyces coelicolor*. *Microbiology* **151**, 855–861.

Edited by: M. Hecker